1-1-2017

The Effects Of Bone Marrow Adipocytes On Metabolic Regulation In Metastatic Prostate Cancer

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THE EFFECTS OF BONE MARROW ADIPOCYTES ON METASTATIC PROSTATE CANCER CELL METABOLISM AND SIGNALLING

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2017

MAJOR: CANCER BIOLOGY

Approved By:

Advisor	Date

DEDICATION

To my Family, Friends, and Wally

ACKNOWLEDGMENTS

When I joined the Podgorski laboratory in April of 2014, I had finished my rotations and spent some time in a collaborating laboratory honing my technical and creative thinking skills to become a valuable asset to her team; however, I was still unprepared for the exciting journey it would be through Izabela's laboratory over the last three years. I was extremely lucky to have landed in Dr. Podgorski's laboratory and will be forever thankful for the tremendous support she has given me to aid in my development as an independent investigator. Along, with Dr. Podgorski, there are many other members of the Wayne State University community that I would also like to extend my thanks to.

Advisors:

I would like to thank *Dr. Izabela Podgorski* for accepting me into her laboratory and her continuous support for me and my research endeavors. Izabela has always been there when I need to ask her questions about my project, about future career goals and how to achieve them, aiding my scientific writing and presenting skills, and has also been a great friend over the course of my PhD training. I will truly miss sitting with Dr. Podgorski and discussing ideas for various projects within the lab and am extremely thankful that she gave me the opportunity to be creative and explore new ideas. Also, she allowed me to present my data at multiple domestic and international conferences that have helped me network and hone my presentation abilities. Lastly, her help with my scientific writing has helped me obtain a NIH/NCI F31 predoctoral grant, setting me up for a successful career in academia. She has been an excellent mentor and model academic advisor and I can only hope that I will be as successful as she is in my future.

Dr. Manohar Ratnam, thank you for all of your help and guidance as a co-mentor on my studies and always being there for me for questions or guidance whether it be at committee meetings or me just dropping by your office. You are a brilliant scientist and have always had my best interest in mind as you guided me through my project and gave me career advice. You have been an invaluable member of my research team and I am both lucky and thankful to have you as a co-mentor.

Committee Members:

Dr. Karin List, thank you for your help with my project and being such a great mentor and friend to me throughout my PhD training. I will truly miss your sense of humor and stopping by your office to talk to you about anything and everything. You kept me sane whenever things seemed to fall apart and also kept me laughing every day. Additionally, your expertise in mouse models provided instrumental input for the design of my *in vivo* work.

Dr. Sokol Todi, thank you for all of your help throughout my graduate studies. You always have a surprisingly vast knowledge base of just about everything and I know I can come to you with any question and you are able to help me through it. Thank you for being both critical and constructive, helping me to think "outside of the box." Additionally, I will miss being reminded by you how bad I am at cutting my own hair and wearing clothes that match.

Dr. Elisabeth Heath, thank you for all of your help throughout my graduate studies and helping me not forget the translational side of research. It is easy to forget that what we do can make a difference and your clinical input is extremely beneficial when developing a project with the potential to be clinically significant. Thank you for letting me present

my work at the Prostate Cancer Research Team meeting and letting me expand my knowledge into renal cancer as we collaborated on a grant submission. It is always a pleasure talking with you and I will always be impressed with how you juggle so many different responsibilities!

Lab Members:

Erandi, thank you so much for being patient with me and my awful jokes and as I stole all of your TBST and Western gels over the years! Thank you for teaching me the techniques in the laboratory and answering all of my questions. Thank you for knowing where literally everything in lab is at any given time. I will miss hearing about your kids and getting to try new foods that you bring in, but you are one of the hardest working people I have ever met and I will miss you, even if you won't miss me!

Mackenzie, thank you so much for putting up with me over the last couple of years. You re-taught me cell culture and how to set up adipocyte cultures and are always there to bounce ideas and questions off of. It has truly been a pleasure staying in lab until midnight with you! I think I saw you more than any other person over the last 3 years. I'm lucky you have a sense of humor and helped me transition into a more liberal mindset! I will miss getting coffee with you every day!

Rebecca, thank you for being a wonderful addition to our team in the Podgorski laboratory. Your hard work and dedication to science will be rewarding and I have full faith that you will be successful in your career endeavors. It has really been a pleasure getting to "mentor" you and I will miss showing you songs on the guitar that I learned and talking to you every time you sit down to work!

Collaborators:

Dr. Siddhesh Aras, thank you for teaching me the proper assays for mitochondrial work and assisting in my preparation and analysis of the Seahorse machine. You are always incredibly knowledgeable and I am lucky to have had you as a collaborator through my training.

Dr. Gargi Mahapatra, thank you for always being so kind to me and assisting in my preparation and analysis of the Seahorse machine. I have truly missed you since you graduated and I am sure you will be successful in your career in science!

Dr. Maik Huttemann, thank you for your help and guidance throughout my PhD and learning everything there is to know about mitochondria and glucose metabolism. From letting me present in your lab meeting to co-sponsoring my F31 grant, I am truly appreciative of everything you have done for me.

Cancer Biology Program:

Dr. Larry Matherly, thank you for welcoming me into the program and all of your help finding a lab to call home. I am proud to graduate from the strong program that you have developed here at WSU and your dedication to the students has been instrumental for our success. Thank you for being a co-sponsor on my F31 and also meeting with me to discuss both scientific questions and also career choices. I am truly honored to have you as a director and personal friend.

Dr. George Brush, thank you for your help with the academic requirements of the program. It's always a pleasure sitting down and talking with you about everything from questions I have about curriculum to guitars. I really appreciate your help and support throughout my training and will miss our talks.

Nadia Daniel, thank you for keeping the programming going. I have relied on you for reimbursements for conference travel and you have always been extremely patient as I gathered all of my receipts and information for you.

Cancer Biology Steering Committee, thank you for allowing me to transition into Izabela's laboratory and awarding me the T32 training fellowship in 2015 and the DeRoy Fellowship in 2016.

Cancer Biology Students, thank you for being great colleagues and, more importantly, friends. The friendships I have developed here I will maintain and hope to keep in contact with as future collaborators across the world.

Others:

Dr. Abdo Najy, thank you so much for being a great friend and mentor. You got me through some of the hardest times in graduate school and helped train me both technically and to think independently. You won the award for the smartest person in the world and you earn that title every day. You are always there for me and I am truly honored to call you a friend. I hope one day to be half the scientist and mentor that you are.

Dr. Bonnie Sloane, thank you so much for being so supportive of me and always willing to sit down and talk to me and give me advice about my future in academia. I am truly grateful to have you in the Pharmacology Department and will miss our talks.

Family:

Lastly, I would like to thank my parents for their continuous support over the course of my PhD training and encouragement to reach my goals and never give up. Also, my twin brother and my older sister for always being there for me and giving me constant support and motivation.

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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION: FIFTY SHADES OF FAT

Adipose tissue is a versatile organ, crucial for maintaining homeostasis by storing and dispersing energy, producing and releasing adipokines and cytokines, with the ability to influence other cells of a body in autocrine, paracrine and endocrine fashion [1]. This highly metabolically active tissue is distributed throughout the body in discrete depots, and its development, expansion and energy balance are regulated by an integrated network of genetic, environmental, epigenetic and pharmacological factors [1, 2]. When unbalanced, or when caloric intake exceeds energy expenditure, adipose tissue becomes problematic and can detrimentally affect physiological processes.

1.2 Different types of adipose tissue: Brown vs. White Fat

Long-thought to have homogenous characteristics throughout the entire body, adipose tissue actually exhibits depot-specific differences in metabolic profiles, and these variations appear to correlate with susceptibility to obesity and specific metabolic disorders [1]. In addition to its localization-based classification, adipose tissue is also commonly categorized based on its coloration, and is divided into brown, white and beige tissues with distinct functional, metabolic and endocrine differences [1].

The main role of brown adipose tissue (BAT) is to provide non-shivering thermogenesis by the means of energy expenditure. mitochondria and cytochrome content are abundant in BAT, a characteristic that attributes, in part, to the color and name of BAT [3]. Brown adipocytes are multilocular, meaning they contain multiple fat droplets. Uniquely, they express an uncoupling protein-1 (UCP1), the function of which is to uncouple respiratory chain proteins in the abundant cellular mitochondria. The

uncoupling of respiratory chain proteins results in the metabolic substrates being oxidized purely for the purpose of heat energy dissipation [3]. The progenitors of BAT can be traced through the expression of myogenic factor 5 (Myf5) that is also expressed in skeletal myocytes [4]. In humans, BAT develops during the fetal stage and is the most abundant throughout the body at infancy and throughout the first decade of life. It eventually declines in its abundance and retires predominately to areas surrounding vital organs such as suprarenal and para-aortic [3, 5] and the supraclavicular area [6]. Cold temperature [6-8] and β-adrenergic stimulation [9, 10] can trigger the expression of UCP1, induction of substrate oxidation and activation of BAT. Increased expression of UCP1 in rodents caused by overfeeding sprouted a theory of the relevance of BAT in evading obesity. The fact that activity of BAT declines in overweight individuals [7, 11] supports the evidence of inverse correlation between propensity to obesity and abundance of BAT [12, 13].

In contrast to BAT, the white adipose tissue (WAT) development begins in utero and continues to evolve throughout life [14, 15]. WAT serves as primary energy storage and based on its location in the body, it is often referred to as subcutaneous or visceral (intra-abdominal) fat that includes mesenteric, epididymal and perirenal depots. In humans, subcutaneous fat develops prior to visceral [16] and can be distinctively different in its pathophysiological processes [17]. Importantly, the morphology of white adipocytes differs from brown as they are unilocular cells containing less mitochondria and do not express UCP1 [18].

"Browning" is a phenomenon described when adipocytes located in the typical WAT sites switch from anabolic to catabolic mechanism producing "beige" adipocytes

[19]. They do so by developing multilocular morphology with increased number of mitochondria and expression of UCP1. This occurrence has been well described in rodents and is triggered by cold exposure and β3-adrenergic stimulation [20-22]. Although closely resembling the brown fat morphology, BAT occurring in the WAT depots does not express the same lineage marker, Myf5, as a "classic BAT" [23]. There has been a growing interest in understanding the capacity of brown and beige adipocytes to counteract obesity, diabetes and other metabolic diseases [19]. Strategies are being developed to selectively enhance respiratory uncoupling in adipose tissue to induce weight loss and reverse obesity-driven pathological processes.

1.3 Bone marrow fat and its roles in physiological processes and disease

Bone marrow fat, known as yellow adipose tissue (YAT), represents a depot dispersed throughout the bone marrow with primary localization to trabecular cavities [24, 25], and often viewed as having mixed characteristics of both WAT and BAT [25-27]. No longer considered just a "space-filler", YAT is recognized as a highly active organ, functions of which extend far beyond the storage of triglycerides and lipid metabolism, and they include systemic energy regulation and management of insulin sensitivity [28, 29]. Importantly, the systemic changes related to adipose tissue homeostasis critically affect glucose and energy balance [30] and reciprocally influence bone health. If the stability in signaling pathways that integrate bone remodeling and energy metabolism gets perturbed by metabolic events related to age and obesity, the physiological processes in the bone, like osteogenesis and hematopoiesis are critically affected [25, 28]. The latter results in susceptibility to pro-inflammatory events and dysregulated bone remodeling [24].

It is well-established, that during the normal aging process, healthy, hematopoietically active red marrow of the bone is progressively replaced by the fatty yellow marrow [24-26]. There is also increasing evidence that obesity and associated metabolic pathologies can have detrimental effects on bone health that go beyond age-driven changes in skeletal homeostasis [24, 31, 32]. Until recently, obesity was thought to have a protective effect on bone metabolism due to positive impact of body weight on bone formation [31, 33]. Current evidence suggests that percent body fat, waist circumference, and waist-to-hip ratio correlate with the risk of osteoporotic fractures, especially in men, who have larger amounts of marrow fat than age-matched women [34-36]. These epidemiological data are mirrored by the results of animal studies, where marrow adiposity has been shown to result in decreases in trabecular bone volume and overall reduced bone mineral density (BMD) [31, 37, 38]. Despite these findings, the relationship between adiposity and bone turnover remains controversial and additional, controlled studies are needed to truly understand the effects of obesity on bone health in humans.

There is growing clinical and epidemiological evidence that metabolic syndrome (MetS), a cluster of metabolic abnormalities that include abdominal obesity, hypertriglyceridemia, low high-density lipoprotein (HDL) cholesterol, high blood pressure, and glucose intolerance [39-41] is a strong contributor to marrow adiposity. This condition is highly prevalent in the United States as demonstrated by its presence in approximately 25-30% of adults over the age of 18 years [42, 43], and is a strong risk factor for cardiovascular disease, diabetes and stroke [44, 45]. A study of metabolic syndrome in normal-weight individuals with only regional accumulation of fat (visceral/abdominal and inter-muscular) was associated with fasting hyperinsulinemia, a risk factor for type 2

diabetes mellitus (T2DM) [46, 47]. Importantly, general obesity was shown to correlate with accumulation of marrow fat in both control and diabetic individuals [48]; however, only in diabetic patients, marrow adiposity was correlated with visceral adipose tissue (VAT) [48]. This finding pinpoints the potential importance of visceral fat depot in bone health, and its implications for development of diabetes. This also underlines the importance of distinguishing VAT from other adipose tissues in studies investigating the impact of obesity and metabolic disorders on skeletal health, because using the central obesity measures continues to lead to inconsistent results [49].

One specific metabolic consequence of excess adiposity is diabetes, a condition highly linked with marrow adiposity [48, 50] and profound effects on bone health [51, 52]. Numerous reports suggest that the following are potential biological links between obesity and diabetes: changes in insulin levels, altered calcium metabolism, reduced renal function, vitamin D regulation, higher concentrations of inflammatory molecules and collagen glycation products, polypeptides, such as osteocalcin and osteopontin, and certain adipokines [27-29, 53, 54]. Increases in circulating levels of bone resorption markers such as Tartrate Resistant Acid Phosphatase (TRAP 5b) and Cathepsin K (CTSK) have been reported in diabetic patients [55] and animal experimental models of diabetes [56, 57]. Serum levels of osteocalcin, an osteoblast-specific polypeptide, were reported to be inversely correlated with adiposity and measures of insulin resistance [58, 59]. In contrast, a positive association with insulin sensitivity and HDL cholesterol was demonstrated for osteoprotegerin, a known inhibitor of bone resorption, further evidence of clear association between the metabolic features and bone degradation [60]. It is noteworthy, similar to other fat depots, adipogenesis in the bone marrow is under the

regulation of PPARγ [28]. This has led to serious concerns in terms of treatment with anti-diabetic thiazolidinedione drugs, which were shown to induce bone marrow adiposity [61], likely even further exacerbating the environment already altered by diabetes itself. It is also important to keep in mind that marrow adiposity associated with diabetes appears to be characterized by low unsaturation and high saturation levels of fats [48, 62]. This suggests that perhaps apart from overall increase in adiposity, the composition of marrow fat might be a more important factor in bone health, and potentially other physiological processes, a phenomenon that warrants further investigations.

1.4 ADIPOCYTE ARTILLERY

Contrary to the previous view of adipocytes being metabolically inert, growing evidence from the last decades of research has revealed that they are in fact metabolically active cells highly involved in the uptake, production, and secretion of many different factors with systemic implications [63]. Through the production of lipids and secretion of hormones, cytokines or adipokines, adipocytes have the ability to influence neighboring cells within their microenvironment and throughout the body as a whole, working as a functional paracrine and endocrine tissue [1, 2, 63]. \

1.4.1 Hormones

The two most commonly studied hormones secreted by adipocytes are adiponectin and leptin. Adiponectin is a protein hormone responsible for regulating multiple metabolic processes [64]. This hormone is secreted primarily by adipocytes and is released into the bloodstream where it binds to adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), and has the potential to bind a membrane receptor, T-cadherin [65, 66]. Adiponectin-receptor binding results in activation of AMP-activated protein kinase

(AMPK) and subsequent signaling through peroxisome proliferator-activated receptor (PPAR)-α transcription factor [67-69]. Adiponectin levels have been associated with many different metabolic diseases. Interestingly, adiponectin is shown to be down-regulated in patients with obesity and/or diabetes and is upregulated upon treatment with insulin-sensitizers [70, 71]. It was recently discovered that bone marrow adipose tissue (MAT), in response to caloric restriction and chemotherapy, secretes adiponectin at a much larger scale comparing to the levels secreted by WAT, suggesting that MAT-derived adiponectin is circulated throughout the body, exhibiting endocrine and metabolic effects on cells [72]. Circulating adiponectin is shown to be decreased in patients with T2DM, cardiovascular disease, liver disease, and hypertension [73-75]. In addition, adiponectin binding to AdipoR1 and AdipoR2 has been shown to have anti-diabetic effects, which further underlines positive effects of this hormone on metabolic homeostasis [76].

Leptin, also known as the “satiety hormone,” is the other most commonly studied factor produced and secreted by adipocytes [77, 78]. Canonical leptin signaling occurs through the leptin receptor which, upon the binding of its ligand, dimerizes and induces phosphorylation and activation of Janus tyrosine kinase-2 (JAK2) [79]. This leads to STAT3 phosphorylation and downstream transcription of leptin target genes. Mutations in the gene encoding leptin or its receptors in the hypothalamus result in disturbed leptin signaling and consequently promote hyperphagic obesity, diabetes mellitus, and neuroendocrine dysfunctions [80]. Interestingly, although leptin is secreted by adipocytes to inhibit hunger, this adipokine is produced and secreted at high rates in obese individuals [81]. Enhanced leptin signaling has been implicated in many different cancers [82]. Leptin binding to its receptor on mammary cancer cells has been shown to

play a role in maintaining cancer stem cell phenotype and promoting stem cell-like properties of triple-negative breast cancers [83].

1.4.2 Inflammatory Cytokines

Obesity is characterized as a state of chronic inflammation. It has been speculated that expansion of adipose tissue occurring in obesity results in oxygen deprivation of adipocytes which are most distant from the capillary network [84]. This hypoxia triggers the activation of hypoxia inducible factor 1-alpha (HIF-1α), which in turn, signals for the macrophage infiltration and induction of inflammation [85]. There is growing evidence that multiple pro- and anti-inflammatory cytokines in obese adipose tissue form a functional circuitry that regulates local and systemic glucose tolerance and insulin sensitivity [86]. These cytokines are secreted by adipocytes, macrophages and other cell types residing in the inflamed tissue [86, 87].

TNF-α was one of the first identified WAT-derived proinflammatory cytokines, thought to be primarily secreted by myeloid cells via activation of MAPK and NFκB signaling pathways and stimulating the release of other inflammatory cytokines, such as IL-1β and IL-6 [88]. It has since been determined that adipocytes themselves are a significant source of TNF-α, whose induction in fat cells occurs in response to free fatty acids (FFA), and activation of JNK signaling pathway [89]. TNF-α, in turn, via activation of ERK signaling pathway stimulates lipolysis, a process resulting in a positive feedback mechanism that further contributes to the chronic state of obesity-induced inflammation [90]. The abundant secretion of this TNF-α has been directly linked to obesity-associated insulin resistance [89, 91] and tumorigenesis [92].

IL-6 is a pleiotropic cytokine, that is released in response to hypoxic stimulation of adipocytes [93] and its secretion is associated with insulin resistance [94], immune responses and host defense mechanisms [88], as well as tumorigenesis and metastatic potential [95]. Approximately 30% of circulating IL-6 levels are thought to originate from adipose tissue, categorizing it as an adipokine [88]. Circulating levels of IL-6 appear to correlate with increased body mass, waist circumference and FFA concentrations; however, its functions in obesity and insulin resistance in regards to tissue and metabolic rate remain controversial [88].

IL-1β is another important regulator of inflammatory responses whose levels are elevated in obesity and associated metabolic disorders [86]. A blockade of IL-1β activity in animal models and human subjects with neutralizing antibodies to this cytokine or its receptor improve insulin sensitivity and help to treat T2DM [86, 96, 97]. However, IL-1β-deficient animals have reported glucose intolerance, while IL-1RA-null mice are resistant to diet induced obesity, findings revealing the need for further investigations of pro-inflammatory axes in obesity and metabolic disorders.

1.4.3 Lipolysis

Key components released by adipocytes that can influence metabolic processes in neighboring cells are glycerol and FFA. In times of excess energy, fatty acids are stored as triglycerides, forming lipid droplets that are housed in the specialized domains within the endoplasmic reticulum [98, 99]. Fat cells are constantly both synthesizing triglycerides, and breaking them down to glycerol and fatty acids during a catabolic process known as lipolysis [100]. This process is driven by activation of the rate-limiting enzyme, adipose triglyceride lipase (ATGL), phosphorylation and activation of hormone-

sensitive lipase (HSL), and monoacylglycerol hydrolysis by monoglyceride lipase (MGL). Lipolysis and its rates are regulated by hormonal and biochemical signaling. The process of lipid breakdown is stimulated through the binding of catecholamines, epinephrine and norepinephrine, to β-adrenergic receptors 1 and 2 and the α-adrenergic receptor [101]. A key process for lipolysis and lipase regulation occurs through activation or suppression of protein kinase A (PKA) [102, 103]. PKA has the ability to both activate HSL and also facilitate the trafficking of proteins involved in lipolysis [104]. ATGL, on the other hand, is not a direct target of PKA and has high affinity for triacylglycerides and no activity against either diacylglycerides or monoacylglycerides [105]. HSL-null mice exhibit severely impaired glycerol release and large accumulation of DAG in several tissues, confirming that this lipase is a rate-limiting enzyme in DAG hydrolysis [106]. In contrast to HSL, ATGL deficiency leads to severe lipid-associated phenotype with high lipid accumulation, poor lipid mobilization, reduced biochemically-induced lipolysis, and myopathy [107-112], indicating its essential function in lipolysis. Absence of ATGL reduces fatty acid release from adipose tissue by 75% and a mutation in ATGL gene in humans causes lipid storage dysfunction called neutral lipid storage disease with myopathy (NLSDM) [113, 114]. Because lipolysis is such a fundamental and crucial process for energy homeostasis and metabolism, dysfunction in this process has been suggested as a hallmark to the onset or maintenance of obesity [115].

1.5 OBESITY-CANCER LINK: THE CONCERNING PROBLEM

Currently, obesity is a global epidemic characterized by excess adipocyte size and numbers. Recent reports indicate that more than two thirds of Americans are overweight or obese and this number has been increasing for decades [116, 117]. Obesity is a

serious health concern and a major risk for the development and onset of a multitude of different cancers [118-120]. Studies have demonstrated that the fraction of patients that have cancer caused by excess weight has reached about 20% of all cancers [120]. The Million Women Study reported that around 50% of cancers in postmenopausal women are linked to obesity [121]. For the high-risk obese patients in general, the most common malignancies appear to be esophageal adenocarcinoma, colorectal, postmenopausal breast, prostate, and renal cancers [122, 123]. Malignant melanoma, thyroid cancers, leukemias, non-Hodgkin's lymphomas, and multiple myelomas have been associated with obesity but to a lesser extent [124, 125].

1.5.1 Role of Circulating Adipokines in Tumorigenesis and Tumor Progression

As experimental and epidemiological evidence linking obesity with cancer risk or recurrence increases, the mechanisms behind this association are still largely unknown. It is becoming increasingly accepted that dysregulation of adipocyte function and obesity-driven chronic inflammation are the main culprits in adiposity-induced tumorigenesis [118, 126]. This is particularly evident in cancers that grow in adipocyte-rich environments like breast carcinomas, or cancers that have propensity to metastasize to fat-rich sites, such as ovarian or gastric malignancies [127]. In addition to acting as local paracrine signaling molecules, adipokines also exert systemic effects and allow for communication with distant sites. The increased levels of adipose tissue-derived factors, such as TNF-α, IL-6, IL-8, macrophage chemoattractant protein (MCP-1), and leptin and their role in tumor progression have been well-documented [82, 127].

Levels of circulating leptin are enhanced in obese individuals, and elevated leptin is a poor prognostic factor for breast cancer patients, underlining the role of this adipokine

in tumor progression [128]. Leptin expression is higher in patients that have prostate cancer compared to benign prostate hyperplasia and higher in patients with advanced, metastatic disease compared to patients with localized, early stage prostate cancer, implicating leptin expression as a biomarker for prostate cancer staging and prognosis [129, 130]. Notably, a polymorphism associated with an overexpression of the mutated leptin in some patients has been suggested as a risk factor for prostate cancer [131]. Furthermore, increased levels of leptin receptor were reported in breast cancer tissue as compared to normal tissue and suggested to correlate with immune response, angiogenesis, reproduction, growth factor signaling and lipid metabolism pathways [132-135]. In gastric cancer, leptin has been shown to increase tumor invasiveness by activating Rho/ROCK signaling pathways [136] while inhibitory effects of this adipokine on mitochondrial respiration have been linked with colon cancer progression [137].

In contrast to leptin, adiponectin, an adipokine with insulin-sensitizing effects, has been suggested to have anti-tumor effects [127, 138]. Low levels of adiponectin, as observed in obese individuals, have been correlated with an increased risk of prostate cancer [139]. Treatment with recombinant adiponectin has resulted in anti-tumor effects in some cancer types such as fibrosarcoma, myelomonocytic leukemia, and breast carcinoma [140-143]. Similarly, inhibitory effects of adiponectin on survival and proliferation of prostate cancer cells was reported, with anti-tumor effects linked to the high molecular form (HMW) of this adipokine, which is known to be responsible for its biological activity [144, 145]. These results were shown both in androgen-dependent LNCaP-FGC cells and androgen-independent DU145 cells, indicating a global effect on prostate cancer cells regardless of androgen receptor status.

1.5.2 Bone Marrow Adipocytes and Skeletal Metastases

Although numerous studies have identified obesity as a risk factor for various cancers [146-149], it is only recently that accumulation of bone marrow fat has emerged as a risk factor for the development and progression of skeletal metastases, particularly from prostate cancer [24, 150]. Specifically, we and others have shown that marrow adipocytes mediate translocation of the lipids to the metastatic cancer cells [151, 152]. These adipocyte-supplied lipids serve as an energy source for cancer cells, and consequently induce tumor cell proliferation, motility and invasion [150, 153]. Moreover, fatty acid binding protein 4 (FABP4), a lipid transporter expressed predominantly in adipocytes, macrophages, and endothelial cells [154], and originally identified as a key mediator of adipocyte-tumor interactions in ovarian cancer [127, 155], is highly upregulated in metastatic prostate cancer cells interacting with adipocytes [150]. We have shown that through its interplay with PPARγ and IL-1β, FABP4 is involved in driving the aggressiveness of prostate tumors in bone [150]. Our studies have also demonstrated that additional pro-inflammatory factors such as cyclooxygenase-2 (COX-2) and MCP-1 are highly induced in metastatic tumor cells under conditions of high marrow adiposity [24]. This underlines the interaction between the lipid-driven and the inflammatory pathways in bone and offers new avenues for investigation of mechanisms behind development and progression of skeletal metastases.

1.6 FEEDING THE ENEMY

1.6.1 Warburg Effect

The role of adipocytes in regulating tumor metabolism is largely understudied and not well-understood. The growth-, proliferation-, and survival-promoting effects of fat cells

on the tumor cells have been clearly demonstrated in breast, prostate, gastric, colon and ovarian cancers [127]; however, little is known about the contribution of altered tumor metabolism to these effects. A recent publication by Nieman et al. showed that ovarian cancer cells utilize adipocytes to gain energy for rapid division by inducing fat cell-driven lipolysis and increasing availability of lipids for uptake by the tumor cells [155]. Subsequent to lipid uptake, there is an overexpression of fatty acid transporter, FABP4, and significant elevation of β-oxidation, which can be blocked by the treatment with inhibitor of carnitine-palmitoyltransferase 1 (CPT-1), etomoxir [155]. Notably, β-oxidation has also been shown to be a main source of energy in prostate cancer cells [156], further suggesting that metabolic reprogramming may be playing an important role in tumorigenesis.

For most of the normal cells in the human body, glucose is an essential energy source. In the presence of oxygen glucose is broken down to pyruvate, which enters the mitochondria and is further oxidized to carbon dioxide with the release of energy in the form of ATP [157]. In the absence of oxygen, normal cells will produce high rates of lactate and undergo a metabolic shift to a more glycolytic phenotype. It has been well documented that unlike normal cells, tumor cells show high rates of glycolysis and lactate production, regardless of the presence or absence of oxygen [158]. This metabolic switch to aerobic glycolysis, also known as the Warburg Effect, provides energy and essential carbon sources for lipogenesis and nutrient production for the rapidly dividing cancer cells [159, 160]. This enhanced glycolytic phenotype was originally postulated to be a direct effect of mitochondrial dysfunction within cancer cells [161]. Under normal physiological conditions ATP is generated through oxidative phosphorylation in the mitochondria in

which acetyl-CoA is oxidized to CO2, releasing energy in the form ATP [162]. It has been shown that oncogenic transformation leads to an increase in glycolytic genes, while tumor suppressor proteins induce expression of oxidative phosphorylation (OxPhos) genes, showing the implications of glycolysis in carcinogenesis [163, 164].

Many of the enzymes responsible for glucose metabolism have significant functions that are non-glycolytic and tumor promoting [165]. Specifically, it was revealed that hexokinase II (HKII) has an anti-apoptotic effect on the mitochondria by binding to the mitochondrial membrane, antagonizing interaction with pro-apoptotic factors Bad and Bax [166-168]. Along the same lines, pyruvate kinase M2 (PKM2) was shown to have non-glycolytic functions in facilitating tumor survival [169, 170]. PKM2 appears to be activated through epidermal growth factor receptor (EGFR) signaling and obese patients have higher levels of serum heparin-binding epidermal-like growth factor, which is able to activate EGFR [171, 172]. Other functions of PKM2 include the phosphorylation of histone H3 and releasing histone deacytelase 3, which leads to induction of many cell cycle genes including cyclin D and metabolic regulator c-MYC [173]. PKM2 is also known to act as a transcriptional regulator through its interactions with Oct4, a transcription factor that drives the expression of many genes in tumorigenesis and nuclear signaling [174, 175].

Glycolysis is much less energy efficient compared to the OxPhos pathway as it generates two net ATP molecules, versus 36 molecules of ATP produced by the OxPhos pathway. Consequently, cancer cells must undergo very high rates of glycolysis in order to generate a large amount of ATP quickly. Interestingly, cells utilizing aerobic glycolysis have high ratios of ATP/ADP and NADH/NAD+ even when proliferating at high rates [176]. The aerobic glycolytic phenotype is important for tumor progression through the

following postulates: 1) high rates of lactate production and secretion can break down and degrade the surrounding extracellular matrix and aid tumor expansion and metastasis; 2) enhanced glycolysis supplies an abundance of ATP to the cancer cells; and 3) associated mitochondrial dysregulation inhibits or reduces apoptosis [177-179]. It has also been shown that aerobic glycolysis creates byproducts that increase the ability of the cells to produce precursors for biosynthesis of multiple different macromolecules essential for rapid division such as lipids, nucleic acids, and proteins [180]. Also, the generated lactate can create a toxic environment for immune cells, contributing to decreased immunosurveillance and thus the ability of the tumor to hide from an immune response within its microenvironment and prevent detection [181]. Excess lactate production has also been implicated in the stimulation of endothelial cells surrounding the tumor to allow vascularization of the tumor and to provide nutrients through the circulation [182, 183].

It is known that the transcription factor c-MYC acts as a master regulator of cellular metabolism by actively transcribing genes associated with glycolysis. Specifically, it has been shown that c-MYC upregulates *lactate dehydrogenase alpha* (LDH-α), an enzyme crucial for the conversion of pyruvate to lactate during the Warburg Effect [184, 185]. The c-MYC transcription factor is also known to regulate key proteins involved in both nucleic acid synthesis and fatty acid synthesis, processes utilized by tumor cells to meet the demands of rapid cellular division, which underlines its role as a crucial regulator of cellular metabolism [186, 187]. Furthermore, overexpression of c-MYC has been correlated with upregulation of pyruvate kinase M2, the splice variant most commonly seen in tumor cells during aerobic glycolysis [188-190]. The M2 isoform of pyruvate

kinase is overexpressed in cancer cells through c-MYC-regulated overexpression of heterogeneous nuclear ribonucleoprotein 1 and 2 (hnRNPA1 and hnRNPA2). These ribonucleoproteins preferentially splice the M2 isoform over the M1 isoform, which is critical for aerobic glycolysis [188, 191]. There is a proposed positive feedback in which PKM2 is upregulated by c-MYC and, in turn, PKM2 is involved in the upregulation of c-MYC [192]. Notably, c-MYC has been shown to be overexpressed in an estimated 50% of all human cancers [193, 194].

Along with an enhanced glycolytic phenotype, tumorigenesis is often associated with mitochondrial dysfunction [195]. Mitochondria become dysfunctional when the mitochondrial DNA (mtDNA) is reduced or mutated and obese individuals have been shown to have a reduction in mitochondrial DNA in adipocytes [196]. Dysregulation in mitochondrial activity has also been shown to play a role in the inhibition of tumor suppressor protein p53, leading to aberrant proliferation checkpoints and tumorigenesis [197]. There is also emerging evidence demonstrating that obesity-induced adipokines promote mitochondrial defects and promote glycolytic phenotype in normal tissue, thereby driving tumorigenesis [198].

1.6.2 HIF-1α Signaling Pathways in Cancer

Anaerobic respiration, a hallmark of tumorigenesis, drives a hypoxic phenotype in cancer cells even in the presence of oxygen [199]. Hypoxic signaling occurs when transcription factor HIF-1α becomes stabilized and translocates to the nucleus where it binds to and activates hypoxic response elements (HRE) [200]. In normoxia, prolyl hydroxylase domain (PHD) proteins hydrolyze HIF-1α, which is then ubiquitinated by Von Hippel-Lindau (VHL) and targeted to the proteasome. However, under hypoxic conditions,

PHD is inhibited and HIF-1α is stabilized and translocated to the nucleus where it dimerizes with HIF-1β to activate hypoxia-responsive genes [201]. In addition to the hypoxic effect seen in low oxygenated tissue, there is also an oxygen-independent HIF-1α signaling in which HIF-1α becomes stabilized and activates target genes in the presence or absence of oxygen [202].

The role of HIF-mediated signaling pathway in tumorigenesis and clinical response to treatments is well established [203]. Many tumors have areas of low oxygenation or intratumoral hypoxia. Patients with poorly oxygenated primary tumors have a higher risk of both metastases and mortality due to a more aggressive cancer phenotype [204]. Elevated HIF-1α expression has been correlated to increased mortality risk in a plethora of different cancers including solid tumors of the bladder, brain, breast, colon, esophageal, head and neck, oropharynx, liver, lung, pancreas, skin, stomach, and uterus as well as acute lymphocytic and myeloid leukemias [205].

HIF-1α acts as a transcriptional activator and regulates the expression of many different glycolytic enzymes involved in metabolic reprogramming. Specifically, LDH-α, the enzyme responsible for the high conversion of pyruvate to lactate in cancer cells, is trans-activated only through HIF-1α transcriptional regulation [206]. Along with HIF-1α, the HIF-2α isoform signals in a similar way, but trans-activates different target genes. A study in renal cell carcinoma cells showed that HIF-1α and HIF-2α signaling converge at genes involved in glucose transport [207], lipid metabolism [i.e., *adipose differentiation-related protein (ADRP)*], pH homeostasis (i.e., *carbonic anhydrase IX (CAIX)*), interleukin responses, (i.e., IL-6), and angiogenesis (i.e., VEGF) [208]. Interestingly, however, there were many significant differences in gene regulation between HIF-1α and HIF-2α. This

study also revealed that HIF-1α but not HIF-2α is highly involved in a glycolytic response, functioning as a trans-activating factor for the following enzymes involved in glycolysis: *hexokinase1*, *hexokinase2 (HK2)*, *phosphofructokinase (PFK)*, *aldolase A (ALDA)*, *phosphoglycerate kinase 1 (PGK1)*, *and LDH*-α [208]. There has also been a proposed feed-forward mechanism in which the Warburg Effect-associated enzyme PKM2 can act as a co-activator for HIF-1α target gene transcription [209]. It was recently demonstrated that Jumonji c domain-containing dioxygenase (JMJD5), is upregulated through HIF-1α signaling and that JMJD5 interacts with PKM2, enhancing its translocation to the nucleus and is recruited to the LDH-α promoter [210, 211]. The inhibition of JMJD5 causes a decrease in glucose metabolism and lactate secretion associated with Warburg Effect [210, 211].

HIFs do not only regulate glycolytic genes to promote a more glycolytic phenotype, but they are also involved in mitochondrial effects and decreased OxPhos activity. Among HIF target genes are microRNAs, particularly miR-210, which has been reported to be overexpressed in hypoxia [212]. MicroRNAs bind to sequences in messenger RNA and either inhibit their translation or, in some cases, initiate their degradation [213]. MiR-210 targets the *iron-sulfur cluster assembly enzyme* (*ISCU)* gene, which is required for the activity of complex I in the mitochondrial electron transport chain during oxidative metabolism, the constituents of cytochrome c oxidase assembly protein (COX10), NADH-dehydrogenase 1a subcomplex 4 (NDUFA4), and subunit D of succinate dehydrogenase complex (SDHD) [214-218]. Accordingly, miR-210 has been labeled as a biomarker of tumor hypoxia, and its high levels have been implicated in poor patient prognosis for several cancers [219]. Because many of the targets of miR-210 affect mitochondrial

activity, it is evident that this molecule plays a central role in cellular metabolism and homeostasis. Notably, it was also reported that there is a positive feedback loop between miR-210 and HIF-1α in human lung cancer cell lines, where miR-210 stabilizes HIF-1α [220]. This leads to increased transcription of HIF-1α target genes and establishment of hypoxic tumor microenvironment. In addition, lung cancer cells that overexpress miR-210 have been shown to have decreased mitochondrial activity and increased glycolytic phenotype, and exhibit elevated resistance to radiotherapy [217]. This decrease in β-oxidation in parallel with increased HIF activity is important for tumor cell survival because it prevents malignant cells from developing high levels of reactive oxygen species (ROS) and allows them to survive under hypoxic stress.

Another important effect of hypoxia on tumor cells is the initiation of angiogenesis, a process of formation of new blood vessels from pre-existing vasculature. Tumor growth and metastatic progression depend heavily on angiogenesis for the continuing supply of nutrients [221]. Accordingly, studies have shown that tumors with functional angiogenesis grow much larger than those without proper vascularization and blood supply, and that reduced blood supply often results in necrosis or apoptosis [222, 223]. The most well-known pro-angiogenic factor regulated by HIF-1α is vascular endothelial growth factor (VEGF) [224]. It has been shown that VEGF transcript and protein levels are upregulated in response to hypoxia and that targeting HIF-1α with small-interfering RNA (siRNA) significantly reduces VEGF gene and protein expression [225]. Interestingly, we have previously demonstrated that treatment of tumor cells with media conditioned by bone marrow adipocytes *in vitro*, as well as *in vivo* establishment of skeletal tumors under conditions of high marrow adiposity result in significant upregulation of oxidative stress

markers and VEGF, suggesting potential activation of HIF-1α [150]. Particularly important to the obesity-cancer link might be the evidence, that obese patients have higher levels of HIF-1α activity in adipocytes due to rapid proliferation and expansion of the fat cells and an increase in VEGF expression [226-230]. VEGF alone has been implicated in tumorigenesis through the stimulation of proliferative signaling pathways through the vascular endothelial growth factor receptor (VEGFR) and through cancer stem cell maintenance [231, 232]. Further studies are required to elucidate the role of adipose tissue hypoxia and VEGF secretion in obesity on tumor initiation and maintenance.

1.6.3 Adipocyte Artillery and their Effects on Metabolism

Few studies have demonstrated the effects of adipocyte-derived factors on tumor metabolism. The majority of reported studies focus on TNF-α, leptin, and lipids or lipolysis products [233, 234]. A study utilizing genetically obese *ob/ob* mice showed an association between TNF-α secretion and OxPhos dysregulation [235]. A marked decrease in mitochondrial respiratory chain activity in liver cells of *ob/ob* mice was reported. Also reported were elevated levels of TNF-α, inducible nitric oxide synthase (iNOS), and tyrosine nitrated proteins correlated with increased adiposity. It was determined that *ob/ob* mice not only have diminished activity of OxPhos system, but also a reduction in the assembly of the OxPhos subunits in the mitochondria to about 50-60% [236]. Most of the decreases were seen in subunits transcribed by mitochondrial-DNA, which was reduced by approximately 60% relative to control mice.

Along with TNF-α, adipocyte-secreted factors such as leptin and Wnt peptides can also cause mitochondrial impairment [237, 238]. The observed effects of leptin appear to not only result from canonical leptin signaling, but also from its non-canonical signals

associated with crosstalk with both the phosphatidylinositol 3-kinase (PI3-K) pathway and the Ras-dependent pathways [239]. These pathways are both commonly deregulated in cancers and affect cellular survival, growth, and metabolism [240, 241]. PI3-K activation leads to subsequent activation of the downstream target protein Akt, and PI3-K/Akt signaling has been shown to directly regulate cellular metabolism [241]. Induction of this pathway leads to the expression of glucose, amino acid, lipoprotein, and iron transporters at the cellular surface [158]. Additional effects include stimulation of glycolytic enzymes hexokinase and phosphofructokinase, increased transcription of glycolytic genes, and relative increases in protein synthesis essential for rapid cellular division [242, 243].

It has been recently demonstrated, that Wnt signaling, which, when elevated, is commonly associated with tumorigenesis and tumor survival, suppresses mitochondrial respiration and cytochrome C oxidase activity [244]. An enhanced Wnt signaling through the β-catenin pathway leads to the inhibition of cytochrome C oxidase subunits COXVIc, COXVIIa, and COXVIIc, and this inhibition of mitochondrial activity results in an enhanced glycolytic phenotype. It has been postulated that with higher adipocyte content, levels of Wnt ligands are increased, leading to enhanced positive correlation with Wnt signaling in neighboring cells [245, 246].

Little is known to date on how adipocyte-derived lipids directly influence tumor metabolism; however, there is increasing evidence that lipids generated by the tumor cells during lipogenesis modulate metabolic pathways in cancer cells and stimulate the Warburg Effect. One consequence of the Warburg Effect is an increase in lipid biosynthesis, and *de novo* lipogenesis is, in fact, performed at high rates in cancer cells [236]. An example of a bioactive lipid with implications for prostate tumorigenesis is

sphingosine-1/2 phosphate (S-1/2P) [247, 248]. S1P is activated by the phosphorylation of sphingosine by sphingosine kinase 1 or 2 (SK1 or SK2). S1P has been shown to induce cell growth, survival, and migration and to play a role in variety of cancers [249]. Similarly, upregulation of SK1 has been associated with glioblastomas, lung, thyroid, and breast cancers [250-254]. Notably, S1P has also been shown to highly present in obese patients compared to lean patients [255] and its potential involvement in obesity-driven tumorigenesis calls for further investigations.

1.7 CURRENT THERAPEUTIC OPTIONS IN TARGETING TUMOR METABOLISM

1.7.1 Tools to Regulate Glycolysis

Because tumor metabolism is deregulated in almost all cancers, targeting glycolytic intermediates has become a hot topic in therapeutic research. One of the first inhibitors developed to target glycolysis was 2-deoxyglucose (2-DG), a glucose analog that downregulates glucose metabolism through competitive inhibition [256]. 2-DG is transported into the cell and phosphorylated by hexokinase to 2-deoxy-glucose-phosphate (2-DG-P). 2-DG-P cannot be further metabolized and accumulates in the cells, leading to competitive inhibition of hexokinase during glycolysis [257]. *In vitro* studies have shown that this effect causes a decrease in cellular ATP production, and leads to the blockage of cell cycle progression and subsequent cell death [258]. A recent study has demonstrated that a combination of 2-DG treatment with photodynamic therapy induces tumor cell death in a synergistic manner [259]. Decreased cellular proliferation and increased apoptosis of cancer cells was also demonstrated upon 2-DG treatment in the N-diethylnitrosamine-induced rat hepatocarcinoma model [260]. Along with a decrease in glycolysis, there was an observable decrease in the tricarboxylic acid (TCA)

cycle activity, fatty acid and cholesterol biosynthesis, and ATP production, all pathways associated with tumor progression and metabolism. Other studies have shown that inhibiting glyoxylase 1, an enzyme responsible for the conversion of the glycolysis byproduct methylyglyoxyl to D-lactate, in a highly metabolically active tumor cells leads to an increase in apoptosis and a decrease in cellular proliferation [261, 262].

Targeting glycolysis in order to reverse the Warburg Effect has sparked interest as a potential anti-cancer therapy and has led to recent breakthroughs in therapeutics. One particularly intriguing target gaining a significant amount of attention from the pharmaceutical industry is LDH-α, an enzyme converting pyruvate to lactate, and a biomarker of advanced disease, poor prognosis, and resistance to therapy in many different cancers [263-265]. LDH-α inhibitors have a high specificity for cancer cells because of the high demand for lactate production in cancer cells during aerobic glycolysis [266]. It was recently reported that inhibition of LDH-α reduced ATP levels and led to an accumulation of reactive oxygen species (ROS) in lymphoma cells [267]. This accumulation of ROS resulted in increased incidence of apoptosis, suggesting that LDH-α is critical for tumor maintenance and cancer cell metabolism [268, 269]. These results have been recapitulated in a variety of diverse cancer types including lung cancer, renal cancer, breast cancer, hepatocellular carcinoma, nasopharyngeal carcinoma, and pancreatic cancer [267, 270-276].

There has been clinical success with drugs designed to target other enzymes in the glycolysis pathway, such as hexokinase II (HKII), phosphofructokinase (PFK), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), and pyruvate kinase M2 (PKM2). Lonidamine, a selective HKII inhibitor, reached phase III trials in the 1990's as a

therapeutic option for patients with lung cancer and was mildly successful but had toxic side effects [277, 278]. A novel approach by Wang et al. used the natural compound curcumin as a potential drug that could target HKII specifically and was shown to have anti-cancer effects *in vitro* [279]. The authors reported that colorectal cancer cells treated with curcumin *in vitro* have decreased mitochondria-associated anti-apoptotic HKII, leading to enhanced cell death. 3-bromopyruvate (3-BrPA), a selective inhibitor of another glycolytic enzyme, glyceralaldehyde-3-phosphate dehydrogenase (GAPDH), has been shown to downregulate PI3K/Akt signaling axis, leading to induced apoptosis in breast cancer cells [280, 281]. This finding suggests potential benefits of dual targeting of glycolysis enzymes and PI3K/Akt-mediated cellular metabolism. 3-BrPA has been FDA approved for phase I clinical trials as a selective glycolysis inhibitor [282-284] and was shown to induce ER stress, inhibit global protein synthesis and thereby induce tumor cell death [285]. Attempts have also been made to target phosphofructokinase, particularly PFKB3, the isoform commonly upregulated in cancers. Specifically, the use of selective PFKB3 inhibitors, such as 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) shown that inhibition of PFKB3 leads to autophagy and could be an effective anti-tumor therapy [286]. 3PO is currently being tested in clinical trials [287].

1.8 CONCLUSIONS

With obesity toll spreading to pandemic levels, it is critical that the underlying mechanisms linking obesity to metabolic pathologies and tumorigenesis are elucidated. It is clear that adipocyte-derived factors and lipolysis products have the capacity to alter cellular homeostasis in neighboring cells. Abnormal adiposity and chronic inflammation in obesity can lead to the secretion of a multitude of factors, all of which can influence

tumor metabolism. Our understanding of tumor metabolism over the last century has revealed a complex, integrated network of enzymes and metabolites cooperating together to facilitate tumor cell growth and survival. The dynamic functions of metabolic proteins make tumor metabolism an intricate and attractive field of research. Further understanding of the interactions between these metabolites and their oncogenic nature will provide insight into elucidating targetable mechanisms and development of novel therapies. Many leaps have been made in cancer therapies in a context of tumor metabolism. It remains crucial to advance our understanding of adipose tissue and disease in order to determine the molecular mechanisms behind adiposity and pathologies and, specifically, cancer.

Table 1: Current, on-going clinical trials targeting different aspects of cellular metabolism in different cancer types.

Target	Drug	Clinical Use	Clinical Trial Reference ID
Phosphofructokinase Isofrom 3 (PFKB3)	ACT-PFK-158	Advanced Solid Malignancies	NCT02044861
MYC	Lenalidomide	B-Cell Lymphoma Multiople Myeloma Hodgkin's Lymphoma	NCT02213913 NCT01380106 NCT01460940
Pyruvare Dehydrogenase Kinase 1 (PDK1)	Dichloroactetate	Brain Cancer Head and Neck Cancer	NCT00540176 NCT01163487
mTOR	Rad001	Prostate Cancer Triple Negative Breast Cancer Glioma Gastric Cancer	NCT00657982 NCT01939418 NCT00823459 NCT01514110
Hexokinase II (HK2)	Curcumin	Breast Cancer Prostate Cancer Leukemia/Lymphoma	NCT01975363 NCT01917890 NCT02100423
Phosphoinositol 3-Kinase	GDC-0980/GDC-0941 BKM120 BKM120	Breast Cancer Advanced Solid Tumors Head and Neck Cancer	NCT01437566 NCT01540253 NCT01816984
Wnt	LGK974 CWP232291 PRI-724	Multiple Cancers Acute Myeloid Leukemia Advanced Myeloid Malignancies	NCT01351103 NCT01398462 NCT01606579
TNFα	L19TNFα	Advanced Solid Tumors	NCT02076620
HIF-1α	Digoxin Ganetespib CRLX101 Phenelzine	Breast Cancer Multiple Cancers Ovarian/Tubal/Peritoneal Cancer Prostate Cancer	NCT01763931 NCT02192541 NCT01652079 NCT01253642

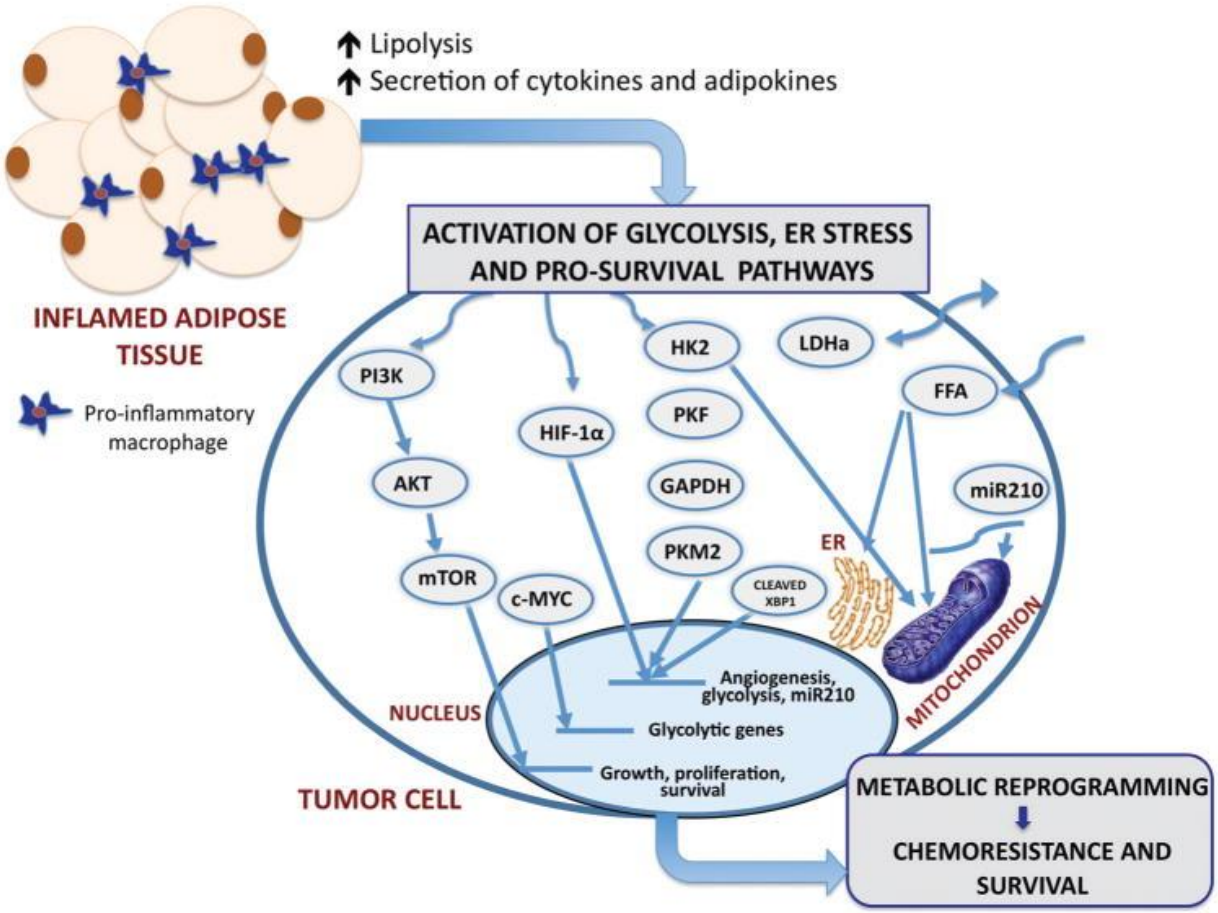

Figure 1: The proposed schematic of possible mechanisms of metabolic regulation of tumor cells by dysfunctional adipocytes in obesity. The major consequence of obesity is adipose tissue inflammation and associated increases in circulating levels of lipolysis-generated lipids and pro-inflammatory cytokines and adipokines. Through paracrine, autocrine, and endocrine effects, adipocyte-derived factors activate metabolic pathways in tumor cells and facilitating growth and survival. Pathways of interest include the following: phosphoinositol 3-kinase (PI3-K) signaling cascade, hypoxia-inducible factor 1α (HIF-1α), increased glucose uptake and enhanced glycolysis, and the potentially oncogenic endoplasmic reticulum (ER stress) pathway. The stimulation of PI3-K pathway leads to downstream activation of Akt and mTOR, enhancing the transcription of genes involved in growth, proliferation and survival. PI3-K signaling can also activate c-MYC and lead to the induction of glycolytic genes. A potential crosstalk between the glycolysis pathway and the HIF-1α signaling axis potentiates HIF activity, and exacerbates the glycolytic and hypoxic phenotypes. HIF-1α signaling leads to the expression of miRNA-210, which disrupts mitochondrial integrity, affecting cellular metabolism. Additionally, FFA have the ability to disrupt mitochondrial and ER membrane integrity and cause mitochondrial dysfunction and ER stress. The interactions of the ER stress response protein, XBP-1, with HIF-1α drive the expression of HIF- and glycolysis-targeted genes. This adipocyte-driven dynamic network of events results in metabolic adaptation of tumor cells, implicating adiposity in tumor aggressiveness and chemoresistance to therapy.

PI3K, phosphoinositide 3-kinase; AKT, protein kinase b; mTOR, mammalian target of rapamycin; c-MYC, myc proto-oncogene; HIF-1α, hypoxia-inducible factor 1; HK2, hexokinase 2; PKF, phosphofructokinase; GAPDH, glyceralaldehyde-3-phosphate dehydrogenase; PKM2, pyruvate kinase isoform; LDHα, lactate dehydrogenase; FFA, free fatty acids; miR-210, micro RNA 210.

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

Bone is a preferential site of metastasis from prostate cancer (PCa). Around 90% of patients with metastatic disease present with skeletal lesions. Although there have been many advances in therapeutic options for patients suffering from metastatic PCa, this disease remains incurable with an estimated five-year survival of 33%. To design effective therapeutic interventions for metastatic PCa, it is essential that we elucidate the molecular mechanisms in which tumor cells adapt to and thrive within the bone metastatic niche. **Age and obesity**, conditions that increase **adipocyte** numbers in **bone marrow**, are risk factors for skeletal metastases from PCa. Marrow fat has a potential to influence neighboring cells in paracrine and endocrine fashion by releasing a plethora of molecules including lipids, cytokines, hormones, complement factors, fatty acids, and free glycerol. We have shown previously that the progression of experimental intraosseous tumors is accelerated in mice with increased marrow adiposity. We have also demonstrated that tumor cells can utilize adipocyte-supplied lipids to support their proliferation and invasiveness. Our novel preliminary results indicate that exposure of tumor cells to marrow adipocytes *in vitro* and *in vivo* enhances their **glycolytic** phenotype and promotes tumor **hypoxia** driven by **HIF-1α.** Stemming from these findings, our overall **hypothesis** is that *adipocyte-supplied lipids within the bone microenvironment cause a metabolic switch to glycolysis in metastatic PCa cells by activation of HIF-1α, leading to increased aggressiveness and survival*. We proposed to test this hypothesis in two specific aims:

Specific Aim 1: Establish the contribution of adipocyte-supplied factors to metabolic changes in tumor cells. *Our working hypothesis is that PCa cells stimulate lipid release and lipolysis in marrow adipocytes, a process that increases lipid availability for tumor cells and leads to an enhanced glycolytic phenotype and tumor hypoxia. We*

propose to examine the interactions between adipocytes and PCa cells in vivo and in vitro to answer the following questions:

a) *Do adipocyte-supplied lipids affect cellular glycolysis and β-oxidation in tumor cells?*

b) *Are the metabolic effects of adipocytes on PCa cells driven through the activation of HIF-1α?*

Specific Aim 2: Determine the adipocyte-induced changes in the PCa cell fatty acyl lipidome and identify key lipid metabolites contributing to altered tumor metabolism and hypoxia. *Our working hypotheses are that: 1) PCa cells adapt to adipocyte-rich bone marrow and alter their lipidome to support own growth and survival; and 2) PCa cells influence the secretome of marrow adipocytes and stimulate them to release factors that promote tumor survival through metabolic regulation. Based on our findings, we proposed to:*

a) Characterize fatty acyl lipidomic profiles of metastatic tumor cells in the absence and presence of adipocyte-derived factors;

b) Define the lipid secretome of marrow adipocytes interacting with tumor cells;

c) Elucidate fatty acyl lipidomic signature associated with glycolytic and hypoxic phenotype in tumor cells

The proposed aims were designed to unravel specific aspects of bone marrow adipocyte involvement in PCa progression in bone. Findings from this study demonstrated a functional relationship between marrow adiposity and tumor growth, metabolic adaption and survival in the metastatic niche, and implicated adipocyte-supplied lipids and tumor metabolism as novel potential therapeutic targets in metastatic disease. This work

establishes a basis for future exploration of 1) the mechanisms behind adipocyte-driven metabolic adaptation and chemoresistance of skeletal tumors, and 2) the potential options for targeting of tumor metabolism for improved therapy and/or prevention of aggressive disease. This work will have high relevance beyond PCa and extend to other bone-trophic cancers.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium and other chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO). HyClone fetal bovine serum (FBS) was from ThermoFisher (Pittsburg, PA). Trypsin-EDTA, collagenase, BODIPY (493/503), Gentamicin (G418), Alexa Fluor 488-conjugated goat anti-rabbit IgG, and rabbit anti-human FABP4 were from Invitrogen (Carlsbad, CA). PureCol® collagen type I was from Advanced Biomatrix (San Diego, CA). Mouse monoclonal E7 Beta tubulin antibody was from Developmental studies Hybridoma Bank (Iowa City, IA). StemXVivo Adipogenic Suppliment, was from R&D Systems (Minneapolis, MN). Rabbit anti-human/mouse ß-actin antibodies were from Novus Biologicals (Littleton, CO). Mouse anti-human neuron-specific Enolase was from Dako-Agilent Technologies (Denmark). Rabbit anti-human-pyruvate dehydrogenase kinase, lactate dehydrogenase alpha, rabbit anti-human hexokinase 2 were from Cell Signaling Technologies (Beverly, Massachusetts). Rabbit anti-human phosphorylated pyruvate dehydrogenase, rabbit monoclonal anti-carbonic anhydrase 9 antibody, and the fluorometric L-lactate detection kit were from Abcam (Cambridge, UK). Adipolyze lipolysis detection kit was from Lonza (Switzerland). RNeasy Mini Kits were from Qiagen (Valencia, CA). Immunoblotting "Western Lightning ECL Plus" detection kits were from Perkin Elmer LLC (Waltham, MA). Rosiglitazone and the Triglyceride Colorimetric Assay kit were from Cayman Chemical (Ann Arbor, MI). ImmPACT NovaRED Peroxidase Substrate and ImmPRESS Anti-Rabbit Peroxidase Reagent kit were from Vector Laboratories (Burlington, CA). Cobalt Chloride was from MP Biomedicals (Solon, OH). Atglistatin was from Axon Medchem (Groningen, Netherlands).

3.2 Cell lines

PC3 cell line, derived from a bone metastasis of a high-grade adenocarcinoma [288], and DU145 cells, derived from human prostate adenocarcinoma metastatic to the brain [289], were purchased from American Type Culture Collection (ATCC; Manassas, VA). The ARCaP(M), an Androgen-Repressed Metastatic Prostate Cancer Cells M ('Mesenchymal' Clone) [290] were purchased from Novicure Biotechnology (Birmingham, AL). The human prostate cancer C4-2B cell line was kindly provided by Dr. Leland W. K. Chung, Cedars-Sinai Medical Center (Los Angeles, CA). PC3 and DU145 cells were grown in DMEM medium with 10% FBS, ARCaP(M) cells in RPMI medium with 5% FBS, and C4-2B cells in RPMI medium with 10% FBS. Cells were maintained in a 37°C humidified incubator ventilated with 5% CO2.

Primary mouse bone marrow stromal cells (mBMSC) were isolated from femurs and tibiae of 6- to 8- week old FVB/N mice according to previously established protocols [291]. To induce bone marrow adipocyte differentiation, mBMSC cells were plated in 3D collagen I gels, grown to confluency for 48-72 hours and treated with adipogenic cocktail (30% StemXVivo Adipogenic Suppliment, 1 μM insulin, 2 μM Rosiglitazone; DMEM and 10% FBS) for 8-10 days as previously described [150]. Differentiated bone marrow adipocyte cultures were washed 3 times with PBS and used in experiments.

3.3 Animals

All experiments involving mice were performed in accordance with the protocol approved by the institutional Animal Investigation Committee of Wayne State University and NIH guidelines. *In vivo* xenograft studies were performed in male mice in the FVB/N

background with homozygous null mutation in the Rag1 gene [FVB/N/ N5, Rag-1−/−]. All mice were bred in-house.

3.4 Diets

At 5 weeks of age, mice caged in groups of 4 were started on either a low-fat (LFD) diet (10% calories from fat; Research Diets no. D12450Ji) or a high-fat (HFD) diet (60% calories from fat; Research Diets no. D12492i). D12450Ji is a standard matched control diet for D12492i as recommended by Research Diets. Mice were maintained on respective diets for 8 weeks prior to the tumor implantation and continued on the diets for additional 6 weeks (PC3 tumors) or 8 weeks (ARCaP(M) tumors). Where indicated, mice were switched from HFD to LFD at time of tumor implantation and maintained on LFD for the remainder of the experiment.

3.5 Intratibial and subcutaneous injections of prostate cancer cells

Intratibial tumor injections were performed under isoflurane inhalational anesthesia according to the previously published procedures [150, 291, 292]. Briefly, a cell preparation containing 5 ×105 of PC3 /ARCaP(M) cells in PBS (20 µl, right tibia), or PBS alone (control, 20 µl, left tibia) was injected into the bone marrow. Mice were euthanized six weeks (PC3 cells) or eight weeks (ARCaP(M) cells) post-injection, and control and tumor-bearing tibiae were removed. For microenvironmental control, separate groups of LFD and HFD mice were injected subcutaneously with 50 µl of PC3 cell suspension (5 x105 cells in PBS/Cultrex). Half of the intratibial tumor samples from each group and half of each subcutaneous tumor were fixed in Z-fix, bone tumors were decalcified, and all samples were embedded in paraffin. Remaining tissues were snap-frozen in liquid

nitrogen, powderized using a tissue pulverizer and RNA was isolated using Trizol and RNeasy Mini Kit.

3.6 TaqMan RT-PCR analyses

The cDNA from cells and *in vivo* samples was prepared from 1-2 µg of total RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The analyses of genes associated with glycolysis, lipolysis, hypoxia and mitochondrial markers were performed using TaqMan® Individual Gene Expression assays for Human *ENO2* (Hs00157360), *LDHa* (Hs00855322), *HK2* (Hs00606086), *PDK1* (Hs01561850), *GLUT1* (Hs00892681), *CS* (Hs 02574374), *IDH2* (Hs00158033), *HIF-1α* (Hs00153153), *CA9* (Hs00154208), *MAGL* (Hs00200752), *CD36* (Hs01567185), *Perillipin 2* (Hs00605340), *VEGF* (Hs00900055), *Il-1β* (Hs01555410), *EP1* (Hs00909194), and *SPHK1* (Hs00184211). Assays were done on three biological replicates using TaqMan® Fast Universal PCR Master Mix and 50 ng of cDNA/well and all reactions were run on an Applied Biosystems StepOnePlus™ system. Three biological replicates of each sample were pooled together and assays were run in at least triplicate. The same assays (*ENO2, LDHa, PDK1, HK2, GLUT1, CA9* and *VEGF*) were performed on triplicate samples of PC3 bone tumors from LFD and HFD mice and normalized to human epithelial cell marker *CD326* (*EPCAM*) (Hs00901885). Specificity of each Taqman probe was cross-checked against RNA from control mouse bones and murine adipocytes (Supplementary Table 4). For all human genes *in vitro*, data were normalized to hypoxanthine phosphoribosyltransferase (*HPRT1*; Hs02800695). For assessment of adipocyte-specific genes in adipocytes grown in co-culture with tumor cells, the following murine Taqman assays were used: *HSL* (Mm00495359), *ATGL* (Mm00503040), *COX-2* (Mm00478374),

COX-1 (Mm00477214), *SPHK1* (Mm004488416). Data were normalized to *Adiponectin* (Mm00456425). DataAssist™ Software (Applied Biosystems) was used for all analyses. *CA9* and *CD326* primers (IDT, Coralville, IA) for PCR were used according to manufacturer's protocol. Human *CA9* forward and reverse primer sequences are as follows: Forward: 5'-GGGTGTCATCTGGACTGTGTT-3'; Reverse: 5'-CTTCTGTGCTGCCTTCTCATC-3'. *CD326* forward and reverse primers are as follows: Forward: 5'- CTG GCC GTA AAC TGC TTT GT-3'; Reverse: 5'-AGC CCA TCA ATT GTT CTG GAG-3'. *EP1* forward and reverse primers are as follows: Forward: 5'- CTT GTC GGT ATC ATG GTG GTGTC-3'; Reverse: 5'- GGT TGT GCT TAG AAG TG GCT GAGG-3'. *EP2* forward and reverse primers are as follows: Forward: 5'- CCA CCT CAT TCT CCT GGCTA-3'; Reverse: 5'- CGA CAA CAG AGG ACT GAA CG-3'; *EP3* forward and reverse primers are as follows: Forward: 5'- CTT CGC ATA ACT GGG GCA AC-3'; Reverse: 5'- TCT CCG TGT GTG TCT TGC AG-3'; *EP4* forward and reverse primers are as follows: Forward: 5'- TGG TAT GTG GGC TGG CTG-3'; Reverse: 5'- GAG GAC GGT GGC GAG AAT-3'. *S1PR* primers were generously provided Dr. Meng-Jer Lee at Wayne State University [293].

***In vitro* models**

3.7 Transwell co-culture

The mBMSC cells were embedded in Collagen, plated in 6-well plates, differentiated into adipocytes, and tumor cells were seeded on top of a Transwell filter (0.2 μm pore size) to allow sharing of soluble factors between the two cell types. After 48 hours, tumor cells were washed with PBS, trypsinized and harvested for RNA and protein extraction. Adipocytes were collected using 1% collagenase. For protein analyses,

lysates were re-suspended in SME buffer with protease and phosphatase inhibitors [150, 294]. For RT PCR analyses, cells were collected into RLT buffer and RNA was purified using RNeasy Mini Kit [150, 294].

3.8 Direct co-culture

Adipocytes embedded in Collagen I were differentiated in 100 mm dishes as previously described [29]. 600,000 PC3 or ARCaP(M) cells were plated in co-culture with adipocytes and on top of Collagen I without adipocytes as control. After 48 hours, 1% collagenase was used to break down the Collagen I and isolate the cells. Human specific qPCR probes were used to measure transcriptional responses in glycolytic genes.

3.9 Co-culture CM treatment

Conditioned media was obtained from either adipocytes alone (Adipo CM) or from PCa-adipocyte direct co-cultures (CCM) and either stored in -80 °C or used fresh after collection. PCa cells were seeded at 200,000 cells per well in 6-well plates 24 hours prior to treatment, then treated with either fresh DMEM containing 10% FBS Adipo CM, or CCM. After 24 hours of treatment, the cells were washed with PBS and collected for RNA and protein as previously described.

Tumor conditioned media was collected the identically for treatment of adipocytes with PC3 or ARCaP(M) conditioned media. Adipocytes were treated with either PC3/ARCaP(M) conditioned media or fresh DMEM containing 10% FBS and 1% P/S and collected for RNA as described above.

3.10 Immunoblot analyses

Lysate and media samples were loaded based on DNA/protein concentrations and the corresponding lysates were electrophoresed on 12% or 15% SDS-PAGE gels,

transferred to PVDF membranes and immunoblotted for human ENO2 (1:1,000), LDHa (1:1000), PDK1 (1:500), p-PDH (1:1000), HK2 (1:1,000), FABP4 (1:500), p-GSK3β (1:1000), total GSK3β (1:1000), β-catenin (1:1000), laminin A/C (1:500), cyclin D (1:1000), phosphorylated-Akt (1:1000), phosphorylated-ERK (1:1000), VDAC/Porin (1:1000) Tubulin (1:1000), and β-actin (1:1,000). To analyze the adipocyte protein expressions mouse COX-2 (1:1,000) was used. All horseradish peroxidase-labeled secondary antibodies were used at 1:10,000. Quantification and analyses of bands were performed using a Luminescent Image Analyzer LAS- 1000 Plus (Fujifilm, Stamford, CT).

3.11 CA9 immunohistochemical analyses

Tumor-bearing tibiae from LFD and HFD mice were fixed, decalcified, and embedded in paraffin. Deparaffinized and rehydrated tissues were then analyzed by immunohistochemistry for expression and localization of CA9 (rabbit anti-human CA9; 1:250). ImmPRESS Anti-Rabbit Peroxidase Polymer Detection systems along with a NovaRED kit as a substrate were used for the peroxidase-mediated reaction.

3.12 Immunofluorescence analyses

Cells were plated on coverslips (50,000 per coverslip) in a 24-well plate, allowed to attach for at least 4 hours, and transferred to control or transwell wells. After 48 hours, cell were stained with BODIPY (493/503) by washing with PBS, fixing with 3.7% formaldehyde at RT for 40 minutes, and incubating with BODIPY (493/503) (1:1000) at RT for 1 hour. Coverslips were washed and mounted onto slides using Vectashield with DAPI (Vector Laboratories). Images were taken using a Zeiss LSM 510 META NLO confocal microscope (Carl Zeiss AG, Göttingen, Germany) and a 40 × oil immersion lens. For CA9 staining, cells were washed with PBS and fixed with cold methanol. Coverslips

were stained with rabbit monoclonal anti-CA9 antibody (1:50) at 4°C overnight. Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000) was used as a secondary antibody, and DAPI was used as a nuclear stain. Coverslips were mounted using Vectashield and imaged using a Zeiss LSM 510 META NLO confocal microscope using a 63 × oil immersion lens.

3.13 ATP analysis

Cells were seeded in 6-well dishes, cultured overnight and treated with either fresh media (control conditions) or CCM. At 12 and 24 hours, the cells were washed and scraped into PBS. The cells were collected in a timely manner to ensure reliability of the sample, snap frozen in liquid nitrogen and stored in -80 ºC. The ATP Bioluminescence Assay Kit HS II (Roche Applied Science) and the boiling method for ATP release were used [124]. Briefly, 700 µl of incubation buffer was added to the cells (100 mM Tris-Cl, pH 7.75, 4 mM EDTA) and the solution was immediately transferred to a boiling water bath for 2 minutes. Samples were diluted 1:25 and 40 µl aliquots were used to determine the ATP concentration following the manufacturer's protocol. The experiments were done as biological duplicates, and then two aliquots were taken from each sample and assayed in triplicate. The concentrations were normalized to total protein using NanoDrop 2000 (Thermofisher Scientific). Data are shown as mean ± SD.

3.14 Assessment of lactate levels in media

Conditioned media was obtained from PC3 and ARCaP(M) cells alone and in transwell with adipocytes after 48-hour co-culture. The media was heat-inactivated at 65 °C for 8 minutes. Abcam's L-lactate Detection Kit was used and conditioned media was assayed using a TECAN plate reader (535nm/590nm) according to manufacturer's

instructions. Data were normalized to the total DNA or RNA concentrations in cell lysates. Experiments were done in triplicate and reported as mean ± SD.

3.15 Seahorse analysis

PC3 and ARCaP(M) cells were plated on a Collagen I matrix at concentrations of 30,000 cells per well in XF24 Cell Microplates and cultured overnight. The following day, cells were treated with fresh DMEM medium or Co-culture CM (CCM) for 12 and 24 hours. One hour prior to reading the plate, the media was changed to DMEM containing 10 mM glucose and 2 mM glutamine. Basal readings were performed on the Seahorse analyzer and the third reading of each experiment was used. Experiments were done in triplicate with at least three wells per sample condition and reported as mean ± SD.

3.16 Determination of mitochondrial membrane potential/intrinsic apoptosis

The JC-1 probe (Thermofisher Scientific) was used to assess mitochondrial membrane potential as a measure mitochondrial integrity [125] Cells were plated in 96-well black plates at a density of 5,000 cells/well and grown overnight. The following day, media was replaced with either fresh DMEM or CCM and cells were allowed to incubate for 12 and 24 hours. JC-1 probe was then added at a final concentration of 1 μM to the media and the plates were incubated for 20 minutes. The plates were then read at excitation and emission wavelengths of 535 nm and 595 nm, respectively, for the red fluorescence and excitation and emission wavelengths of 485 nm and 535 nm, respectively, for the green fluorescence. Data were normalized based on cell viability. Experiments were done in triplicate with quadruplicate wells per condition at each time point and shown as mean ± SD.64872

3.17 Determination of cell viability

Calcein AM Assay (Trevigen) was used to assess cell viability. Cells were seeded in black-walled 96- well plates at a density of 5,000 cells/well and grown overnight. The following day, media was removed and fresh DMEM or CCM was added to the wells and the cells were incubated for 12 or 24 hours. The plate was then read at excitation and emission wavelengths of 490 nm and 520 nm, respectively. Experiments were done in at least triplicate with quadruplicate wells analyzed per experiment and shown as mean ± SD.

3.18 Free glycerol assay analysis

Conditioned media was obtained from adipocytes alone, adipocytes in transwell with PC3 or ARCaP(M) cells, or adipocytes treated with conditioned media from PC3 or ARCaP tumor cells and analyzed using manufacturer's protocol for the AdipoLyze Lipolysis Detection Kit (Lonza). Experiments were done in triplicate and reposted as mean ± SD.

3.19 Triglyceride assay

Tumor cells were grown in transwell with adipocytes in the presence or absence of 10 μM Atglistatin. After 48 hours, adipocytes were collected as previously indicated and re-suspended in the Standard Diluent (provided in the Triglyceride assay kit; Cayman Chemical). Samples were sonicated and centrifuged. The supernatant was then used for the assay. All steps were performed according to manufacturer's protocol. Experiments were done in triplicate and reported as mean ± SD.

3.20 Activation of HIF-1α *in vitro*

PC3 cells were pre-plated in 6-well plates and allowed to settle overnight. For pharmacological HIF-1α activation, cells were treated the following day with 150 μM

cobalt chloride (CoCl2). After 24 hours, cells were lysed, and processed for RNA analyses as described above. For establishment of hypoxic cultures, cells were plated in 6-well plates and allowed to settle overnight in normoxia, and then either maintained in normoxia (control cells) or grown in Biospherix hypoxia chamber (Biospherix, Parish, NY) under 1% O2 (hypoxic cells). After 24 hours, all cells were processed for RNA isolation as previously described.

3.21 siRNA Approaches

PC3 or ARCaP(M) cells were pre-plated in 6-well plates or on Transwell filters and grown overnight. The following day, when the cells reached ~70% confluency, a unique 27mer siRNA duplex targeting HIF-1α transcripts (OriGene-SR302102) or Trilencer-27 Universal scrambled negative control (Origene-SR30004) were added using RNAiMAX transfection reagent (Thermofisher Scientific) at a final concentration of 20 μM (based on manufacturer's protocol). After 6 hours, cells were washed and moved into transwell co-culture with differentiated bone marrow adipocytes or grown alone. After 24 hours, cells were collected and processed for RNA analyses as described above. Two unique 27mer siRNA duplexes that efficiently knocked down HIF-1α transcripts were used.

Following the same protocol, we used a unique 27mer siRNA duplex targeting IL-1β transcripts (OriGene-XM_017003988) and the Trilencer-27 Universal scrambled negative control (OriGene-SR30004).

3.22 *In silico* analyses

The Oncomine database (OncomineTM v4.5: 729 datasets, 91,866 samples) was used for the analysis of primary (P) *vs*. metastatic (M) tumors by employing filters for selection of conditions and genes of interest (prostate cancer; metastasis *vs*. primary;

genes). Data were ordered by 'overexpression' and the threshold was adjusted to *P*-value < 1E-4; fold change, 2 and gene rank, top 10%. For each database, only genes that met the criteria for significance were reported.

3.23 Statistical analyses

Data were presented as means ± SD and statistically analyzed using unpaired student *T*-test. For three or more groups, one-way analysis of variance was used.

3.24 Lipidomics analyses

PC3 and ARCaP(M) cells were plated in 6 well plates or transwell inserts for co-culture with bone marrow adipocytes and allowed to grow for 48 hours in complete media. After 48 hours, the media was changed to serum-free DMEM overnight. The subsequent day, media was collected from tumor cells alone, adipocytes alone, and tumor/adipocyte co-cultures and delivered to the lipidomics core facility at Wayne State University. LC-MS methods available through our Lipidomics core (http://lipidomics.wayne.edu) cover the entire range of fatty acyl lipidome. Using Information Dependent Acquisition (IDA) mass spectrometry, we collected the mass spectra of differentially biosynthesized lipids for further structural characterization and identification. The data was analyzed by MarkerView (a multivariate analysis software to analyze the mass spectral data by ABSCIEX) to identify compounds that significantly ($p<0.05$) differ between samples. Additionally, cell lysates were given to the lipidomics core to measure intracellular sphingosine-1-phosphate levels. Cells were washed 3 times with PBS, trypsinized, collected, spun down, washed once with PBS, and then snap-frozen with liquid nitrogen and delivered to the core for analysis.

3.25 IL-1β Treatment and Inhibition

A-9-11-08 cells were differentiated in 6 well plates (See **Cell Lines** section above) into mature adipocytes. Adipocytes were then treated with 5ng/mL recombinant IL-1β (R&D Systems) for 48 hours and collected for RNA analyses as described above.

To assess IL-1β inhibition with a blocking antibody or receptor antagonist, PC3 cells were plates alone in a 6 well plate or in transwell co-culture in complete media in the presence or absence of an IL-1β Blocking Antibody (R&D Systems; 1 µg/ml) or IL-1R Antagonist (Sigma, 1 µg/ml). Adipocytes were also grown in alone conditioned in complete DMEM with or without the blocking antibody or receptor antagonist in order to assess changes in adipocytes in response to IL-1β inhibition.

3.26 EP Receptor Inhibitors

PC3 cells were grown in 6 well plates or in transwell inserts with bone marrow adipocytes as previously described in the presence or absence of the EP1-3 inhibitor, AH6809 (Cayman, 2 µM), the EP2 inhibitor, TG4-155 (Cayman, 1 µM), or the EP4 inhibitor GW628368X (Cayman, 1 µM) for 48 hours. Cells were collected for RNA and protein analysis as previously described.

3.27 PGE2 and 15-(S)-15-Methyl PGE2 Treatment

PC3 cells were plated in 6 well plates and allowed to settle overnight in complete DMEM. The following day, the media was removed and serum-free media was added after washing the cells with PBS. The subsequent day cells were treated with either PGE2 (Cayman, 1 µM) or 15-(S)-15-Methyl PGE2 (Cayman, 1 µM) or DMSO control in the presence or absence of the EP receptor inhibitors (described in **6.26**) for 24 hours. After

24 hours of treatment, cells were collected and processed for RNA or protein expression as described above.

3.28 SPHK1 Inhibition

PC3 cells were grown in 6 well plates or in transwell inserts overnight and allowed to settle. Adipocytes were pre-treated with SKI2 (Sigma, 5 µM) overnight. The following day, the tumor cells grown in the transwell inserts were moved into co-cultures with the adipocytes and fresh, complete media with the addition of DMSO or 5 µM SKI2 and allowed to grow for 48 hours. Cells were harvested and analyzed for RNA and protein expression as described above.

3.29 S1P Receptor Inhibition

PC3 cells were grown alone or in transwell inserts in co-culture with bone marrow adipocytes in complete medium containing DMSO, JTE-013 (Cayman, 1 µM), a selective S1PR4 antagonist, or VPC23019 (Cayman, 20 µM), a S1PR1-3 antagonist for 48 hours. After 48 hours, the cells were collected and processed for RNA and protein expression as previously detailed.

3.30 S1P Treatment/S1PR Agonists

S1P (Cayman) was resuspended in ethanol in 20 µL aliquots of 10 µM. Samples were placed in a SpeedVac for 2 hours or until liquid evaporated. Dry pellets of S1P were stored in -20°C and fresh S1P was used for each experiment. For experimental treatment with S1P, aliquots resuspended in 20 µL of 0.04% BSA in PBS and sonicated for 5 seconds to form micelles and added to PC3 cells plated in 6-well plates in serum-free media. For receptor agonist treatment, PC3 cells were grown in 6 well plates overnight and allowed to settle in complete medium. The following day, the media was removed

and, after a PBS wash, was replaced with serum-free media overnight. The following day, agonists for S1PR1 (SEW2857, Cayman, 1 µM), S1PR2 (CYM5520, Cayman, 3 µM), S1PR3 (CYM5541, Cayman, 1 µM), S1PR4 (CYM50308, Cayman, 1 µM), S1P, or DMSO were given to the cells for 24 hours. Also, to assess the optimal concentration of S1P treatment, PC3 and ARCaP(M) cells were grown in 6 well plates overnight and allowed to settle. The following day the complete medium was replaced with serum-free media after a PBS wash. The next day, cells were treated with 100 nM, 1 µM, or 10 µM S1P for 24 hours and collected for RNA analysis as previously discussed. To assess S1PR signaling, PC3 and ARCaP(M) cells were grown in 60 mm dishes overnight and allowed to settle. The following day the complete medium was replaced with serum-free media after a PBS wash. The next day, cells were treated with 10 µM S1P for 0, 10, 20, and 30 minutes and collected for protein analysis as previously described. Lastly, PC3 cells were grown in complete medium, complete medium with 10 µM S1P, Adipo CM, Adipo CM + 10 µM S1P, or CCM for 24 hours and collected for RNA analysis.

3.31 Stable SPHK1 Overexpression in Tumor Cells

PC3 cells were plated in 6-well plates. The following day, when the cells reached ~70% confluency, a plasmid encoding an overexpressing vector for SPHK1 (Generously provided by Dr. M. Lee, WSU) was added using Lipofectamin3000 transfection reagent (Thermofisher Scientific) at a final volume of 3.75 µL or 7.5 µL (based on manufacturer's protocol). After 48 hours, the cells were trypsinzed and re-plated into a 100 mm dish and the selecting agent, neomycin (Thermofisher Scientific), was added. Cell growth was monitored and single cell clones were selected using cloning rings and re-plated into 24-well plates with neomycin. Once they became confluent in the 24-well plates, the cells

were trypsinized and re-plated into a 6-well plate with neomycin and grown to confluency. At confluency, the cells were collected for RNA expression as previously described.

3.32 Colony Formation Assays

PC3 and ARCaP(M) cells were plated in 6 well-plates or transwell inserts with bone marrow adipocytes in complete media. After 48 hours grown in alone conditions or with adipocytes, cells were trypsinized and re-plated in 6-well plates at a density of 1,000 cells/well. Cells were monitored and after 5 days of growth, media was changed to replenish nutrients and allowed to grow for 5 more days. At the end of the 10 days, Crystal Violet™ stain (Sigma-Aldrich) was used to stain the cells and the colonies were imaged.

3.33 Mitochondrial Fractionation

PC3 cells were plated in 6-well plates or in tanswell inserts with bone marrow adipocytes in complete DMEM. After 48 hours cells were harvested for mitochondrial fractionation. To isolate mitochondria, cells were trypsinzed and washed with PBS and centrifugated at max speed for 5 minutes to produce a cell pellet. 90 µL of Tris-buffer (10 mM Tris. pH 7.6) was added to the cell pellet and sonicated to homogenize the mixture for 15 seconds. Immediately after homogenization, 20 µL of 1.5 M sucrose in SEKT buffer (250 mM sucrose, 40 mM KCl, 20 mM Tris-HCL, 2 mM EGTA, pH 7.6) was added to the mixture. Then, centrifuge at 600xg for 10 minutes at 4°C and remove the supernatant which is the mitochondrial fraction. Add 70 µL of SEKT buffer to the remaining pellet and collect as the “non-mitochondrial” fraction. Then, centrifuge supernatant mitochondrial fraction at 10,000Xg for 10 minutes at 4°C and discard the supernatant. We then resuspended the mitochondrial pellet in 500 µL of SEKT buffer and centrifuged for an

additional 10 minutes at 10,000Xg at 4°C. Finally, the mitochondrial pellet was resuspended in 70 µL of SEKT buffer and stored in -80°C.

3.34 Chemotherapy Treatment

PC3 cells were plated in 96-well plates at a density of 5,000 cells/well and grown overnight. The following day, Docetaxel (Generously provided by Dr. Elisabeth Heath) was given to the cells at various concentrations. The cells were treated for 48 hours in normoxia or hypoxia (1% O_2) and Calcein AM viability assays were performed (See Above).

CHAPTER 4: BONE MARROW ADIPOCYTES PROMOTE THE WARBURG PHENOTYPE IN METASTATIC PROSTATE CANCER CELLS THROUGH THE ACTIVATION OF HIF-1α

4.1 Introduction:

Altered metabolic phenotype and the ability to adapt and thrive in harsh microenvironments are features that distinguish cancer cells from normal cells [295, 296]. It is well-accepted that most tumor cells rely on accelerated glucose metabolism for support of anabolic processes such as lipid, protein and nucleic acid syntheses, and consequently for growth and survival [159, 297]. This phenomenon, known as the "Warburg Effect" is one of the hallmarks of cancer, and the glycolytic fueling of growth is thought to be the key feature behind the progression of most tumors [298]. However, it is becoming increasingly apparent that the metabolic phenotype of a cancer cell can vary depending on the tumor type and the stage of the disease. The possession of a distinct metabolic phenotype is especially evident in primary prostate cancers, which unlike other solid tumors, do not undergo the classical "glycolytic switch" [127, 299]. Instead, these tumors generally exhibit activation of β-oxidation pathways as the means of supporting tumor cell viability under conditions of energy stress [156, 300-302]. Primary prostate cancer cells have unique abilities to exploit fatty acid metabolic pathways to foster malignant transformation. The uptake of lipids from the microenvironment, aberrant *de novo* lipid synthesis and alterations in fatty acid catabolism and steroidogenesis pathways are now emerging as key mechanisms linking dysregulated lipid metabolism in the primary prostate tumor with subsequent progression and reduced survival [299, 303, 304]. In contrast to the primary disease, however, metabolic phenotype of metastatic prostate cancers is not well-understood. The acquisition of a glycolytic phenotype in

advanced stages of prostate cancer has been suggested by the reports of increased accumulation of fluorodeoxyglucose (FDG) [305] and the immunohistochemical evidence of expression of glycolytic markers and monocarboxylate transporters in advanced tumors. The mechanisms contributing to metabolic adaptation and progression of metastatic prostate tumors in bone has not, however, been previously explored and are not known.

Metastatic growth in bone is a complex process involving reciprocal interactions between the tumor cells and the host bone microenvironment. One of the most abundant, yet overlooked components of the metastatic marrow niche are the bone marrow adipocytes [24, 25, 27]. Adipocyte numbers in the marrow increase with age, obesity and metabolic disorders [26, 27, 52, 189, 306, 307], all of which are also risk factors for metastatic disease [308-312]. We and others have shown previously that marrow fat cells, as highly metabolically active cells, can serve as a source of lipids for cancer cells, and promote growth, invasion, and aggressiveness of metastatic tumors in bone [24, 150, 313]. Based on the growing evidence from cancers that grow in adipocyte-rich tissues, it is becoming apparent that one way adipocytes can affect tumor cell behavior is through modulation of cancer cell metabolism [314]. Although direct effects of adipocyte-supplied lipids on tumor metabolism have not been investigated in a context of metastatic prostate cancer, there have been studies in other cancers demonstrating that some lipids do have the ability to enhance the Warburg Effect in tumor cells [315-319]. Reciprocally, tumor cells have been shown to act as metabolic parasites by inducing lipolysis in adipocytes [155, 320]. This is important in the context of tumor metabolism regulation as the lipolysis-generated glycerol can feed into the glycolytic pathway [321-323] and the released fatty

acids can be oxidized through β-oxidation [324, 325]. As active and vital components of the bone tumor microenvironment, adipocytes are likely to be involved in the metabolic adaptation of tumors in the metastatic niche. However, the concept of metabolic coupling between marrow adipocytes and tumor cells leading to metabolic reprogramming in the tumor has not been explored before.

One of the principal mechanisms behind metabolic reprogramming is hypoxic stress and activation of hypoxia inducible factor (HIF) [326]. HIF-1 stimulates the conversion of glucose to pyruvate and lactate by upregulating key enzymes involved in glucose transport, glycolysis, and lactate extrusion and by decreasing conversion of pyruvate to acetyl-CoA through transactivation of pyruvate dehydrogenase kinase (PDK1) and subsequent inhibition of pyruvate dehydrogenase (PDH) [326]. Regulation of lactate dehydrogenase (LDHa) and PDK1 by HIF-1 keeps the pyruvate away from mitochondria, and thus depresses mitochondrial respiration [297]. Under normoxic conditions HIF-1 is rapidly degraded by the ubiquitin-proteasome pathway [327]. Decreased oxygen availability prevents HIF-1 hydroxylation leading to its stabilization and activation of downstream pathways [296]. In cancer cells, HIF-1 stabilization and activation can occur during normoxia via multiple oxygen-independent pathways [328]. This phenomenon, termed “pseudohypoxia” is thought to facilitate adaptation of tumor cells to harsh conditions and to promote survival and resistance to therapy [329-331]. Whether HIF-1-dependent signaling plays a role in metabolic reprogramming of prostate tumor cells in bone is not known.

The objective of this study was to elucidate the role of bone marrow adiposity in the modulation of tumor metabolism and adaptation within the bone microenvironment.

Using *in vivo* models of diet induced marrow adiposity in combination with *in vitro* models of paracrine, autocrine, and endocrine signaling between bone marrow adipocytes and prostate cancer cells, we show that bone marrow adipocytes are responsible for enhancing the glycolytic phenotype of metastatic prostate cancer cells. We demonstrate that bidirectional interaction between adipocytes and tumor cells leads to increased expression of glycolytic enzymes, increased lactate production, and decreased mitochondrial oxidative phosphorylation in tumor cells via necessary cancer cell-initiated paracrine crosstalk. We also reveal that the observed metabolic signature in tumor cells exposed to adipocytes mimics the expression patterns seen in patients with metastatic disease. These results offer potential mechanisms underlying metabolic adaptation of metastatic tumors in bone and implicate bone marrow adipocytes, a cell type so abundantly present in the skeleton especially in advanced age and obesity, as viable culprits in the progression of this currently incurable disease.

4.2 RESULTS

4.2.1 *In silico* analysis of glycolysis-associated genes in prostate cancer patients

The metabolic phenotype of primary prostate tumors has been well-described [156, 300-302]; however, its characteristics in relation to the glycolytic pathway at the metastatic site are not well-understood. Therefore, we first performed an Oncomine analysis of primary and metastatic prostate tumors and compared mRNA expression of genes that encode for enzymes/proteins known to be involved in different aspects of glucose metabolism and Warburg metabolism. Specifically, thirteen available Oncomine datasets were examined for the expression of genes covering a broad spectrum of metabolic responses and associated with glucose transport

[*glucose transporter 1* (*GLUT1*)], glycolysis [*hexokinase 2* (*HK2*) and *enolase 2* (*ENO2*)], Warburg metabolism [*pyruvate dehydrogenase kinase 1* (*PDK1*) and *lactate dehydrogenase* (*LDHa*)], and hypoxia [*carbonic anhydrase 9* (*CA9*) and *vascular endothelial growth factor* (*VEGF*)]. Our analyses revealed significant differences in the metabolic phenotype between primary and secondary sites observable in several prostate cancer datasets (Table 1). The Grasso Prostate dataset, which contains most metastatic samples, showed the most significant upward changes in the expression of *PDK1, ENO2, HK2, GLUT1,* and *LDHa* (Figure 1A), as well as many other genes associated with the glycolysis pathway (Table 1). Additional analyses of prostate datasets available through cbioportal.com revealed that copy number alterations/mutations/deletions in these genes are infrequent in prostate cancer (Figure 2), pointing to the mRNA overexpression as the main mechanism behind the acquisition of metabolic phenotype. In addition to glycolytic markers, HIF-1 target genes, *CA9* and *VEGF* were also significantly upregulated in metastatic tissue (Figure 1A, Figure 2, and Table 2). Since HIF-1 is well-known to regulate glycolysis [326], these results further underscored the apparent metabolic differences between primary and secondary prostate cancer and prompted us to investigate the contribution of the metastatic environment to the tumor metabolic phenotype in bone.

4.2.2 Bone marrow adiposity contributes to the *in vivo* glycolytic phenotype in prostate bone tumors

One important cell type credited with the ability to alter tumor metabolism is the adipocyte, whose effects on the phenotype of a tumor cell have been predominantly reported for colorectal and ovarian cancers [315, 343, 344]. Given the abundance of adipocytes in bone marrow, we hypothesized that they are likely to have similar

Table 1: Oncomine gene analysis of 13 prostate datasets comparing upregulated genes involved in glycolysis and hypoxic response in prostate cancer patients with primary and metastatic disease. *Enolase* (*ENO2*), *hexokinase 2* (*HK2*), and *glucose transporter 1* (*GLUT1*) were upregulated in 3/13, 4/9, and 5/13 available datasets, respectively. Warburg-associated enzymes *lactate dehydrogenase* (*LDHa*) and *pyruvate dehydrogenase kinase 1* (*PDK1)* were upregulated in metastatic sites of patients compared to primary prostate cancer in 8/13 and 8/12 of the datasets, respectively. Hypoxic responsive genes *carbonic anhydrase 9* (*CA9*) and *vascular endothelial growth factor alpha* (*VEGFA*) were upregulated in 6/12 and 5/13 metastatic tumors when compared to primary tumors. (n = number of samples; P = Primary site; M = Metastatic site).

Gene	Prostate Cancer	Fold Change	P Value	n
ENO2 (3/13)	Grasso [332]	2.22	4.33E-4	P: 59; M: 35
	LaTulippe [333]	1.45	0.034	P: 23; M: 9
	Varambally [334]	6.122	0.009	P: 7; M: 6
LDHa (8/13)	Grasso [332]	1.60	0.001	P: 59; M: 35
	LaTulippe [333]	1.78	0.008	P: 23; M: 9
	Holzbeierlein [335]	1.73	0.009	P: 40; M: 9
	Chandran [336]	1.68	0.003	P: 10; M: 21
	Ramaswamy 2 [337]	3.06	0.003	P: 10; M: 3
	Ramaswamy [338]	2.60	0.009	P: 10; M: 4
	Yu [339]	3.052	2.47E-7	P: 64; M: 24
	Varambally [334]	2.176	7.25E-4	P: 7; M: 6
PDK1 (8/12)	Grasso [332]	2.96	1.27E-7	P: 59; M: 35
	LaTulippe [333]	2.78	0.005	P: 23; M: 9
	Chandran [336][336][336][336][336][336][336][336][336][336]	1.44	0.002	P: 10; M: 21
		1.63	0.015	P: 62; M: 9
		1.114	0.005	P: 27; M: 5
	Lapointe [340]	1.370	9.99E-8	P: 64; M: 24
	Vanaja [341]	2.152	0.021	P: 23; M: 12
	Yu [339]	2.167	0.030	P: 7; M: 6
	Tamura [342]			
	Varambally [334]			
HK2 (4/9)	Grasso [332]	4.755	3.53E-9	P: 59; M: 35
	Varambally [334]	4.427	4.59E-4	P: 7; M: 6
	Ramaswamy 2 [337]	9.507	0.021	P: 10; M: 3
	Chandran [336]	3.291	3.40E-6	P: 10; M: 21
GLUT1 (5/13)	Varambally[334]	1.633	5.74E-5	P: 7; M: 6
	Ramaswamy[338]	2.049	0.017	P: 10; M: 4
	Ramaswamy 2[337]	2.132	0.019	P: 10; M: 3

	Yu [339]	1.088	4.63E-4	P: 64; M: 24
	Grasso [332]	2.048	1.51E-5	P: 59; M: 35
CA9 (6/12)	Ramaswamy [338]	10.458	0.010	P: 10; M: 4
	Ramaswamy 2 [337]	11.031	0.010	P: 10; M: 3
	Varambally [334]	3.131	0.003	P: 7; M: 6
	Yu [339]	1.109	0.003	P: 64; M: 24
	Grasso [332]	3.363	4.79E-6	P: 59; M: 35
	Chandran [336]	1.487	1.22E-4	P: 10; M: 21
VEGFA (5/13)	Grasso [332]	7.552	1.55E-14	P: 59; M: 35
	Varambally [334]	3.750	1.10E-5	P: 7; M: 6
	Tamura [342]	2.443	0.014	P: 23; M: 12
	Yu [339]	1.594	5.14E-5	P: 64; M: 24
	Chandran [336]	2.488	9.27E-4	P: 10; M: 21

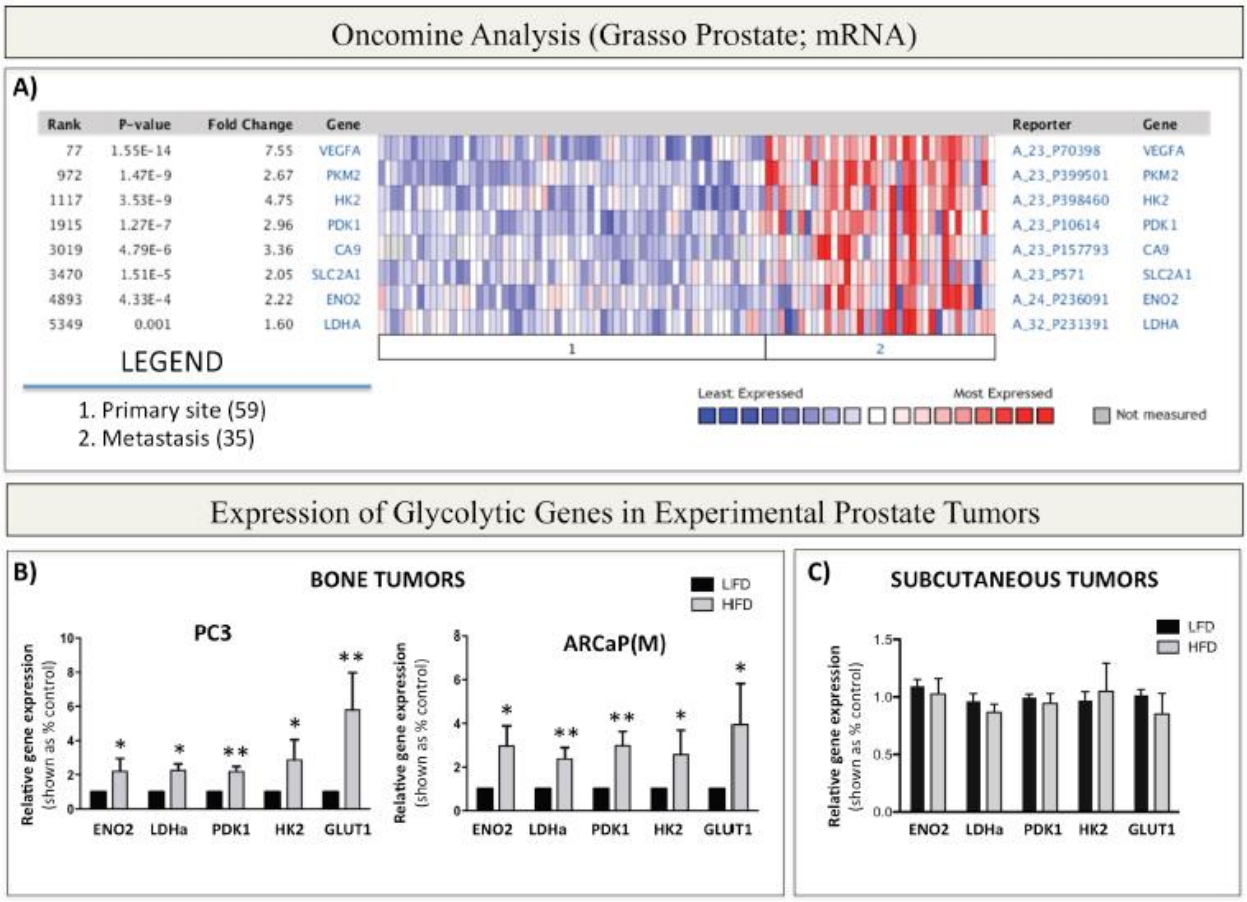

Figure 1: Warburg effect-associated genes are upregulated in patients with metastatic prostate cancer and in bone tumors from mice with enhanced marrow adiposity. A. Oncomine gene analysis comparing the expression of metabolic genes (*VEGF*, *PKM2*, *HK2*, *PDK1*, *CA9*, *SCL2A1* (*GLUT1*), *ENO2*, *LDHA*) in patient samples collected from metastatic or primary sites. Data were ordered by "overexpression" and the threshold was adjusted to *P*-value < 1E-4; fold change, 2 and gene rank, top 10%. **B.** Taqman RT-PCR (Life Technologies) analysis of expression of Warburg Effect-associated genes *ENO2*, *LHDa*, *PDK1*, *HK2*, and *GLUT1* in PC3 (left) and ARCaP(M) (right) bone tumors or **C.** subcutaneous tumors from LFD- and HFD-fed mice. Data were normalized to human *EPCAM* and represent a mean of a minimum of 3 mice/group ± SD. Values * $P < 0.05$; ** $P < 0.01$ are considered statistically significant.

Table 2: Oncomine gene analysis of Grasso Prostate database comparing upregulated genes involved in glycolysis in prostate cancer patients with primary and metastatic disease. Significantly upregulated genes are shown with a p value of $p < 0.05$.

GENE	FOLD CHANGE	P-VALUE
Triosephosphate Isomerase 1 (TPI1)	2.31	2.30E-13
Pyruvate Kinase M2 (PKM2)	2.67	1.47E-9
Hexokinase 2 (HK2)	4.75	3.53E-9
Enolase 3 (ENO3)	2.85	1.05E-8
Glucose-6-Phosphate Isomerase (GPI)	2.01	1.43E-8
Aldolase C (ALDOC)	2.69	2.41E-8
Dihydrolipoamide 5-Acetyltransferase (DLAT)	1.95	3.02E-8
Phosphoglycerate Kinase 1 (PGK1)	6.77	8.17E-8
Aldolase A (ALDOA)	1.85	1.16E-7
Transmembrane Protein 54 (TMEM54)	1.99	3.10E-7
Pyruvate Dehydrogenase Alpha 1 (PDHA1)	1.69	6.20E-7
Aldolase B (ALDOB)	12.85	1.99E-6
Oxoglutarate Dehydrogenase (OGDH)	1.55	1.84E-5
Phosphoglycerate Mutase 1 (PGAM1)	1.69	2.12E-5
Hexokinase Domain Containing 1 (HKDC1)	2.37	1.23E-4
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	1.57	2.63E-4
Enolase 2 (ENO2)	2.22	4.33E-4
Phosphoglycerate Mutase 2 (PGAM2)	1.79	4.99E-4
Lactate Dehydrogenase Alpha (LDHA)	1.60	0.001
Phosphofructokinase, Platelet (PFKP)	1.63	0.001
Hexokinase 3 (HK3)	1.45	0.004
IQ Motif Containing D (IQCD)	1.56	0.004
Pyruvate Kinase, Liver and RBC (PKLR)	1.29	0.013
Glucokinase (GCK)	1.28	0.020
Glyceraldehyde-3-PhosphateDehydrogenase, Spermatogenic (GAPDHS)	1.68	0.021
Lactate Dehydrogenase A-Like 6A (LDHAL6A)	1.64	0.023

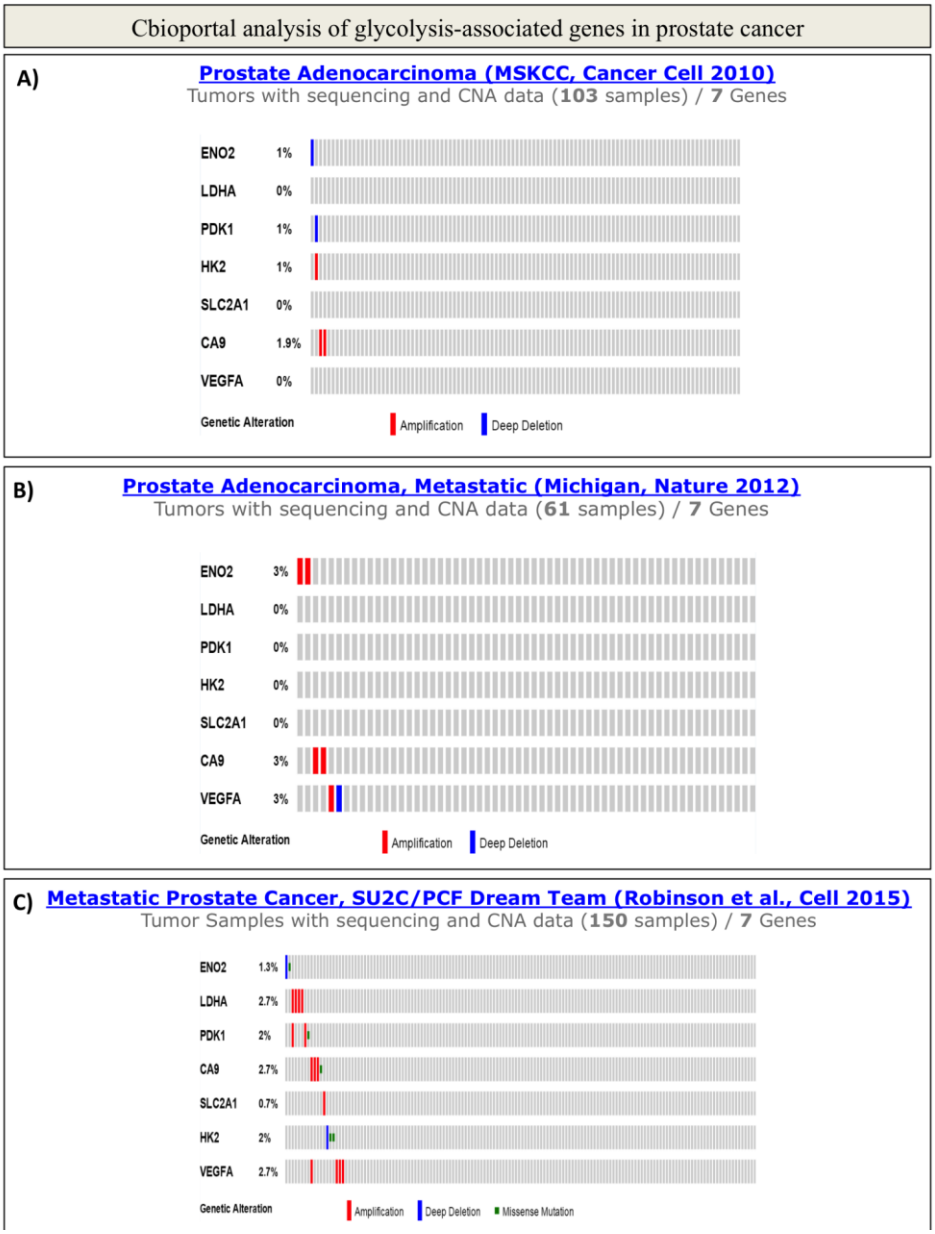

Figure 2: CBioPortal analysis of genetic copy number alterations (CNA) and mutations in glycolysis associated genes *enolase* (*ENO2*), *lactate dehydrogenase alpha* (*LDHa*), *pyruvate dehydrogenase kinase 1* (*PDK1*), *hexokinase 2* (*HK2*), and *glucose transporter 1* (*SLC2A1*), and hypoxic response genes *carbonic anhydrase 9* (*CA9*) and *vascular endothelial growth factor A* (*VEGFA*) across three different datasets of metastatic prostate cancer patients (126, 137-140). Vertical bars represent individual patients. If there are genetic alterations within the gene of interest, a red bar represents amplification of the gene, blue bars depict deep deletions within the gene, and green bars signify mutations within the gene. Gray bars represent unaffected patients.

metabolism-modulating effects on metastatic prostate cancer cells. To study effects of marrow fat cells on prostate tumor growth and progression in bone, we utilized a well-documented approach of inducing marrow adiposity with high fat diet (HFD) [27, 37, 292, 345]. We have shown previously that intratibial implantation of prostate cancer cells into this model results in accelerated tumor growth and extensive bone destruction, suggesting potential tumor-supportive effects of marrow adipocytes [294, 345]. To determine whether this adiposity-driven tumor progression in bone is associated with an altered metabolic phenotype, we analyzed mRNA expression of glycolysis-associated genes in intratibial PC3 and ARCaP(M) tumors from low fat diet (LFD) and HFD mice using human-specific Taqman probes. Our results revealed significantly increased transcript levels of *PDK1, ENO2, HK2, GLUT1,* and *LDHa* in tumors grown under conditions of HFD-induced marrow adiposity (Figure 1B), whereas the levels of mitochondrial enzymes *citrate synthase* (*CS*) and *isocitrate dehydrogenase 2* (*IDH2*), remained unaffected by diet-induced marrow adiposity (Figure 10). Notably, this enhanced glycolytic phenotype was also observed in bone tumors from mice, in which marrow adiposity was induced with HFD, but the animals were switched to LFD upon tumor implantation into the tibia (Figure 3). This approach allowed for tumor growth in adipocyte-rich marrow without systemic effects of HFD and revealed that expression of Warburg genes in tumor cells does not appear to be a direct effect of HFD. Furthermore, in contrast to bone tumors, expression of glycolysis-associated genes was not significantly altered in subcutaneous tumors from HFD mice in comparison to LFD mice (Figure 1C), despite the fact that HFD enhanced the growth and progression of these tumors, as we have demonstrated previously [345]. Collectively, these findings suggest

that Warburg metabolism might be especially important for prostate tumor progression in bone and implicate marrow adiposity as a potential regulator of metabolic adaptation in the skeleton.

4.2.3 Bone marrow adipocytes alter the metabolism of prostate cancer cells *in vitro*

To determine if the glycolytic phenotype observed in intratibial tumors *in vivo* is indeed a direct effect of bone marrow adipocytes and to specifically investigate the mechanisms behind this metabolic regulation, we utilized *in vitro* models of tumor cell-adipocyte interactions. First, using human-specific Taqman RT PCR probes, we examined the expression of glycolytic markers *ENO2*, *LDHa*, *PDK1, HK2*, and GLUT1 in PC3 and ARCaP(M) cells grown in direct contact co-culture with bone marrow adipocytes (Figure 4A). Transcript levels of nearly all investigated markers were significantly increased in tumor cells grown in co-culture as opposed to those cultured alone (Figure 4B). Next, to determine if this change in metabolic phenotype requires direct interaction with adipocytes, we employed a transwell system in which adipocytes were differentiated in the bottom chamber and tumor cells were then plated on top of the insert and cultured together for 48 hours. This allowed the two cell types to share the media without direct interaction (Figure 4C). Mirroring the findings from the direct co-culture, gene expression of *ENO2*, *LDHa*, *PDK1, HK2*, and *GLUT1* was significantly increased in both PCa cell lines co-cultured with marrow adipocytes (Figure 4D). Notably, PCa cells grown in transwell co-culture with bone marrow stromal cells that were not induced to differentiate into adipocytes had no effect on the expression of glycolysis-associated genes (Figure 5), suggesting that this observed metabolic switch in tumor cells is indeed adipocyte-driven. This enhancement of a glycolytic phenotype upon interaction with adipocytes was

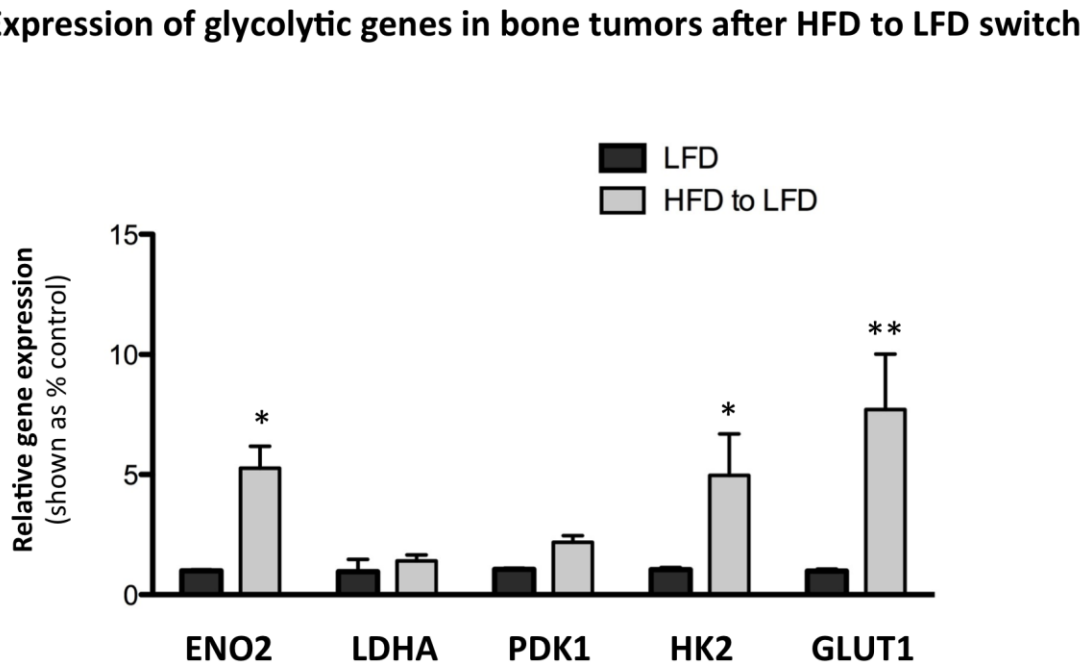

Figure 3: Comparative RT-PCR analysis of Warburg Effect-associated genes in bone tumors from mice on LFD vs. mice with HFD-induced marrow adiposity that were switched to LFD upon tumor implantation into the tibia (HFD to LFD). Data, showing persisting glycolytic phenotype in 'HFD to LFD' mice were normalized to human *EPCAM* and represent a mean of a minimum of 3 mice/group ± SD. Values * $p < 0.05$; ** $p < 0.01$ are considered statistically significant.

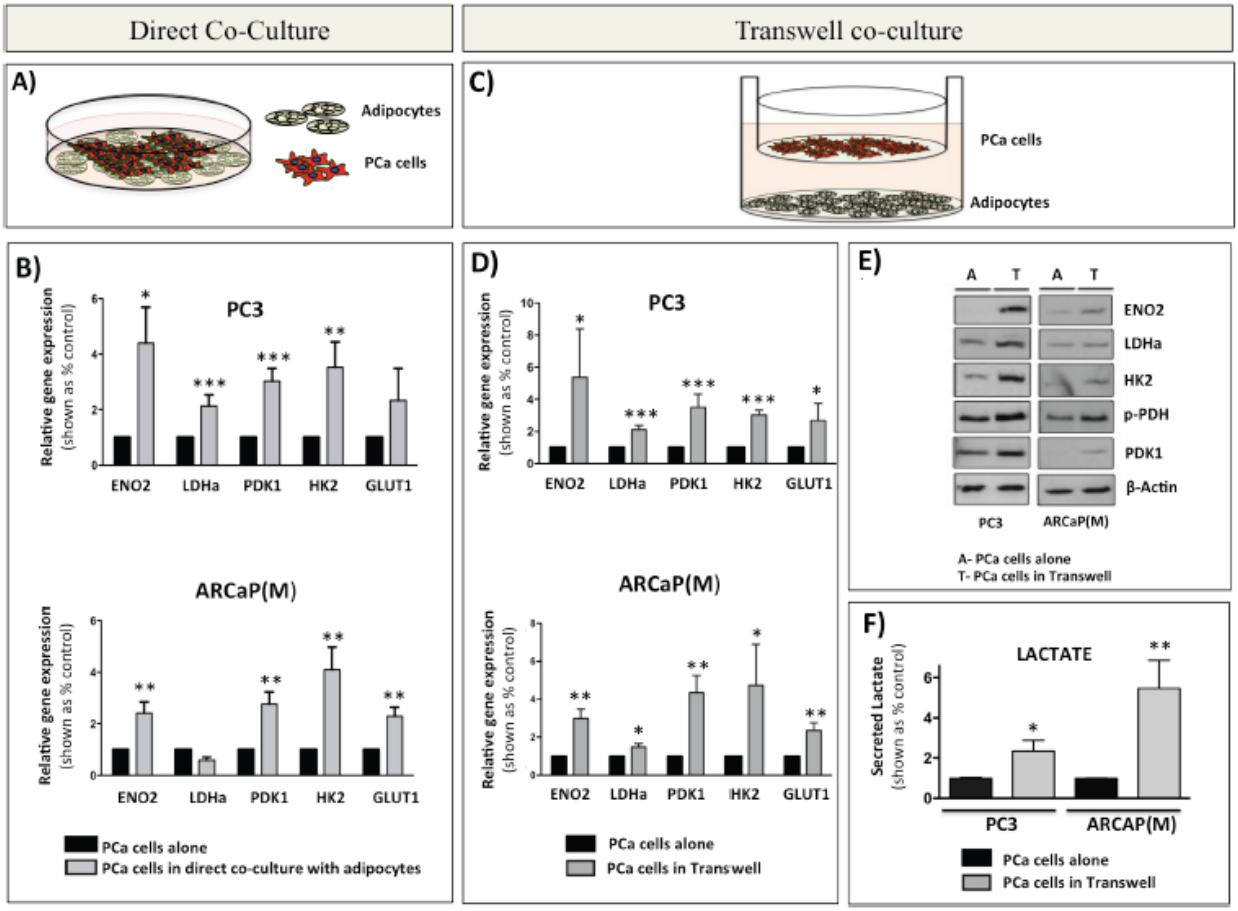

Figure 4: Bone marrow adipocytes enhance a glycolytic phenotype of prostate cancer cells in direct co-culture and in transwell co-culture in vitro. **A.** Schematic representation of a direct co-culture of tumor cells and bone marrow adipocytes. **B.** Taqman RT-PCR analysis of *ENO2, LDHa, PDK1, HK2*, and *GLUT1* expression in PC3 (Top) and ARCaP(M) (Bottom) cells cultured directly with bone marrow adipocytes. Data are normalized to *HPRT1* and shown relative to control. **C.** Schematic representation of transwell co-cultures of tumor cells and bone marrow adipocytes. **D.** Taqman RT-PCR of *ENO2, LDHa, PDK1, HK2*, and *GLUT1* expression in PC3 (top) and ARCaP(M) (bottom) in transwell co-culture. **E.** Western blot for ENO2, LDHa, HK2, phospho-PDH, and PDK1 in PC3 (left) and ARCaP(M) (right) exposed to bone marrow adipocytes in transwell co-culture. Beta-actin was used as loading control. **F.** Analysis of lactate secreted (Abcam) by PC3 (left) and ARCaP(M) (right) cells exposed to bone marrow adipocytes in transwell co-culture. Results represent a mean of at least 3 independent experiments ± SD. Values * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ are considered statistically significant.

confirmed by the marked increases in the protein expression of ENO2, LDHa, PDK1, and HK2 (Figure 4E). We also observed increased levels of phosphorylated pyruvate dehydrogenase (p-PDH) in cells grown in transwell co-culture, which indicates elevated PDK1 activity and a shift in glucose metabolism from pyruvate to lactate (Figure 4E). To test this functionally we performed lactate analyses of media conditioned by the tumor cells in the absence or presence of adipocytes as a conventional, well-accepted approach for measuring extracellular acidification and glycolytic shift [346, 347]. Our results revealed significant increases in lactate secretion by the tumor cells exposed to adipocytes (Figure 4F), while contribution of adipocytes to lactate secretion was not significant (data not shown). This provided further evidence of acquired Warburg phenotype in tumor cells exposed to adipocytes. We also observed an augmented expression of glycolytic genes in other prostate cell lines (i.e., DU145 and C4-2B) grown in transwell co-culture with fat cells (Figure 6), confirming the important contribution of marrow adipocytes to the metabolic phenotype of prostate tumors in bone.

The fact that both direct and transwell co-culture with adipocytes induced a glycolytic phenotype in tumor cells suggested that this process does not require physical interaction between the tumor cells and adipocytes. Therefore, we next examined whether the media conditioned by the marrow adipocytes alone (Adipo CM; Figure 7A) can bring on similar metabolic changes in tumor cells as observed in transwell co-culture. Interestingly, no changes in the mRNA expression of *ENO2*, *LDHa*, *PDK1, HK2,* and *GLUT1* were observed in either of the PCa cell lines in response to Adipo CM (Figure 7B). However, when the adipocytes were directly co-cultured with PC3 or ARCaP(M) cells for 24 hours prior to the collection of conditioned media, and then the co-

culture conditioned media was used to treat the tumor cells (Adipo CCM; Figure 7C), a significant upregulation of glycolysis-associated genes was observed in both tumor lines (Figure 7D). This altered gene expression was mirrored by the increased levels of glycolysis-associated proteins (Figure 7E), suggesting that paracrine signaling is required between the adipocytes and tumor cells for the subsequent metabolic shift towards the glycolytic phenotype. Interestingly, inactivation of proteins in the co-culture media by boiling did not reduce the expression of glycolytic genes (Figure 8), suggesting that the observed Warburg phenomenon might be driven by lipid rather than protein mediators.

4.2.4 Functional evidence of enhanced glycolytic phenotype in response to marrow adipocytes

The increased expression of glycolytic genes and proteins in tumor cells exposed to adipocytes, and significantly elevated levels of lactate in transwell co-cultures, clearly indicated augmented glycolytic activity in tumor cells interacting with adipocytes. An enhanced glycolytic phenotype in cells undergoing Warburg metabolism can often be associated with dysfunction in mitochondrial activity and consequently reduced rates of oxidative phosphorylation (OXPHOS) [348]. To determine if this is true in our system we performed an XF^e Seahorse analysis in tumor cells grown in the absence or presence of Adipo CCM and used oxygen consumption rate (OCR) as a tool to quantify OXPHOS. Significantly reduced OCR was detected in both PC3 and ARCaP(M) cells exposed to Adipo CCM for 12 hours. (Figure 9A). A decrease in OCR was also observable at 24 hours and did not appear to be due to a reduction in mitochondrial integrity, since there were no significant changes in JC-1 fluorescence, indicating that membrane matrices remained intact (Figure 9B). This was further supported by the lack of significant changes

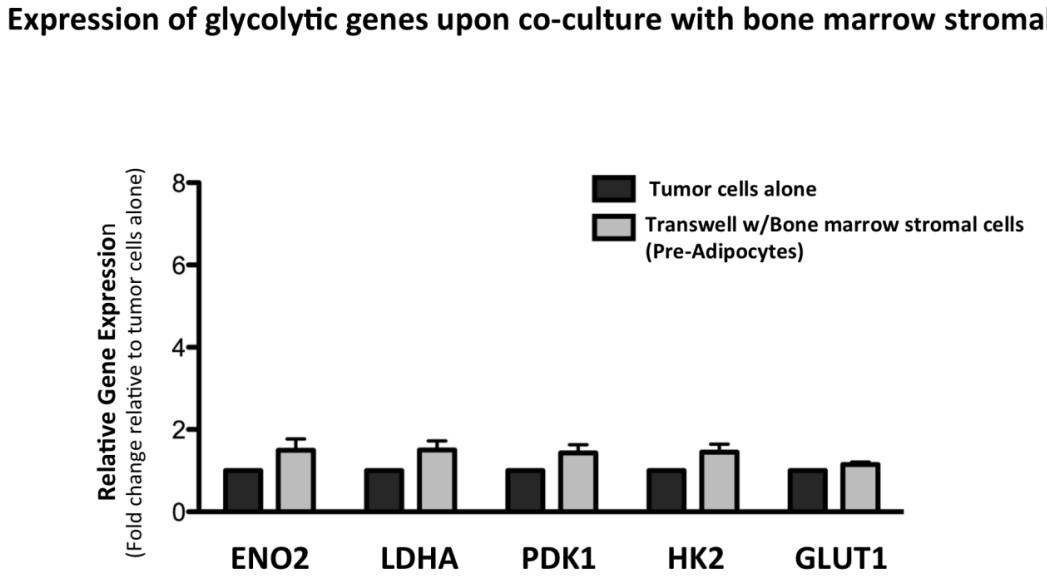

Figure 5. Bone marrow stromal cells (pre-adipocytes) do not induce Warburg Effect-associated genes. Taqman RT-PCR analysis of *ENO2, LDHa, PDK1, HK2*, and *GLUT1* in PC3 cells grown in transwell co-culture with pre-adipocytes. Data are normalized to *HPRT1* and shown as increase relative to control. Results represent a mean of at least 3 independent experiments ± SD.

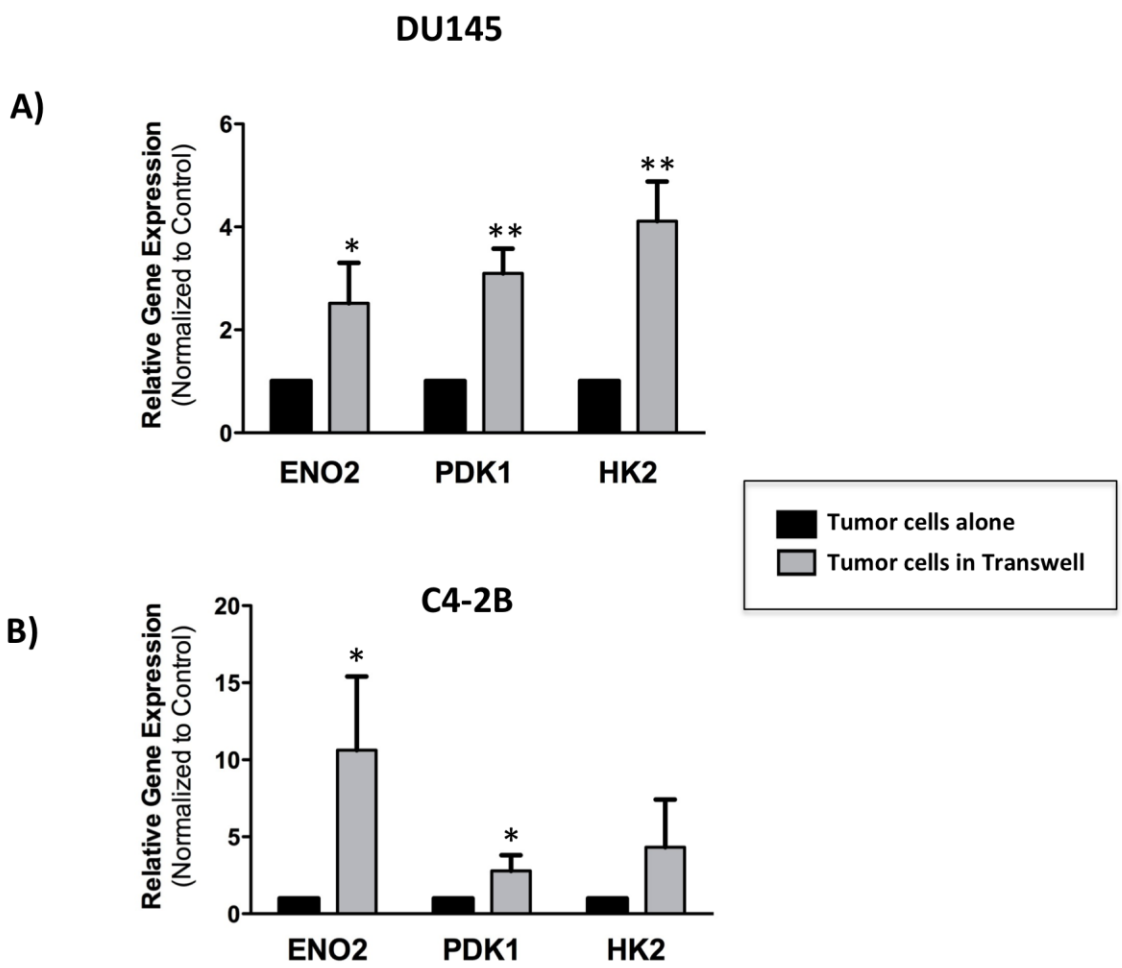

Figure 6. Bone marrow adipocytes enhance Warburg Effect in DU145 and C4-2B cells. Taqman RT-PCR analysis of *ENO2, PDK1,* and *HK2* in DU145 (top) and C4-2B (bottom) cells cultured alone or in transwell co-culture with bone marrow adipocytes. Data are normalized to *HPRT1* and shown as increase relative to control. Results represent a mean of at least 3 independent experiments ± SD. Values * $p < 0.05$; ** $p < 0.001$ are considered statistically significant.

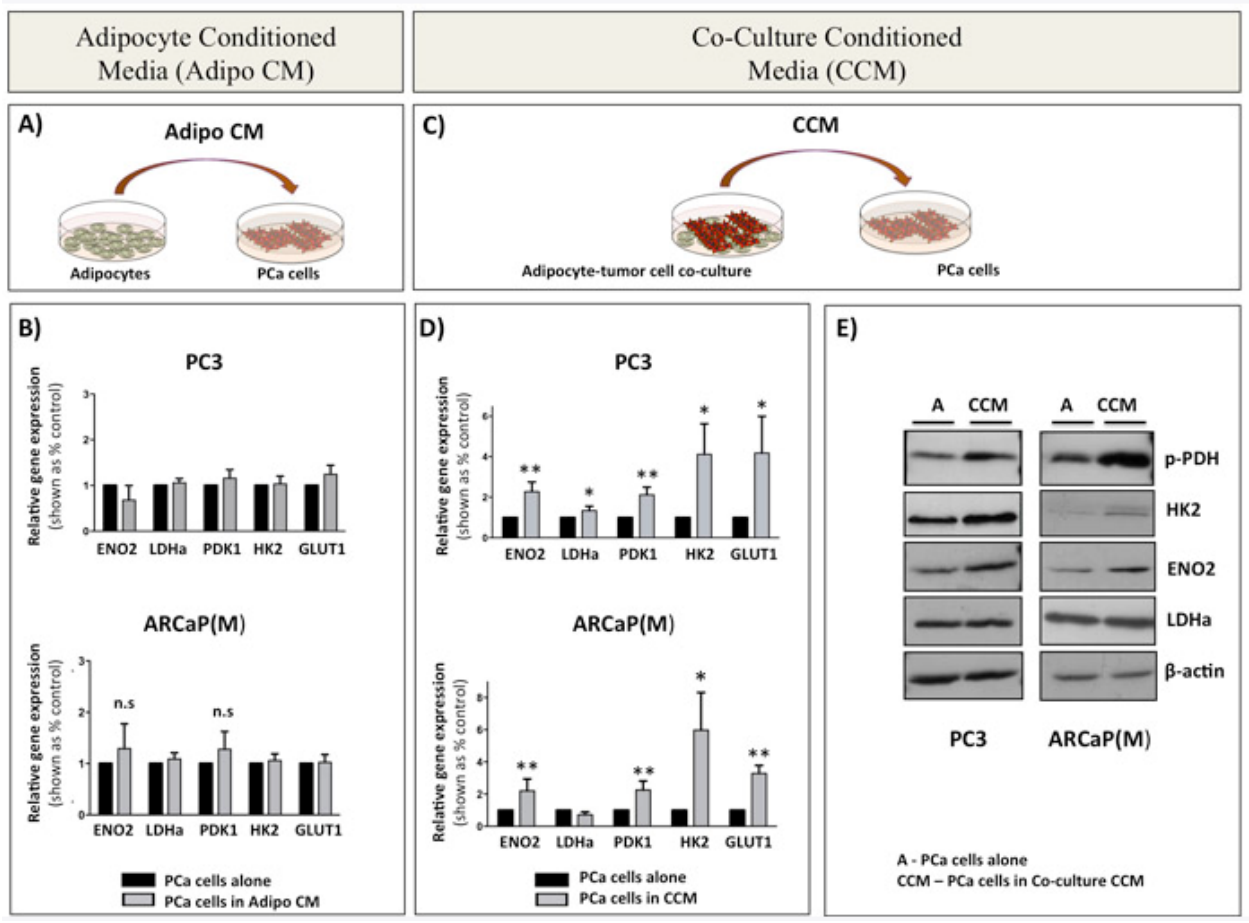

Figure 7: Paracrine signaling between PCa cells and bone marrow adipocytes is required for the induction of glycolytic gene and protein expression in PCa cells. A. Schematic representation of tumor cells treated with media conditioned by bone marrow adipocytes (Adipo CM). B. Taqman RT-PCR analysis of ENO2, LDHa, PDK1, HK2, and GLUT1 in PC3 (top) and ARCaP(M) (bottom) cells in the presence or absence of Adipo CM. Data are normalized to HPRT1 and shown as increase relative to control. C. Schematic representation of tumor cell- adipocyte co-culture system (CCM). D. Taqman RT PCR analysis of mRNA expression of ENO2, LDHa, PDK1, HK2, and GLUT1 in PC3 (top) and ARCaP(M) (bottom) cells in the presence of CCM. E. Western blot analysis of ENO2, LDHa, HK2, and phospho-PDH in PC3 (left) and ARCaP(M) (right) in the presence of CCM. Beta-actin was used as a loading control (bottom). Results represent a mean of at least 3 independent experiments ± SD. Values * $P < 0.05$; ** $P < 0.01$ are considered statistically significant.

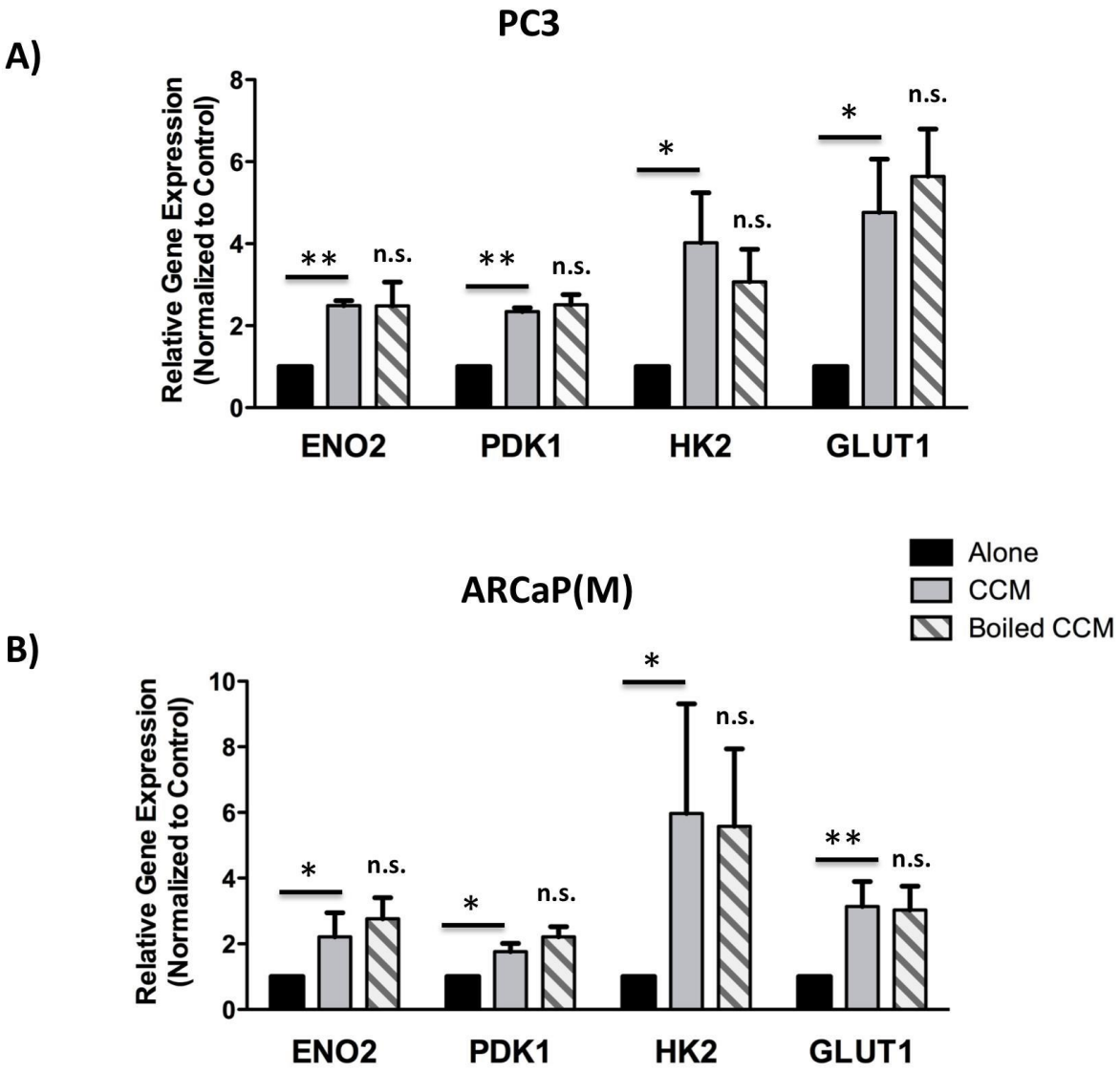

Figure 8. Warburg phenotype in prostate cancer cells is driven by adipocyte-derived lipids. Taqman RT-PCR analysis of *ENO2, PDK1, HK2*, and *GLUT1* in PC3 (top) and ARCaP(M) (bottom) in the presence or absence of co-culture conditioned media (CCM) and boiled co-culture conditioned media (boiled CCM). Data are normalized to *HPRT1* and shown as increase relative to control. Results represent a mean of at least 3 independent experiments ± SD. Values * $p < 0.05$; ** $p < 0.01$ are considered statistically significant.

in expression of two mitochondrial enzymes, *CS* and *IDH2* in PCa cells exposed to Adipo CCM (Figure 9C), a result mirroring their unaltered expression *in vivo* (Figure 10).

Since oxidative phosphorylation is much more efficient at producing copious amounts of ATP than glycolysis, a decrease in OXPHOS activity should expectedly result in a depletion of cellular ATP levels. Indeed, exposure of PC3 and ARCaP(M) cells to Adipo CCM for 12 hours led to a significant decrease in ATP concentration; however, further exposure to Adipo CCM for up to 24 hours led to a rescue of cellular ATP further suggesting an enhanced glycolytic phenotype upon Adipo CCM treatment (Figure 9D). This was further confirmed by additional recovery of ATP levels with 48-hour exposure to Adipo CCM (Supplementary Figure 8). It is important to note that the reduction in cellular ATP levels at 12 hours was not due to an enhanced proliferation induced by Adipo CCM as the Calcein AM assay showed no significant differences in cell numbers or viability of Adipo CCM-treated cells compared to cells grown under control conditions (Figure 9E). The uncompromised viability of Adipo CCM-treated tumor cells at 12 or 24 hours was further confirmed by the apoptosis assay showing no differences between control and Adipo CCM-treated cells (Figure 9F).

4.2.5 Prostate cancer cells stimulate lipolysis in adipocytes

Adipocytes store triglycerides and hydrolyze them into glycerol and free fatty acids *via* the process of lipolysis [320] and lipolysis-generated glycerol can feed into the glycolytic pathway [321-323]. Based on the above-presented evidence that bone marrow adipocytes induce metabolic changes in tumor cells, and the fact that these changes appear to require paracrine interaction between the two cell types, we sought to investigate whether this could be due to tumor cell-induced lipolysis in adipocytes, as

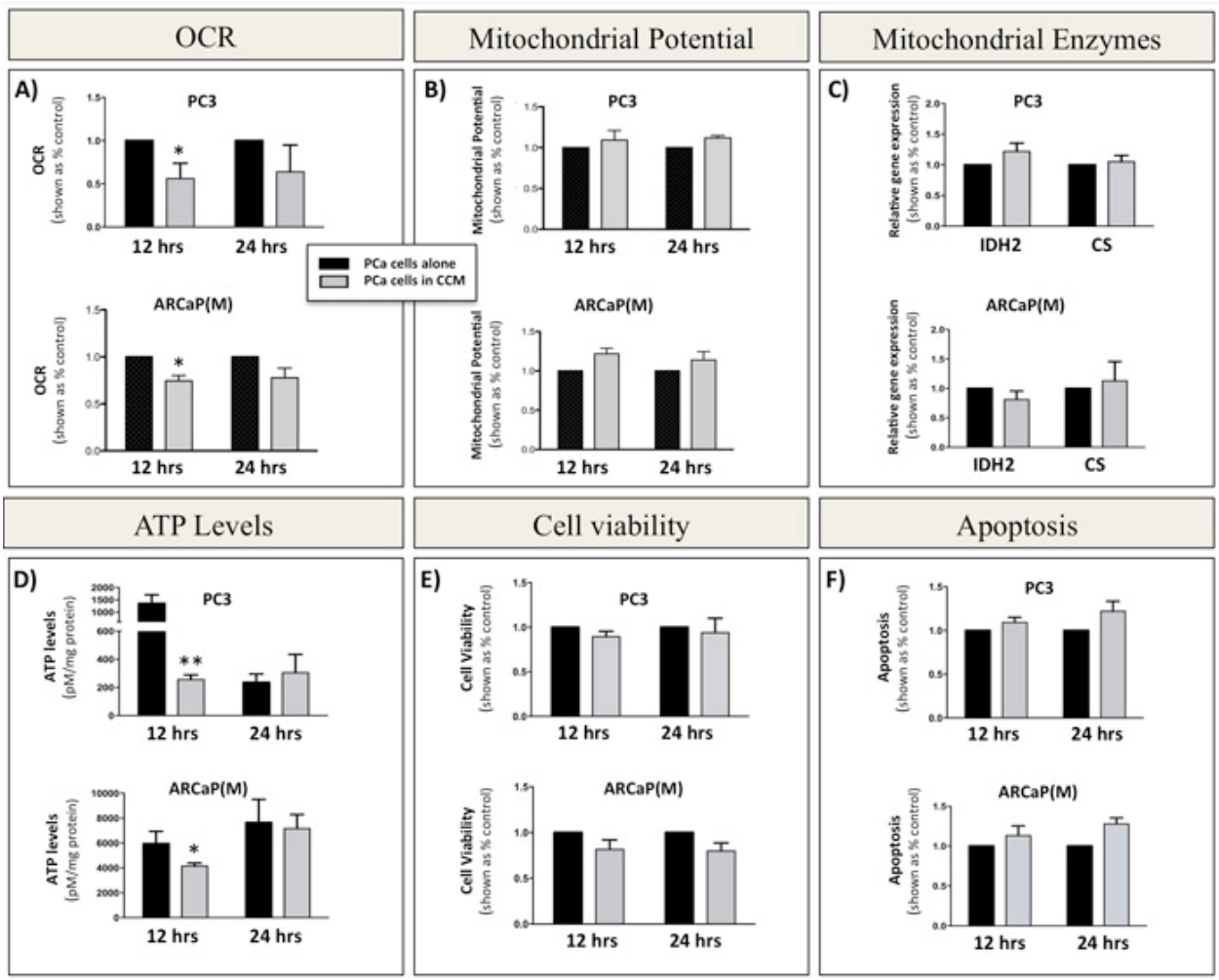

Figure 9: Decreased oxidative phosphorylation in prostate cancer cells exposed to bone marrow adipocyte-derived factors. A. Seahorse XFe24 analyzer (Seahorse Bioscience) analysis of the oxygen consumption rate (OCR) in PC3 (top) and ARCaP(M) (bottom) cells upon 12- and 24-hour incubation in the absence or presence of CCM. B. Mitochondrial membrane potential measured via JC-1 fluorescence. C. Taqman RT-PCR analysis of oxidative phosphorylation genes isocitrate dehydrogenase 2 (IDH2) and citrate synthase (CS) after 12 and 24 hours in culture in the absence or presence of CCM. Data are normalized to HPRT1 and shown relative to control. D. ATP levels in PC3 (top) and ARCaP(M) (bottom) cells cultured in the absence or presence of CCM. Significant decrease in ATP levels was observed after 12 hours. CCM exposure had no effect on viability as shown by Calcein AM assay E. and JC-1 apoptosis analyses F. Values *P < 0.05; **P < 0.01 are considered statistically significant.

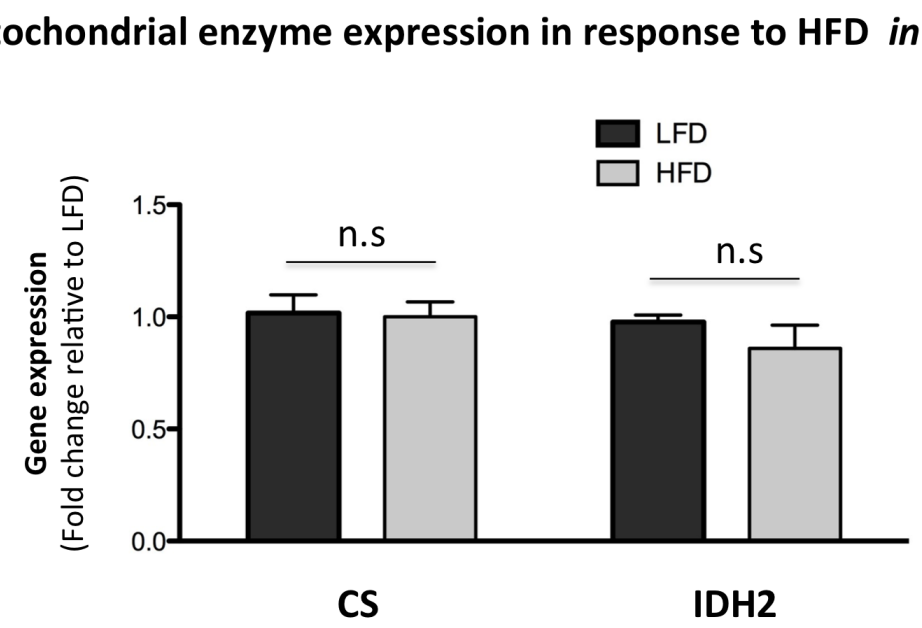

Figure 10: Expression of mitochondrial enzymes in intratibial prostate tumors is not affected by HFD-induced marrow adiposity. Taqman RT-PCR analysis of *citrate synthase* (*CS*) and *isocitrate dehydrogenase 2* (*IDH2*) in PC3 bone tumors. Data are normalized to *EPCAM* and shown as increase relative to control. Results represent a mean of at least 3 independent experiments ± SD.

previously demonstrated in ovarian cancer [343]. We have shown previously that exposure to adipocyte-derived factors leads to lipid accumulation by prostate tumor cells [345] and lipids have been shown to contribute to the Warburg phenotype in tumor cells [315-319]. Indeed, our analysis of media from marrow adipocytes grown alone or in a transwell co-culture with tumor cells revealed significant increases in free glycerol levels under co-culture conditions (Figure 11A). Similar changes were observed when adipocytes were treated with media conditioned by PC3 or ARCaP(M) cells (Figure 11B). The master regulator and the rate-limiting enzyme driving lipolysis in adipocytes is adipose triglyceride lipase (ATGL) [349, 350]. Our analysis of gene expression of *ATGL* in adipocytes co-cultured with tumor cells or exposed to tumor cell-conditioned media showed significant upregulation indicating an induction of a lipolytic phenotype (Figure 12) and suggesting that tumor cells may be secreting factors that induce lipolysis in fat cells. We next utilized a selective ATGL inhibitor Atglistatin, known to effectively block lipolysis in adipocytes [351] and recently shown to attenuate the growth of cancer cells [352]. A complete abrogation of free glycerol release by 10 μM Atglistatin (Figure 11C), mirrored by an accumulation of un-hydrolyzed triglycerides (Figure 11D) was observed for fat cells cultured in the absence of tumor cells. A very effective (~80%), but not absolute, reduction in free glycerol levels was also observed in transwell co-cultures (Figure 11C). This incomplete inhibition of lipolysis in adipocytes grown in transwell co-cultures was reflected in overall lower triglyceride levels as compared to adipocytes grown alone (Figure 11D). This suggests a dynamic, paracrine interaction between the two cell types that results in ongoing hydrolysis, uptake and release of lipids.

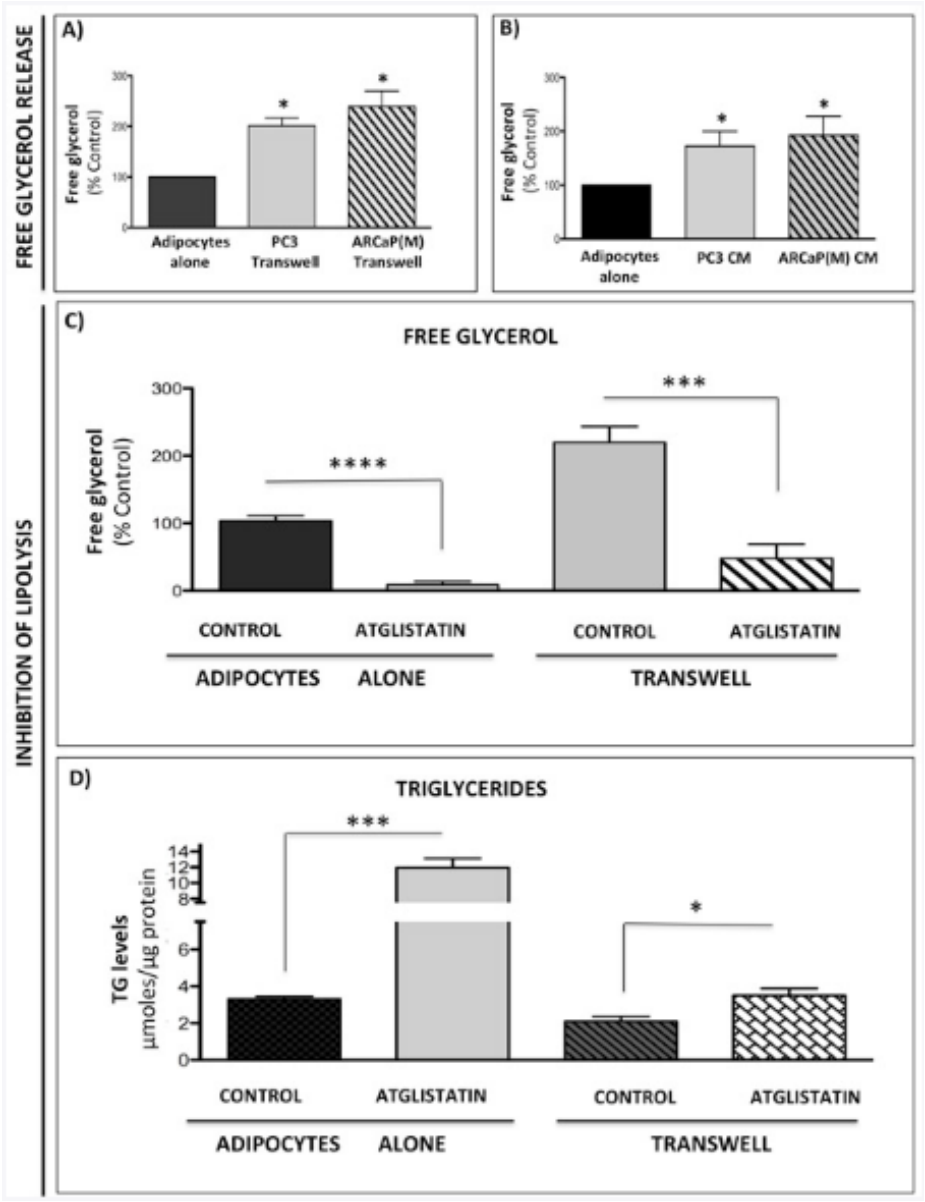

Figure 11: Prostate cancer cells stimulate lipolysis in bone marrow adipocytes. Free glycerol release from adipocytes in transwell co-culture with PC3 or ARCaP(M) cells A. or adipocytes treated with conditioned media from PC3 and ARCaP(M) cells B. C. Free glycerol release by adipocytes cultured alone or in transwell with tumor cells in the absence or presence of 10µM Atglistatin. Samples were measured in triplicate and are representative of three separate experiments (shown as percent control). Data are shown as the mean ± SD. D. Intracellular triglyceride (TG) levels were measured in adipocytes cultured alone or in transwell with PC3 cells. Measurements were done in triplicate and are representative of three separate experiments. Data are shown as µmoles TG/µg protein in cell lysates (Mean ± SD). Values *P < 0.05; *** P < 0.001, and ****P < 0.0001, are considered statistically significant.

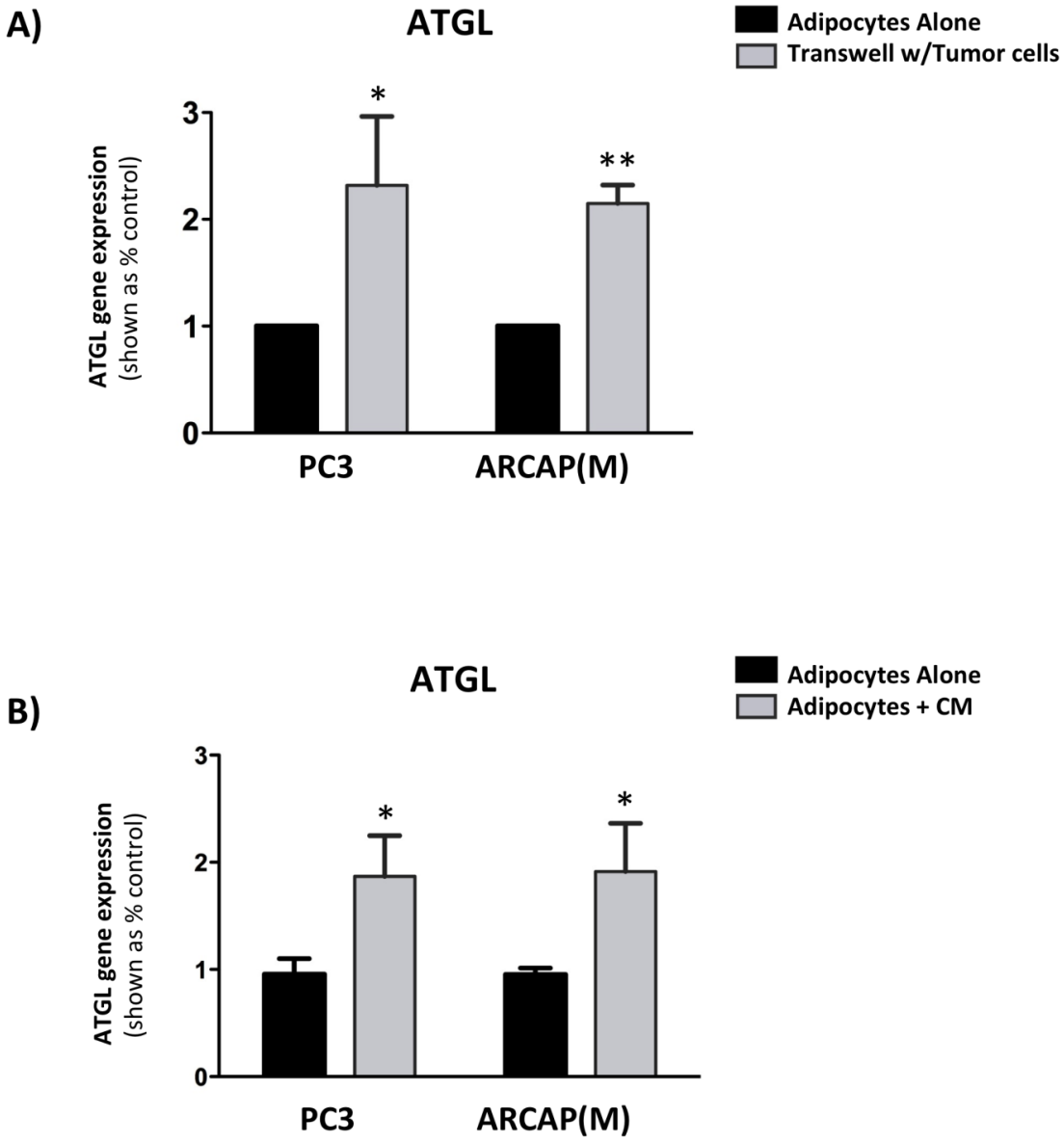

Figure 12. Taqman RT-PCR analysis of *adipose triglyceride lipase (ATGL)* expression in adipocytes grown in transwell co-culture with PC3 or ARCaP(M) cells (**A**) or treated with PC3 or ARCaP(M) conditioned media (**B**). RT-PCR data are normalized to mouse *adiponectin* and shown relative to control. Results represent a mean of at least 3 independent experiments ± SD. Values * $p < 0.05$; ** $p < 0.01$ are considered statistically significant.

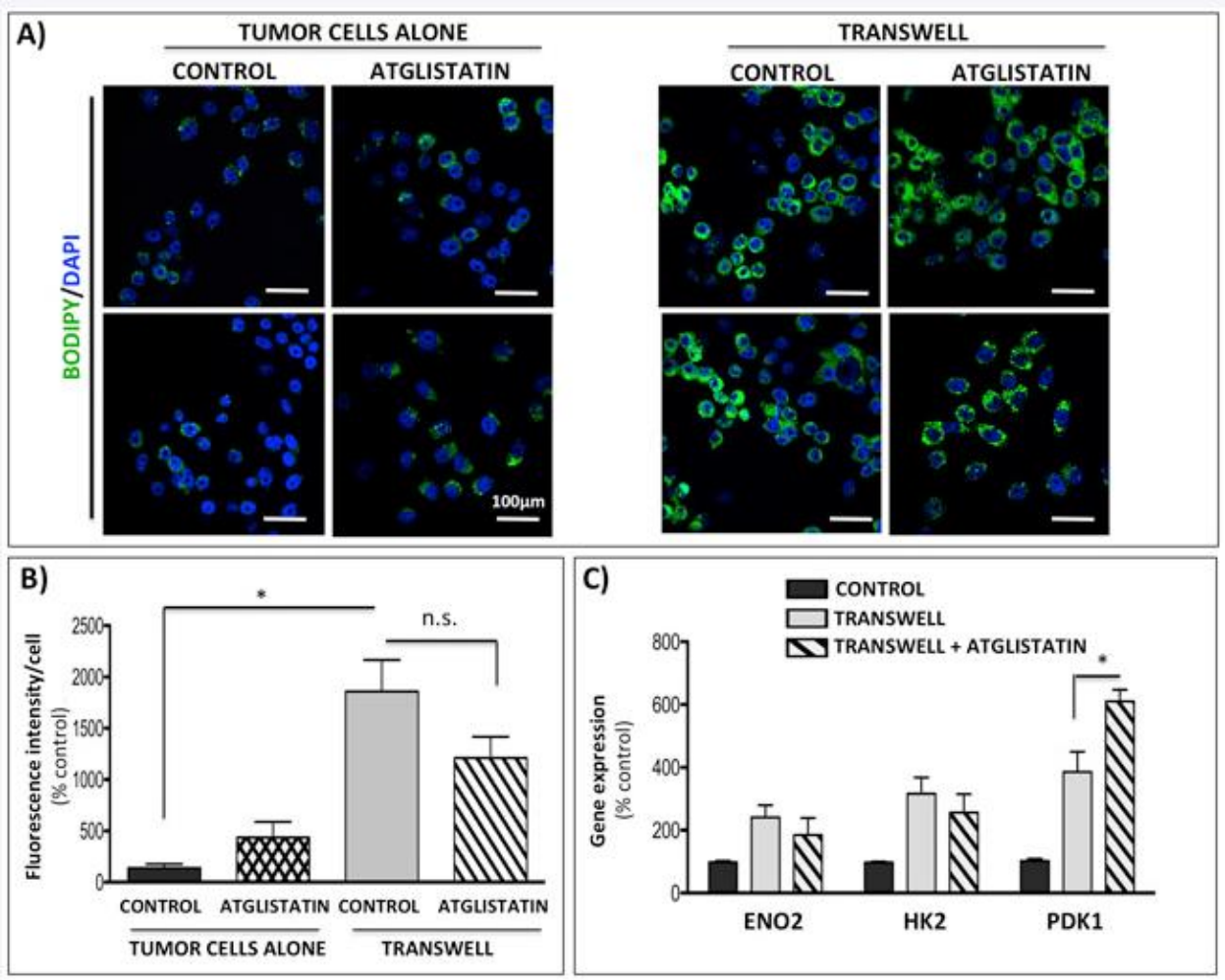

Figure 13: Atglistatin does not prevent lipid accumulation by the tumor cells and is not sufficient to prevent the induction of Warburg phenotype. A. Immunofluorescence imaging of lipid droplets (BODIPY 493/503 nm) in PC3 cells alone (left panels) or in transwell co-culture with bone marrow adipocytes (right panels) and in the presence or absence of 10µM Atglistatin. DAPI was used as a nuclear stain; 40x images. Bar 100µm. B. Fluorescent intensity of the BODIPY 493/503 staining was quantified using Volocity (Perkin Elmer, Waltham, MA) and shown relative to PC3 cells alone. Results represent a mean of at least 3 independent experiments ± SD. C. Taqman RT-PCR analysis of ENO2, HK2, and PDK1 in PC3 cells in transwell co-culture in the absence or presence of 10µM Atglistatin. Data are normalized to HPRT1 and shown relative to control. Values *P < 0.05 are considered statistically significant.

Since lipolysis-generated glycerol can incorporate into the glycolytic pathway, we went on to determine whether inhibition of adipocyte ATGL with Atglistatin could reverse the Warburg phenotype in tumor cells. Our previous studies have shown that prostate tumor cells are capable of taking up adipocyte-supplied lipids [345]. To determine if this uptake can be reduced by inhibitors of lipolysis, we treated the tumor cells grown alone or in transwell co-culture with Atglistatin and performed BODIPY staining (Figure 13A). In agreement with our previous results [345], significantly increased lipid labeling was observed in tumor cells exposed to adipocytes in transwell co-culture (Figure 13A, right panels). Interestingly, treatment with Atglistatin had little effect on adipocyte-induced lipid uptake, as demonstrated by sustained BODIPY fluorescence (Figure 13A and 13B). This was further confirmed by significantly increased gene expression of lipid droplet marker *perilipin 2* and lipid transporter *CD36* in tumor cells exposed to adipocytes both in the absence and presence of Atglistatin (Figure 14A).

Consistent with limited effects of adipocyte ATGL inhibition on lipid uptake by the tumor cells, only modest reduction in mRNA levels of *ENO2* and *HK2* was revealed indicating limited impact on glycolytic phenotype in the tumor cells. Even more surprisingly, the presence of Atglistatin in transwell co-cultures led to a small, but significant increase in the expression of *PDK1* (Figure 13C). This upregulation at the gene level corresponded to sustained higher levels of p-PDH at the protein level, suggestive of enhanced PDK1 activity in tumor cells interacting with adipocytes (Figure 15). Interestingly, in addition to the effects on PDK1, Atglistatin treatment increased the expression of lipid transporter fatty acid binding protein 4 (FABP4) in tumor cells grown in transwell with adipocytes (Figure 15). We have shown previously that FABP4 levels

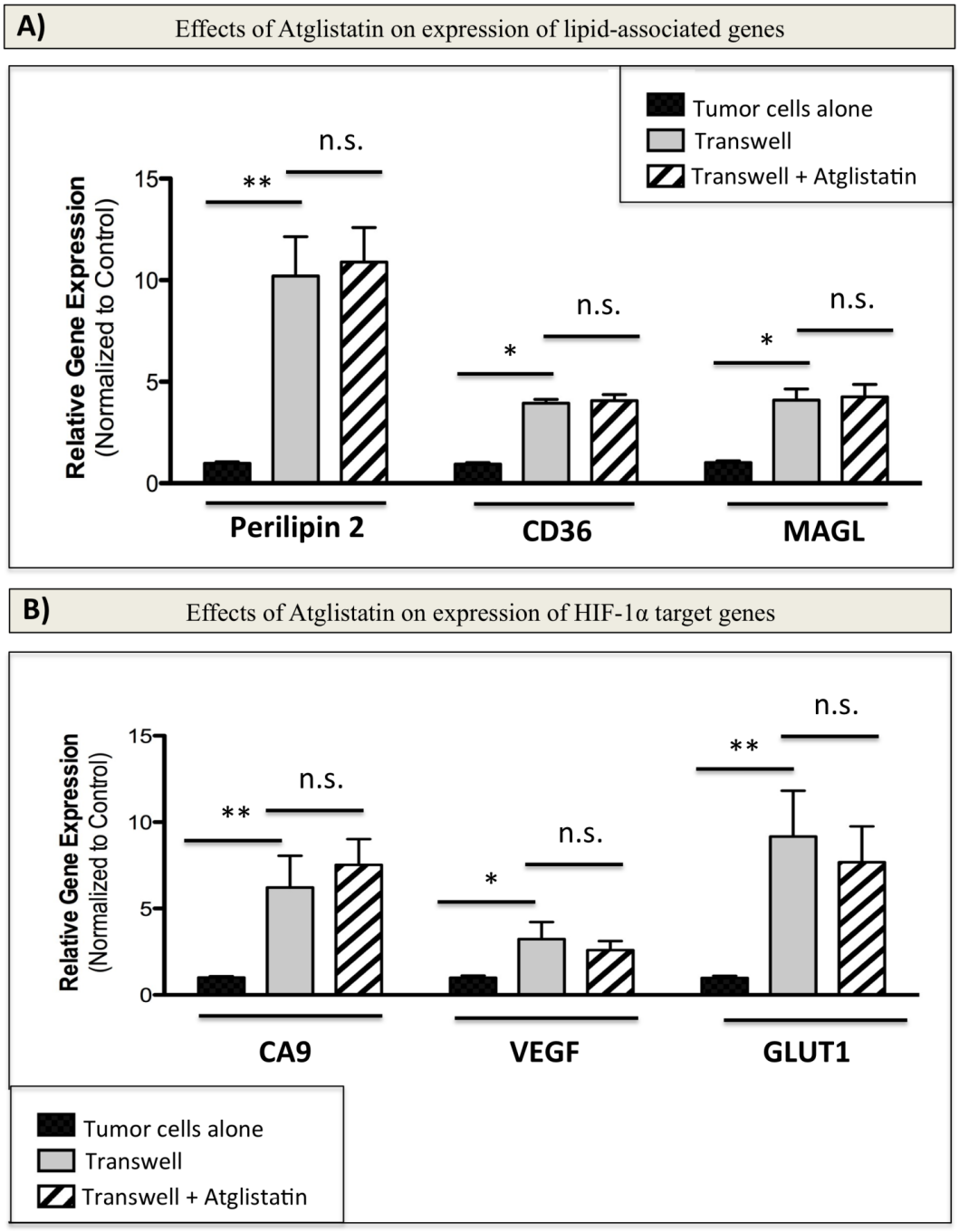

Figure 14. Inhibition of adipocyte lipolysis does not reduce the expression of lipid transporters and hypoxia-associated genes in tumor cells. Taqman RT-PCR analysis of *Perilipin 2, CD36,* and *MAGL*, expression (**A**) and *CA9, VEGF*, and *GLUT1* expression (**B**) in PC3 cells alone or in transwell co-culture with bone marrow adipocytes. Cells were cultured in the presence or absence of 10 μM Atglistatin. Data are normalized to *HPRT1* and shown as increase relative to control. Values * $p < 0.05$; ** $p < 0.01$ are considered statistically significant.

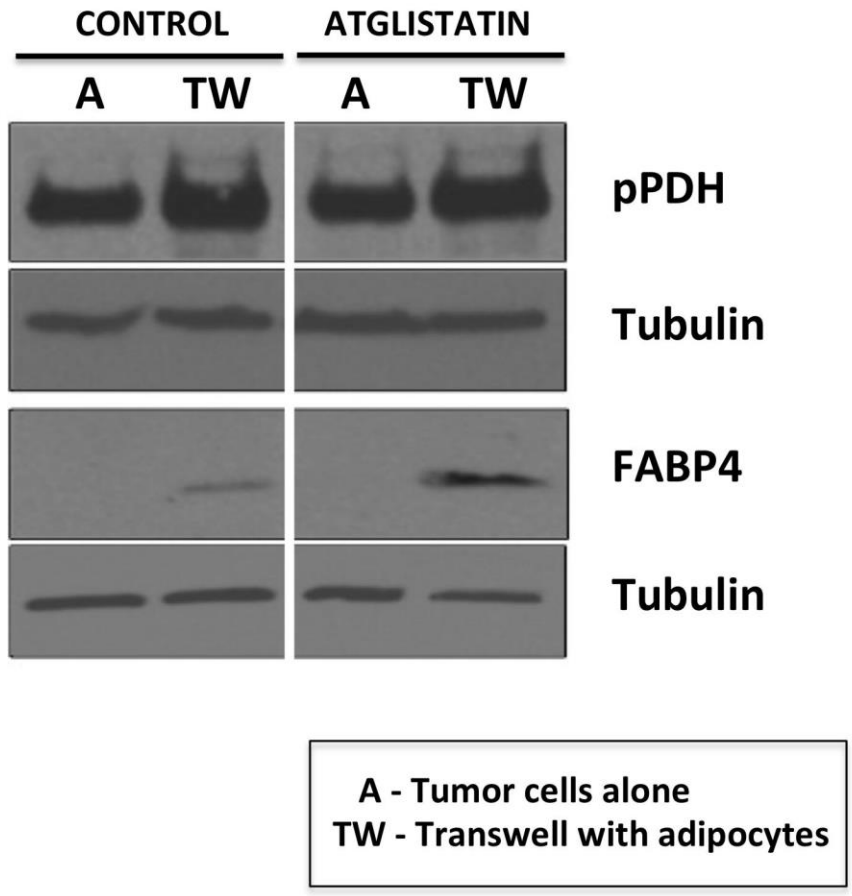

Figure 15. Inhibition of lipolysis with Atglistatin enhances phosphorylation of PDH and increases expression of lipid transporter FABP4. Western blot analysis of phosphorylated PDH (top) and FABP4 (middle) in PC3 cells alone or in transwell co-culture in the absence or presence of 10 μM Atglistatin. Tubulin was used as a loading control (bottom).

are significantly induced in tumor cells exposed to adipocyte-derived factors [345]. Given the known role of FABP4 in lipid transport and hydrolysis [353], its apparent induction by the inhibitors of lipolysis suggests a potential feedback response by tumor cells overwhelmed with adipocyte-supplied lipids. It is noteworthy that the expression of tumor-derived *monoacyl glycerol lipase (MAGL)*, a lipase previously implicated in prostate cancer progression [354, 355], was also induced in response to adipocytes and persisted upon inhibition of adipocyte ATGL with Atglistatin, suggesting an additional possible compensatory mechanism in tumor cells that might be contributing to the adipocyte-driven metabolic phenotype (Figure 14A).

4.2.6 Marrow adipocytes induce HIF-1α signaling in prostate tumor cells

One of the major mechanisms behind metabolic re-programming towards a glycolytic phenotype is the activation of HIF-1α signaling [326]. Hypoxia has been linked with aggressiveness and metastatic progression in prostate cancer [356] and we have shown previously that *HIF-1α* gene expression is increased in prostate bone tumors from HFD mice as compared to LFD mice [345]. To determine whether bone marrow adiposity might be contributing to HIF-1α activation in the bone microenvironment, we analyzed the mRNA levels of HIF-1α target genes, *CA9* and *VEGF,* in intratibial PC3 tumors from LFD- or HFD-fed mice. Both target genes were significantly upregulated in bone tumors from mice on HFD as compared to LFD mice (Figure 16A), a result that complemented a significant increase in the levels of *GLUT1* (Figure 1B), another direct target of HIF-1α activity [202]. Notably, no difference in *CA9* and *VEGF* expression between LFD and HFD conditions was observed in subcutaneous tumors (Figure 16B), in line with our earlier finding demonstrating that an increase in *GLUT1* and augmented levels of other

glycolysis-associated genes are observable only in metastatic tumors but not in primary tumors in PCa patients (Figure 1A), and in bone tumors but not in subcutaneous tumors in mice (Figure 1B and 1C). We next performed an immunohistochemical analysis of CA9 expression (Figure 16C-16G). Our results showed weak, diffuse CA9 staining in bone tumors from LFD mice (Figure 7C, 7D), whereas an abundance of CA9 protein with its typical membrane localization was detected in tumors from HFD mice (Figure 16E, 16F), a result further confirming a glycolytic phenotype of bone tumors under conditions of high marrow adiposity.

To determine whether bone marrow adipocytes are in fact capable of activating HIF-1α in PCa cells, we examined the expression of *CA9* and *VEGF* in PC3 cells under transwell conditions. Indeed, expression of both genes was highly increased in cells grown in transwell co-culture with adipocytes (Figure 17A, 17B). In addition, immunofluorescence analysis of CA9 protein revealed a significant increase in expression and typical membrane localization of CA9 in PC3 cells exposed to adipocytes (Figure 17C-17G). Notably, adipocyte treatment with Atglistatin had no effect on *CA9, VEGF* or *GLUT1* expression in PC3 cells (Figure 14B), suggesting that inhibition of adipocyte lipolysis is not sufficient to reverse adipocyte-driven HIF-1α activation in tumor cells and offers a potential explanation for the persisting glycolytic phenotype.

4.2.7 HIF-1α knockdown inhibits acquisition of a glycolytic phenotype in PCa cells exposed to adipocytes

Activation and stabilization of HIF-1α is known to be a major event in metabolic transformation to a glycolytic phenotype [326]. Indeed, culture of PC3 cells under hypoxic (1% oxygen) conditions or treatment with HIF-1α inducer $CoCl_2$ (Figure 18A and 18B)

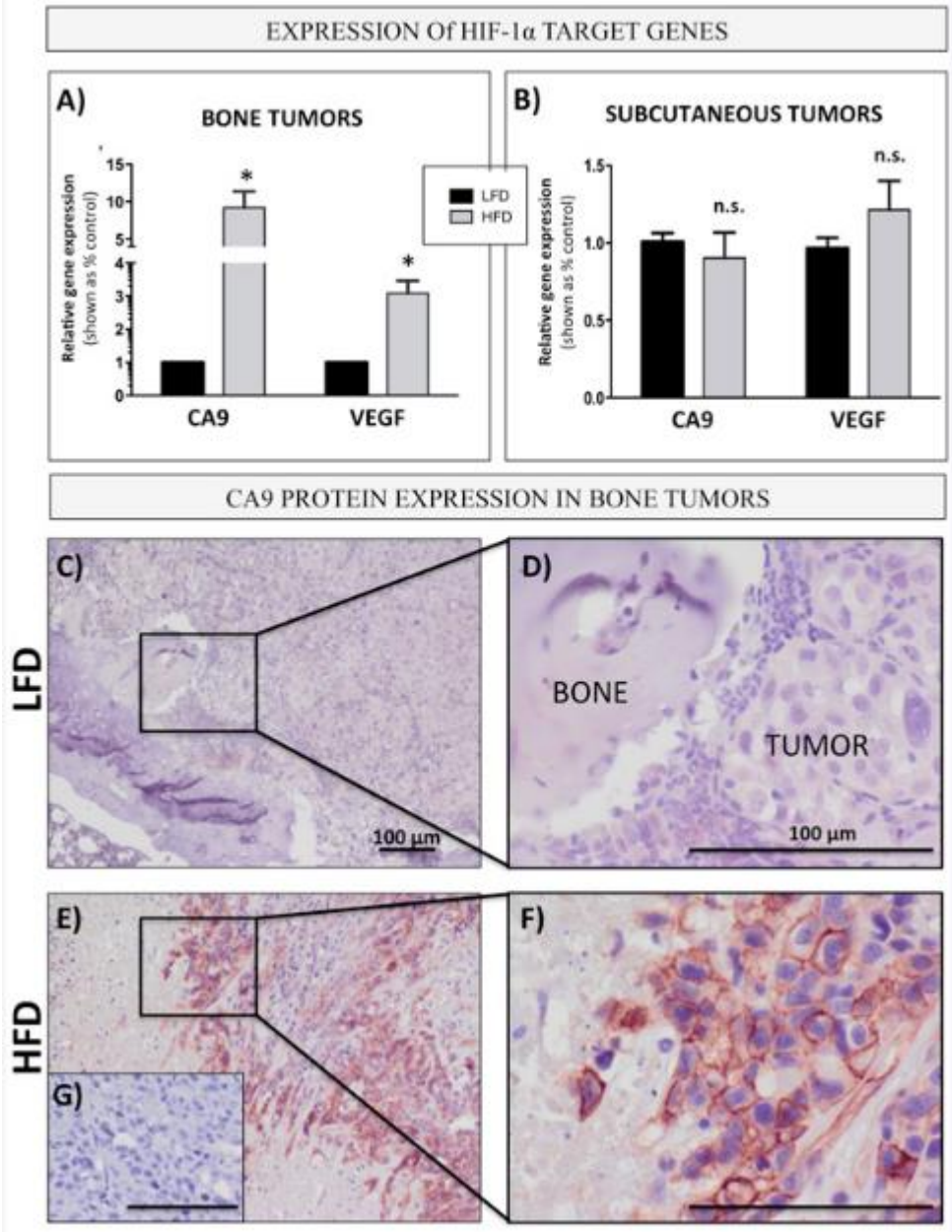

Figure 16: Bone marrow adiposity enhances HIF-1α signaling in PCa cells in vivo prostate bone tumors. Taqman RT-PCR analysis of CA9 and VEGF in PC3 cells grown intratibially A. and subcutaneously B. in LFD and HFD fed mice. Data are normalized to EPCAM and shown relative to LFD tumors. C.-G. Immunohistochemical (NovaRED) staining for CA9 protein in prostate bone tumors from mice on LFD C. and HFD E., 10x images D.,F. High magnification (40x) images depicting membrane CA9 localization in HFD F. but not LFD D. tumors. G. No primary antibody control. Bar, 100µm.

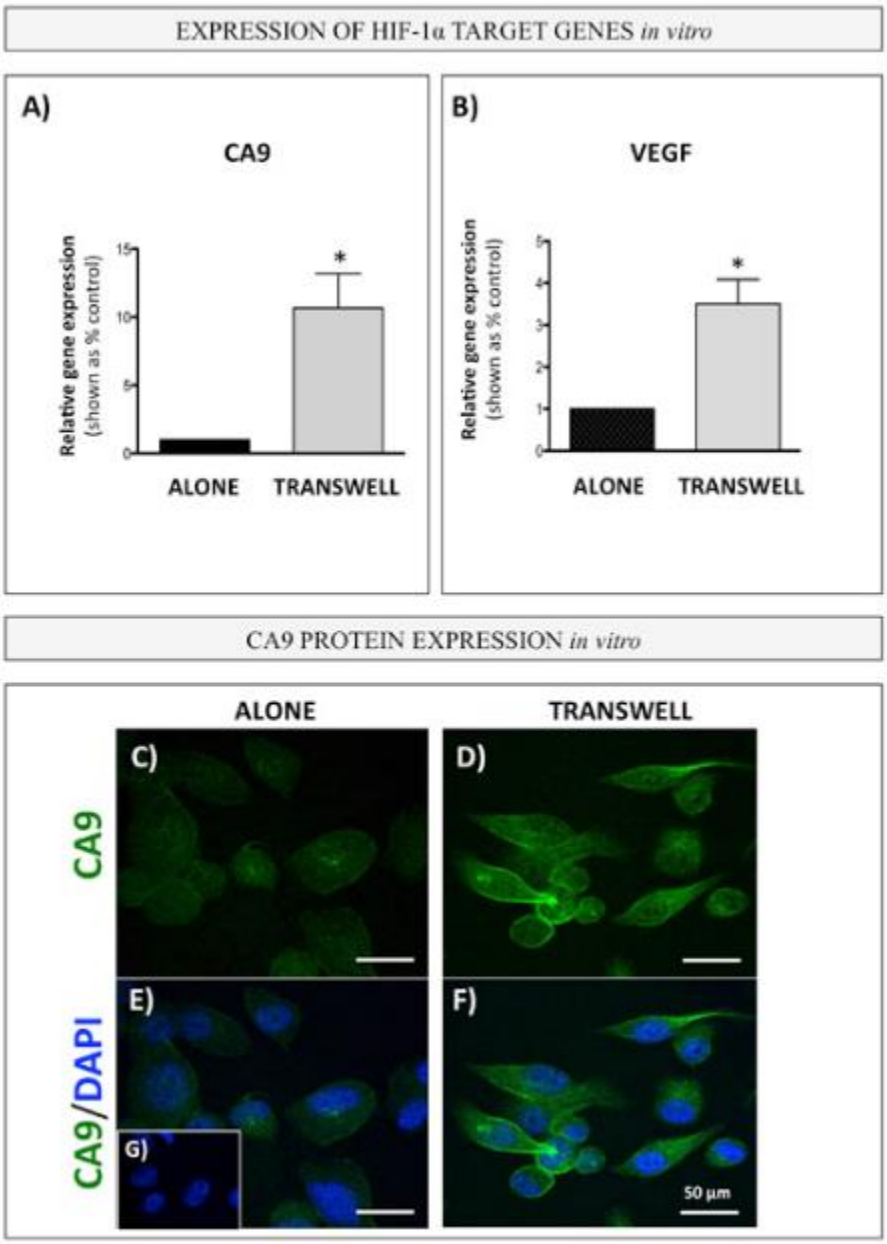

Figure 17: Bone marrow adipocytes activate HIF-1α signaling in PCa cells in vitro. Carbonic anhydrase 9 (CA9; A. and VEGF B. gene expression (Taqman RT PCR) in PC3 cells in transwell co-culture with bone marrow adipocytes. Data are representative of at least 3 separate experiments, normalized to HPRT1 and shown relative to tumor cells cultured alone. Values *P < 0.05 are considered statistically significant. C.-G. Immunofluorescence staining of CA9 (green fluorescence) in PC3 cells grown alone (left) or in transwell co-culture with bone marrow adipocytes (right); DAPI (blue) was used as nuclear dye; 63x images. Bar, 50µm. G. No primary antibody control was used as a control.

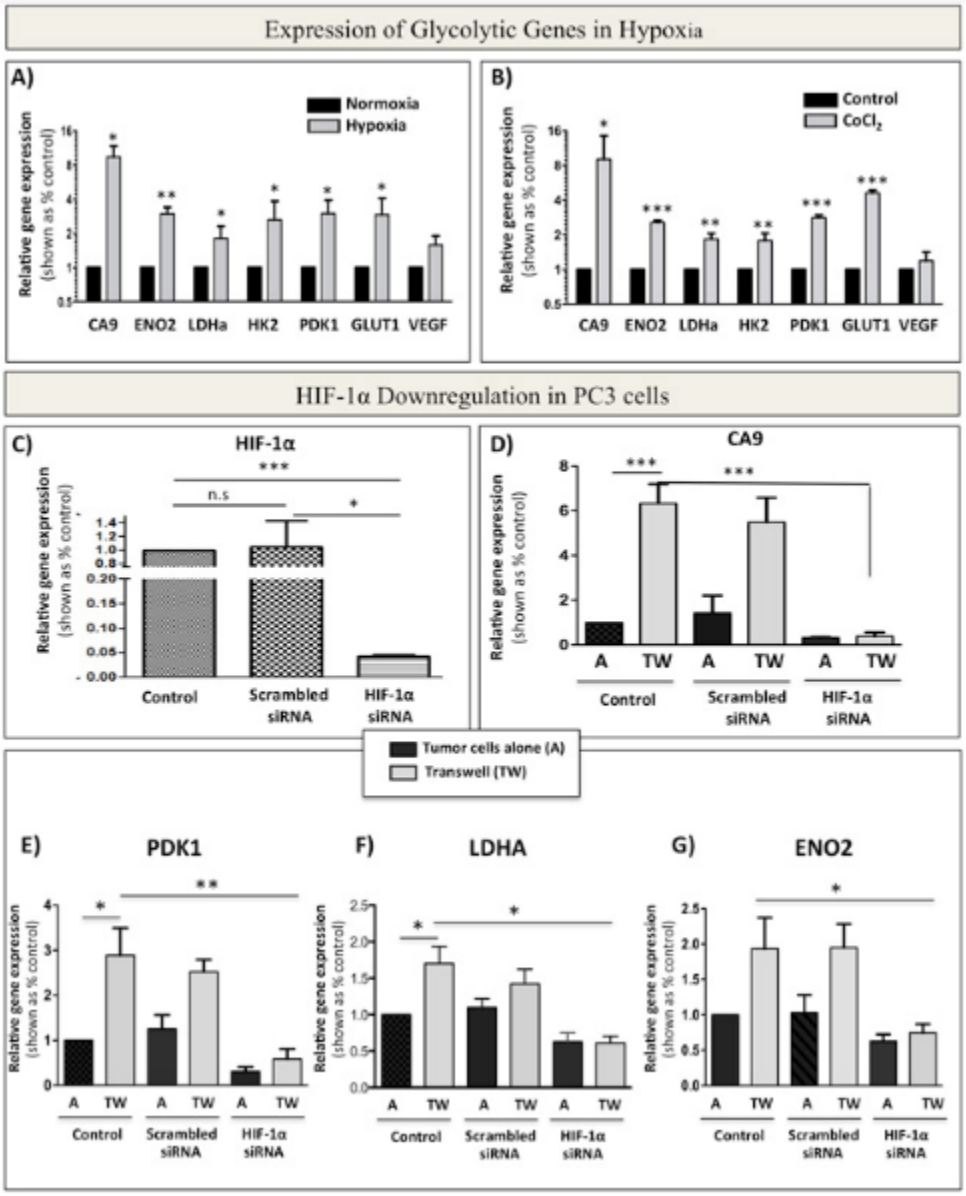

Figure 18: siRNA-mediated knockdown of HIF-1α abrogates bone marrow adipocyte-induced Warburg phenotype in PC3 cells. A. Taqman RT-PCR analysis of CA9, ENO2, LDHa, HK2, PDK1, GLUT1, and VEGF in PC3 cells cultured in normoxia (20% O2) or hypoxia (1% O2) B. mRNA expression of CA9, ENO2, LDHa, HK2, PDK1, GLUT1, and VEGF in response to treatment with 150 μM CoCl2. C. mRNA levels of HIF-1α in PC3 cells grown under control conditions or treated with 20 μM scrambled siRNA, or 20 μM HIF-1α siRNA. D. Taqman RT-PCR analysis of the expression of HIF-1α target gene CA9 to further confirm HIF-1α knockdown from cells grown in the presence or absence of adipocytes. E-G: Effect of HIF-1α knockdown on the mRNA expression of glycolysis associated genes: PDK1 E., LDHA F., and ENO2 G. Data are the mean of analyses with 2 different siRNA constructs done in triplicate. Values *P < 0.05; **P < 0.01, and ***P < 0.001 are considered statistically significant.

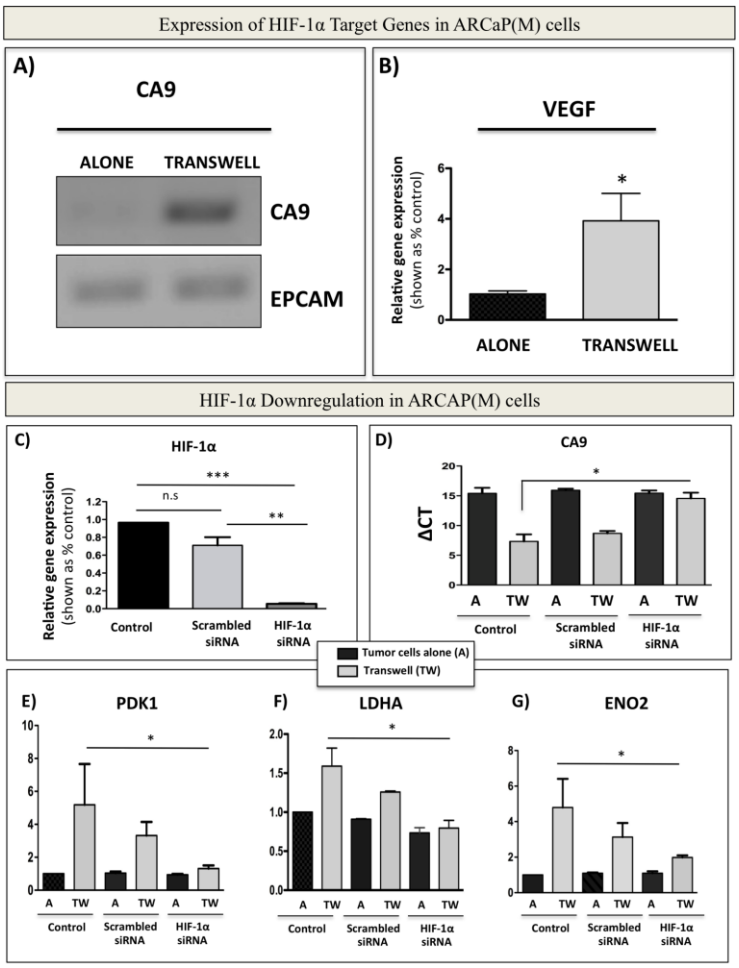

Figure 19. Adipocytes induce HIF-1α signaling in ARCaP(M) cells. A: *CA9* expression in ARCaP(M) cells alone or in transwell co-culture with bone marrow adipocytes as measured by semi-quantitative PCR. PCR products were resolved on 2% agarose gel. *EPCAM (CD326)* is used as a loading control (bottom). *CA9* expression is not detectable under control conditions. **B:** Taqman RT-PCR analysis of *VEGF* expression in ARCaP(M) alone or in tranwell co-culture with bone marrow adipocytes. **C:** mRNA levels of *HIF-1α* in ARCaP(M) cells grown under control conditions or treated with 10 μM scrambled siRNA, or 10 μM HIF-1α siRNA. **D:** Taqman RT-PCR analysis of the expression of HIF-1α target gene *CA9* to further confirm HIF-1α knockdown fromcells grown in the presence or absence of adipocytes. Changes in CA9 mRNA levels are shown as ΔCT due to its low baseline expression under control conditions and upon HIF-1□ knockdown in ARCAP(M) cells. **E-G:** Effect of HIF-1□ knockdown on the mRNA expression of glycolysis associated genes: *PDK1* **(E),** *LDHA* **(F),** and *ENO2* **(G).** Data are the mean of analyses with 2 different siRNA constructs done in triplicate. Data are normalized to *HPRT1* and shown as increase relative to control. Values * $p < 0.05$; ** $p < 0.01$, and *** $p < 0.001$ are considered statistically significant.

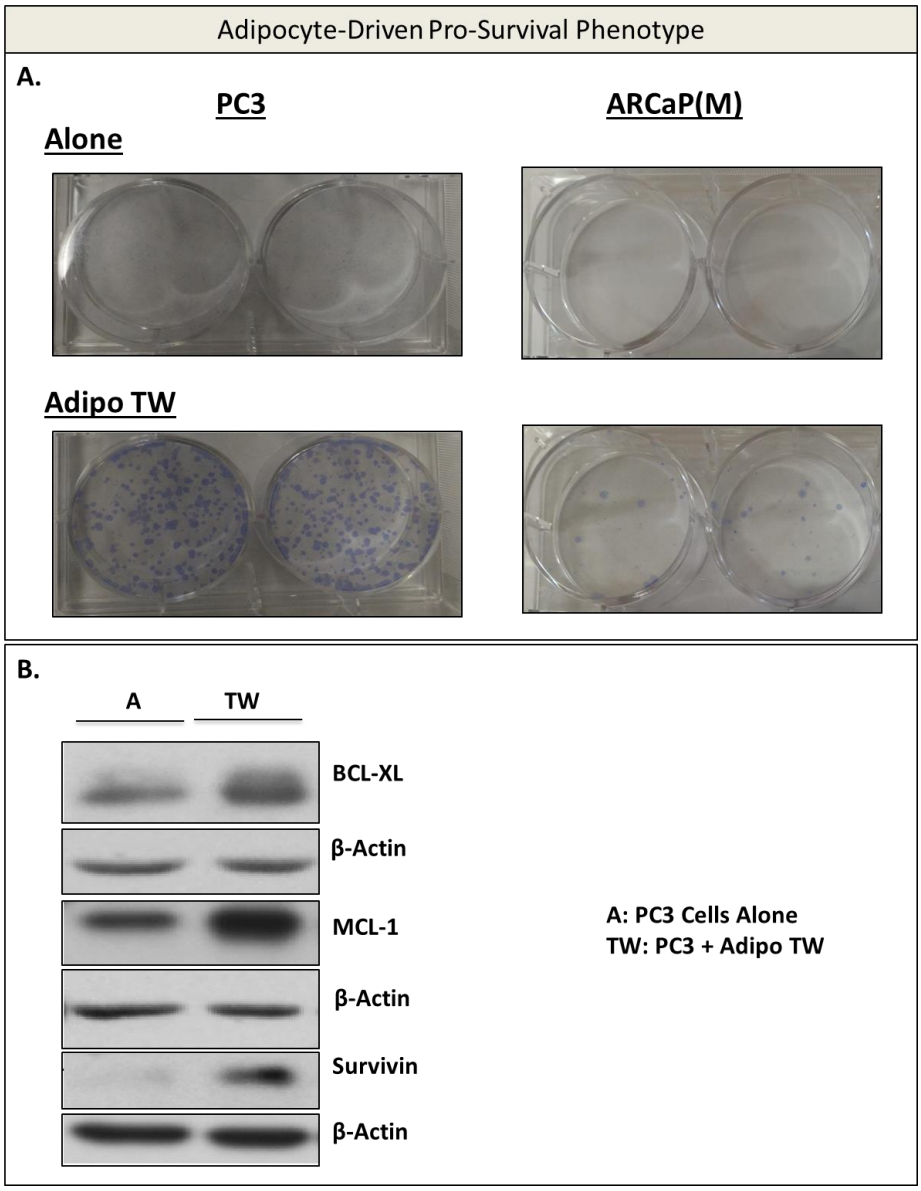

Figure 20: Tumor cells exposed to bone marrow adipocytes have increased clonogenic potential. A. Colony formation assay of PC3 (Left) and ARCaP(M) (Right) cells after grown alone or in transwell co-culture with bone marrow adipocytes after 10 days. **B.** Western blot analysis of pro-survival proteins in PC3 cells cultured alone or in transwell with adipocytes.

efficiently increase the expression of glycolytic genes to levels comparable to those observed in tumor cell-adipocyte co-cultures (Figure 4B). This suggests that adipocyte-driven Warburg phenotype in tumor cells is likely a downstream effect of HIF-1α activation under normoxic conditions. To test this, we downregulated HIF-1α in PC3 cells by siRNA and cultured control and knockdown cells alone or in transwell with marrow adipocytes (Figure 18C, 18D). A significant reduction in HIF-1α activity in siRNA-treated cells was evident by almost complete abrogation of *CA9* expression (Figure 18D). This coincided with reduced expression of glycolytic genes *PDK1, LDHA* and *ENO2* (Figure 17E-17G). Analogous to PC3 cells, ARCaP(M) cells also showed HIF-1α activation upon exposure to adipocytes, as evidenced by the increases in *CA9* mRNA expression, which is otherwise undetectable under control conditions (Figure 19A). Exposure to adipocytes also led to augmented expression of *VEGF* (Figure 19B), as well as increased *GLUT1* (Figure 4B). Upon siRNA-mediated knockdown of HIF-1α (Figure 19C), expression of *CA9, PDK1, LDHA* and *ENO2* was significantly reduced (Figure 19D-19G), further underscoring the importance of HIF-1α signaling in marrow adipocyte-driven metabolic adaptation of PCa tumors in bone.

4.2.8 Adipocyte-Driven Pro-Survival and Chemoresistance in PCa Cells

Metabolic adaptation within the bone microenvironment is important for prostate cancer growth and survival. Stemming from the work showing the PCa cells exposed to bone marrow adipocytes exhibit enhanced Warburg metabolism and activation of HIF-1α, we sought to interrogate pro-survival pathways affected by enhanced glycolysis and hypoxic signaling. We first performed a clonogenic colony formation assay and observed that tumor cells exposed to adipocytes have higher clonogenic potential indicative of the

activation of pro-survival pathways (Figure 20A), result confirmed by the increased expression of pro-survival factors Bcl-XL, Mcl1 and surviving (Figure 20B) Interestingly, many of the glycolytic enzymes not only perform functions in glycolysis, but have other functions that can affect cellular survival [357, 358]. One enzyme of particular importance, hexokinase 2 (HK2), has a known role of inhibiting apoptosis by binding to voltage-dependent anion channel (VDAC) as a competitive inhibitor of pro-apoptotic factors such as Bax [359]. As has been shown herein, HK2 expression is increased in metastatic tumors in patients compared to primary sites, in tumor cells intratibially injected into mice with elevated marrow adiposity compared to control mice, and in tumor cells exposed to bone marrow adipocytes *in vitro* through the activation of HIF-1α [360]. This led us to investigate the localization and binding of HK2 to the mitochondria in tumor cells exposed to adipocytes as a mechanism of survival within the bone microenvironment. We isolated mitochondria from PC3 cells grown alone or exposed to adipocytes in transwell, and immunoprobed for HK2. We observed that HK2 levels increased in tumor cells in transwell co-culture with adipocytes in the non-mitochondrial fraction and, importantly, the mitochondrial fractions (Figure 21A). Additionally, this was shown in hypoxic conditions as well, mimicking the pseudohypoxic phenotype in tumor cells exposed to adipocytes (Figure 21B).

Stemming from the induction of this pro-survival phenotype observed, we then assessed the sensitivity of these prostate cancer cells to standard chemotherapy agent for patients with metastatic disease, docetaxel [361-363]. Our preliminary data show that culture of PCa cells under hypoxic conditions makes them less sensitive to Docetaxel and that in hypoxia, Warburg genes are induced at similar levels as seen when cultured with

adipocytes, suggesting the activation of HIF-1α does confer chemoresistance to Docetaxel in these cells (Figure 22).

4.3 Discussion:

Adipocytes are metabolically active cells with the ability to regulate the phenotype and function of neighboring cancer cells through the processes of lipid transfer and lipolysis [127, 152, 343, 364, 365]. They have been linked to metabolic reprogramming and tumor progression in a handful of cancers, including tumors of breast, ovaries and colon, all with tendencies to grow in fat-enriched sites [127, 343, 366-368].In the context of prostate cancer, adipocytes from visceral and periprostatic tissues have been linked to the progression of localized disease [127, 369]. The data have been lacking, however, on how adipocytes that occupy bone marrow space might be influencing the metabolism and consequently the progression of prostate tumors that have colonized this fat-enriched metastatic niche. The results presented above reveal an important contribution of bone marrow adipocytes to the metabolic phenotype of metastatic PCa tumors. We show that marrow fat cells are capable of inducing the glycolytic phenotype in PCa cells through paracrine upregulation of glycolytic enzymes, increases in lactate secretion and reduction in oxidative phosphorylation. We also demonstrate that tumor cells are able to modulate the metabolism of a fat cell. They do so by stimulating adipocyte lipolysis in the effort of utilizing the fat cell-supplied lipids to fuel the glycolytic pathway. This speaks to the importance of the supportive host microenvironment in tumor progression and demonstrates the metabolic coupling between the tumor cells and host adipocytes. This adipocyte-tumor cell interaction ultimately shapes the metabolism of the tumor cell allowing for the adaptive survival in the metastatic niche [314].

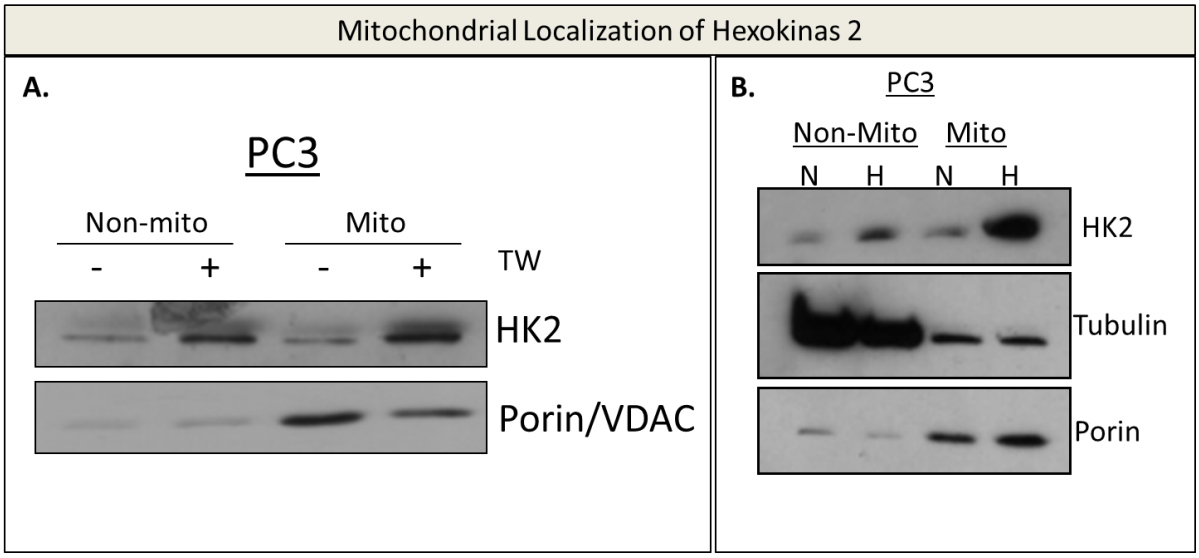

Figure 21: Hexokinase 2 localizes to the mitochondria in tumor cells exposed to bone marrow adipocytes or under hypoxic conditions. **A.** Immunoblot analysis of HK2 expression and localization in PC3 cells alone or in transwell co-culture with bone marrow adipocytes in mitochondrial and non-mitochondrial fractions. **B.** Western blot analysis of HK2 expression and localization in normoxia and hypoxia (1% O2) in PC3 cells in mitochondrial and non-mitochondrial fractions. VDAC/Porin was used as a loading control for mitochondrial fractions. Tubulin was used as a loading control for non-mitochondrial fractions.

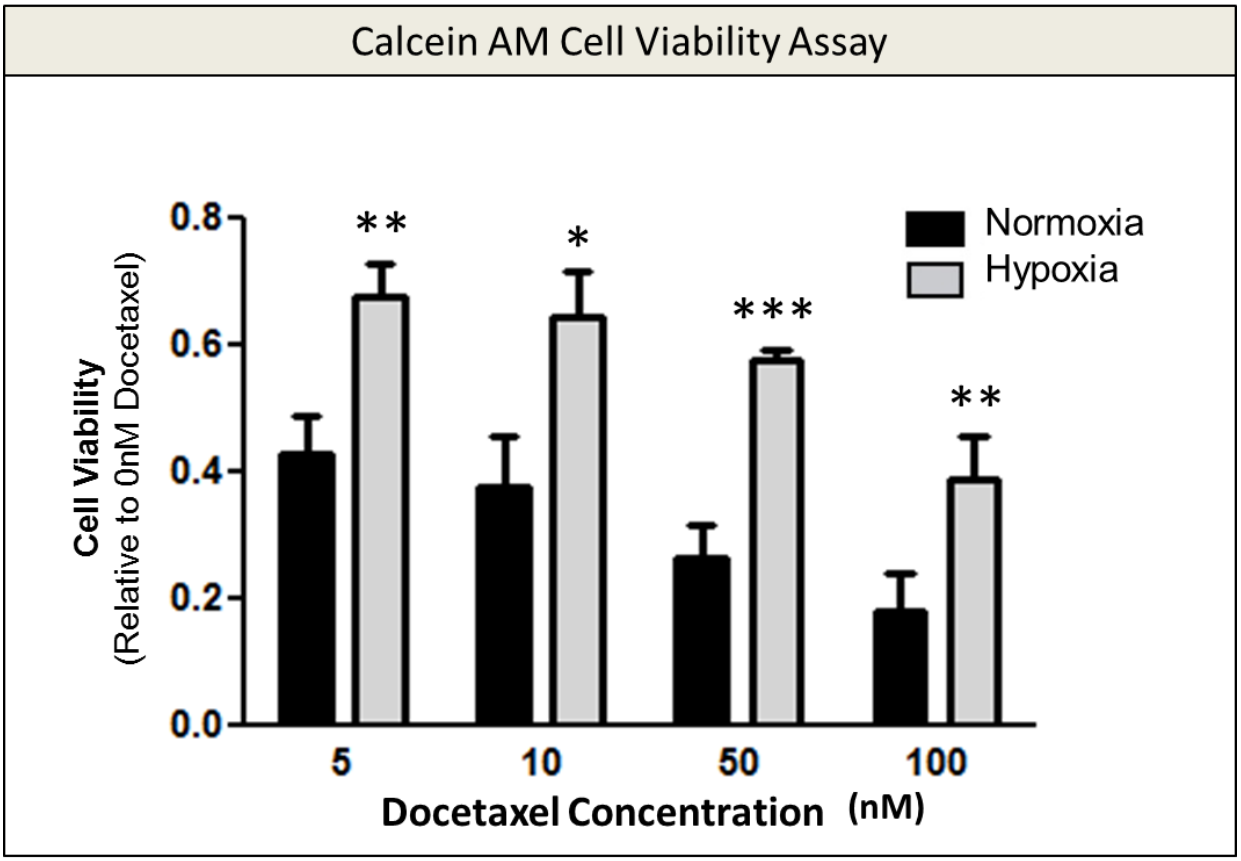

Figure 22: PC3 cells are more resistant to Docetaxel treatment under hypoxic conditions. Calcein AM viability assay of tumor cells grown in normoxia or hypoxia treated with 5, 10, 50, or 100 nM Docetaxel.

We focused on metabolism because of the important selective advantage an enhanced glycolytic phenotype can have on tumor aggressiveness and survival within a harsh metastatic niche [370, 371]. Glycolysis is not the most effective way, but it is the quickest way of creating ATP that allows the tumor cells to efficiently gain metabolic autonomy in the tumor microenvironment [371]. It permits the continuous supply of nutrients for biosynthetic processes, protection from oxidative stress, and, potentially, an activation of survival pathways [372, 373]. Warburg metabolism is often associated with a more hypoxic tumor signature, which is also a very well-documented facilitator of tumor aggressiveness and chemoresistance [374-377]. Glycolytic enzymes, such as ENO2, LDHa, PDK1, and HK2, and proteins involved in glucose uptake, such as GLUT1, are all regulated through HIF-1α [378-380]. In hypoxia, HIF-1α compromises oxygen-consuming OXPHOS by inducing the expression of PDK1 and preventing conversion of pyruvate into acetyl-CoA [381, 382]. The resulting production and secretion of lactate by highly glycolytic cells is known to increase tumor invasion, but it can also serve as an alternative carbon source for surrounding oxygenated cells [383]. Hypoxia is also known to induce acidosis *via* increased acid load in the tumor microenvironment, a process that leads to upregulation of enzymes, such as carbonic anhydrase 9 (CA9) that can regulate extracellular pH allowing the tumor cells to thrive in the acidic microenvironment [371]. It is the membrane-bound CA9, whose expression correlates with aggressive disease and poor survival in many cancers [371], that is thought to modulate pH through the interaction with bicarbonate transporters on the cell surface [384]. Notably in our study, evidence of clearly increased CA9 expression in human metastatic prostate cancer samples and experimental bone tumors from HFD mice, together with immunocytochemical data

showing its surface expression in response to adipocytes, suggest that metastatic PCa cells might be utilizing CA9-dependent mechanisms to adapt and grow in the metastatic niche.

Bone tissue is intrinsically hypoxic [385, 386], with O_2 concentrations ranging from ~1.3 to 3% based on the proximity to the vessels and distance from the endosteum [387]. This makes bone an environment already prone to hypoxic stress. Our data presented herein show that PCa cells exposed to low oxygen concentration in a hypoxia chamber show the same glycolytic phenotype as cells interacting with marrow adipocytes under normoxic conditions. This suggests that adipocytes promote oxygen-independent mechanism of HIF-1α activation in PCa cells, known as 'pseudohypoxia' [331, 388, 389]. HIF-1α, under some circumstances, can be directly activated in well-oxygenated microenvironments [390], or its activation and stabilization can be a consequence of mutations in metabolic genes [331, 391]. The mechanisms behind its regulation by bone marrow adipocytes are currently unknown and are subject of ongoing investigations in our laboratory.

One important consequence of hypoxia is the induction of HIF-1α-mediated accumulation of lipid droplets in tumor cells [392]. Hypoxic tumor cells have been recently shown to bypass lipogenesis and to rely on scavenging of unsaturated lipids from the microenvironment [393, 394].

Hypoxia has also been linked to the upregulation of proteins that stabilize the integrity of lipid droplets, such as perilipin and adipose differentiation-related protein (ADRP), as well as members of the FABP4 family of lipid transporters [392]. This is of relevance to our study, as hypoxia and glycolytic phenotype in our PCa cells interacting with

adipocytes coincide with both an enhanced lipid uptake and an upregulation of lipid transporters and lipid droplet markers. Whether HIF-1α activation is a cause or a consequence of lipid accumulation in metastatic tumor cells remains to be uncovered. It is plausible that the initial exposure to adipocyte-supplied lipids triggers HIF- 1α stabilization and that consequent activation of HIF-1α signaling leads to further lipid uptake, perpetuating the hypoxic and glycolytic phenotype in tumor cells. The fact that hypoxia-mediated effects persist even upon the inhibition of adipocyte-driven lipolysis speaks to the importance of HIF-1α signaling in driving the Warburg phenotype in tumor cells. The mechanism by which tumor cells exposed to adipocytes in the presence of ATGL inhibitor continue to accumulate and utilize fat cell-supplied lipids remains to be elucidated. One potential player in this process might be the MAGL, an enzyme implicated in lipid remodeling and scavenging by tumor cells and shown recently to be associated with aggressive phenotype of PCa cells [354, 355, 395].

Although the acquisition of a glycolytic phenotype appears to be the predominant metabolic change in PCa cells in response to marrow adipocytes, it is important to mention that some of the glycolytic enzymes we see upregulated in response to fat cells are also known to have non-glycolytic functions that are important for tumor cell growth and survival. Specifically, hexokinase-2 (HK2), an enzyme critical for first step of glycolysis, elicits its functions by binding to the outer mitochondrial membrane protein voltage-dependent anion channel (VDAC). This allows for receipt of newly synthesized ATP and rapid and efficient production of glucose-6-phosphate, which contributes not only to the glycolytic pathway but also to metabolite synthesis in the pentose-phosphate pathway and TCA cycle, both important for tumor growth and proliferation [396-399].

Intriguingly, interaction of HK2 with VDAC prevents pro-apoptotic proteins such as BAX and BAD from binding to the mitochondrial pores to facilitate apoptosis resulting in cells that are more resistant to cell death and chemotherapy [400-402]. Because around 80% of total HK2 is reported to be bound to the mitochondrial VDAC [403], and because we see elevated levels of HK2 in tumor cells that are exposed to bone marrow adipocytes, it is plausible to expect that the mitochondrial binding of HK2 is occurring in PCa cells, a process that could be promoting tumor cell survival *via* inhibition of intrinsic apoptosis in response to adipocyte-supplied factors.

In this study we utilized an intratibial model of intraosseous tumor growth, a widely used *in vivo* experimental system designed to specifically study tumor-bone interactions, and tumor growth and expansion in the bone microenvironment [404-406]. We and others have used this system previously in combination with diet induced obesity (DIO) models to study effects of marrow adiposity on tumor progression in bone [24, 345, 407]. The DIO model is a well-documented approach to induce marrow adiposity [27, 37, 292, 345] and we have previously shown that eight-week exposure to HFD significantly augments adipocyte numbers in this system [24, 345]. We do acknowledge we cannot exclude potential systemic consequences of the diet itself on both the tumor growth and metabolic phenotype in bone. There is an ongoing debate on the role of dietary lipids in prostate cancer development and progression [408, 409] and future studies utilizing genetic models of obesity and age-induced models of marrow adiposity will provide a more detailed understanding of adipocyte impact on metabolic adaptation and survival of tumor cells in the bone marrow niche. An additional value will be added by the comparative

metabolic profiling of experimental bone prostate tumors and orthotopic primary prostate tumors.

The rationale for our study was based on the Oncomine analyses of human prostate cancer samples suggesting clearly distinct metabolic phenotype of metastatic sites as compared to primary tumors. We recognize that currently available datasets do not allow for distinction of bone metastases from other potential metastatic sites. However, given the fact that more than 80% of metastatic patients present with bone lesions, it is highly likely that majority of these tumors represent skeletal lesions. Limited availability of bone metastatic tissues is certainly an ongoing, unresolved issue in prostate cancer research. We believe that this distinct metabolic phenotype in metastatic tissues revealed by our Oncomine analyses provides an important starting point for future studies investigating the contribution of tumor metabolism to progression and survival of metastatic prostate tumors in the bone microenvironment.

Metabolic requirements of a tumor cell are much more complex than previously appreciated and they likely involve multiple pathways and nutrients that aid in malignant transformation and progression [410]. There is also no doubt that metabolic adaptation and consequent growth and survival of a tumor is the result of a complex interaction between the cancer cell and the surrounding host microenvironment. Data presented herein reveal marrow adipocytes as important players involved in shaping tumor metabolism in bone. To our knowledge, this is the first study demonstrating the importance of bi-directional interactions between marrow fat cells and tumor cells in activating HIF-1α signaling and driving the Warburg phenotype in metastatic prostate cancer cells. Adipocyte-supplied factors have been shown to enhance glycolysis in

primary cancer cells and render them more aggressive and resistant to therapy [167, 411-413]. Understanding the molecular mechanisms behind this metabolic regulation in bone is of critical importance in terms of potential treatment options for metastatic disease.

CHAPTER 5: BONE MARROW ADIPOCYTE-DERIVED PROSTAGLANDIN E2 ACTIVATES CANONICAL PGE2 SIGNALING METASTATIC PROSTATE CANCER CELLS

5.1 Introduction

The uptake of lipids from the microenvironment, aberrant *de novo* lipid synthesis and alterations in fatty acid catabolism and steroidogenesis pathways are now emerging as key mechanisms linking dysregulated lipid metabolism in the primary prostate tumor with subsequent progression and reduced survival [299, 303, 304]. These lipids have the propensity to regulate signaling networks within cancer cells through receptor-mediated signaling pathways [414, 415]. Previous work stemming from our laboratory has shown that metastatic prostate tumors in bone both induce lipolysis in bone marrow adipocytes and also utilize lipids from adipocytes to fuel their own metabolic processes that are largely lipid-driven [345, 360]; therefore, it is crucial to understand what lipids are responsible for tumor growth and survival in bone.

Prostaglandin E2 (PGE2), a bioactive lipid that has been implicated across many different cancer types in facilitating many of the hallmarks of cancer such as cell proliferation, angiogenesis, inflammation, immune surveillance, and apoptosis [416-418], is secreted by adipocytes [419-421]. PGE2, as a prostaglandin, is a member of the eicosanoid family of lipids and can be produced by all cell types within the body [422]. Its synthesis is catalyzed by a multiple step process involving the conversion of arachidonic acid (AA) to prostaglandin H2 (PGH2) by cyclooxygenases (COX) and then PGH2 to PGE2 with the aid of the enzyme prostaglandin E synthase [423]. There are two distinctly different cyclooxygenases, COX-1 and COX-2. COX-1 is constitutively expressed in almost all tissues, while COX-2 needs stimuli in order to be induced [424]. Because COX-

1 is constitutively expressed, its functions are thought to be more in maintenance of tissue homeostasis by regulating basal levels of prostaglandins. The inducible COX-2, however, has a variety of stimuli such as cytokines, growth factors, and tumor-derived factors [425-427]. Interestingly, we have shown previously that tumor cells secrete large amounts of IL-1β in response to exposure to adipocytes [154, 345] and IL-1β is a known inducer of COX-2 [428]. PGE2 produced from COX enzymes can signal in a paracrine or autocrine manner where it activates its receptors EP1-4 [429, 430]. EP receptors are G protein coupled receptors that are expressed ubiquitously throughout the body. Deregulated EP receptor activity has been shown across many cancers including breast [431], colorectal [432, 433], and esophageal cancers [416, 434].

Importantly, there have been some studies in prostate cancer showing PGE2/EP receptor signaling to play critical roles in proliferation [435], vascular endothelial growth factor expression and angiogenesis [436], and invasion [437]. Extending from our previous work, we have shown that bone marrow adipocytes induce *vascular endothelial growth factor* (*VEGF*) expression in metastatic prostate cancer cells and have increased invasion, both downstream indicators of EP receptor signaling [345, 360]. We have shown that HIF-1α is activated in tumor cells exposed to bone marrow adipocytes [360] and *VEGF* is regulated by HIF-1α signaling [438]. Intriguingly, PGE2 signaling has been shown to have the propensity to activate HIF-1α signaling through EP1, EP2, and EP4 receptors [439, 440].

The objective of this study was to elucidate the role of bone marrow adiposity in the modulation of tumor signaling networks within the bone microenvironment. Using *in vivo* models of diet induced marrow adiposity in combination with *in vitro* models of

paracrine signaling between bone marrow adipocytes and prostate cancer cells, we show that tumor-derived IL-1β activates COX-2 in bone marrow adipocytes. COX-2 then is responsible for the synthesis and secretion of prostaglandin E2 that activates the EP receptors in tumor cells, leading to the induction of downstream signaling pathways. These results offer potential mechanisms underlying metabolic adaptation of metastatic tumors in bone and implicate bone marrow adipocytes, a cell type so abundantly present in the skeleton especially in advanced age and obesity, as viable culprits in the progression of this currently incurable disease.

5.2 Results

5.2.1 *In silico* analysis of prostaglandin E2 receptors EP1-4 and EP receptor signaling in metastatic prostate cancer patients

Because COX-2 has been shown to be elevated in PCa and its signaling pathways are implicated in many cellular processes required for cancer cell survival and growth [441], we first performed an Oncomine analysis and compared mRNA expression of genes linked to PGE2 signaling in primary and metastatic prostate cancer samples (Figure 1A). A significant upregulation of the gene encoding the *EP1* receptor was revealed in patients with metastatic disease compared to those with primary prostate cancer (Figure 1A). In addition to the PGE2 receptor, genes involved in downstream signaling of EP1 activation such as *cyclin d1* (*CCND1*) and *c-myc* (*MYC*) were also significantly upregulated in metastatic tissue. These data link our previous observations showing a hypoxic phenotype in metastatic PCa compared to primary PCa, indicative of HIF-1α activation, suggesting a correlation between EP receptor signaling and HIF-1α activity in these patients [442]. These results further underscore the importance of

eicosanoid signaling between primary and secondary prostate cancer that might be playing a role in metastatic progression.

5.2.2 Adipocytes exposed to intratibially injected prostate cancer cells have increased expression of COX-2 *in vivo*

Although previous reports from our laboratory have focused on how bone marrow adipocytes are affecting PCa cells, it is of critical importance to study the effects of tumor cells on adipocytes as well. To examine the effects of metastatic prostate cancer cells on bone marrow adipocytes, we utilized a well-documented approach of inducing marrow adiposity with high fat diet (HFD) [27, 37, 150, 292]. We have shown previously that intratibial implantation of prostate cancer cells into this model results in accelerated tumor growth and extensive bone destruction compared to normal diet (LFD), suggesting potential tumor-supportive effects of marrow adipocytes and that the tumor metabolism is altered to a more glycolytic and hypoxic state [150, 294]. To determine whether this observed pseudohypoxic phenotype in the tumor cells exposed to adipocytes was correlated with increased PGE2 secretion by adipocytes and EP receptor activation of HIF-1α signaling in the tumor, we analyzed mRNA expression of mouse *COX-1* and *COX-2* genes in adipocytes exposed to intratibially injected PC3 and ARCaP(M) tumors from LFD and HFD mice. Our results revealed significantly increased transcript levels of both *COX-1* and *COX-2* in adipocytes in tumor bearing bone compared to control bone and in the effect was exacerbated in the HFD model compared to the LFD model, suggesting contribution of adipocytes to tumor-induced COX-1/2 expression in the bone [443]. These findings implicate tumor cells as a potential regulator of adipocyte PGE2 production

through the COX-2 pathway in bone and led us to further investigate the mechanism of this regulation *in vitro.*

5.2.3 Adipocytes exposed to PCa cells have increased expression of COX-1 and COX-2 *in vitro*

We utilized *in vitro* models of tumor cell-adipocyte interactions to determine if the activation of COX-2/PGE2 axis in adipocytes exposed to PCa cells as observed in intratibial tumors *in vivo* is a direct effect of tumor cells. We first examined COX-1/-2 expression in adipocytes exposed to PCa cells by employing a transwell system in which adipocytes were differentiated in the bottom chamber and tumor cells were then plated on top of the insert and cultured together for 48 hours. This allowed the two cell types to share the media without direct interaction. *COX-2*, the inducible cyclooxygenase involved in prostaglandin synthesis was significantly increased in adipocytes co-cultured with PCa cells (Figure 2A). COX-2 was also upregulated at the protein level in adipocytes exposed to tumor cells (Figure 2B). Interestingly, although COX-1 is constitutively expressed, its levels are still induced in adipocytes exposed to PC3 and ARCaP(M) prostate cancer cells (Figure 2C). Activation of COX1/2 signaling was further supported by significantly elevated levels of Prostaglandin E2 in media conditioned by transwell co-cultures as opposed to adipocytes or tumor cells grown in alone conditions (Figure 2D).

The fact that transwell co-culture with tumor cells caused the activation of COX-1 and COX-2 and PGE2 production in adipocytes suggested that this process does not require physical interaction between the tumor cells and adipocytes. Therefore, we next examined whether the media conditioned by the PCa cells alone (PC3/ARCaP(M) CM)

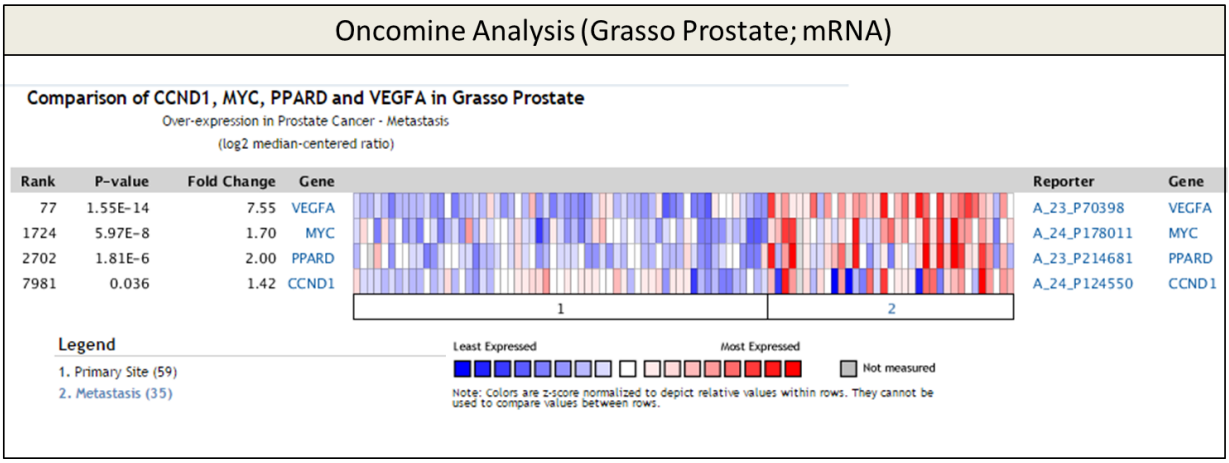

Figure 1: Gene targets of GSK3β/β-catenin signaling through the prostaglandin receptors are upregulated in patients with metastatic prostate cancer. A. Oncomine gene analysis comparing the expression of GSK3β/β-catenin target genes (*VEGF*, *MYC*, *PPARD*, and *CCND1*) in patient samples collected from metastatic or primary sites. Data were ordered by "overexpression" and the threshold was adjusted to *P*-value < 1E-4; fold change, 2 and gene rank, top 10%. **B.** Taqman RT-PCR analysis of expression of *COX-1* and *COX-2* in adipocytes exposed to PC3 and ARCaP(M) tumors in LFD- and HFD-fed mice. Data were normalized to human *EPCAM* and represent a mean of a minimum of 3 mice/group $\pm$ SD. Values * $P < 0.05$; ** $P < 0.01$ are considered statistically significant.

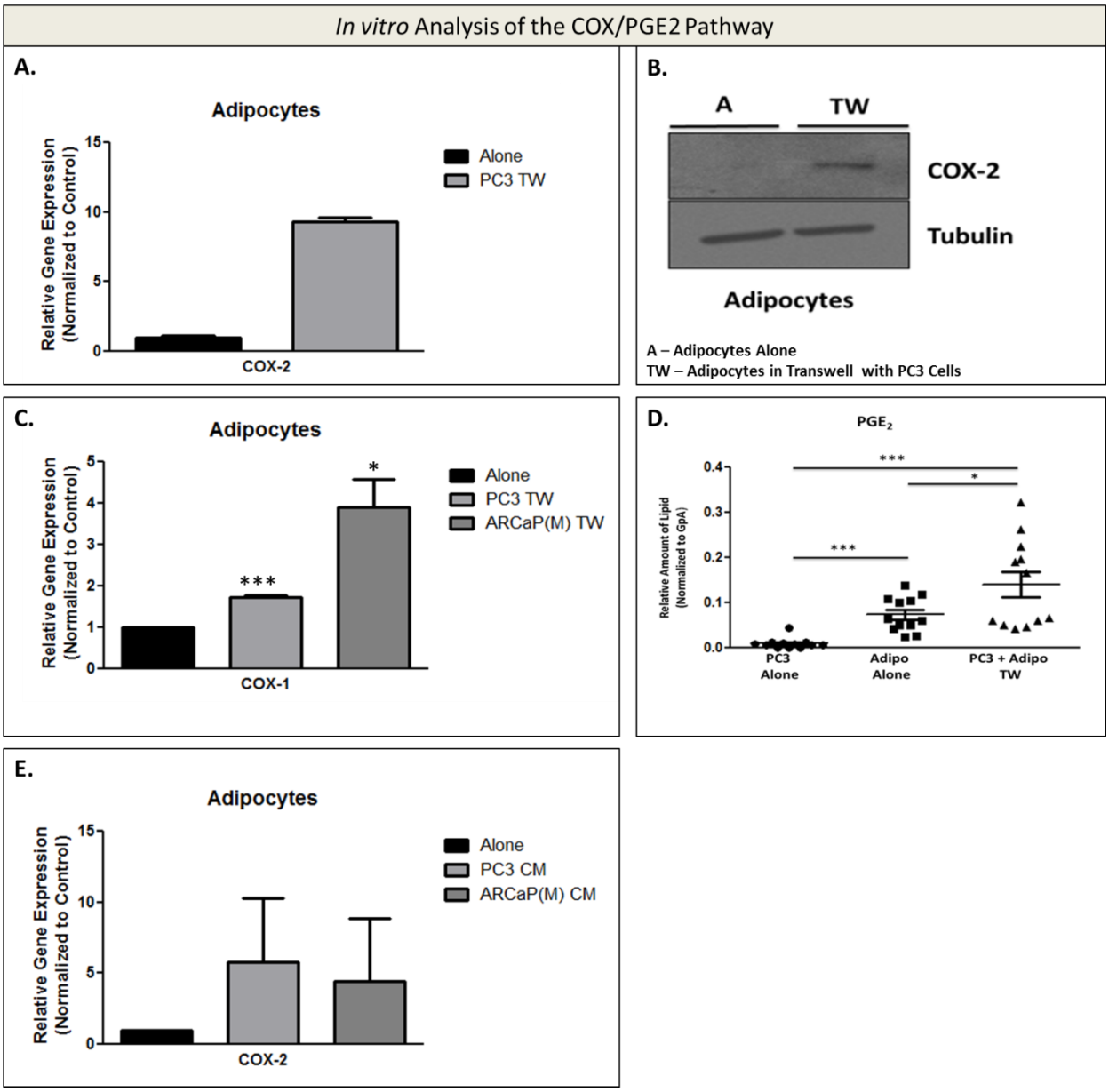

Figure 2: COX-2 expression and activity increases in bone marrow adipocytes exposed to tumor cells *in vitro*. A. Taqman RT-PCR analysis of *COX-2* in adipocytes exposed to PC3 prostate cancer cells in transwell co-culture. Data were normalized to mouse *Adiponectin* and shown as increase relative to control. **B.** Immunoblot analysis of COX-2 in adipocytes alone or in transwell co-culture with PC3 cells. **C.** Taqman RT-PCR of *COX-1* expression in adipocytes exposed to PC3 or ARCaP(M) cells in transwell co-culture. **D.** Lipidomics analysis of PGE2 levels in media conditioned by PC3 cells alone, adipocytes alone, or PC3 cells and bone marrow adipocytes cultured together in transwell. **E.** Taqman RT-PCR of *COX-2* expression in adipocytes treated with tumor cell conditioned media. Results represent a mean of at least 3 independent experiments ± SD. Values * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ are considered statistically significant.
can bring on similar changes in adipocytes as observed in transwell co-culture.

Interestingly, treatment with either PC3 or ARCaP(M) conditioned media induced expression of *COX-1* and *COX-2* in adipocytes (Figure 2E), suggesting that paracrine signaling is required between the adipocytes and tumor cells for the subsequent phenotype.

5.2.4 Adipocyte COX-1 and COX-2 are regulated by PCa cell secreted IL-1β stimulation of lipolysis

Because treatment of adipocytes with tumor cell conditioned media increases the expression of COX-1 and COX-2 and adipocytes exposed to tumor cells secrete elevated levels of PGE2, we then sought to determine what factors released from the tumor cells activate the COX/PGE2 axis. Previously, our laboratory has published that prostate tumor cells secrete high levels of IL-1β when exposed to adipocytes [150]. Additionally, IL-1β has been shown to have the propensity to activate COX-1 and COX-2 and increase PGE2 production [428]. With this knowledge, we then treated adipocytes with recombinant IL-1β and observed a marked increase in *COX-1* and *COX-2* expression, suggesting the increased activation of the COX/PGE2 axis in adipocytes is driven by tumor-secreted IL-1β (Figure 3A). We then used blocking antibodies against IL-1β (R&D Systems, 1μg/mL) and an IL-1R receptor antagonist (Sigma-Aldrich, 1μg/mL) to determine if increased expression *COX-1* and *COX-2* were due to IL-1β signaling between prostate cancer cells and bone marrow adipocytes. Intriguingly, using the blocking antibody against IL-1β or receptor antagonist against IL-1R did not abrogate the induction of *COX-1* or *COX-2* (Figure 3B); however, levels of *IL-1β* increased in tumor cells grown in transwell with adipocytes as a possible negative feedback mechanism to compensate for inhibition of IL-1β.. To resolve this issue, we

then performed a siRNA-mediated knock down of IL-1β (OriGene) and observed a substantial knockdown of IL-1β (Figure 3C). Studies utilizing this siRNA-mediated knockdown of IL-1β in tumor cells to determine the requirement of IL-1β on *COX-1* and *COX-2* expression in adipocytes are currently ongoing.

5.2.5 Adipocyte-derived PGE2 activates the GSK3β/β-catenin pathway through the EP receptors

After confirming that the COX/PGE2 pathway is activated in adipocytes exposed to prostate cancer cells through tumor-secreted IL-1β activation of COX-1 and COX-2, we then examined the effects of PGE2 release on tumor cell signaling. There are four receptors that can be activated by PGE2 (PTGER1-4) [444] and we first performed a screen looking at the receptor expression in PCa cells grown alone and exposed to adipocytes in transwell co-culture (Figure 4A) using primers for each specific gene (Table 1). Our results showed that *PTGER1* (*EP1*) was upregulated in PCa cells exposed to adipocytes in transwell co-culture, while there were no observable differences in expression of *PTGER2* (*EP2*) or *PTGER4* (*EP4*), and *PTGER3* (*EP3*) was not detected in any of our samples. Importantly, the increased expression of *EP1* in PCa cells exposed to adipocytes *in vitro* was also observed in PCa cells intratibially injected in HFD mouse tibia compared to LFD tibia (Figure 4B), supporting our observations that adipocyte-derived PGE2 is playing a role in tumor cell signaling. Taqman RT-PCR confirmed this increase in *EP1* expression in both PC3 and ARCaP(M) cells in tumor cells exposed to adipocytes in transwell co-culture *in vitro* (Figure 4C) and *in vivo* in HFD tumors compared to LFD tumors (Figure 4D).

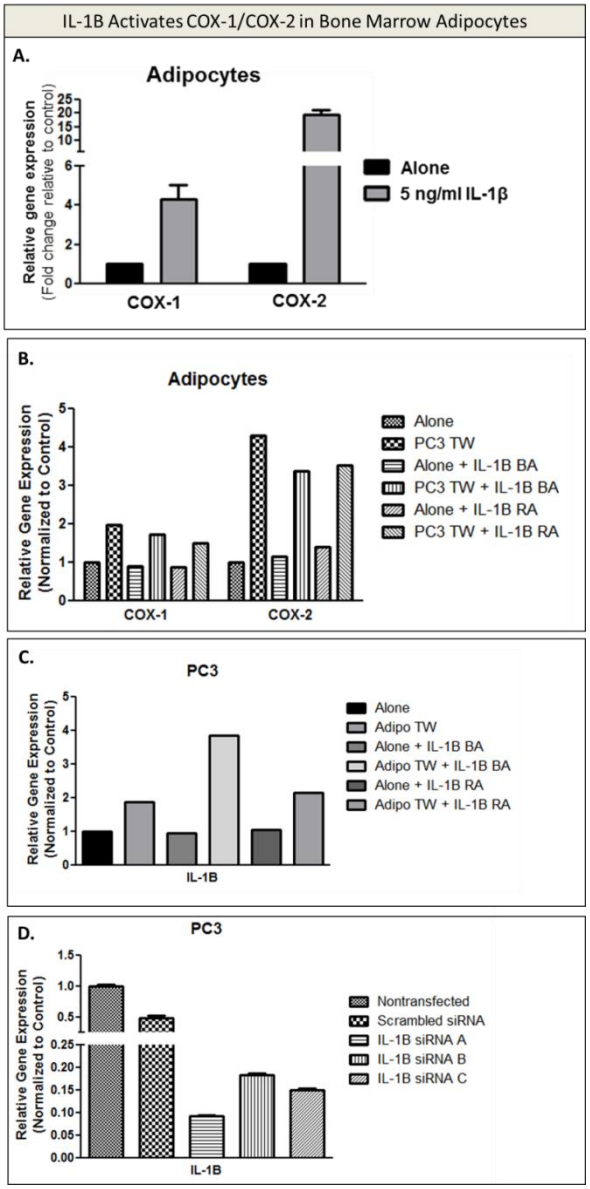

Figure 3: *COX-1* and *COX-2* are regulated by tumor-secreted IL-1β. A. Taqman RT-PCR analysis of *COX-1* and *COX-2* expression in bone marrow adipocytes treated with 5ng/mL IL-1β. **B.** Gene expression of *COX-1* and *COX-2* in adipocytes alone or exposed to PC3 cells in transwell co-culture in the absence or presence of an IL-1β blocking antibody (BA) or receptor anatagonist (RA). **C.** Taqman RT-PCR of *IL-1β* in tumor cells alone or in transwell co-culture with bone marrow adipocytes with or without the IL-1β blocking antibody (BA) or IL-1R receptor antagonist (RA). **D.** IL-1β gene expression upon knockdown using three different siRNA constructs compared to the nontransfected control and scrambled siRNA control.

We then examined the canonical PGE2 signaling in tumor cells exposed to adipocytes by looking at the activation of GSK3β and β-catenin and cyclin D. We showed that tumor cells exposed to adipocytes have increased GSK3β activation as shown through a decrease in phosphorylated GSK3β (Figure 5A) and increased nuclear β-catenin (Figure 5B). Additionally, cyclin D, a target of β-catenin, was shown to be upregulated in tumor cells exposed to fat cells in transwell co-culture (Figure 5C).

Lastly, to determine which receptor the PGE2 signaling is occurring through, we used specific inhibitors that target either EP1-3 (AH6809) or EP4 (GW627368X) in tumor cells exposed to adipocytes in transwell co-culture. Looking at phosphorylated GSK3β as a marker of PGE2 signaling, there was an observable decrease in phospho-GSK3β in tumor cells exposed to adipocytes and with the inhibition of EP4 there was a slightly abrogated effect with little reduction in phospho-GSK3β, while AH6809 treatment completely inhibited the activation of GSK3β, showing this signaling pathway is mainly activated through EP1-3 (Figure 5D).

5.2.6 Activation of the EP1 receptor in tumor cells by adipocyte-derived PGE2 leads to HIF-1α activation

Stemming from previous work from our laboratory showing that tumor cells exposed to adipocytes have enhanced HIF-1α activity [360] and knowing that there is a mechanism for β-catenin stabilization of HIF-1α [439, 445, 446], we then looked at PGE2 signaling activating a hypoxic response in tumor cells similar to effects observed in tumor cells exposed to adipocytes in transwell co-culture. We first treated PCa cells with PGE2 and its non-metabolizable form 15-S-15 Methyl-PGE2, which is a more potent activator of PGE2 signaling, and assessed HIF-1α activation by analyzing expression of HIF-1α

target genes *VEGF* and *CA9* [447]. Taqman real-time PCR analysis showed increased expression of *CA9* in PCa cells treated with PGE2 and the methylated PGE2 metabolite, demonstrating that activated PGE2 signaling in our cells increases hypoxic signaling and HIF-1α activation (Figure 6A). Unfortunately, however, inhibition of EP1 receptor with AH6809, EP2 receptor with TG4-155, or EP1-3 receptors with GW627368X did not alter *CA9* expression levels, indicating inhibition of the receptors themselves is not sufficient to halt the psuedohypoxic phenotype in tumor cells induced by PGE2 signaling (Figure 6B).

5.3 Discussion

Adipocytes are metabolically active cells with the ability to regulate the phenotype and function of neighboring cancer cells through the processes of lipid transfer and lipolysis [127, 152, 343, 364, 365]. In the context of prostate cancer, adipocytes from visceral and periprostatic tissues have been linked to the progression of localized disease [127, 369]. The data have been lacking, however, on how adipocytes that occupy bone marrow space might be influencing tumor cell signaling and consequently the progression of the disease for tumors that have colonized this fat-enriched metastatic niche. The results presented above reveal an important contribution of bone marrow adipocytes to the activation of the GSK3β/β-catenin pathway through PGE2-mediated EP receptor activation in metastatic PCa tumors. We show that marrow fat cells are capable of secreting prostaglandin E2 which activates the EP1, EP2, and EP4 receptors on the tumor cell surface, leading to the downstream activation of GSK3β/β-catenin pathway and a stabilization of HIF-1α. We also demonstrate that tumor cells are

Table 1: Primer sequences for PCR analysis of EP receptors 1-4 in human prostate cancer cells exposed to bone marrow adipocytes in transwell co-culture.

Gene	Forward Sequence	Reverse Sequence
EP1 (PTGER1)	CTTGTCGGTATCATGGTGGTGTC	GGTTGTGCTTAGAAGTGGCTGAGG
EP2 (PTGER2)	CCACCTCATTCTCCTGGCTA	CGACAACAGAGGACTGAACG
EP3 (PTGER3)	CTTCGCATAACTGGGGCAAC	TCTCCGTGTGTGTCTTGCAG
EP4 (PTGER4)	TGGTATGTGGGCTGGCTG	GAGGACGGTGGCGAGAAT

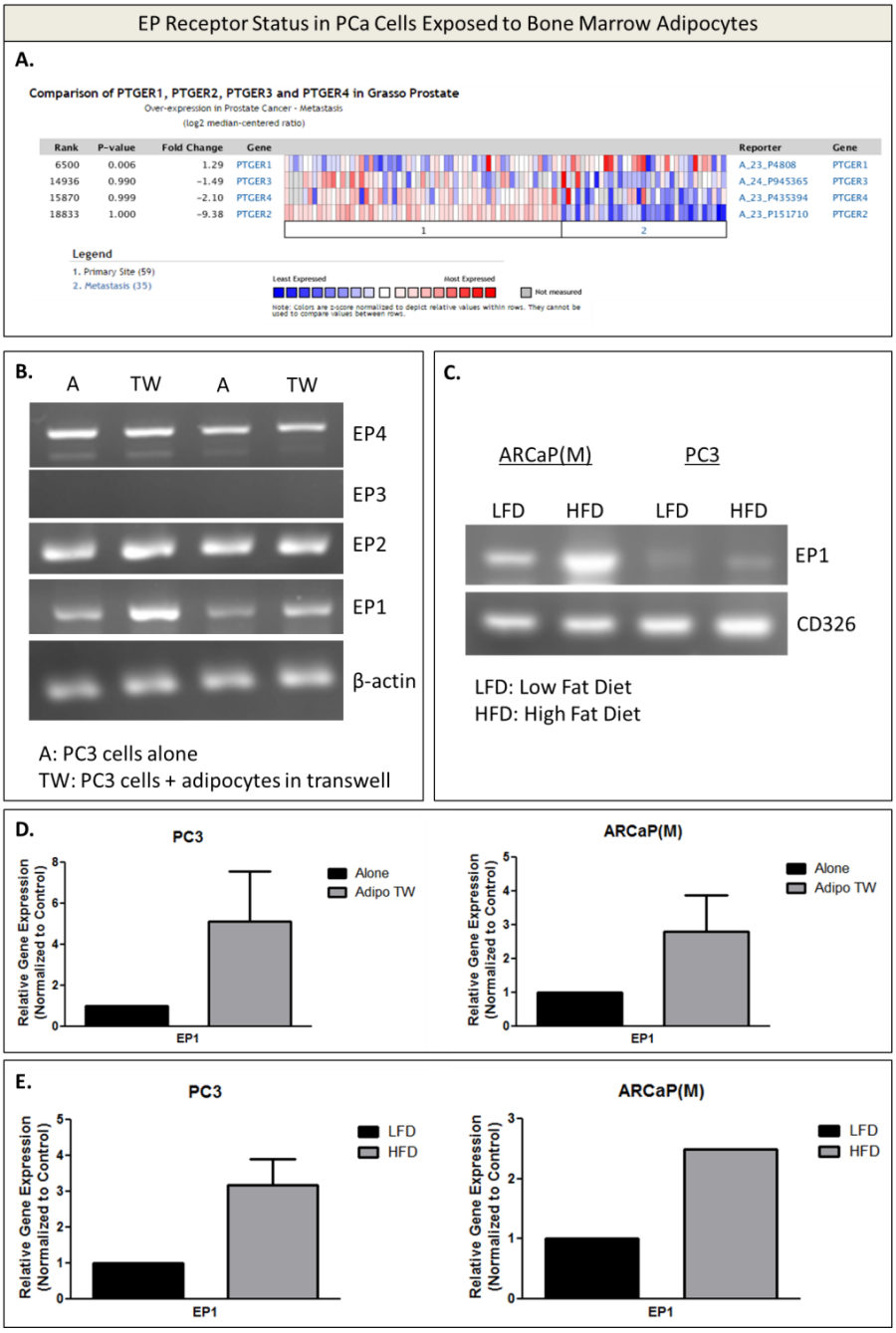

Figure 4: *EP1* is upregulated in PC3 and ARCaP(M) cells exposed to bone marrow adipocytes *in vitro* and *in vivo*. A. Oncomine gene analysis comparing the expression of EP receptors 1-4 (*PTGER1*, *PTGER2*, *PTGER3*, *PTGER4*) in patient samples collected from metastatic or primary sites. Data were ordered by "overexpression" and the threshold was adjusted to *P*-value < 1E-4; fold change, 2 and gene rank, top 10%. **B.** PCR analysis of *EP1-4* expression status in PC3 cells alone or in transwell with adipocytes. β-actin was used as a loading control. **C.** Gene expression analysis of *EP1* in tumor cells from LFD- or HFD-fed mice. **D.** Taqman RT-PCR expression examination of PC3 cells (Left) or ARCaP(M) cells (Right) alone or in transwell co-culture with adipocytes or in LFD- or HFD-fed mice (**E.**).

able to modulate the secretion of PGE2 from a fat cell. They do so by releasing interleukin-1β (IL-1β), which then induces the expression and activity of cyclooxygenase-2 (COX-2). This speaks to the importance of the supportive host microenvironment in tumor progression and demonstrates the crosstalk between the tumor cells and host adipocytes. This adipocyte-tumor cell interaction ultimately shapes the metabolism of the tumor cell allowing for the adaptive survival in the metastatic niche [314].

We focused on the COX-2/PGE2 pathway because activation of the EP receptors by PGE2 has been shown to facilitate tumor aggressiveness and survival within a harsh metastatic niche by inducing the expression of many proteins involved in cell survival [448, 449], proliferation [418], and hypoxia [439]. Activation of the β-catenin pathway has been associated with tumor aggressiveness and chemoresistance [450, 451]. Additionally, activation of HIF-1α has been linked to a poor prognosis and higher rates of chemoresistance [377, 452, 453]. The role of bone marrow adipocyte in mediating chemoresistance in PCa cells is currently unknown and is subject of ongoing investigations in our laboratory.

Our study was founded upon the Oncomine database analysis of human prostate cancer samples of metastatic sites compared to primary sites. Using this database allowed us to observe noticeable and significant increases in downstream targets of GSK3β/β-catenin signaling in metastatic prostate tumor, illuminating a clear translational relevance to the work presented within. Because the five-year survival of patients with metastatic disease drops from close to 99% in patients with localized disease to around 29% in metastatic patients, it is of upmost importance to understand

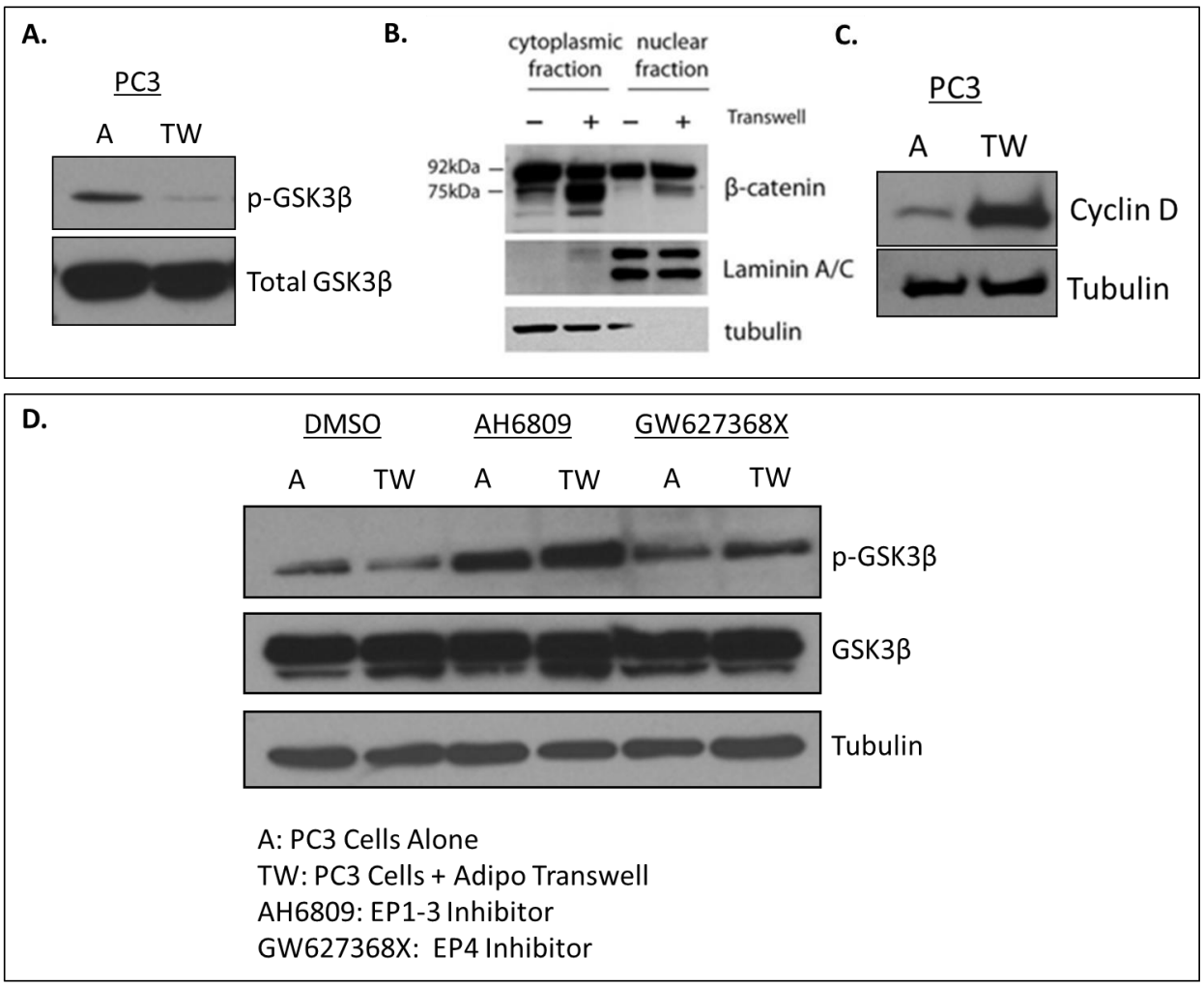

Figure 5: Bone marrow adipocytes activate the GSK3β/β-catenin pathway in PCa cells through the tumor EP receptors. A. Western blot analysis of phosphorylated and total GSK3β in PC3 cells alone or in transwell co-culture with bone marrow adipocytes. **B.** Immunoblot expression examination of β-catenin in cytoplasmic or nuclear fractions in PC3 cells grown alone or in transwell with adipocytes. Laminin A.C was used as a control for nuclear fractions and tubulin for cytoplasmic fractions. **C.** Cyclin D expression in PC3 cells cultured with or without adipocytes in transwell. Tubulin was used as a loading control. **D.** Western blot analysis of phosphorylated and total GSK3β in tumor cells grown alone or in transwell co-culture with adipocytes in the presence or absence of EP1-3 receptor inhibitor AH6809 and EP4 receptor inhibitor GW627368X. Tubulin was used as a loading control.

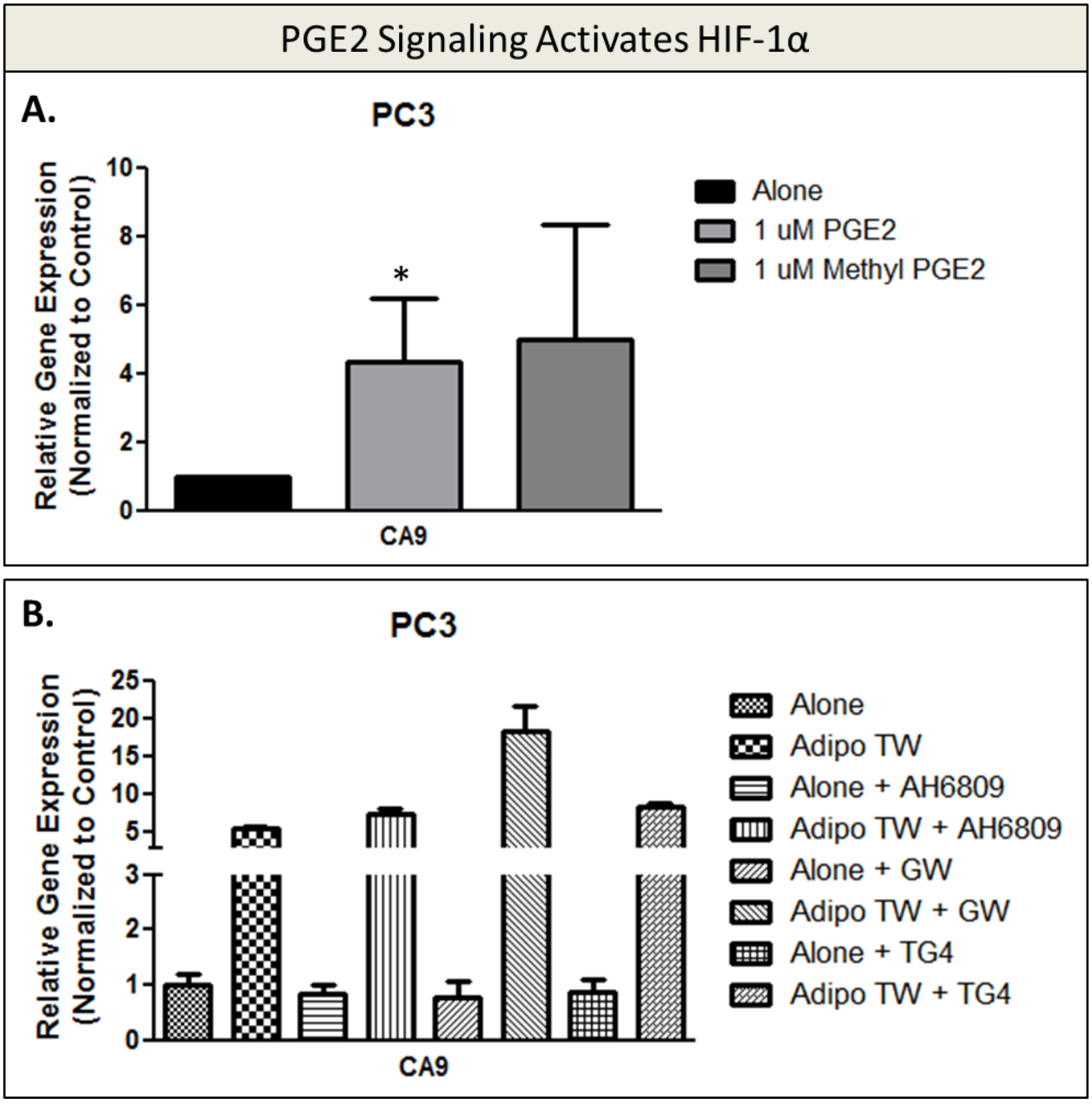

Figure 6: Activation of the EP1 receptor in PCa cells leads to the activation of HIF-1α signaling. A. Taqman RT-PCR analysis of *CA9* and *VEGF* in tumor cells alone or treated with PGE2 or 15-S-15 Methyl PGE2. **B.** Taqman RT-PCR analysis of *CA9* in tumor cells frown alone or in transwell with adipocytes in the presence or absence of EP receptor inhibitors AH6809 or GW6627368X or TG4-0155.

the signaling pathways responsible for the promotion of tumor growth and survival within the bone[454]. We believe that this distinct signaling network mediated by adipocyte-provided PGE2 activation of the GSK3β/β-catenin pathway in metastatic tissues revealed by our Oncomine analyses provides an important starting point for future studies investigating the contribution of the COX-2/PGE2 axis to progression and survival of metastatic prostate tumors in the bone microenvironment.

Data presented herein reveal marrow adipocytes as important players involved in shaping tumor cell signaling known to be involved in cell survival, proliferation, and chemoresistance in bone. To our knowledge, this is the first study demonstrating the importance of bi-directional interactions between marrow fat cells and tumor cells in activating tumor-secreted IL-1β activation of the COX-2/PGE2 axis in adipocytes, causing an activation of the tumor EP receptors and downstream signaling networks. Understanding the molecular mechanisms behind this regulation in bone is of critical importance in terms of potential treatment options for metastatic disease.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

Metastatic prostate cancer (PCa) is currently a deadly disease that affects many men throughout the world. When prostate cancer cells metastasize to the bone microenvironment, they come in contact with many different cell types present such as the commonly studied osteoblasts, osteoclasts, fibroblasts, and immune cells; however, one largely understudied component of the marrow space that tumor cells interact with are the bone marrow adipocytes. Bone marrow fat cells were largely thought to be "filler" in the bone space but increasing evidence has shown that these cells have endocrine, and paracrine effects on neighboring cells [497, 498]. The original work presented within this thesis was the first to investigate the interaction between metastatic prostate cancer cells and bone marrow adipocytes in the context of adipocytes altering the metabolism of PCa cells through lipid signaling. For our first project interrogating the effects of bone marrow adipocytes on tumor metabolism we were able to make the following conclusions: 1) Patients with metastatic disease have increased expression of glycolytic and hypoxic genes compared to primary PCa tumors; 2) tumors grown intratibially *in vivo* in diet-induced models of high marrow adiposity have increased expression of glycolytic and hypoxic genes compared to mice with fewer marrow adipocytes; 3) paracrine interactions between tumor cells and adipocytes *in vitro* induce expression of glycolytic and hypoxic proteins in tumor cells; 4) PCa cells exposed to adipocytes with increased expression of glycolytic markers exhibit enhanced Warburg metabolism with increases in lactate production, decreases in oxidative phosphorylation, and decreases in ATP production without perturbation of mitochondrial integrity or cellular viability; 5) tumor cells stimulate

lipolysis within adipocytes but the inhibition of lipolysis does not affect adipocyte-driven changes in PCa cell metabolism due to possible compensatory mechanisms; 6) metabolic effects are driven through the activation of HIF-1α in PCa cells as shown by increased expression of hypoxia-responsive genes and the reversal of adipocyte-induced metabolic changes upon knockdown of tumor cell HIF-1α.

Stemming from these results we aimed to elucidate the specific lipid mediators released by bone marrow adipocytes and involved in HIF-1α activation in our tumor cells. Literature searches and lipidomics analyses revealed PGE2 as a candidate bioactive lipid responsible for HIF-1α activation. After investigating the potential role of PGE2 in hypoxia signaling, we were able to deduce the following conclusions: 1) metastatic prostate tumor cells had increased expression of downstream targets of PGE2/EP1-4 axis in patients compared to primary tumors; 2) mice with elevated marrow adiposity had higher levels of COX2, enzyme responsible for PGE2 synthesis; 3) adipocytes exposed to tumor cells *in vitro* had increased expression levels of COX-1 and COX-2 and elevated levels of secreted PGE2 and paracrine signaling was involved in COX-2 regulation by tumor cells; 4) adipocyte expression of COX-1 and COX-2 was regulated by tumor-secreted IL-1B; 5) PGE2 receptor 1 (EP1) was upregulated in metastatic tumors in patients, in PCa cells in mice with elevated marrow adiposity, and in tumor cells co-cultured with adipocytes *in vitro*; 6) PGE2 from adipocytes activated the EP1, EP2, and EP4 receptors in tumor cells leading to downstream GSK3β/β-catenin signaling which are involved in cell growth ad survival; 7) PGE2 activated HIF-1α in tumor cells as shown by the activation of carbonic anhydrase 9.

Lastly, while investigating PGE2 as a candidate lipid, we looked at sphingosine-1-phosphate (S1P) an additional bioactive lipid responsible for the activation of HIF-1α in tumor cells. Unfortunately, our studies with S1P did not yield positive results on HIF-1α activation; however inhibition of SPHK1 does indeed inhibit HIF-1α-mediated metabolic changes, showing that S1P may be a necessary, but not sufficient molecule in metabolic regulation of tumor cells. The following conclusions can be drawn from our work with adipocyte-supplied S1P and S1P receptor signaling in tumor cells: 1) the enzyme that synthesizes S1P from sphingosine, sphingosine kinase 1 (SPHK1) was upregulated in adipocytes exposed to intratibially injected prostate cancer cells and was increased in mice with high marrow adiposity; 2) SPHK1 was upregulated in adipocytes exposed to prostate cancer cells *in vitro* via paracrine interactions with the tumor cells; 3) inhibition of SPHK1 abrogated the glycolytic and hypoxic phenotype of tumor cells exposed to bone marrow adipocytes, suggesting a role of S1P in modulating tumor metabolism; 4) S1P receptors 5 and 2 (S1PR5 and S1PR2) were upregulated in metastatic prostate tumors in patients compared to primary sites, whereas S1PR status did not change and decreases S1PR5 were observed in tumor cells exposed to adipocytes *in vitro*; 5) inhibition of S1PR1-3 did not affect the metabolic response of tumor cells to adipocytes; 6) treatment with S1P did not affect metabolic or hypoxic responses; 7) intrinsic S1P as shown by overexpression of SPHK1, did not make the cells more glycolytic or hypoxic; and 8) SPHK1 induction in adipocytes was shown to be partially mediated through tumor-activated HSL-mediated lipolysis.

6.2 Future Directions

Herein we show that major signaling pathways HIF-1α and GSK3β/β-catenin are activated in tumor cells exposed to adipocytes and is at least partially through prostaglandin E2 synthesis and release from adipocytes into the tumor microenvironment and activation of the EP receptors in tumor cells. The HIF-1α and GSK3β/β-catenin signaling pathways have been implicated in many functions critical to cancer growth, survival, angiogenesis, and chemoresistance [377, 450, 499-503]. The work presented within this thesis can be extrapolated to multiple projects looking at sruvaival of tumor cells through the activation of pro-survival pathways and/or chemoresistance and drug response.

6.2.1 Pro-survival Mechanisms/Chemoresistance

We have shown that interaction of PCa cells with marrow adipocytes increases their clonogenic potential and activates pro-survival pathways (Chapter 4, Fig 21 and 22), and this is coincident with upregulation of HK2, a glycolytic enzyme with pro-survival functions. Future work to extrapolate these findings would be to determine if HK2 bound to the mitochondria is critical for cellular survival through the inhibition of apoptosis. We will be able to address this using apoptosis inducing agents on tumor cells that have been exposed to adipocytes *in vitro* with or without HK2 knockdown via siRNA techniques or using mutated HK2 that can no longer bind to the mitochondria. Expected results would show that HK2 translocation to the mitochondria in tumor cells exposed to fat cells is required for resistance to apoptosis-inducing agents, effect abrogated by the disruption of mitochondrial binding of HK2. Additionally, examining their response specifically to Docetaxel, and its next generation derivative Cabazitaxel, would shed light on possible

adipocyte-mediated mechanisms of chemoresistance and survival of PCa cells within the bone microenvironment.

7.3 Clinical Relevance/Novel Targets

The work presented herein aimed to elucidate novel interactions between disseminated prostate cancer cells that have metastasized to the bone and residing bone marrow adipocytes. There have been many efforts in the clinic to target HIF-1α signaling, the COX-2/PGE2 axis, and the SPHK1/S1P axis that we have shown are dysregulated in tumor cells via adipocyte-driven effects.

Targeting tumor metabolism has been a continuous effort over generations of scientists and has been a hot topic over the recent years [504, 505]. Because we have shown in our studies, that adipocytes activate HIF-1α in PCa cells, modulate their metabolism and increase HK2 binding to the mitochondria, it is imperative to consider HIF-1α and HK2 as potential therapeutic targets in metastatic prostate cancer patients. There are many drugs that have been found to target various steps in HIF-1α signaling such as its stabilization, translocation to the nucleus, binding to HIF-1β, and transcription factor activity [502]. Current therapies for targeting tumor metabolism are highlighted in **Chapter 1** [506].

The COX-2/PGE2 axis has been less of a challenge to target. Given the inflammatory roles of COX-2 and PGE2 synthesis and action, there is extensive data using non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase activity, on cancer progression and prevention. In the context of metastatic prostate cancer, there have been conflicting reports of the benefits of NSAIDS as a treatment option. Recently, it was found that giving NSAIDs to patients with localized disease

reduced the incidence of metastasis [507], while others have shown that NSAID treatment actually was associated with an increase risk in PCa-related mortality [508]. Additionally, a large ongoing STAMPEDE trial reported that adding celecoxib (COX-2 inhibitor) to standard hormone therapy for men with metastatic prostate cancer showed no advantage compared to the control group receiving the standard hormone therapy itself [509]. Collectively, there have been largely inconsistent results from COX-2 inhibition in metastatic prostate cancer patients. *In vitro* assays have shown that NSAIDs induce apoptosis in PC3 and LNCaP cells and G1 arrest [510-512], that COX-2 overexpression in LNCaP cells makes them more resistant to radiation therapy [513], and that celecoxib treatment in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice have delayed tumor growth and progression [514, 515], suggesting the COX-2 axis as a viable targetable network. Additionally, preclinical work has been done using inhibitors of the prostaglandin receptors (EP1-4), showing benefits across many different cancer typed *in vitro* [444].

Lastly, emerging data have pointed to benefits of inhibiting SPHK1 and S1P signaling in cancer therapy. Many of the targets were created for treatment of multiple sclerosis-related inflammation but recent work has shown that using anti-S1P antibodies, small molecule inhibitors of S1P signaling [516], or SPHK1 inhibitors [517], effectively halts tumor angiogenesis [518], growth and survival [519]. Two well-known antibodies have been created to target S1P: Sphingomab and Sonepcizumab. Sonepcizumab (ASONEP) failed in phase II clinical trials for both Renal Cell Carcinoma (NCT01762033) and persistent pigment epithelial detachment in subjects with acute macular degeneration (AMD) or polypoidal choroidal vaculopathy (PCV) (NCT01334255). Additional clinical

studies have attempted inhibiting S1P receptors in multiple sclerosis and have had promising clinical results [520]. FTY720 is now FDA approved as a S1PR antagonist that competitively binds to S1PR1 as a structural analogue of S1P for multiple sclerosis [521] and has been shown to have anticancer roles as a therapy in preclinical work [522]. Specifically FTY720 has been shown to sensitize prostate cancer cells to radiotherapy [470], cause proteasomal degradation of SPHK1 in androgen-independent PCa cells [523], inhibit cell-cycle entry and induce apoptosis in prostate cancer cells [524, 525], inhibit *in vivo* tumor growth of androgen-independent PCa [526], inhibit invasion of androgen-independent PCa cells [527], and suppress overall aggressiveness of PCa cells [528].

Collectively, there have been many reports that suggest HIF-1α signaling, the COX-2/PGE2 axis, and the SPHK1/S1P axis are targetable pathways that have now been implicated in the crosstalk between metastatic prostate cancer cells and bone marrow adipocytes to facilitate tumor growth and survival in the bone niche. Future directions from the work presented within this thesis could use the aforementioned inhibitors to study PCa growth in bone as potential therapeutic targets *in vivo* to characterize the importance of these signaling networks for tumor growth in bone. [497]

APPENDIX

Adipocyte-Derived Sphingosine-1-Phosphate does not affect the Metabolic Phenotype of Metastatic Prostate Cancer Cells

A.1 Introduction

The uptake of lipids from the microenvironment, aberrant *de novo* lipid synthesis and alterations in fatty acid catabolism and steroidogenesis pathways are now emerging as key mechanisms linking dysregulated lipid metabolism in the primary prostate tumor with subsequent progression and reduced survival [299, 303, 304]. These lipids have the propensity to regulate signaling networks within cancer cells through receptor-mediated signaling pathways [414]. Previous work stemming from our laboratory has shown that metastatic prostate tumors in bone both induce lipolysis in bone marrow adipocytes and also utilize lipids from adipocytes to fuel their own metabolic processes that are largely lipid-driven [345, 360]; therefore, it is crucial to understand what lipids are responsible for tumor growth and survival in bone.

There is increasing emerging evidence that the lysosphingolipid sphingosine-1-phosphate (S1P), a bioactive lipid in the sphingolipid family, plays a role in cancer progression [455-457]. S1P is synthesized by the conversion of sphingosine to sphingosine-1-phosphate through its phosphorylation by the enzymes sphingosine kinase 1 (SPHK1) or sphingosine kinase 2 (SPHK2) [458, 459]. Once S1P is synthesized it can be exported out of the cell by specific sphingolipid transporters such as spinster 2 (SPNS2) [460, 461] where it exerts its autocrine and paracrine effects. Secreted S1P has the propensity to bind to five independent receptors known as S1PR1-5 [462]. S1P receptor signaling has been implicated in regulating a multitude of functions important for cancer progression such as autophagy [463], angiogenesis [464, 465], proliferation [466],

and many other processes [467]. S1P has an additional intrinsic function where SPHK1 or SPHK2 can translocate to the nuclear envelope or endoplasmic reticulum and synthesize S1P locally [468, 469].

Importantly, limited studies in prostate cancer have shown S1P receptor signaling to play critical roles in tumor-promoting autophagy through S1PR5 [463], sensitization to irradiation [470] and to Docetaxel, the standard chemotherapeutic agent for hormone-resistant PCa, docetaxel [471]. Re-sensitization of PCa cells was seen through the inhibition of SPHK1 and intracellular S1P production. One study examining effects of osteoblast-derived S1P on metastatic prostate cancer, ,showed its promoting effects on tumor cell growth and survival [472]. S1P has been shown to promote adipogenesis [473] and elevated serum levels of S1P were shown in obese patients compared to lean [474]; however, no studies have looked at the effects of bone marrow adipocyte-supplied S1P on prostate cancer cell growth and survival within the bone. Extending from our previous work we have shown that HIF-1α is activated in tumor cells exposed to bone marrow adipocytes [360]. Intriguingly, S1P signaling has been shown to have the propensity to activate HIF-1α signaling [475] and HIF-2α expression and activity [476].

The objective of this study was to elucidate the role of bone marrow adiposity in the modulation of tumor signaling networks within the bone microenvironment. Using *in vivo* models of diet induced marrow adiposity in combination with *in vitro* models of paracrine signaling between bone marrow adipocytes and prostate cancer cells, we show that tumor-stimulated activation of lipolysis in bone marrow adipocytes leads to induction of *SPHK1* and subsequent secretion of S1P into the tumor microenvironment. S1P then activates the S1P receptors in tumor cells; however, this does not modulate the

metabolism of the tumor cells or activate HIF-1α signaling as we had hypothesized. These results introduce a potential mechanism underlying other functions regulated by S1P in facilitating adaptation of metastatic tumors in bone and implicate bone marrow adipocyte, a cell type so abundantly present in the skeleton especially in advanced age and obesity, as a viable culprit in the progression of this currently incurable disease.

A.2 Results

A.2.1 Sphingosine Kinase 1 (SPHK1) is upregulated in adipocytes exposed to intratibially injected prostate cancer cells *in vivo*

To determine whether the hypoxic phenotype observed in PCa cells exposed to adipocytes was correlated with increased S1P production in adipocytes leading to activation of the HIF-1α, we analyzed mRNA expression of mouse *SPHK1* in adipocytes exposed to intratibially injected PC3 and ARCaP(M) cells in LFD and HFD mice. Our results revealed significantly increased host (mouse) transcript levels of *SPHK1* in bone tumors from HFD mice as compared to LFD mice (Figure 1), suggesting a potential contribution of marrow adipocytes to this process. These findings implicate tumor cells as potential regulators of adipocyte S1P production, which likely occurs through the activation of the SPHK1 enzyme in bone marrow fat cells. This led us to further investigate the mechanism of this regulation *in vitro.*

A.2.2 SPHK1 and S1P are upregulated in adipocytes exposed to prostate cancer cells *in vitro*

We utilized *in vitro* models of tumor cell-adipocyte interactions to determine if the activation of SPHK1/S1P axis observed in intratibial tumors *in vivo* is a direct effect of tumor cell action on adipocytes. We first examined *SPHK1* expression in adipocytes

exposed to PCa cells by employing a transwell system in which adipocytes were differentiated in the bottom chamber and tumor cells were then plated in the traswell insert and cultured together for 48 hours. This allowed the two cell types to share the media without direct interaction. *SPHK1* was significantly increased in adipocytes co-cultured with PCa cells (Figure 2A). Interestingly, lipidomics analyses of S1P levels in PCa cell lysates in transwell with adipocytes showed elevated levels of S1P (Figure 2B) This corresponded with lower levels of intracellular ceramides and increased sphingosines (Figure 2C and 2D). Additionally, adipocytes have elevated levels of sphingosine and S1P as well (Figure 2B) when exposed to PC3 cells in transwell co-culture. Important to note, S1P was only detected in a small amount of samples and further analysis is crucial to conclude that S1P levels are significantly higher in cells PC3 cells or fat cells in co-culture; however, these results suggest that adipocytes are synthesizing more sphingosine which is in turn converted to S1P and that this effect is also happening in tumor cells in which they are decreasing the balance of ceramide to sphingosine in favor of sphingosine synthesis.

The fact that transwell co-culture with tumor cells caused the activation of *SPHK1* and increase S1P production in adipocytes suggested that this process does not require physical interaction between the tumor cells and adipocytes. Therefore, we next examined whether the media conditioned by the PCa cells alone (PC3/ARCaP(M) CM) can facilitate similar changes in adipocytes as observed in transwell co-culture. Interestingly, treatment with either PC3 or ARCaP(M) conditioned media induced expression of *SPHK1* in adipocytes (Figure 2D), suggesting that paracrine signaling is required between the adipocytes and tumor cells for the subsequent phenotype.

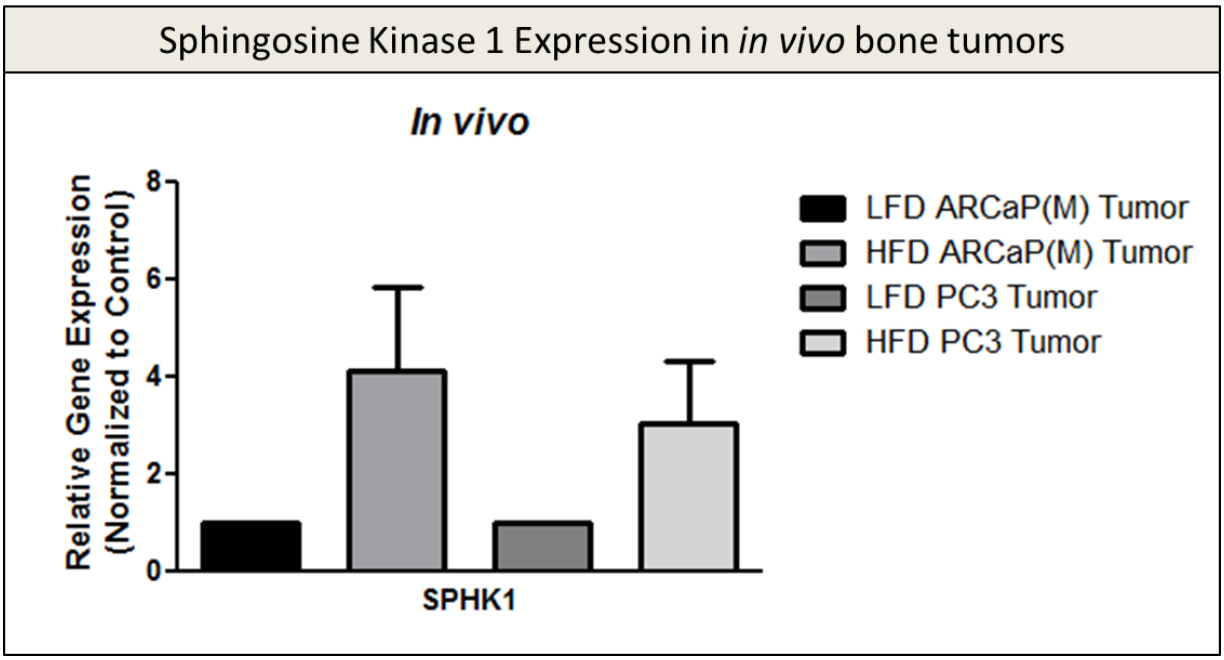

Figure 1: *SPHK1* expression increases in bone marrow adipocytes exposed to prostate cancer *in vivo*. Taqman RT-PCR analysis of mouse *SPHK1* expression in adipocytes exposed to both ARCaP(M) and PC3 cells that were intratibially injected and allowed to grow in the marrow space in high fat diet- (HFD) or low fat diet (LFD) fed-mice. Data were normalized to mouse *Adiponectin* and represent a mean of a minimum of 3 mice/group $\pm$ SD.

A.2.3 Inhibition of SPHK1 abrogates the metabolic effect of adipocytes on PCa cells

Because *SPHK1* is upregulated in adipocytes exposed to tumor cells *in vivo* and *in vitro*, we used a pharmacological inhibitor of SPHK1 activity, sphingosine kinase inhibitor 2 (SKI-2) [207, 477, 478] and assessed the hypoxic and metabolic effects of adipocytes on tumor cells *in vitro*. We have previously shown that bone marrow adipocytes enhance the Warburg phenotype in PCa cells through the activation of HIF-1α [360]; Therefore, we looked at the genes involved in activation of HIF1a and glycolysis in tumor cells interacting with adipocytes upon SPHK1 inhibition. Interestingly, we observed that treatment with SKI-2 abrogated the effects of the adipocytes on tumor metabolism, suggesting a role of S1P in regulating the hypoxic signature of tumor cells exposed to adipocytes (Figure 3A and B). This effect was observed in both PC3 and C4-2B cells (Figure 3C).

A.2.4 Sphingosine-1-phosphate receptor expression status in patients with metastatic prostate cancer compared to primary prostate cancer

S1P is a bioactive lipid that has the propensity to activate cellular signaling through five different S1P receptors (S1PR1-5) [479]. We next performed an *in silico* Oncomine database analysis of S1P receptors in metastatic prostate cancer tumors compared to primary prostate tumors. Of the five receptors, S1PR5 and S1PR2 were significantly upregulated in metastatic tumors (Figure 4A), showing a possible importance of individual S1P receptors in S1P signaling in bone tumors.

A.2.5 S1P receptor 1-5 expression levels in tumor cells exposed to adipocytes *in vitro*

Stemming from the *in silico* analysis fo S1P receptor expression in metastatic tumors compared to primary tumors, we then looked at the S1PR1-5 expression status *in vitro* in tumor cells exposed to adipocytes in transwell co-culture (Figure 4B). Using primers designed to recognize each of the five S1P receptors, we observed no significant increases in S1PR1, S1PR2, or S1PR4, significant decreases in S1PR5, and undetectable levels of S1PR3 in tumor cells exposed to adipocytes in transwell co-culture. Although these results were disappointing, receptor expression levels do not always correlate with receptor activity. Therefore we used antagonists to different S1P receptors and assessed the hypoxic and metabolic activity of the tumor cells upon receptor inhibition. Specifically, we utilized VCP23019 to inhibit the activity of S1PR1 and S1PR3 [480-482] and JTE-013, to selectively target S1PR2 [482, 483]; however, we did not observe any changes in the induction of the hypoxia-associated gene *CA9* or the glycolysis associated genes *ENO2* and *HK2* (Figure 5), suggesting that S1P may not be activating the HIF-1α pathway through S1PR1, S1PR3, or S1PR4.

A.2.6 S1P may act as a necessary, but not sufficient, cofactor for HIF-1α activation in tumor cells

We then sought to determine which of the S1P receptors is involved in signaling leading to HIF1a activation. We used pharmacological receptor agonists as well as synthetic S1P itself, to activate each of the receptors and look at both a hypoxic and metabolic response in the tumor cells (Table 1). Neither treatment with S1P itself nor any of the agonists activated a hypoxic or glycolytic response in the tumor cells (Figure 6A-C), suggesting that S1P signaling through the S1P receptors is not sufficient for HIF-1α activation. S1P has been shown to activate Akt signaling and as a control, we assessed

the phosphorylation of Akt. We observed that upon exposure to S1P, Akt is being activated in the PC3 and ARCaP(M) cells suggesting that S1P is functionally active (Figure 6D). We have previously shown that paracrine interactions between tumor cells and adipocytes are critical for regulation of tumor metabolism. This was determined by treating the tumor cells with media conditioned by a co-culture of tumor cells and adipocytes. Treatment with adipocyte conditioned media alone is not sufficient to induce metabolic changes in tumor cells therefore, we postulated that because tumor cells are required to increase S1P levels from adipocytes through the induction of SPHK1, S1P is the missing factor in adipocyte conditioned media compared to the co-culture conditioned media. Conversely, adipocyte conditioned media induces the expression of lipid transporters that are critical for the uptake of lipids such as S1P. To test this hypothesis, experiments were performed in which tumor cells were treated with S1P that was added to adipocyte conditioned media. We saw that even though lipid transporters were increased under those conditions, they were not responding metabolically to the addition of S1P as us we have observed upon treatments with direct co-culture conditioned media (Figure 6E).

A.2.7 Intrinsic SPHK1 overexpression does not activate a hypoxic or glycolytic phenotype in PCa cells

S1P can signal through its receptors but also has cellular-intrinsic signaling [468]. Because we see *SPHK1* induction in adipocytes and the inhibition of SPHK1 using SKI-2 abrogates the metabolic effects of adipocytes on tumor cells, a phenotype not inhibited by receptor antagonists, we pursued cellular-intrinsic signaling within the tumor

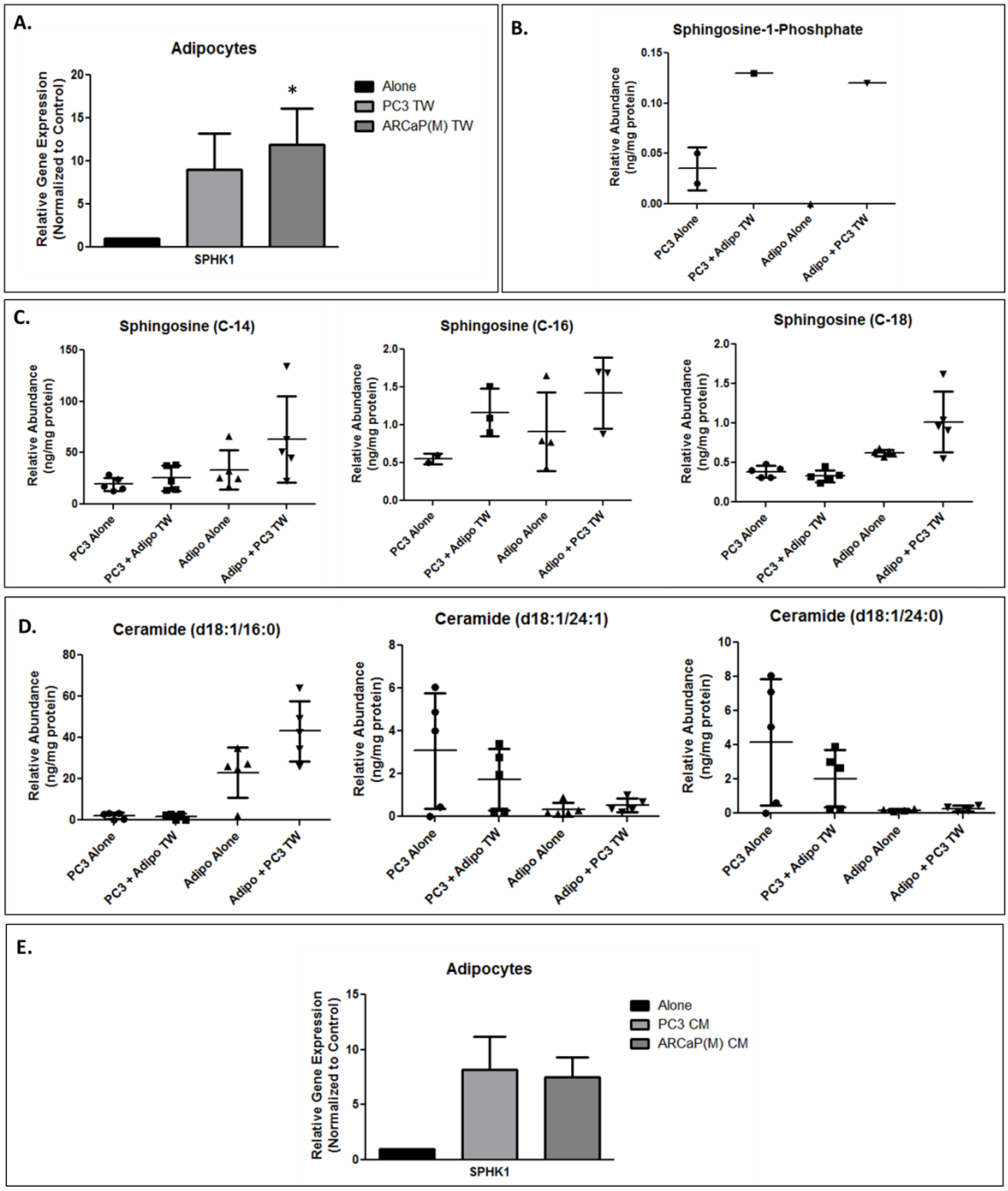

Figure 2: *SPHK1* expression and activity increases in adipocytes exposed to prostate cancer cells *in vitro*. A. Taqman RT-PCR analysis of *SPHK1* expression in adipocytes in transwell co-culture with PC3 and ARCaP(M) cells *in vitro*. Lipidomics analysis of sphingosine-1-phosphate (S1P) levels (**B.**), sphingosines (**C**.), and ceramides (**D.**) in tumor cells and adipocytes alone or in co-culture. **E.** Expression of *SPHK1* in adipocytes treated with PC3 or ARCaP(M) conditioned media.

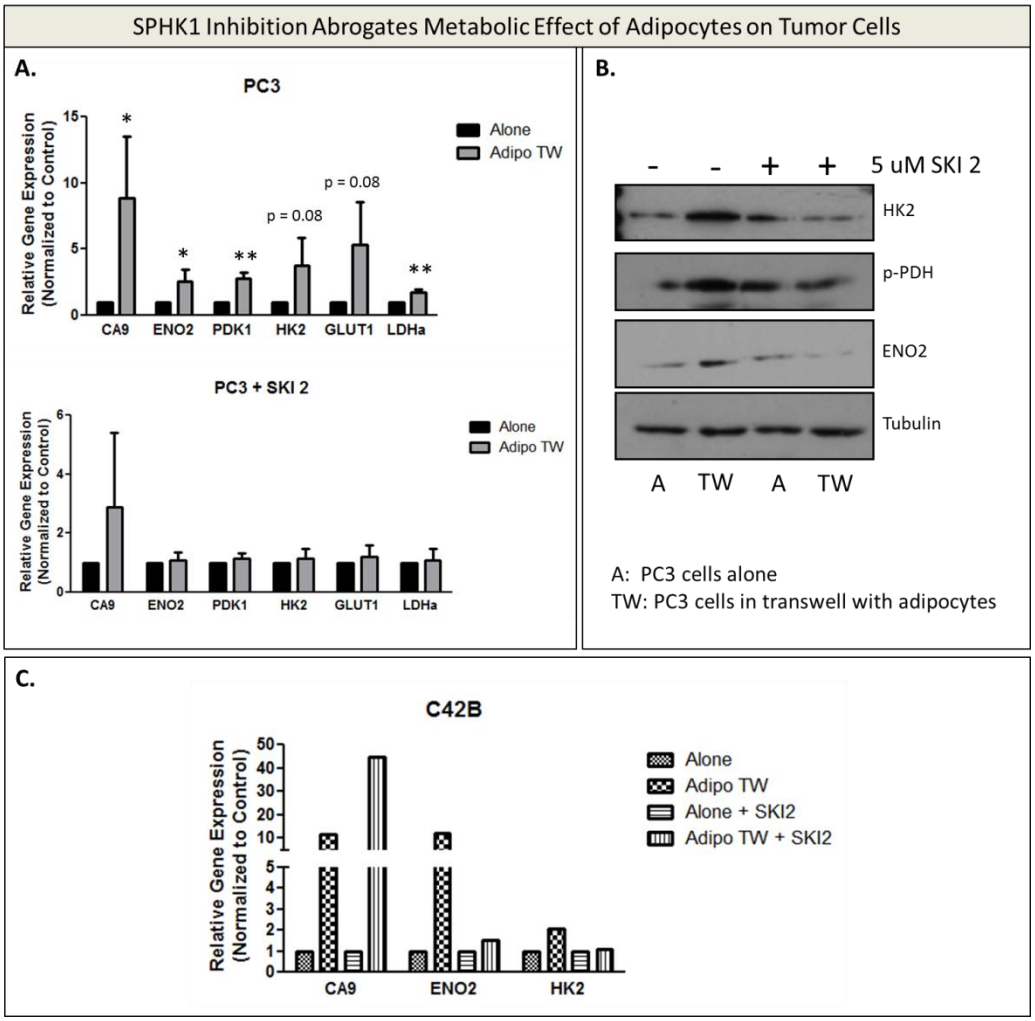

Figure 3: SPHK1 inhibition using SKI2 abrogates metabolic effects of adipocytes on prostate cancer cells. A. Taqman RT-PCR analysis of Warburg Effect associated genes *ENO2*, *PDK1*, *HK2*, *GLUT1*, and *LDHa* and hypoxic marker *CA9* in tumor cells alone or in transwell co-culture with adipocytes in the absence (top) or presence (bottom) of SKI2, a SPHK1 inhibitor. Data are normalized to *HPRT1* and shown relative to control. **B.** Western blot analysis of Warburg Effect associated proteins HK2 and ENO2 and phosphorylated PDH, indicative of enhanced PDK1 activity in PC3 cells alone or in transwell with adipocytes with or without 5 uM SKI2. Tubulin was used as a loading control. **C.** Taqman PCR of hypoxic and glycolytic genes in C4-2B PCa cells alone or in transwell in the presence or absence of 5 uM SKI2. Results represent a mean of at least 3 independent experiments ± SD. Values * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ are considered statistically significant.

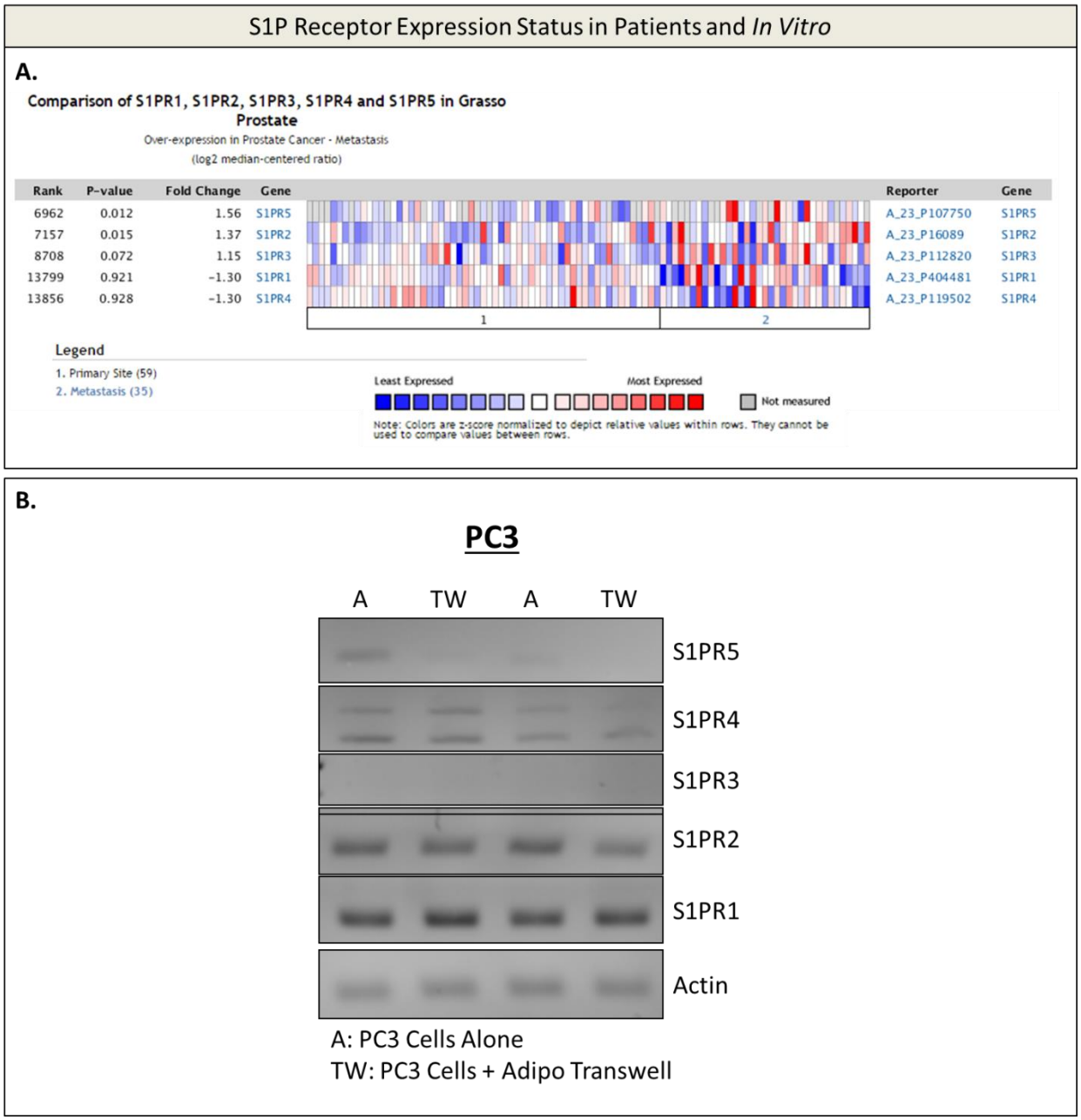

Figure 4: S1P receptor expression status in patients with metastatic prostate cancer compared to primary prostate cancer and PCa cells exposed to bone marrow adipocytes *in vitro*. A. Oncomine gene analysis comparing the expression of metabolic genes (*S1PR1-5*) in patient samples collected from metastatic or primary sites. Data were ordered by "overexpression" and the threshold was adjusted to *P*-value < 1E-4; fold change, 2 and gene rank, top 10%. **B.** Qualitative PCR of *S1PR1-5* in PC3 cells co-culture with adipocytes in transwell. Actin was used as a loading control.

cells that is not receptor-mediated. We first looked at *SPHK1* levels in tumor cells to see if not only the adipocytes are increasing levels of *SPHK1*, but also tumor cells; however, Taqman real-time PCR results showed no significant changes in *SPHK1* levels in PCa cells alone or exposed to adipocytes in transwell co-culture (Figure 7A). We then stably overexpressed *SPHK1* in the tumor cells to increase intrinsic S1P levels (Figure 7B) and assessed the metabolic effects of increasing *SPHK1* expression in tumor cells (Figure 7C). Surprisingly, there was no effect of *SPHK1* overexpression on the metabolic phenotype seen in tumor cells exposed to adipocytes, showing that intrinsic S1P signaling also does not have an effect on tumor metabolism.

A.2.8 Activation of hormone sensitive lipase-mediated lipolysis by PCa cells enhances *SPHK1* expression in adipocytes

Lastly, we pursued the regulation of *SPHK1* in bone marrow adipocytes exposed to PCa cells. Previous literature has shown that *SPHK1* and the production of S1P is regulated by hormone sensitive lipase (HSL-)-mediated lipolysis in white adipose tissue [207]. We tested this in bone marrow adipocytes using a chemical inducer of lipolysis, forskolin. Upon treatment with forskolin, we observed a large increase in *SPHK1* expression in bone marrow fat cells, suggesting that activation of lipolysis increases *SPHK1* levels (Figure 8A). We then used inhibitors of adipose triglyceride lipase (ATGL), Atglistatin, and hormone sensitive lipase, BAY59-9435 (BAY), and saw a marked decrease in *SPHK1* expression with BAY treatment but not with Atglistatin, showing that this regulation of *SPHK1* is mediated by HSL activation during lipolysis (Figure 8B) similar to studies in white adipose tissue [207]. Studies are currently ongoing to determine the contribution of the phosphorylation (activation) of HSL in

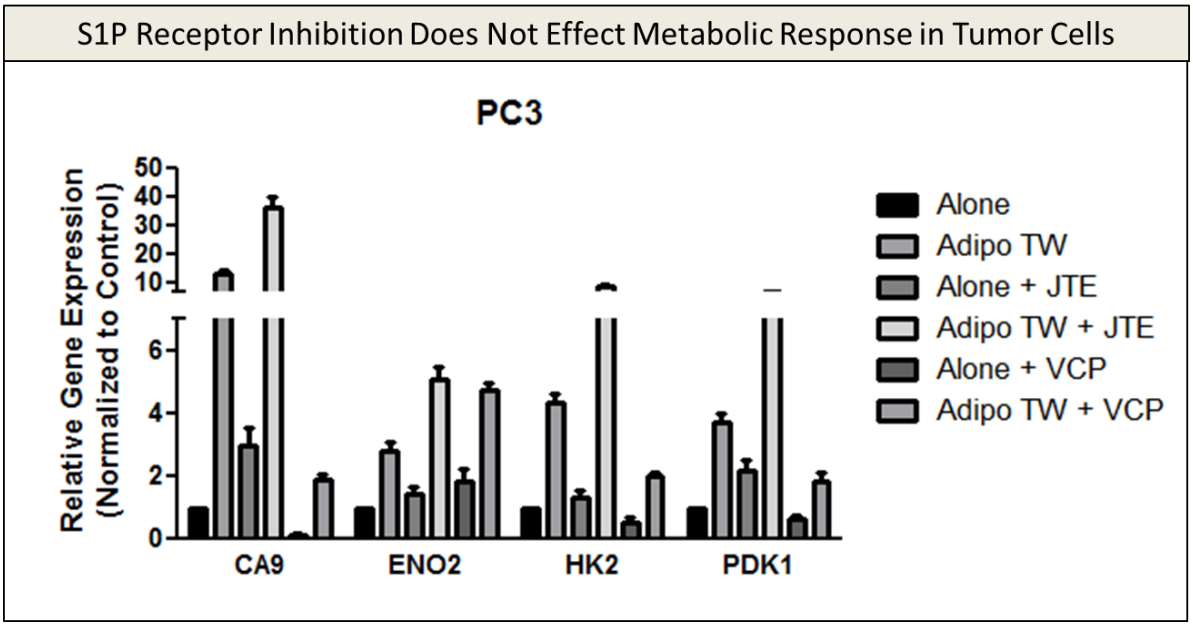

Figure 5: S1PR inhibition does not abrogate effects of adipocytes on tumor metabolism. Taqman RT-PCR analysis of hypoxic response marker *CA9* and glycolytic genes *ENO2*, *HK2*, and *PDK1*. Data were normalized to *HPRT1* and shown as fold change relative to PC3 cells alone. Experiment was done one time and experimental replicates are shown as SD.

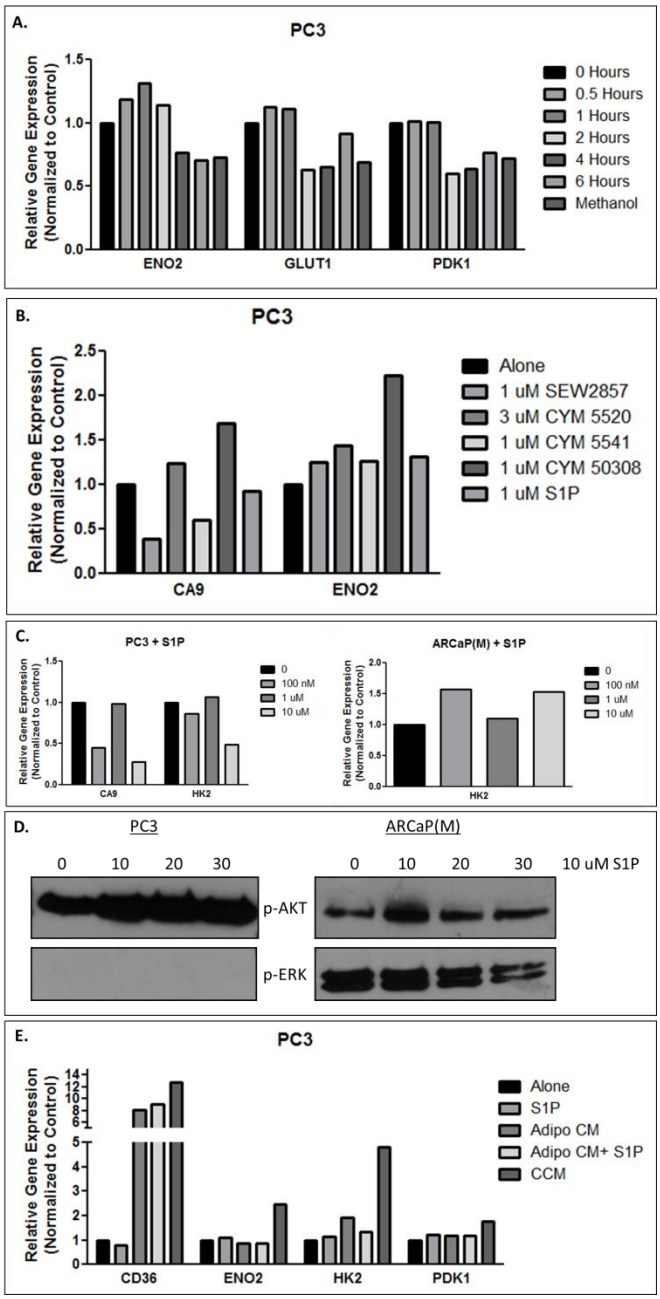

Figure 6: Treatment with S1P is not sufficient to induce Warburg phenotype in PCa cells as seen by bone marrow adipocytes. A. Taqman RT-PCR analysis of glycolysis markers *ENO2*, *GLUT1*, and *PDK1* after treatment with 500 nM S1P for 0, 0.5, 1, 2, 4, or 6 hours. **B.** Gene expression analysis of *CA9* and *ENO2* in tumor cells treated with S1PR agonists or S1P itself. **C.** Taqman RT-PCR of *CA9* and *ENO2* in PC3 cells (Left) or *ENO2* in ARCaP(M) cells (Right) at multiple concentrations of S1P. **D.** Immunoblot measurement of phosphorylated Akt and ERK in tumor cells treated with 10 uM S1P over a time course in PC3 cells (Left) and ARCaP(M) cells (Right). **E.** Taqman RT-PCR of *CD326*, *ENO2*, *HK2*, and *PDK1* in PC3 cells treated with 10 uM S1P, Adipocytes Conditioned Media (Adipo CM), Adipo CM with 10 uM S1P, or Adipocyte tumor co-culture conditioned media (CCM).

adipocytes in co-culture with PCa cells and if this correlates with S1P synthesis. It has been shown that HSL activation leads to upregulated c-Jun N-terminal kinase (JNK)/activating protein-1 (AP-1) signaling and *SPHK1* induction [207], so we will examine the JNK/AP-1 pathway in adipocytes exposed to PCa cells to determine how HSL activation regulates *SPHK1*. Also, we will determine if BAY treatment completely or partially inhibits HSL activation because BAY only partially decreases the robust increase in *SPHK1* expression levels in adipocytes exposed to tumor cells. Additional experiments will be designed to use other specific inhibitors of HSL [484-487] to show the importance of this specific lipase to the regulation of *SPHK1* expression.

A.3 Discussion

The results presented above reveal an important contribution of tumor cell-induced lipolysis in bone marrow adipocytes leading to sphingosine-1-phosphate (S1P) production and a possible regulation of tumor metabolism. We show that marrow fat cells are capable of secreting S1P that either binds to S1PR1-5 or is internalized and used within tumor cells but that S1P alone is not sufficient to cause a metabolic effect within tumor cells, even though it may be necessary. We also demonstrate that tumor cells are able to modulate the secretion of S1P from a fat cell. They do so by inducing lipolysis and the activation of specifically hormone sensitive lipase (HSL). This speaks to the importance of the supportive host microenvironment in tumor progression and demonstrates the crosstalk between the tumor cells and host adipocytes. This adipocyte-tumor cell interaction ultimately shapes the cell signaling within the tumor cell allowing for the adaptive survival in the metastatic niche.

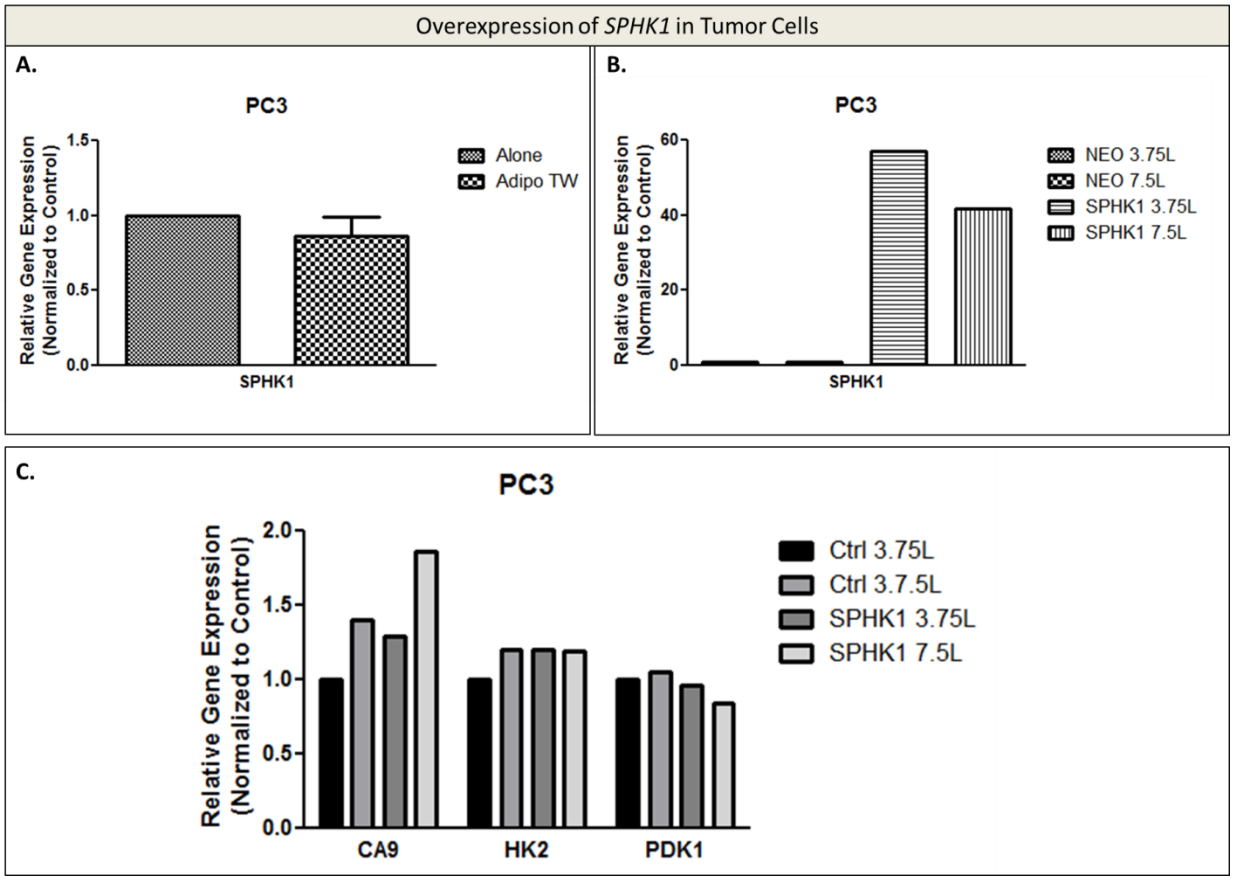

Figure 7: Overexpression of *SPHK1* in PC3 cells does not affect the metabolic phenotype. A. Taqman RT-PCR analysis of *SPHK1* expression in tumor cells alone or in transwell with adipocytes. **B.** Quantitative real time PCR of *SPHK1* expression stably expressed in PC3 cells in control Neomycin vector (Neo) or with *SPHK1* overexpressing vector (SPHK1) with two different concentrations of Lipofectamine3000 (3.75 and 7.5 ul). **C.** Taqman RT-PCR of *CA9*, *HK2*, and *PDK1* in PC3 cells overexpressing *SPHK1* compared to control cells. Data were normalized to *HPRT1* and shown as a fold change relative to control.

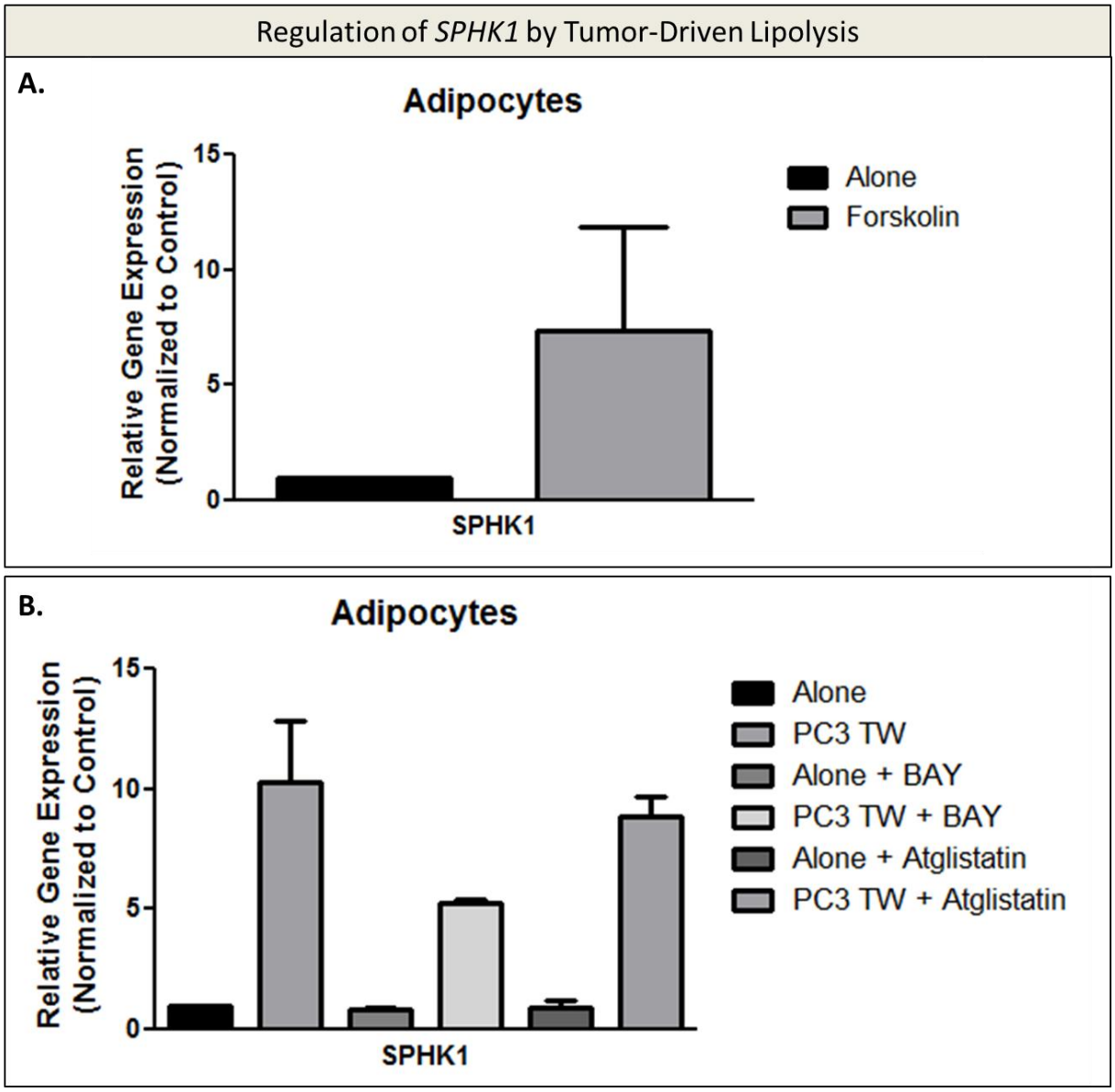

Figure 8: Adipocyte *SPHK1* expression is regulated by the induction of HSL-mediated lipolysis by tumor cells. A. Taqman RT-PCR analysis of *SPHK1* in adipocytes in the presence or absence of lipolysis inducing agent Forskolin. **B.** Gene expression analysis of *SPHK1* in adipocytes exposed to tumor cells in traswell co-culture in the presence or absence of HSL inhibitor (BAY) or ATGL inhibitor (Atglistatin). Data are normalized to *Adiponectin* and shown as fold change relative to control.

We focused on the SPHK1/S1P pathway because activation of the S1P receptors by S1P has been shown to facilitate tumor aggressiveness and survival by inducing the expression of many proteins involved in angiogenesis [465, 488], proliferation [[472[Hu, 2010 #3960], migration [489] and many other hallmarks of cancer [490]. Additionally, microarray studies have shown that high levels of SPHK1 and S1P signaling are correlated with a poor prognosis in breast cancer [491] and other cancers such as lung and ovarian cancer [456]. Importantly, for the context of our studies, it has been shown that S1P can activate HIF-1α [475, 492]. Unfortunately, our data do not support these findings in prostate cancer cells, showing that S1P is not sufficient to activate HIF-1α signaling in PC3 or ARCaP(M) cells.

Although our data did not support our hypothesis, we have shown that sphingosine and S1P levels are increased in both tumor cells and adipocytes in co-culture compared to alone conditions. This S1P can be secreted into the tumor microenvironment and regulate many different signaling networks in tumor cells that aid in growth and survival within the bone. Additionally there are many intracellular targets of S1P [468, 493] and it appears that tumor cells are also decreasing ceramide levels and increasing sphingosine and sphingosine-1-phosphate levels, indicative of a shift in balance toward sphingosine synthesis and away from ceramide. This is important because it has been shown that increased ceramide levels correlate with induction of apoptosis and increasing the sphingosine levels serves as a pro-survival mechanism for tumor cells [494]. Surprisingly, we also observed that adipocytes exposed to tumor cells have increased production of certain ceramide species (Figure 2D). This could possibly be due to the parasitic nature of tumor cells feeding off of the adipocytes inducing lipolysis and lipid transfer [345],

leading to apoptosis of the adipocytes. Experiments will be performed to determine if adipocytes are undergoing early stages of apoptosis after tumor cells deplete them of their lipid and nurients. Future studies will also be utilized to assess the expression and function of ceramidase enzyme sphingolipid delta-4 desaturase (DES1) in tumor cells and adipocytes that are responsible for ceramide catabolism to sphingosine and ceramide synthase that converts sphingosine to ceramide. Collectively, this work will show us if there are enzymes responsible for the accumulation of sphingosine and S1P, but also increases in ceramides in the adipocytes. Also, lipidomics assays will be performed on media conditioned by adipocytes and tumor cells and adipo/tumor co-cultures to determine if this S1P is secreted and possible roles of S1P in the local or systemic tumor microenvironment.

A future avenue that is worth consideration is the functional role of S1P in the activation of autophagy in tumor cells leading to a cytoprotective effect via activation of ER stress responses [463, 495, 496]. In the context of bone marrow adipocyte interactions with prostate cancer cells, S1P levels driven by tumor-induced lipolysis within the adipocytes could contribute to activation of ER stress and autophagy pathways within adipocytes, a crosstalk that is yet to be explored.

The rationale for our study was based on the gene expression analyses of bone marrow adipocyte samples suggesting clearly distinct increases in *SPHK1* expression and extensive literature showing SPHK1/S1P signaling has the propensity to activate HIF-1α as a mechanism to explain the lipid-mediated crosstalk between bone marrow adipocytes and prostate cancer cells within the bone microenvironment. Unfortunately, however, we have shown that S1P itself is not sufficient to activate HIF-1α and induce

metabolic changes in the tumor cells. We believe that showing that the SPHK1/S1P axis is dysregulated, although not sufficient to alter the metabolism of the tumor cells, provides an important starting point for future studies. These findings illuminate novel avenues of investigation for elucidating the contribution of the SPHK1/S1P axis to progression and survival of metastatic prostate tumors in the bone microenvironment.

Data presented herein reveal marrow adipocytes as important players involved in shaping tumor cell signaling known to be involved in cell survival, proliferation, and chemoresistance in bone. To our knowledge, this is the first study demonstrating the importance of bi-directional interactions between marrow fat cells and tumor cells in activating tumor-activated lipolysis, stimulating the expression and activity of the SPHK1/S1P axis in bone marrow adipocytes, causing an activation of the tumor S1P receptors or internalization, and activation of intracellular targets of S1P and downstream signaling networks. Understanding the molecular mechanisms behind this regulation in bone is of critical importance in terms of potential treatment options for metastatic disease.

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ABSTRACT

THE EFFECTS OF BONE MARROW ADIPOCYTES ON METASTATIC PROSTATE CANCER CELL METABOLISM AND SIGNALLING

by

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August 2017

Advisor: Dr. Izabela Podgorski

Major: Cancer Biology

Degree: Doctor of Philosophy

Bone is a preferential site of metastasis from prostate cancer (PCa). Although there have been many advances in therapeutic options for patients suffering from metastatic PCa, this disease remains incurable with an estimated five-year survival of 33%. To design effective therapeutic interventions for metastatic PCa, it is essential that we elucidate the molecular mechanisms responsible for tumor cell adaptation to and the ability to thrive within the bone metastatic niche. Age and obesity, conditions that increase adipocyte numbers in bone marrow, are risk factors for skeletal metastases from PCa; therefore, our laboratory is focused on the interactions between marrow adipocytes and PCa cells.

We initially detailed the metabolic alterations that occur in prostate cancer cells in response to interactions with bone marrow adipocytes in multiple *in vivo* and *in vitro* models. The following conclusions were drawn as a result of these experiments: 1) Patients with metastatic disease have increased expression of glycolytic and hypoxic genes compared to primary PCa tumors; 2) tumors grown intratibially *in vivo* in diet-induced models of high marrow adiposity have increased expression of glycolytic and

hypoxic genes compared to mice with fewer marrow adipocytes; 3) paracrine interactions between tumor cells and adipocytes *in vitro* induce expression of glycolytic and hypoxic proteins in tumor cells; 4) PCa cells exposed to adipocytes with increased expression of glycolytic markers exhibit enhanced Warburg metabolism with increases in lactate production, decreases in oxidative phosphorylation, and decreases in ATP production without perturbation of mitochondrial integrity or cellular viability; 5) tumor cells stimulate lipolysis within adipocytes but the inhibition of lipolysis does not affect adipocyte-driven changes in PCa cell metabolism due to possible compensatory mechanisms; 6) metabolic effects are driven through the activation of HIF-1α in PCa cells as shown by increased expression of hypoxia-responsive genes and the reversal of adipocyte-induced metabolic changes upon knockdown of tumor cell HIF-1α.

Additionally, we found novel signaling pathways are activated in tumor cells due to cross talk between tumor cells and adipocytes. We observed a regulation of COX-2 in adipocytes by tumor-secreted IL-1β that leads to increased PGE2 synthesis and release and this PGE2 signals through the EP receptors on the tumor cells to elicit downstream GSK3β/β-catenin signaling and subsequent HIF-1α activation.

We also observed increased SPHK1 in adipocytes exposed to tumor cells as an effect of tumor-stimulated lipolysis within adipocytes, but that S1P was not sufficient to activate HIF-1α signaling in tumor cells or downstream metabolic alterations.

In summary, we have discovered novel crosstalk between metastatic prostate tumor cells and bone marrow adipocytes that cause activation of many pathways involved in tumor survival and growth within the bone. We have revealed a functional contribution of bone marrow adipocytes to altered tumor metabolism and signaling in bone. The

expected outcome of this research is the validation of the significance of adipocyte-derived lipids in growth and aggressiveness of metastatic PCa in bone. The ultimate goal is utilize findings from this study to explore whether adipocyte-driven metabolic adaptation contributes to chemoresistance of skeletal tumors and whether targeting tumor metabolism offers new options for improved therapy and/or prevention of aggressive disease.

AUTOBIOGRAPHICAL STATEMENT

JONATHAN DIEDRICH

I enrolled in the PhD program at the Wayne State University School of Medicine in the summer of 2012. I ultimately chose to perform my dissertation work in Dr. Izabela Podgorski's laboratory in the summer of 2014. I was interested in Dr. Podgorski's work investigating interactions between prostate cancer cells and bone marrow adipocytes as a means to understand how obesity and bone marrow fat affect tumor progression in bone. Additionally, it was very clear that Dr. Podgorski would set me up for success in academia and would be a fantastic mentor to me through my graduate studies.

Working with Dr. Podgorski, I was able to collaborate with clinical faculty at the Karmanos Cancer Institute (KCI) and a multitude of brilliant independent investigators at Wayne State University, helping to not only strengthen my technical skills and learn the clinical applications of our work, but to network with other scientists and work together toward a common goal.

Dr. Podgorski gave me ample opportunities to present my work at internal, domestic, and international conferences where I have given a poster presentation in Vancouver, BC, Canada, Toledo, OH, New Orleans, LA, Bar Harbor, ME, Indianapolis, IN, Windsor, ON, Canada, and Lille, France and give oral presentations in Memphis, TN, Tampa, FL, and Rotterdam, Netherlands. These presentations have been instrumental in helping me network and hone my presentation skills.

Dr. Podgorski's hard work and valuable mentoring has been paramount for my success and I will always be grateful for everything she has done molding me into an independent investigator.