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AMPK and mTOR Impose Dualistic Regulation of Mitophagy and Autophagy in In Vitro Models of Prostate Cancer

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AMPK and mTOR Impose Dualistic Regulation of Mitophagy and Autophagy in *In Vitro* Models of Prostate Cancer

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SUMMARY

Prostate cancer is the abnormal growth and proliferation of previously normal cells of the prostate and has the second highest incidence in men worldwide. Physiologic manipulation of AMP-activated protein kinase (AMPK), a highly conserved enzyme responsible for regulation of energy homeostasis during metabolic stress, is a potential treatment, especially for metastatic, castration-resistant prostate cancers. AMPK is a known inhibitor of the enzyme mTOR, the final enzyme in the PKT/AKT/mTOR pathway responsible for cell growth and proliferation signaling. The hypothesis of this project is that activation of AMPK leads to increased mTOR-dependent mitophagy and subsequent autophagy in prostate cancer cells. The overall aim of this project is to establish a role between activated AMPK and mTOR dependent mitophagy and autophagy. Although preliminary data remains inconclusive, future research is promising.

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INTRODUCTION

A Brief Introduction to Cancer

Cancer can be defined as the development of abnormal growth and division in derivations of normal cells, leading to cancerous stem cells (CSCs). CSCs are believed to interrupt body-preserving homeostasis (1). Although there are differing theories regarding the origin of cancer cells (1-3), it is widely agreed upon that all cancer cells are similar in that they share a group of characteristics and together these characteristics encourage abnormal growth, proliferation, and migration. For clarification, this project will define growth as in increase in individual cell size, proliferation as an increase in individual numbers of cells within a population via division or other such reproduction, and migration as the movement of those cells throughout their host organism. One review suggests the cardinal characteristics defining cancer cells are the developed abilities to sustain proliferative signaling, evade growth suppressors, activate invasion and metastasis, enable replicative immortality, induce angiogenesis, and resist cell death (4). These may be influenced by a phenomenon known as Warburg's Effect, a drastic change in physiology seen in cancer cells, which aids in their rapid proliferation and migration (5).

Again, opinions differ regarding cancer cell etiology. *The stem cell division theory of cancer,* a review published in 2018, theorizes risk of cancer cell development is correlated with the number of divisions the cell has undergone since its original ancestor, the zygote. This theory, the stem cell division theory, suggests that cancer-associated DNA mutations are only a class of cancer-promoting factors. This review suggests other classes of cancer-promoting factors include pathological and environmental factors (6).

A year later, Niculescu published a review discussing the atavistic and stochastic cancer cell theories. Niculescu's review favored the atavistic theory, which suggests genetic and epigenetic influences, including post-translational modification, non-specifically transform cells, thus activating biochemical mechanisms and population dynamics conserved from the cells' primitive, unicellular ancestors. The stochastic theory suggests cancer occurs in otherwise normal cells due to accumulation of DNA mutations (2). As an interesting side note, the stochastic theory is named after the differential equation it is based upon, which is mathematically analytical in nature and beyond the scope of this project (7). Argument in favor of the atavistic theory includes evidence suggesting that loss of gene expression, the most clearly understood epigenetic influence on cancer cell development, occurs via transcription silencing ten times more often than it occurs via genetic mutations (8).

Yet another research group studying cancer cell origin suggests that for cancer cells to migrate, or become metastatic, the cancerous cell population as a whole must have heterogeneity and stochasticity. These researchers believe individual cells in the population have specific migration capacities and individual cell migration capacities change temporally throughout each cell's existence. Furthermore, these researchers suggest heterogeneity is inherent in a metastatic cancer cell population, leading to unique challenges when developing a model for metastasis (3).

Although the origin of cancer cells is debatable, the following review of cancer investigates aberrant regulation and execution of cell-cycle progression and regulation, DNA

repair, and cell reproduction. This investigation will be focused on normal physiologic processes and the deviations from these pathways related to cancer development and progression.

The cell cycle can be described in layman's terms as the process by which cells of the body, or any eukaryotic cells for that matter, carry out their daily function and divide (9-12). For this study, discussion of the cell cycle will focus on human somatic cells, diploid cells of the body at risk of becoming malignant and from which models of cancer have been previously derived (13). Somatic cell division as a normal means of proliferation, and thus tissue maintenance, is known as mitosis and will be discussed in brief.

The cell cycle is mostly spent in interphase, a time during which cell growth and preparation for division occurs. Cells spend approximately 90% of the cell cycle in interphase, which includes the phases G1, S, and G2; times when growth without DNA replication, DNA replication, and synthesis of proteins to prepare for division take place, respectively (10). During the S phase, when DNA is replicated, each strand of DNA is unwound and copied via highly specific mechanisms to produce two identical copies of that strand. While the DNA is being unwound, replicative machinery travels along each strand of original DNA, ultimately creating two semi-conservative copies of the original DNA strand (14). These two copies are known as sister chromatids, which are connected via a centromere in a cohesin complex to mark the beginning of mitosis (15).

Mitosis is a brief phase of the cell cycle and begins during a vague period of time when chromosomal condensation can be observed under microscope. Chromosomal condensation begins early in the G2 phase of the cell cycle when aurora kinases phosphorylate histones of DNA

in preparation for mitosis, but remains reversible until the first subphase of mitosis, known as prophase. There is a brief period at the end of G2 known as antephase, during which time the cell has not yet completely committed to mitosis. Cell commitment to mitosis usually occurs well into prophase and is physiologically indicated by the accumulation of cyclin dependent kinase 1 and cyclin B complexes in the nucleus and nuclear envelope breakdown (NEB) (16).

The transition from terminal G2 antephase and commitment to mitosis in late prophase switch is regulated by at least two checkpoints. One checkpoint prevents entry into mitosis in response to DNA breaks and the other checkpoint responds to a variety of acute stress factors, ranging from drug treatments to light exposure during microscopy observation. Although different mechanisms are used to do so, both checkpoints prevent mitotic entry by decreasing activity of cyclin B and A dependent kinases. Once committed, the cell must continue through mitosis until the cell is split into two daughter cells in a process known as cytokinesis. Otherwise, the cell may face apoptosis (16).

Complete condensation of chromosomes, centrosome migration, and completion of NEB characterize the first official phase of mitosis, known as prophase (17). Centrosomes are complexes of proteins that migrate to opposite ends of the cell and are responsible for the organization of microtubules (18). During prometaphase, microtubule arrays from centrosomes at both side of the cell specifically attach to cohesive proteins known as kinetochores on each semi-conservatively produced sister chromatid. Specific bonding between microtubules and kinetochores ensures that each sister chromatid of a duplicated chromosome is allocated to a different daughter cell (16, 17). It is important to recognize the spindle assembly checkpoint, a

key in mitotic progression because this checkpoint prevents mitotic progression in cases of insufficient or inappropriate spindle-kinetochore complexing. The spindle assembly checkpoint ensures each daughter cell receives only one sister chromatid from each duplicated chromosome and that this process occurs in a timely manner (19).

During metaphase, condensed chromosomes align centrally within the cell and cohesive proteins between chromatids dissolve, allowing for the separation of sister chromatids during anaphase (17). Telophase occurs once each pair sister chromatids have reached opposing poles of the cell. Telophase involves the development of nuclear envelopes and the decondensation of chromosomes. Soon after, the cell separates equally into two daughter cells in a process known as cytokinesis. The cell then enters G1 and remains particularly sensitive to the presence of nutrients and growth factors for approximately 3 hours, after which time the cell may re-enter the cell cycle if adequate nutrition and signaling is available (17). Mitotic exit, a popular topic among cancer researchers, signals the completion of the cell cycle (16).

Mitigation of DNA mutations is an important concept to understand when trying to understand cancer-associated deviations from normal cell physiology. As discussed above, division of normal cells produces two genetically identical daughter cells; any change in the genetic makeup that occurs, whether due to an error in copying the DNA or from DNA-damaging factors, is a mutation. Compared to their haploid germline counterparts, human somatic cells have 46 chromosomes in 23 pairs, with one chromosome from each pair derived from maternal genes and the other from paternal genes. Mutations that alter the number of chromosomes present in a daughter cell, which often occur during mitosis, produce cells with differing numbers

of chromosomes and this condition can be described as aneuploidy (11). Because of associated chromosomal instability, cancer cell genomes are diverse when compared to their non-cancerous counterparts. Differences between tumors and heterogeneity between cells of tumors present rampant and greatly varying aneuploidy. Aneuploidy can influence tumor development, therapy response, relapse, and many other aspects of cancer development and treatment (20). Checkpoints and DNA repair pathways are two ways the physiologically normal cell decreases the occurrence of and repairs random mistakes made throughout the cell cycle.

(i) **Checkpoints** throughout the cell cycle represent one major means of DNA mutation mitigation (11). Each cell cycle checkpoint consists of a sensor to recognize the problem or mistake, a signal produced by the sensor and propagated by means of signal transduction pathways or other signaling mechanisms, and a response element of cell cycle machinery, targeted to lock cell cycle progression (21). One checkpoint previously discussed in this manuscript is the spindle assembly checkpoint, which mitigates aneuploidy during mitosis.

Two major checkpoints of the cell cycle related to DNA replication include one between G1 and S and another between G2 and mitosis (11, 16). DNA replication checkpoints are most directly regulated by two main proteins: cyclin-dependent kinases (CDKs) and cyclins. When bound to form CDK-cyclin complexes, these proteins function to inhibit processes preventing DNA replication and encourage the synthesis of proteins made for mitosis (10).

An important note is that CDKs, kinases present to phosphorylate other compounds, are always present, but by default are inactive. They activate only when bound to specific

cyclins and cyclins are only produced at certain times in the cell cycle (11). Stringent control of cyclin protein and cyclin dependent kinase binding regulates progression of the cell cycle and deregulation of these processes is correlated with cancer development (22). During G1, cyclins D and E are produced and bind to CDK4 and CDK2, respectively. The CDK4-CyclinD complex phosphorylates and inactivates a protein known as retinoblastoma (Rb), which is a tumor suppressor protein normally responsible for inhibition of DNA replication. During the S phase, cyclin A is produced and binds with CDK 2 to activate DNA replication. In the G2 phase, cyclin B is produced and binds with CDK1 to activate mitosis. Though these pairings of CDK – cyclin complexes may seem nonsensical, a key point in understanding the role of cell-cycle checkpoints is that precise synthesis of cyclin at specific times throughout the cell's cycle is imperative for normal cell growth and proliferation (11).

(ii) **DNA repair pathways** are another means by which the cell naturally prevents excessive DNA damage and subsequent mutation. There are various sub-categories of internal (endogenous) and external (exogenous) sources of DNA damage, with endogenous DNA damage pathways being an aberrant by-product of natural and necessary DNA replication (23).

Sources of endogenous DNA damage include DNA replication errors, base mismatches, topoisomerase-DNA complexes, spontaneous base deamination, development of abasic sites, oxidative DNA damage, and DNA methylation, all of which may warrant a review in and of themselves, though this is beyond the scope of this project (23). Though a variety of factors influence error rates, spontaneous mutations are estimated to occur at a rate of

approximately 10^{-10} mutations per base pair per generation in eukaryotic cells (24). Aberrant geometrical structure of abnormally paired DNA initiates DNA damage response (DDR), the process by which DNA damage is identified and flagged for target by repair mechanisms (23).

In some circumstances, such as alkylation of DNA, damage can be reversed. Knockout of enzymes responsible for reversal of alkylation of DNA is associated with various metabolic diseases and cancers (23). Base excision repair (BER) is the pathway through which small forms of DNA damage can be repaired. For larger errors, the cell employs nucleotide excision repair (NER), mismatch repair (MMR), interstrand cross-link repair (ICL), and/or translesion synthesis (23). Single strand breaks (SSBs), which may be correlated with oxidative damage, abasic sites, and/or activity of DNA topoisomerase 1 (TOP1), require cell engagement in single strand break repair (SSBR); each source of damage having a unique respective SSBR pathway (23).

The Warburg Effect is a phenomenon seen in proliferating and developing cells, including those of tumors, with normally functioning mitochondria and in the presence of adequate quantities of oxygen. Cells experiencing the Warburg Effect alter their metabolism to dramatically increase glucose uptake and fermentation of glucose to lactate (25). Fermentation of glucose to lactate occurs via a process known as aerobic glycolysis (26), a term which has been used synonymously with the Warburg Effect (27). Though this dramatic change in metabolism was discovered and coined in the mid-1920s, the mechanisms, onset, and impact of the effect remain poorly understood (28).

Cells exhibiting the Warburg Effect deviate from normal cell metabolism when pyruvate, the final product of glycolysis, accumulates in the cytosol due to increased glucose uptake by the cell and decreased flux of pyruvate into the mitochondria (25, 29). This and similar examples of imbalanced metabolic supply and demand have been termed overflow metabolism, a metabolic shift that creates substrates for anabolic metabolism in times of limited nutrition (30). For this discussion, overflow metabolism and the Warburg Effect will be distinguished by the observation of overflow metabolism in cancer cells, specifically, during the Warburg Effect. Increased glucose uptake is facilitated via expression of different membrane glucose transporter isoforms, including increased expression of high-affinity GLUT-1 and GLUT-3 (29, 31). Disregarding glycolysis, pyruvate can also be produced in the cytosol via oxidation of lactate by lactate dehydrogenase, reformation of pyruvate from malate via cytosolic malic enzyme, and catabolism of amino acids, primarily via alanine transaminase (32).

An important note is that many cancer cells express a splice variant isoform of pyruvate kinase, the final and pyruvate-producing enzyme involved in glycolysis. The isoform often seen in cancer cells is pyruvate kinase M2, a fetal isoform of the enzyme. Pyruvate kinase M2 has an increased quantity of tyrosine residues. In rapidly growing cells, which are known to exhibit increased activation of tyrosine kinase signaling, the additional tyrosine residues of pyruvate kinase M2 are phosphorylated. Phosphorylated tyrosine residues inhibit stimulation of pyruvate kinase M2 by fructose 1,6-bisphosphate. This results in a slowing of glycolysis and an accumulation of pyruvate within the cytosol (29, 33).

Normally, pyruvate in the cytosol is transported into the mitochondria via a membrane transport system that shuttles pyruvate across the inner mitochondrial membrane (32). Once inside the mitochondria, pyruvate is normally converted to acetyl-coenzyme A (acetyl-coA) via pyruvate dehydrogenase (PDH) (34). During times of nutrient deprivation, cells have also been observed converting pyruvate to acetate via keto acid dehydrogenases within the mitochondria and converting acetate to acetyl-coA via enzymes within a family of acetyl-coA synthetases in the cytosol (34). Acetyl-coA is fed into the tricarboxylic acid (TCA) cycle, which breaks acetyl-coA down via oxidative phosphorylation by the enzyme acetyl-coA carboxylase (ACC), an enzyme responsible in part for lipid metabolism (31). In humans, the isoform ACC1 produces malonyl-coA for lipid metabolism in lipogenic tissues and the isoform ACC2 regulates beta-oxidation of fatty acids (35). Because these ACC enzymes are so heavily involved in lipid metabolism, they have become a target for potential treatments of metabolic disease (35) and cancer (36).

The TCA cycle produces the energy-containing molecule guanosine triphosphate (GTP) and reduces nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD^{2+}) to produce the electron-carrying molecules NADH and $FADH_2$, respectively (31). Electrons carried by NADH and $FADH_2$ are carried to the respiratory chain located within the inner membrane of mitochondria, where electrons are shuttled along a chain of enzymes to produce a proton gradient within the intra-membrane space of the mitochondrion. Due to the gradient, protons from the intra-membrane space flood into the inner mitochondrial space through an enzyme complex known as adenosine triphosphate synthase (ATP synthase), which produces adenosine triphosphate (ATP) via oxidative phosphorylation (37). Cancer cells experiencing the Warburg

Effect display deregulation of genes of the TCA cycle, including upregulation of glucose transporters, lactate dehydrogenase A, fatty acid transporters, fatty acid synthase, f A carboxylase (ACC), adenosine triphosphate citrate lyase, citrate synthase, sodium-coupled amino acid transporters, glutaminase, dihydrolipoamide S-succinyltransferase, and alpha-ketoglutarate dehydrogenase complex and downregulation of OGDH (31). Mutations in isocitrate dehydrogenases 2 and 3, succinate dehydrogenase, and fumarase are seen in cancer as well (31). Researchers have identified deregulation and mutation in genes involved in components of the mitochondrial respiratory chain as well (37).

ATP is produced in the mitochondria via oxidative phosphorylation to fuel viability, growth, and proliferation of cells (37). In normal human somatic cells, oxidative phosphorylation through the TCA cycle and respiratory chain of the mitochondria is the most efficient means of ATP production from glucose in the presence of functioning mitochondria and adequate oxygen (27, 38, 39). Though the Warburg Effect is shown to dissociate glucose influx, pyruvate production, and the TCA cycle (40), the Warburg Effect is shown to benefit cancer cells via increase in the rate of ATP synthesis (27, 38), increased biosynthesis (25, 30, 34, 41-43), enhanced tumor microenvironment (25, 29, 30, 34, 42-44), and sustained cell signaling (25, 29, 43, 45, 46).

Cancer, though hard to define, is an accumulation of aberrations in normal cell physiology that results in rapid growth, proliferation, and possibly metastasis in previously normal cells. Though the origin of cancer cells can be argued, it is clear that a variety of mutations present themselves in cancer cells, including mutations of genes responsible for cell cycle checkpoints, mitosis, DNA repair, and metabolism in general. Although cells deploy DNA checkpoints and

repair pathways to mitigate and resolve issue related to deregulation or mutation of these genes, these errors are still correlated to cancer development. Additionally, cancer cells exhibit a form of overflow metabolism known as the Warburg Effect, which increases glucose uptake and aerobic glycolysis to provide energy and building components of the cell.

Prostate Cancer

Prostate development is marked in the embryo by the interaction of wolffian ducts, müllerian ducts, the urogenital sinus, and the fetal gonads (47). Sexual differentiation in male embryos is a two-part process beginning with development of the male gonads, or testis, from the urogenital ridge. Development of the testis is marked by sufficient differentiation of bi-potential pre-Sertoli cells to Sertoli cells (47-49). The *Sex-determining Region Y* gene, which is expressed by pre-Sertoli cells, is found on the Y chromosome, which is donated to the zygote by the paternal spermatozoan (47, 50). Expression of *Sex-determining Region Y* creates a positive feedback loop between Sox9, an enzyme involved in production of anti-müllerian hormone by Sertoli cells, and Fgf9, an enzyme responsible for repression of the ovarian gene *Wnt4* (49).

In humans, development of the wolffian duct begins between 25 to 30 days after conception and the wolffian ducts initially aid the mesonephros in the secretion of toxins that will eventually be secreted by the fully developed kidney, also known as the metanephros (47, 51, 52). At approximately 43 days after conception, the wolffian duct elongates to link the hindgut, which caudally becomes the cloaca, to the mesonephros and gonads (47, 53). The cloaca is a temporarily present structure in the developing embryo between embryological weeks 4 and 6, after which time the cloaca is divided to form the ventral urogenital sinus and dorsal hindgut

(54). Between embryological days 46 and 48, the urorectal septum forms, dividing the cloaca into the urogenital sinus and hindgut (47, 53, 54). Prostatic ducts, eventually forming the adult prostate, originate from the urogenital sinus (47). Müllerian ducts begin development by elongating parallel to the wolffian ducts at approximately 6 weeks gestation. By the eighth week of gestation, the müllerian duct runs parallel, but between, the wolffian ducts. At approximately the same time, the müllerian duct reaches the urogenital sinus, forming the müllerian tubercle. The müllerian tubercle forms the penile urethra in males (47). This process is the first part of sexual differentiation in male embryos involves the degradation of the müllerian duct system and reinforcement, via androgens, of the wolffian duct system (47, 49).

The second part of sexual differentiation in male embryos begins when Leydig cells are stimulated to produce the androgen hormone testosterone and includes masculinization of the urogenital sinus and external male genitalia, including development of the prostate. In the human fetus, the prostate begins to bud from the urogenital sinus at approximately 10 weeks fetal age (47).

The prostate is a walnut-sized gland found at the base of the urinary bladder in adult male humans (47). The prostate is located within the visceral layer of the pelvic fascia, which is a connective tissue separating the membranous peritoneum from the muscular pelvic walls and floor. The peritoneum lines the abdominal cavity and extends into the pelvic cavity. The right and left coxal/pelvic/hip bones and sacrum bone create the pelvic girdle, which provides skeletal framework for the greater and lesser pelves. The lesser pelvis is surrounded by the inferior pelvic girdle, located between the pelvic inlet and pelvic outlet, which are the large and small circular

openings of the pelvic girdle, respectively. The lesser pelvis surrounds the pelvic cavity and perineum, the two of which are separated by the muscles of the pelvic diaphragm. The prostate is located within the pelvic cavity, which is continuous with the abdominal cavity, and receives blood supply primarily by prostatic branches of the inferior vesical arteries of the internal iliac artery. From an anterior view, the prostate can be located between the internal urethral orifice of the bladder and the external urethral sphincter, surrounding a portion of the urethra commonly referred to as the prostatic urethra. The base of the prostate closely relates to the neck of the bladder, the apex is in contact with the connective tissue superior to the urethral sphincter, and the posterior surface is related to the rectum (55).

The prostate can be divided into three histologically and anatomically unique areas; the prostate-surrounding nonglandular fibromuscular stroma, the glandular peripheral zone, and the glandular central zone. The wedge-shaped central zone creates the base of the prostate and surrounds the ejaculatory canal. The peripheral zone forms the remainder of the prostate and surrounds a portion of the urethra distal to the prostate (47). There is also a small, glandular region surrounding the prostatic urethra, which is known as the transition zone (47).

Additionally, the prostate can be divided into three distinct lobes. The isthmus, or anterior lobe, of the prostate lies anterior to the urethra and contains little glandular tissue. The isthmus separates the right and left lobes of the prostate, which can be subdivided into 4 lobules based on proximity to the urethra and ejaculatory ducts: the inferoposterior, inferolateral, superomedial, and anteromedial lobules. The four lobules of the right and left lobes of the prostate can be distinguished by arrangement of ducts and connective tissue (55).

The glandular regions and zones of the prostate are characterized by epithelial acini, or grape-shaped, glands (56). Epithelial tissue is a type of connective tissue that lines many internal organs of the human body and plays a role in absorption of nutrients, secretion of hormones and enzymes, and forming a protective barrier in these areas (57). The epithelial tissues of the prostate organize into a basal layer containing four cell types: secretory luminal, basal, neuroendocrine, and transit-amplifying (56, 58). When damaged or under the stress of DNA mutation accumulation, epithelial cells activate intrinsic homeostatic mechanisms to replenish epithelial tissue. For these reasons, it is crucial that epithelial cells have a degree of flexibility allowing for significant alterations of homeostatic mechanisms within each individual cell to sustain this vital tissue (57). However, this epithelial plasticity has been implicated in cancer development (56, 57, 59).

The mature and active prostate is considered essential to male fertility and health and is responsible for secretion of glycoproteins, trace elements, and metabolites citrate and spermine that encourage normal spermatozoa function and transport (47). Semen, the ejaculate secreted by men during copulation, is approximately 2-5% spermatozoa and 95-98% fluids secreted by seminal vesicles, prostate epithelium, and bulbourethral glands, collectively known as the male accessory glands (50). The prostate is arguably the most vital male accessory gland with regard to male fertility, owing to its pivotal role in activating pathways leading to ejactulation and subsequent sperm and capacitation (50). One secretory product released by the prostate is prostate-specific antigen, a member of a family of glycoproteins known as kallikreins (60).

Prostatitis, benign prostatic hyperplasia, and prostate cancer are the three major disease of the prostate (47, 50, 60-62). Prostatitis (63) and benign prostate hyperplasia (47, 61, 64, 65) are not cancerous, but prostatitis involves inflammation of the prostate and benign prostatic hyperplasia is non-cancerous growth (47, 50, 63, 65) The transition zone of the prostate is the most common site of prostatitis and prostate carcinoma (47). In conditions such as benign prostatic hyperplasia and cancerous prostate growth, prostate-specific antigen bypasses the blood-testis barrier and can be detected in the serum plasma of affected individuals (47, 60, 61, 66). However, it is important to note disagreement within the scientific and medical communities regarding the use of prostate-specific antigen screening for diagnosis of prostate pathology (67, 68).

Prostate cancer is described as abnormal and uncontrolled growth of previously normal cells of the prostate (69). According to the American Cancer Society, prostate cancer is the second leading cause of death in men (69). Postmortem studies conducted regardless of cause of death estimate 80% of men 80 years and older have histopathological evidence of prostate cancer, regardless of their diagnostic status (70). Symptoms of prostate cancer include painful urination (dysuria), blood in the semen (hematuria), bone pain, and erectile dysfunction (ED) (66). Furthermore, estimates in the United Kingdom suggest the average patient may accrue nearly $70000 in additional healthcare costs yearly once diagnosed with cancer and a society may spend nearly $400,000 per million *capita* to adopt only one new chemotherapy agent (71). Given this evidence, prostate cancer has an astronomical impact on the physical, emotional, and financial

wellbeing of numerous patients and researchers are behooved to find a safe, efficient means of prostate cancer treatment.

Common treatments (72) for localized prostate cancer include removal of the malignant tissue (radical prostatectomy) (73), chemotherapy (74), and radiotherapy (75). Radical prostatectomy results in lower rates of overall prostate cancer mortality while chemotherapy and radiotherapy are more useful treatment for cases of metastatic, relapsing, or castration-resistant prostate cancers (76).

Though the origin of prostate cancer remains uncertain, there is evidence suggesting prostate cancer develops in correlation with an accumulation of mutations in rapidly dividing epithelial cells of the glandular prostate. In most prostate cancers, the lumens of prostatic acini glands have lack of or disrupted basal membranes. Additional evidence supports the theory that prostate cancer arises from basal cells of the prostate versus epithelial cells (56). There is also growing evidence defining a relation between inflammation and the development of prostate cancer, which may play a role in the accumulation of DNA mutations (77).

As implied above, prostate cancer has a variety of clinical presentations. Some ways to describe prostate cancer are as primary, metastatic, relapsing, and/or castration-resistant, with the development of each unique prostate cancer influenced by the patient's genomic and environmental factors (78-80). Androgen deprivation therapy (ADT), which lowers testosterone levels, may be prescribed to a patient to physiologically treat the cancer. Tumors that grow and proliferate despite ADT are described as castration-resistant (78). Metastatic cells, cells able to travel throughout the body and establish tumors outside of the original cancer tumor, are

especially malignant and are the primary cause of death from cancer (81). Once a primary prostate cancer shows castration-resistance and continues to spread throughout the body, it can be classified as metastatic castration-resistant prostate cancer (mCRPC) (78).

Genomic variability between prostate cancer genomes is believed to influence presentation and progression of the disease (65, 82-84). Furthermore, genomic variability between prostate cancers may be driven by DNA mutations and malfunction of DNA repair pathways (83), mechanisms which have been discussed above.

Integrative Clinical Genomics of Advanced Prostate Cancer, published in 2015, exhibits genomic analysis on 150 mCRPC biopsy samples previously treated with combination strategies involving inhibition of poly(adenosine diphosphate [ADP]–ribose) polymerase (PARP) by abiraterone acetate or enzalutamide and/or inhibition of aurora kinase (78). Poly(adenosine diphosphate [ADP]–ribose) polymerase (PARP) is a family enzymes heavily involved in DNA repair that is responsible for detection of single-strand DNA breaks and orchestration of single strand break repair (85). Aurora kinase inhibitors were used to treat cancer due to the overexpression of aurora kinase A in metastatic neuroendocrine prostate cancers. Aurora kinase is a cell-cycle kinase that complexes to the transcriptional factor N-Myc, which is also overexpressed in some prostate cancers. When complexed to aurora kinase A, N-Myc is stabilized, allowing the oncogene to suppress androgen receptor activity and encourage androgen-independent prostate cancer progression (86).

To be considered for analysis, researchers involved in *Integrative Clinical Genomics of Advanced Prostate Cancer* study design required each biopsy sample contain at least 20% tumor

content (78). Then, researchers collected DNA content to create an RNA transcriptome. The transcriptome was sequenced for analytical use and was ultimately compared to the human reference genome (GRCh37/hg19) (78, 87). Data from this study identify multiple gene, chromosome, and aneuploidy mutations in prostate cancers. These cases had an average mutation rate of 4.4 mutations/Mb and an average number of relevant genetic mutations per case of 7.8, with the most common mutations occurring in androgen receptor (AR), TP53, and Phosphatase and Tensin Homolog (PTEN) genes. Relevant to this project, Robinson *et al*. also showed a substantial number of mutated PI3K pathway genes, *vide infra*. (78).

The role of androgen receptor signaling in prostate cancer can be traced back to the key role of androgen receptor signaling in prostatic development (47) and has been researched extensively (88-90). Androgen deprivation therapy (ADT) utilizes agonists and antagonists of luteinizing hormone-releasing hormone and/or *orchiectomy*, a medical procedure removing one or both testicles, (91-93) and has been shown to decrease blood serum testosterone levels by as much as 98% (88). Even after androgen deprivation therapy, prostate cancer cells can be seen inappropriately restoring androgen receptor synthesis. These cells are considered castration-resistant prostate cancer (CRPC) (78, 93). Previous research regarding the role of TP53, a tumor suppressor gene, in androgen deprivation therapy resistance suggests malfunction of TP53 results in the transition of epithelial-like, androgen receptor-dependent cancer cells to androgen receptor-independent, basal-like cells. The ability of a cell to alter the phenotypic expression of its lineage has been termed *lineage plasticity* (94). PTEN is a tumor suppressor gene involved in regulation of the PI3k-AKT pathways, *vide infra* (95). Given known roles of AR, TP53, and PTEN

genes and the association of aberrations of these genes with nmCRPC and mCRPC cases of prostate cancer, it is clear that further understanding of these genes and pathways is crucial to advancement in the understanding and treatment of prostate cancer.

***In vitro* models of prostate cancer** must be considered to promote clinical applicability of *in vitro* research findings; it is imperative to use a model mimicking the target clinical pathology of research outcomes. To mimic clinical pathology as closely as possible, it is prudent to use *in vitro* models of prostate cancer in prostate cancer research. However, this becomes challenging when one considers the various clinical presentations of the disease, *vide supra* (96).

According to Risbridger *et al*., "patient derived preclinical models", or samples of prostate cancer tissue sourced from human cases, can include xenografts, explants, and organoids. To elaborate, a patient derived-explant (PDE) is a sliced cancerous tissue that can be prepared with mesenchyme to create a patient-derived xenograft (PDX) or suspended in a cell solution to create patient-derived organoid (PDO) (96). Mesenchyme is an epithelium-derived tissue responsible for replenishment of the extracellular matrix, the cellular framework from which the body is built (97). It is important to note that PDX, while considered the "gold standard" preclinical testing model, are particularly time and labor intensive for prostate cancer tissues (96). So much so, in fact, that only approximately one percent of PDX models of human cancer for preclinical studies held in the Jackson Laboratory for Genomic Medicine PDX catalog are of prostate cancer (96, 98). The most conclusive collection of patient-derived prostate cancer cell line models is held by the Living Tumor Laboratory, totaling approximately 45 unique samples as of 2018 (96, 99). Considering the primary limitations of this project being time and resources, the focus of this

discussion will be on cell line models because they are relatively inexpensive and convenient to study when compared to PDX, mice, and/or human clinical research models.

In the past, localized prostate cancer was routinely harvested during radical prostatectomy procedures. With freezing and fixing techniques, previous research on these samples provided genomic data of the cells at time of harvest (96) . However, research conducted on frozen or fixed cells gives only a static snapshot of the cell at time of harvest, and prostate cancer is known to progress and metastasize, often leading to fatality. Additionally, an observable decline in progressive, localized samples has been seen due to early screening and effective treatment programs that mitigate disease progression. Metastatic samples, on the other hand, have been historically harvested primarily from bone tissue. Presently, modern tissue harvest practices have provided a variety of prostate cancer cell lines, each with unique genetic and phenotypic properties (96).

AMP-Activated Protein Kinase

AMP-activated protein kinase (AMPK) is an enzyme found in nearly every eukaryote and it is responsible for regulation of cell metabolism in response to stress. AMPK allows cells to maintain a high and relatively constant concentration of ATP by sensing the ratio of ADP to ATP. Consequently, cells can increase catabolic and decrease anabolic pathways in response to ATP requirements (100). Altering metabolic pathways allows the cell to conserve ATP during various conditions including low energy store, diabetes, cancer, and inflammation (101-103). Because of this, AMPK has become a target for therapeutic treatments of these various conditions.

To understand the complex function and regulation of AMPK, one must have a basic understanding of enzyme's structure. AMPK is a heterotrimeric complex with one each of alpha, beta, and gamma subunits. In mammals, there are two alpha, two beta, and three gamma isoforms which combine to form 12 unique AMPK isozyme complexes (100, 104, 105). Isozyme expression is species and tissue dependent. To elaborate, each isoform of AMPK is expressed in different concentrations depending on the species and tissue type of the cell expressing the isoform (100). Positions of various subunit domains are determined by ligand binding, but a core of the enzyme is always formed by the gamma subunit, the alpha C-terminal domain (CTD), and the β- C-terminal domain. The β-C-terminal domain is located between the alpha and beta subunit (105).

The alpha subunit of AMPK is considered the catalytic component because it contains a serine/threonine kinase domain, an autoinhibitory domain (AID), and a serine/threonine rich area harboring phosphorylation sites known as the "ST loop", and all of these components are involved in enzyme activity (104-106). Humans express alpha-1 and alpha-2, which are encoded by the *PRKAA1* and *PRKAA2* genes, respectively (104). There is a key regulatory threonine residue (T172) located in the kinase domain. T172 phosphorylation by upstream kinases results in activation of AMPK (104-106). The α-C-terminal domain interacts with the beta subunit and is the location of the ST loop. The ST loop is where phosphorylation sites for upstream indicators of cellular energy status are located (104).

The beta subunit functions both as a scaffolding protein between and as a regulatory unit of the enzyme. The β-C-terminal domain functions as a scaffold to connect the gamma subunit,

the α-CTD, and a β-linker loop, the latter of which connects the β-CTD with the CBM (105). The beta subunit contains a region known as the carbohydrate binding module (CBM) or glycogen binding domain (GBD) (100, 105, 107). Humans express beta-1 and beta-2, encoded by the genes PRKAB1 and PRKAB2, respectively (104).

The gamma subunit is responsible for nucleotide-dependent regulation of AMPK. Each gamma subunit isoform has an isoform specific N-terminus and an adenine nucleotide-binding domain (104-106). Humans express γ1, γ2 and γ3, encoded by *PRKAG1*, *PRKAG2,* and *PRKAG3* genes, respectively (104). The adenine nucleotide binding domain contains binding sites for adenine nucleotides AMP, ADP, and ATP. These sites are known to be four motifs in the gamma subunit known as cystathionine-β-synthase (CBS) repeats. The four repeats are named according to which ribose ring of adenine nucleotides AMP, ADP, and ATP the repeat binds to. AMP, ADP, and ATP are thought to competitively bind to cystathionine-β-synthase repeats 1 and 3, suggesting this repeat may serve a potential target for AMPK activation (104-106, 108, 109).

Activation of AMPK is primarily achieved via phosphorylation of threonine 172 (T172), which is within the kinase domain of the alpha subunit, and/or by binding of AMP and/or ADP to the gamma subunit (101, 104, 105, 108, 109). The binding of AMP and/or ADP to the gamma subunit directly and allosterically activates AMPK and structurally prevents dephosphorylation of T172, which then increases AMPK activity by more than 200% (100, 110). Two upstream enzymes responsible for T172 phosphorylation of AMPK are liver kinase B1 (LKB1; also known as STK11) and Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CaM kinase kinase) (111-116).

Two additional methods to activate AMPK include binding of AMP analogs and endocrine influence. AICAR, or 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl-5-monophosphate, is a natural by-product of *de novo* purine synthesis in humans. In certain human pathologies, the enzyme adenylosuccinate lyase (ASL) converts the molecule succinyl-AICAR (SAICAR) to AICAR. As AICAR accumulates, negative feedback occurs by AICAR on adenylosuccinate lyase (117). Additionally, certain hormones, including ghrelin from fasting individuals and adiponectin from fat tissue of lean individuals, are thought to activate AMPK (108, 109, 118).

Inhibition of AMPK can occur through presence of ATP, which binds cystathionine-β-synthase repeat 3 and prevents an alpha subunit region called the alpha-hook or alpha regulatory subunit interacting motif (α-RIM2) from interacting with cystathionine-β-synthase repeat 3. Interaction between the α-hook and cystathionine-β-synthase repeat 3 stabilizes the active conformation of AMPK when ATP is not bound to cystathionine-β-synthase repeat 3 (100, 109). Although there are a variety of AMPK-activating compounds, only Compound C has been identified as a cell-permeable inhibitor of AMPK (ref). However, Compound C is relatively nonspecific and is believed to affect various kinases. Therefore, Compound C is not ideal for studies of AMPK activity (119). One hormone thought to inhibit AMPK is leptin, which is secreted from adipocytes of obese individuals (108, 109, 120).

Downstream targets of AMPK have a net downstream effect of activation of catabolic pathways and inhibition of anabolic pathways to conserve intracellular ATP concentrations (100, 104, 105, 108, 121, 122). Basic and hydrophobic residues near phosphoacceptor sites are

thought to be important in recognition of substrates by AMPK (109). In rats, the first substrate identified for AMPK was acetyl-CoA carboxylase-1 (ACC1) (123). In humans, AMPK phosphorylates ACC1 at S80 and ACC2 at S222 (36). Other possible targets of AMPK include, but are not limited to, 2-hydroxy-3-methylglutaryl-coA (HMG-CoA) reductase, glycogen synthase and tuberous sclerosis gene products TSC1 and TSC2 (108, 121, 122, 124). TSC1 and TSC2 regulate the GTPase Rheb, which directly interacts with and inhibits mTORC1 (124). This relationship between AMPK and mTORC1, led to the exploration of the relationship between AMPK, the mTOR pathway, and inhibition of cancer cell growth.

The mTOR Pathway

Another highly conserved serine/threonine kinase involved in cancer metabolism includes the mammalian target of rapamycin (mTOR), which is a 289 kDa serine/threonine kinase that forms the complexes mTORC1 and mTORC2 (125, 126). The mTOR pathway, also known as the phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling cascade, involves the role of growth factors, nutrient availability, and energy availability in cell viability, proliferation, and motility. Inhibition of the mTOR pathway was first discovered using the compound rapamycin and is thought to play a role in prevention of tumor progression and metastasis (125-127).

Phosphatidylinositol 3-kinase (PI3K) represents a group of kinases normally involved in translating extracellular growth signals of nutrient availability so that the cell may begin macromolecule synthesis and initiate cell cycle progression. However, PI3K enzymes have been identified as oncogenes due to the ability of cancer cells to increase PI3K activity independent of

systemic regulation (128). There are three classes of PI3K enzymes, with all forms being composed of a PI3K signature motif containing a C2 domain, a helical domain, and a catalytic domain. PI3K classification is based on additional protein domains and how they interact with regulatory subunits (129). Class I PI3K enzymes can be divided into two categories, with class IA associated with receptor tyrosine kinases (RTK) and class IB associated with G-protein coupled receptors. RTKs have extracellular domains believed to bind growth factors including epidermal growth factors, platelet-derived growth factors, and insulin. The class IA catalytic subunit isoform p110α is essential for proliferation during embryogenesis and oncogenic transformation. Interestingly, *PIK3CA*, the gene encoding for p110α, is mutated and/or amplified in many tumors. The regulatory subunit of class IA enzymes, p85α, is encoded by the gene *PIK2R1* and contains a domain, Src homology 2, which allows binding of phosphorylated tyrosines to proteins. This suggests p85 regulatory domains allow RTKs to associate with the p110 catalytic domain. Many tumor-specific mutations in *PIK3R1* and *PIK3CA* prevent or decrease p110α inhibition by p85α, thus increasing PI3K signaling. When p85-p110 heterodimers bind to insulin receptor-insulin-receptor substrate protein complexes, an important second messenger known as phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is produced at the plasma membrane (128).

The production of PIP_3, which recruits proteins to the plasma membrane via a target motif called the pleckstrin-homology (PH) domain, results in PIP_3 recruiting members of the AKT family of serine-threonine kinases to the plasma membrane (ref). Binding of PIP_3 to AKT PH domains allows AKT enzymes to meet another PH domain–containing protein kinase, 3-phosphoinositide–dependent protein kinase 1 (PDK1), which then phosphorylates T308 on AKT to activate the AKT

enzyme (128) When localized to membranes, AKT can also be activated via phosphorylation at S473 by mTORC2. AKT enzymes are mutated in many cancer cells and involved in many cell processes leading to tumorigenesis (130) Activated AKT is known to inhibit apoptosis and encourage proliferation. One mechanism of AKT influence includes AKT phosphorylating and inactivating TSC1/2, which causes Ras homologue enriched in brain (RHEB) bound to GTP to accumulate and, subsequently, stimulate mTORC1 activity (131).

The complexes mTORC1 and mTORC2 contain raptor and rictor, respectively (132). The isoform mTORC1 is activated by growth factors and adequate supply of amino acids, but the exact mechanism of activation of mTORC1 remains unclear. The isoform mTORC1 stimulates protein synthesis through phosphorylation and inactivation of the initiation factor known as 4E binding protein 1 (4E-BP1), a repressor of mRNA translation (131). Although 4E-BP1 is phosphorylated at seven sites, phosphorylation of T37 is considered a priming event in the mTOR pathway (131, 133-135). There is evidence suggesting mutations in eukaryotic translation initiation factor 2B (eIF2B), which complexes with 4E-BP1 for activation of both enzymes, is implicated in development of mitochondrial malfunction and cancer development (132, 133, 135, 136).

Inhibition of mTOR typically occurs through a lack of nutrients and growth factors and induces protein breakdown by autophagy, *vide infra* (132). The mTORC1 enzyme can be inhibited by lack of growth factors and/or nutrients, and/or through various compounds, including rapamycin, the compound after which the enzyme is named, and its analogues everolimus (125, 126, 131, 132, 137, 138). AMPK has also been shown to inhbit mTORC1 via phosphorylation of TSC2, *vide supra* (124, 139). There are over 23,000 pure and mixed isoprenoids and both types

have been shown to inhibit tumor cell proliferation, some through inhibition of mTOR (140, 141) Isoprenoids, also known as terpenes or terpenoids, are composed of multiple five carbon isoprene units. Isoprenoids are secondary products of mevalonate metabolism and lead to cancer cell death (140, 141). Some pure isoprenoids, including perillyl alcohol and geraniol, suppress HMG-CoA reductase by decreasing efficiency of HMG-CoA reductase transcript translation (140-142).

In summary, energetic stress within a cell results in binding of ADP and, preferentially, AMP to the gamma subunit of AMPK, which activated the enzyme. Additionally, upstream kinases such as LKB1 and CaM kinase kinase phosphorylate AMPK at T172, which results in more than a two-fold increase in activation of the enzyme. AMPK then phosphorylates TSC2, which blocks Rheb, an enzyme involved in mTOR activation. AMPK can also directly inhibit phosphorylation and activation of mTORC1. AMPK also inhibits mTORC1. The enzyme mTORC1 is responsible for phosphorylation and activation of S6K, an enzyme responsible for phosphorylation and activation of ribosomal subunits. The enzyme mTORC1 is also responsible for inhibition of 4E-BP1, an mRNA translation factor involved in tumor suppression. In contrast, growth factors are recognized by the enzyme PI3K, which then activates AKT. AKT activates mTOR. These metabolic pathways have been implicated in a variety of metabolic diseases and cancers and have been extensively studied in cancer research, but little research exists regarding the dualistic role AMPK and mTOR impose upon mitophagy and autophagy.

Mitophagy and Autophagy

Autophagy is the degradation of cytosolic organelles and protein for use in recycling of cellular products during nutrient deprivation. Autophagy occurs under times of extreme metabolic stress, but is repressed by mTOR during times of cell growth and proliferation. In mammals, it is proposed that autophagy is initiated by activation of the enzymes Bcl2/E1B 19 kDa-interacting protein 3-like protein (BNIP3), Nix (also known as BNIP3L), unc-51 like autophagy activating kinase 1 (ULK1), and autophagy gene (Atg) proteins (100, 101, 108, 109, 143-149). When autophagosomal machinery becomes overwhelmed, the cell is believed to initiated programmed cell death via apoptosis (150, 151).

AMPK and mTOR have been shown to impose opposing regulation on autophagic machinery through the ULK1 and Atg pathways (101, 109, 139, 148, 149, 152-155). In short, the Atg family of protein complexes is responsible for initiation of autophagy. Atg6, which contains PI3K, and Atg1, which is suppressed by mTOR, are essential for the formation of pre-autophagosomal structures. These structures are then ubiquitinated within the cell, which is followed by maturation of pre-autophagosomal structures to autophagic membranes and machinery. Atg7 and Atg8 are heavily involved in the latter process (148).

In mammals, AMPK promotes and mTOR inhibits autophagy by direct phosphorylation of ULK1 (139, 152, 153, 155). One experiment showed evidence that AMPK directly phosphorylates ULK1 at S317 and S777. Controls in these experiments included AMPK inhibition by ATP rich environments and compound C (139) Unfortunately, these experiments were not repeated with

human cells, which may have ULK1 phosphorylation sites in different locations. Further research is needed to assess AMPK phosphorylation of ULK1 in human cells.

Mitophagy is the process wherein defective or unnecessary mitochondria are degraded by autophagic machinery (156, 157). The mitochondria are cell organelles responsible for production of ATP and essential for cell metabolism, viability, and proliferation (158, 159). In addition, mitochondria are the primary source of endogenous reactive oxygen species (ROS) production and the release of these ROS molecules may be correlated with cancer development (160, 161). In normal cells, the re-construction of mitochondria may occur as a response to mitochondrial malfunction, which may be a normal and necessary response to everyday events as simple as exercise induced stress (162, 163). This reconstruction, termed mitochondrial biogenesis, is paired with the continuous breakdown of inefficient mitochondria, mitophagy (162, 164, 165).

Mitochondrial malfunction has been identified in many human pathologies, including metabolic and age-related diseases (136, 160, 161, 166, 167). During mitophagy, the mitochondrion is surrounded by the autophagosome so that it may be transferred to the lysosome for hydrolytic degradation. If the volume of damaged mitochondria surpasses that manageable by autophagic machinery, cell death initiates (41, 156, 157, 164, 165, 168). However, some cancer cells have been able to bypass this natural response to overwhelming damage through adaptation, including the Warburg Effect (25, 28, 40, 41, 43, 45, 156, 157, 164, 165, 168).

Interestingly, little development has occurred regarding quantitative measurement of mitophagy *in vivo* (144-146). Qualitative analysis of mitophagy can occur via transmission electron microscopy, wherein one can expect to observe the development of double-membrane autophagosomes. Furthermore, pieces of or entire cell organelles, including mitochondria, can be found in autophagosomal machinery (144-146, 169). Quantitative assessment of mitophagy in cells *in vitro* includes fluorescence microscopy assays, immunoblotting for mitophagy proteins, and mitochondrial mass assays, among other means (144-146).

AIMS AND GOALS

Previous Research

Peffley et al. published a study regarding isoprenoids. Researched treated PC3 cells with 400 uM of perillyl alcohol or geraniol for 16 hours before western blot analysis, including analysis of 4E-BP1 phosphorylation, a final substate of the mTOR pahtway. Data from this experiment suggest the isoprenoid perillyl alcohol decreases proliferation of PC3 cell lines in part via inhibition of the PI3K/AKT/mTOR pathway (140).

Rae et al. published a study which observed the role of AICAR in activation of AMPK in PC3 and LNCaP cell lines. These researchers treated PC3 and LNCaP cells with .5, 1, and 3 mM solutions of AICAR suspended in media before western blot analysis of phosphorylation of acetyl-coA. They also used a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) toxicity assay to assess viability of treated cells. Results show a concentration-dependent increase in phosphorylated ACC and decrease in cell viability (170), though there is no mention as to which ACC1 phosphorylation site was probed for.

The above evidence shows that AMPK activation via phosphorylation at T172 may occur with the perillyl alcohol, geraniol, and/or AICAR in the prostate cancer cell line PC3, this cell line was the first investigated.

Hypothesis

The hypothesis of this project is that activation of AMPK leads to increased mTOR-dependent mitophagy and subsequent autophagy in prostate cancer cells.

Specific Aim 1

The first aim of this project is to determine whether the treatment of prostate cancer cell lines (PC-3) with perillyl alcohol, geraniol, and/or AICAR mediate increased activation of AMPK. The goal of this aim is to determine compounds for use in future experiments that increase activation of AMPK.

The hypothesis of this aim is that PC-3 cells treated with perillyl alcohol, geraniol, AICAR will exhibit a dosage-dependent increase in T172-phosphylated AMPK and S80-phosphorylated ACC1 when controlled against these same enzymes not specifically phosphorylated at these sites. Furthermore, a dosage-dependent decrease in T37-phosphorylated 4E-BP1 is expected because activated AMPK has been shown to inhibit mTORC1 and mTORC1 phosphorylates and inactivates 4E-BP1. Rapamycin, a direct mTOR inhibitor, will be used as a control treatment for inhibition of the PI3K/AKT/mTOR pathway.

In this aim, PC-3 cells will be directly treated with various concentrations of perillyl alcohol, geraniol, AICAR, or rapamycin. Timing and dosage of treatment with these compounds

will be dictated by previous research (140, 170). Varying concentrations of these compounds will be used to identify optimal activation conditions for AMPK. Activation of AMPK will be evaluated via western blot analysis of the enzymes AMPK, ACC, and 4E-BP1 and their phosphorylated counterparts p-AMPK (T172), p-ACC (S80), and p-4E-BP1 (T37). With both experiments, expected results include an increase in p-AMPK (T172) and p-ACC (S80) and a decrease in p-4E-BP1 (T37) when compared to their non-phosphorylated forms. These experiments will establish the role of perillyl alcohol, geraniol, and/or AICAR in activation of AMPK and the relationship that activation of AMPK has to the mTOR pathway.

Specific Aim 2

The second aim of this project is to determine whether pharmaceutically induced activation of AMPK leads mitophagy in prostate cancer cell lines. The goal of this aim is to lay a framework for the understanding of potential pathways and mechanisms through which AMPK influences mitophagy through autophagy. Previous research, *vide supra*, suggests mitochondrial malfunction may enlist autophagic machinery to dispose of the mitochondria via mitophagy. The autophagic machinery may be met with an overwhelming quantity of damaged mitochondria, in which cause the cell self-induces autophagy and subsequent apoptosis. AMPK and mTOR have been shown to impose opposing regulation on mitophagy via regulation of autophagic machinery by the NIX, ULK1, and autophagic gene (Atg) pathways.

The hypothesis of this aim is that activation of AMPK will activate mitophagy in PC3 cells. In addition, activation of AMPK will inhibit mTORC1 influence on mitophagy pathways in PC3 cells.

To test this hypothesis, PC-3 cells will be directly treated with optimal concentrations of perillyl alcohol, geraniol, and/or AICAR. Timing and dosage of treatment with these compounds will be dictated by previous research and results of specific aim 1. AMPK influence on mitophagy in PC3 cells will first be analyzed via transmission electron microscopy. Expected results will include observed development of double-membraned autophagosomes and the envelopment of organelles, including mitochondria, by autophagosomes (144, 169). After confirmation of mitophagy development via qualitative analysis, mitophagy will then be analyzed by fluorescence microscopy for mitochondria-autophagosome colocalization. Mitotracker will be used for live-cell imaging of mitochondria and GFP-LC3 will be used for identification of autophagosomal structures (171, 172). Expected results include the movement of mitochondria and/or autophagosomal machinery into contact with each other. Finally, mitophagy will be assessed via immunoblotting assay probing for proteins that are autophagosomal, involved in mitophagy, and regulated by AMPK and/or mTOR. The enzymes that will be probed for include NIX, ULK1, and Atg proteins Atg1, Atg7, and Atg8. However, research regarding the AMPK-dependent activation of these enzymes remains to be explored. These experiments will further elucidate the relationship between AMPK activation and mitophagy.

Specific Aim 3

The third aim of this project is to determine whether pharmaceutically induced activation of AMPK in pc3 cells leads to autophagy. The goal of this aim is to determine whether activation of AMPK leads to self-destruction in prostate cancer cells.

The hypothesis of this aim is that AMPK-activated mitophagy leads to subsequent autophagy in prostate cancer cells.

To test this hypothesis, PC-3 cells will be directly treated with optimal concentrations of perillyl alcohol, geraniol, and/or AICAR. Timing and dosage of treatment with these compounds will be dictated by previous research and results of specific aim 1. Autophagy will first be confirmed via transmission electron microscopy analysis as described in specific aim 2. Protein samples from treated cells will then be used to perform western blot analysis on baseline and autophagy-dependent changes in NIX, ULK1, and autophagic gene (Atg) proteins. Expected results include an increase in active autophagic machinery in cells treated with AMPK activators. This aim should help to further clarify how AMPK activation inhibits prostate cancer progression and induces tumor cell death.

METHODS

Reagents and cell lines. PC-3 cell lines (ATCC CRL-1435) acquired from American Type Culture Collection (ATCC, Manassas, VA) and stored in a vapor-phase liquid nitrogen freezer. Perillyl alcohol (product ID #8578S), geraniol (product ID #163333), and AICAR (product ID #A9978) were sourced from Sigma-Aldrich (St. Louis, MO). Each compound was stored according to manufacturer instruction.

***Cell culture*.** All work performed with live cells and reagents for cell culture occurred under a sterile, laminar flow hood. The working hood was sterilized with ultraviolet light for 15 minutes followed by wiping with 70% ethanol from a spray bottle and a sterile tissue. During the 15-minute sterilization of hood with ultraviolet light, Roswell Park Memorial Institute Medium (RPMI-1640) medium with L-glutamine (Sigma-aldrich, St Louis, MO), fetal bovine serum (FBS), and a solution of combined antibiotic (Penicillin and Streptomycin solution, 100x, Mediatech Inc., a Corning subsidiary, Manassas, VA) and antimycotic (Amphotericin B, Thermo Fisher Scientific, Waltham, MA) were placed in 37°C bath. The antibiotic and antimitotic solution contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B. Complete media was created using 500 mL RPMI-1640, 50 mL FBS, and 5 mL antibiotic and antimycotic solution. A 50 mL syringe was used to force the complete media through a 22-micron media filter. Filtered complete media was distributed into 50 mL aliquots and stored the aliquots at 8^{o}C for later use. For resuscitation of cells from liquid nitrogen storage, 10 ml of complete medium was first added to a T75 flask. Both the T75 flask and the 15 mL conical were placed in the incubator for equilibration temperature (37°C) and carbon dioxide (5%) levels. The 1mL cell

culture cryotube was sterilized, opened under the hood, closed tightly, and warmed in a 37°C bath with gentle agitation for 2 minutes. The T75 flasks were then removed from the incubator, sterilized with 70% ethanol, and placed in the hood. Cells suspended in cryopreservation solution were transferred to the 15 mL conical tube containing 9 mL complete media. The student then centrifuged the conical containing media, cryopreservation solution, and cells at 130 x g for 10 minutes at 25°C. The student used a 1000 uL pipette with a sterile tip to remove supernatant before resuspending the cells in 1 mL of complete growth medium. Finally, 1 ml of cell suspension was added to the 10 mL of complete medium in the T75 flask. Cell cultures were examined after 24 hours and an exchange of media occurred every 48 hours until the cells reached approximately 80-90% confluence within the t75 vessel.

Cell treatment assays. Each cell culture passage was grown until approximately 80-90% confluency. Cells appearing in exponential growth were seeded at approximately 700,000 cells per 60 mm polystyrene tissue culture plate for use in experiments. Triplicates for each treatment were plated. Once seeded, cells grew in the incubator for 24-48 hours or until 80% confluency before media exchange for treatment. Controls for cell treatment assays consisted of cells incubated in only complete media. Control treatments were conducted in triplicate and simultaneously with cell treatments consisting of perillyl alcohol, geraniol, and AICAR, treatments. Cell treatment were also performed in triplicate. Isoprenoids, including perillyl alcohol and geraniol, were used to treat cells for 24 hours according to the protocol outlined in previous research, *vide infra* (140). Pipettes with sterile tips were used to suspend perillyl alcohol (PA) or geraniol (G) in complete media. Cells were treated with 400 uM of PA and/or G for approximately 24h before harvest. AICAR cell treatment was conducted according to the

methods set forth in previous research, *vide* infra (170). Twelve to fifteen culture plates were seeded for each cell treatment assay: 3 replicates each of negative control, vehicle control (cell culture-grade DMSO, if used), .5 mM, 1 mM, and 3 mM of AICAR. Cells were treated with AICAR for 24h.

Cell lysis, protein harvest, and bicinchoninic acid (BCA) protein assay. The remainder of the protocols described in these methods were conducted outside of the sterile hood on the bench top. For cell lysis, control and treatment media was removed from experimental plates using pipettes and sterile tips. The plates were immediately placed directly on ice in an ice bucket and/or pan and covered with their respective lids. Three mL of ice-cold PBS was used to gently wash each plate. Then 200 µl of RIPA Lysis Buffer with Inhibitors (Source, city, state) was added to each flask, swirled to distribute buffer, and the plate incuated on ice for 15 minutes. A plastic sterile cell scraper was used to remove adherent cells in lysis solution A pipette and sterile tips were used to transfer the cell lysate to a 1.5 mL Eppendorf tubes. The tubes were then centrifuged at 13000 x g for 5 minutes at 4°C. The cell lysate supernatant, referred to as the protein concentrate from this point forward, was removed from each 1.5 mL Eppendorf tube and transferred to a new, sterile tube. Protein concentration density of each sample was determined using a Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo-Fisher Scientific), per manufacturer instructions. Protein concentrates were aliquoted into Eppendorf vessels based on protein concentration prior to storage at -20°C.

***Western blot analysis*.** Protein concentrates used for western blot analysis were removed from the -20°C freezer and allowed to thaw on ice. With variable volumes depending on protein

concentration of each treatment group, protein concentrate was aliquoted such that each gel well would have a constant mass of proteins. Protein concentrate was combined with 4x laemlli buffer and DI water before being heated to 85°C for 5 minutes. Each sample was loaded into a 4-20% Mini-PROTEAN TGX Gels (Bio-Rad Labratories, Inc., Hercules, Ca). Negative and positive AMPK control lysates from C2C12 cells (product ID #9158, Cell Signaling Technology, Danvers, MA) were used in addition to lysates from control treatments of PC3 cells. The western blot apparatus was set to 100 volts until the samples passed through stacking gel portion of gel, then voltage was raised to 180 volts. Polyvinylidene fluoride (PVDF) membranes were soaked 100% ethanol for 15 minutes before being stacked with the gel in transfer solution. The stack was then placed in a Turboblot (Bio-Rad) cassette to run at a constant 25 volts for 14 minutes. The PVDF membrane was then placed in blocking buffer solution (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature. Afterwards, the membrane was washed 3 times with PBS-T for 10 minutes with gentle agitation on the rocker. All PVDF membranes were probed with mouse anti-B-actin (1:5000 ratio, Cell Signaling) in blocking buffer solution. Each "A" labeled PVDF membrane was stained with rabbit primary antibodies against 4E-BP1, AMPKa, and ACC (1:1000 each, Cell Signaling) in blocking buffer solution. Each "B" labeled membrane was stained with rabbit primary antibodies against p-4E-BP1, p-AMPKa, and p-ACC (1:1000, Cell Signaling) in blocking buffer solution. Primary antibodies were stained overnight at 4°C. Afterwards, membranes were again washed three times with PBST for 10 minutes with gentle agitation. Membranes were then incubated with anti-mouse (red) and anti-rabbit (green) secondary antibodies (1:10000, Cell Signaling) in blocking buffer with 0.1% tween and 0.01% SDS for 30

minutes at room temperature. Lastly, cells were rinsed 3 more times with PBST and transferred to PBS solution before imaging with LI-COR imaging machinery.

RESULTS

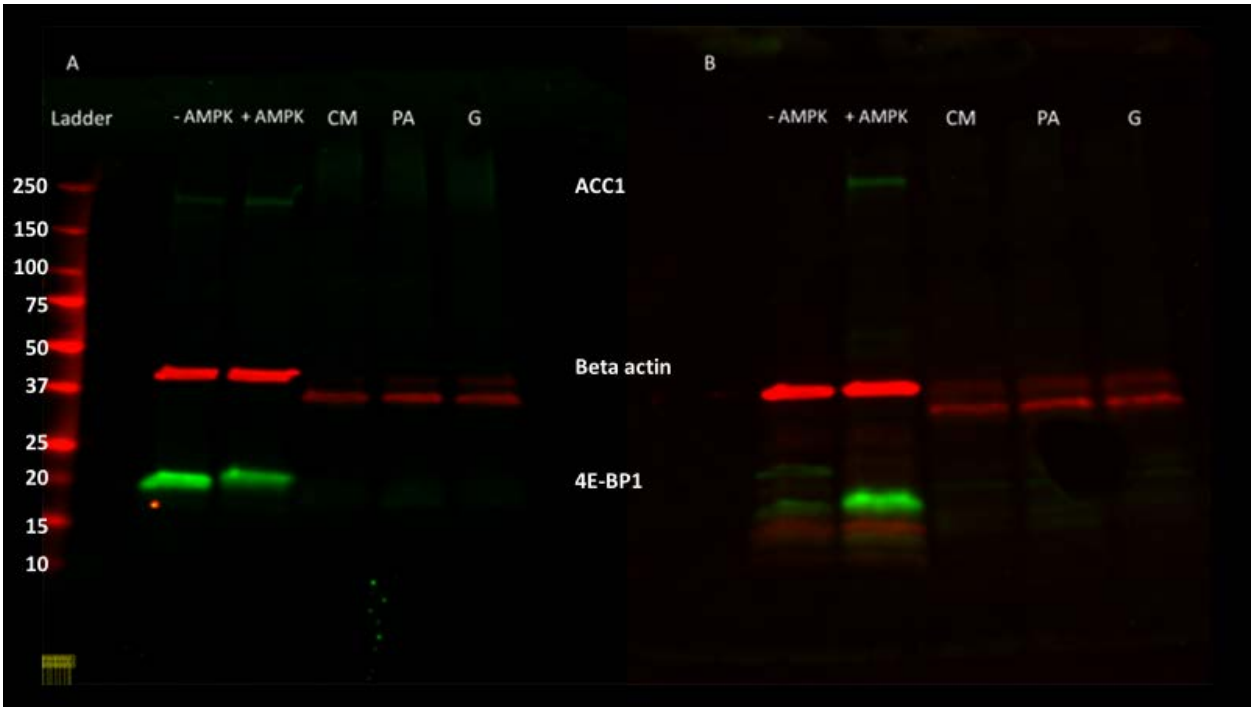

Figure 1: Western blot comparison of the effects of isoprenoids perillyl alcohol and geraniol on site-specific phosphorylation of 4E-BP1, AMPK, and ACC (A) and their phosphorylated counterparts PC3 cells (B).

This experiment was conducted to confirm previous data suggesting the isoprenoids perillyl alcohol and geraniol decrease proliferation of PC3 cell lines in part via inhibition of the PI3K/AKT/mTOR pathway (140). The hypothesis of this experiment is that PC3 cells treated with 400 uM perillyl alcohol or geranial solutions for 24h would exhibit an increase in T172-phosphorylated AMPK and S80-phosphorylated ACC1 and a decrease in T37-phosphorylated 4E-BP1 when controlled against these same enzymes not specifically phosphorylated at these sites.

Although largely inconclusive, results show increased expression of 4E-BP1, a protein in the mTOR pathway, in the positive control AMPKa lysate samples (+AMPK). This requires further

investigation considering the hypothesis in that activation of AMPK should inhibit mTOR and thus decrease expression of T37-phosphorylated 4E-BP1. Additionally, there seems to be some degradation in the beta actin housekeeping gene, illustrated by a double-band appearance not otherwise expected.

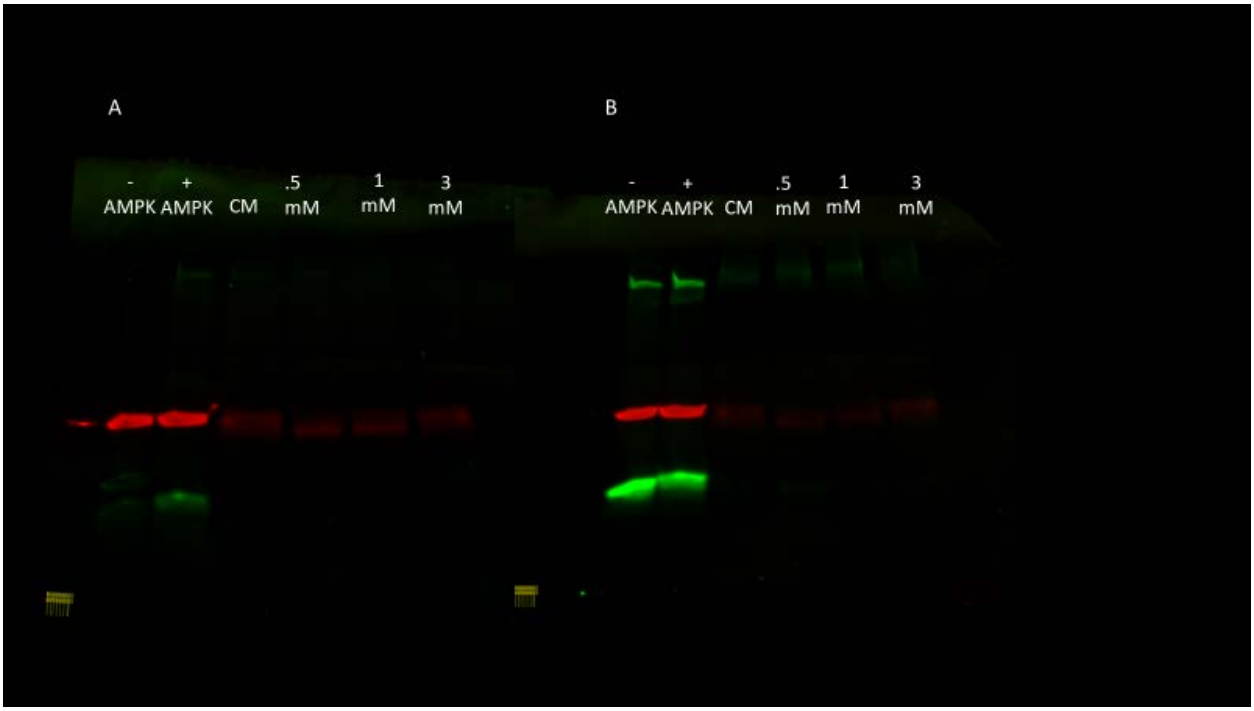

Figure 2: Western blot comparison of the effects of AICAR on site-specific phosphorylation of 4E-BP1, AMPK, and ACC (A) and their phosphorylated counterparts PC3 cells (B).

This experiment was conducted to confirm previous data suggesting the AMP-analogue AICAR activates AMPK in PC3 cells. The hypothesis of this experiment is that PC3 cells treated with .5, 1, and 3 mM AICAR solutions for 24h would exhibit a dose-dependent increase in T172-phosphylated AMPK and S80-phosphorylated ACC1 and decrease in T37-phosphorylated 4E-BP1 when controlled against these same enzymes not specifically phosphorylated at these sites.

Although difficult to see, results show expression of T-80 phosphorylated ACC1 in PC3 cells, including in control treatments. The double-banded expression of beta actin remains.

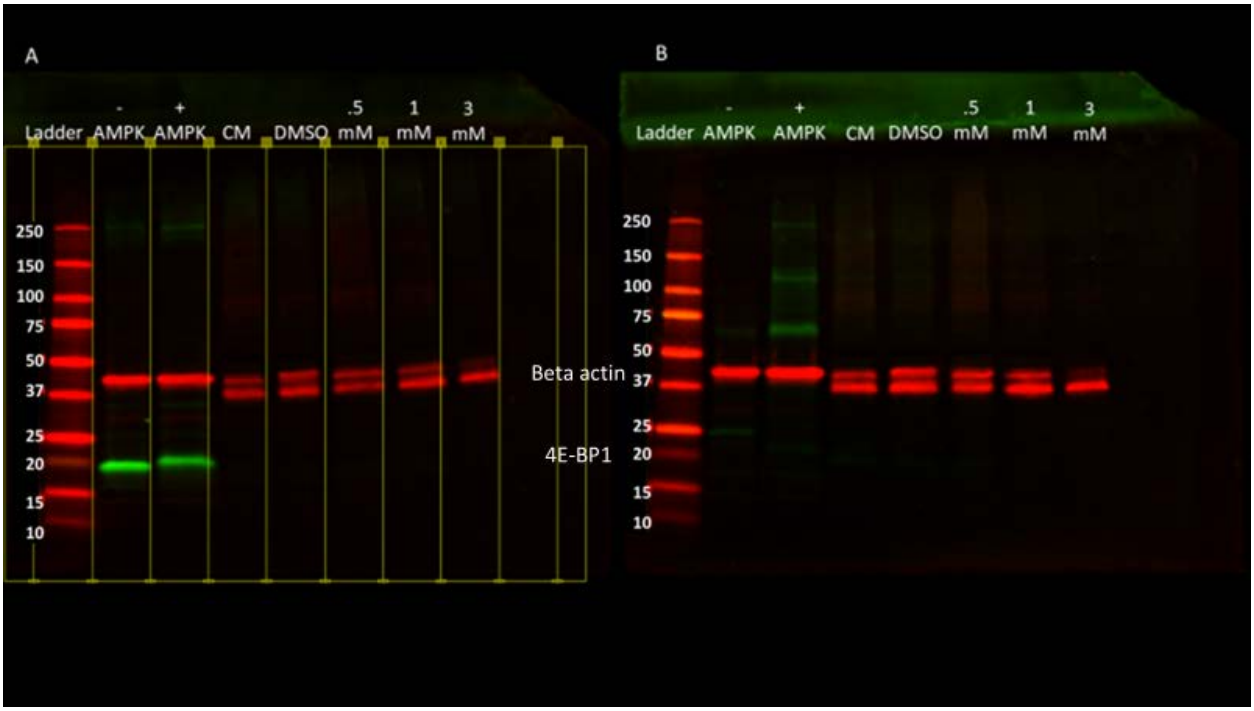

Figure 3: Western blot comparison of the effects of AICAR on site-specific phosphorylation of 4E-BP1, AMPK, and ACC (A) and their phosphorylated counterparts PC3 cells (B).

This experiment was conducted to confirm previous data suggesting the AMP-analogue AICAR activates AMPK in PC3 cells. The hypothesis of this experiment is that PC3 cells treated with .5, 1, and 3 mM AICAR solutions for 24h would exhibit a dose-dependent increase in T172-phosphylated AMPK and S80-phosphorylated ACC1 and decrease in T37-phosphorylated 4E-BP1 when controlled against these same enzymes not specifically phosphorylated at these sites.

Results of this experiment were inconclusive. The double band appearance of the housekeeping gene beta actin remains.

DISCUSSION

As outlined in the introduction of this manuscript, research regarding the dualistic roles AMPK and mTOR impose on mitophagy and autophagy in *in vitro* models of prostate cancer is complex. Although AMPK and mTOR are well established regulators of cell metabolism and growth, research regarding the dualistic role between the two enzymes on mitophagy and autophagy remains largely unexplored. The following discussion will explore implications of results, scrutinize the theory and technique of this project, and investigate future directions of research.

Results from initial experiments could not be analyzed because there were no apparent results from protein concentrates of PC3 cell lines after perillyl alcohol, geraniol, and/or AICAR treatment. However, control cell lysates seemed to exhibit an increase in ACC1 phosphorylation in serum starved C2C12 AMPK control cell lysates. This shows that AMPK activation via serum starvation leads to increased phosphorylation of ACC1, but says nothing about the compounds used in this experiment. An important note to be made regarding AMPK control lysates is that these lysates from C2C12 cells were used in comparison to lysates from control treatments of PC3 cells. This was done in an attempt to ensure functionality of primary and secondary antibodies. However, C2C12 are derived from mouse muscle cell lines, and therefore these AMPK control cell lysates should not bind to anti-human AMPKα1 (ATCC CRL-1772). Results using anti-human AMPK alpha 1 antibodies against these cell lysates cannot be reliably assessed. Additionally, positive AMPK cell lysate controls exhibited an increase in T37-phosphorylated 4E-BP1, bringing into question the hypothesis that activated AMPK mediates PC3 mitophagy and

autophagy through the mTOR pathway in mouse muscle cells. One interesting take from initial experiments was the apparent double bands seen in beta actin proteins. Though results from this experiment were inconclusive, a protease and phosphatase inhibitor cocktail will added to future cell lysate solutions.

From a foundational level, it is important to have a solid understanding of research theory and technique. Though some concepts, including western blot analysis, have been used for decades, the technique and application of experimental analysis is continuously evolving.

Though cell culture was conducted according to supplier recommendations and under presumably sterile conditions, repeated infection of cell culture by what appeared to be a fungal source remained a significant barrier to productive research during this project. Initially, PC3 cells were incubated with 1% antibiotic solution immediately after resuscitation of cell culture directly from the distributor. The antibiotic solution contained the antibiotics penicillin and streptomycin, both shown to prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria, respectively (173, 174). However, treatment of cell cultures with antibiotics has been implicated in a variety of undesired outcomes, including genomic and morphological changes (175).

Cells were treated with antibiotic solution in hopes of mitigating bacterial contamination and based on previous research with PC3 cells (140). However, PC3 cell cultures showed repeated evidence of fungal contamination across multiple passages. Amphotericin B has been shown to prevent fungal contamination of cultures due to the compound's inhibition of multi-cellular fungus and yeast growth (176, 177). However, amphotericin B has been shown to have

undesired effects on cells *in vitro*, including alteration of proteomes (176-179). In the future, it will be imperative to investigate best practice for mitigation of bacterial and/or fungal contamination and adhere best practice as strictly as possible (175, 179-181).

A major improvement in cell treatment assays would include further exploration into time- and dosage-dependent outcomes of prostate cancer cell treatment with perillyl alcohol, geraniol, and/or AICAR to activate AMPK. Once the optimal time and dosage of treatment to activate AMPK is determined, optimal concentrations can be used for manipulation of AMPK-regulated pathways. As mentioned in aim 1, implementation of this investigation can be as simple as preliminary research comparing various dosages and treatment times of compounds in benign prostatic and prostatic cancer cell lines.

Western blot analysis is a three-part protocol beginning with cell lysis, protein harvest, and protein density assays. During this time, a variety of reagents are used to break open the cell, store un-altered protein, and determine the concentrations of these protein samples. The unexpected double banded appearance of the housekeeping gene beta actin suggests an issue may have occurred during cell lysis or protein harvest. Because of this, a protease and phosphatase inhibitor should be added to future samples.

The second part of western blot analysis requires that these proteins are electrically pulled through a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. In order to successfully pull these proteins through the gel and accurately analyze the results, it is important that proper care be taken to load appropriate and constant levels of protein to each well. In preliminary

experiments, varying concentrations and volumes of protein concentrate samples were used in analysis. In future experiments, it is imperative to establish a standard volume and concentration of protein lysate solution. Additionally, although pre-cast gels were used in preliminary experiments, it may be appropriate to hand-cast gels with the specific protein to be probed for in mind (182).

The final component of western blot analysis includes the production of densitometric data and computational analysis of that data. Densitometric data is produced digitally by comparing the brightness of proteins on a western blot images first to the background fluorescence, then to proteins of other samples (182). Unfortunately, the low reliability and accuracy of western blot results from preliminary experiments prevented the generation and statistical analysis of data. However, it is imperative that an appropriate and reliable statistical analysis comparing treatments and outcomes be established.

Future Research

Because evidence shows that AMPK activation via phosphorylation at T172 may occur with the perillyl alcohol, geraniol, and/or AICAR in the prostate cancer cell line PC3, this cell line was the first investigated. However, future research should include *in vitro* studies of AMPK in human benign prostatic and prostate cancer cell lines. For prostate cancer cell line research, it may be helpful to classify prostate cancer cell lines by androgen-dependent or androgen-independen because these differing physiologies significantly alter prostate cancer cell line outcomes (80, 88-91, 93, 183, 184). Inclusion of androgen-dependent studies would include the

cell line LNCaP and androgen-independent would include the cell lines PC3 and DU-145 (185-188).

One experiment showed evidence that AMPK directly phosphorylates ULK1 at S317 and S777 in mouse cells. Controls in these experiments included AMPK inhibition by ATP rich environments and compound C (139). However, neither this study nor a subsequent literature search produce identification of the serine or threonine residue phosphorylated by AMPK on ULK1 in human prostate cancer cells. It is important to consider that AMPK may act on serine/threonine residues in a species- and tissue- specific manner (104, 105, 124, 139, 152, 153, 170). For this reason, future research should include studies of ULK1 phosphorylation by AMPK in human benign prostatic and prostate cancer cell lines.

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FIGURE LEGEND

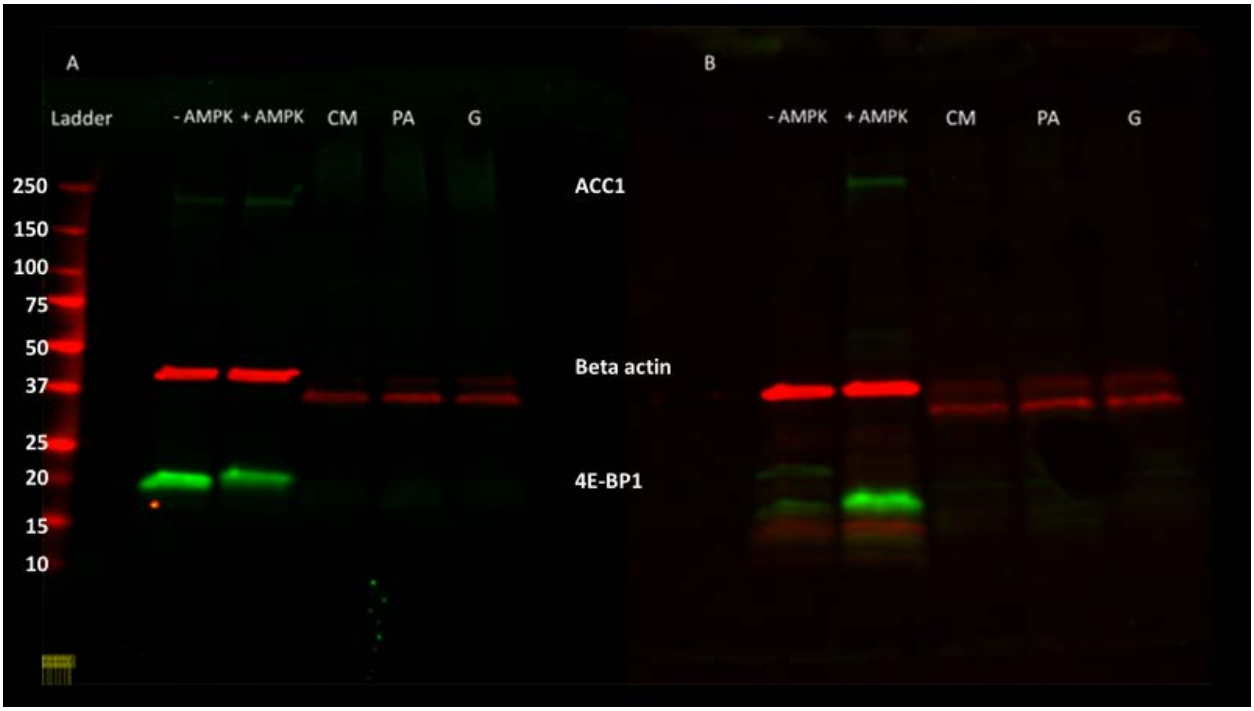

Figure 1: Western blot comparison of the effects of isoprenoids perillyl alcohol and geraniol on site-specific phosphorylation of 4E-BP1, AMPK, and ACC (A) and their phosphorylated counterparts PC3 cells (B). Cells were treated with 400 uM of PA and G for approximately 24h. Rabbit anti-human primary antibodies were used to probe PVDF membranes. The membrane labeled A was probed, using rabbit anti-human primary antibodies, for 4E-BP1, AMPKa, and ACC (A). The membrane labeled B was probed for phosphorylated 4E-BP1, AMPKa, and ACC, phosphorylated at T37, T172, and S80, respectively (B). Both membranes were also probed with mouse anti-antihuman B-actin, which serves as a housekeeping control in these experiments. Beta actin should be visualized at approximately 42 kDa (red). AMPK should be visualized at approximately 63 kDa (green). 4E-BP1 has a molecular weight of 15 kDa (green). ACC has a molecular weight of 265 kDa.

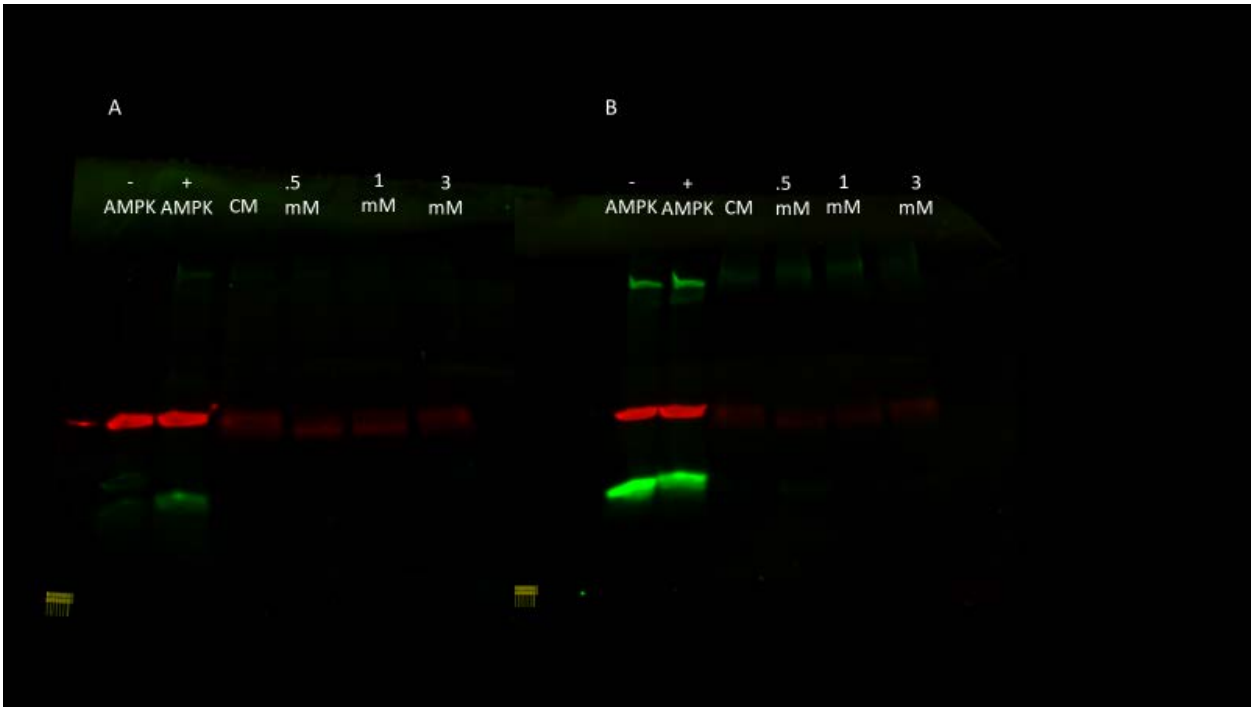

Figure 2: Western blot comparison of the effects of AICAR on site-specific phosphorylation of 4E-BP1, AMPK, and ACC (A) and their phosphorylated counterparts PC3 cells (B). Cells were treated with control, .5, 1, or 3 mM of AICAR for 24h. Rabbit anti-human primary antibodies were used to probe PVDF membranes. The membrane labeled A was probed, using rabbit anti-human primary antibodies, for 4E-BP1, AMPKa, and ACC (A). The membrane labeled B was probed for phosphorylated 4E-BP1, AMPKa, and ACC, phosphorylated at T37, T172, and S80, respectively (B). Both membranes were also probed with mouse anti-antihuman B-actin, which serves as a housekeeping control in these experiments. Beta actin should be visualized at approximately 42 kDa (red). AMPK should be visualized at approximately 63 kDa (green). 4E-BP1 has a molecular weight of 15 kDa (green). ACC has a molecular weight of 265 kDa (green).

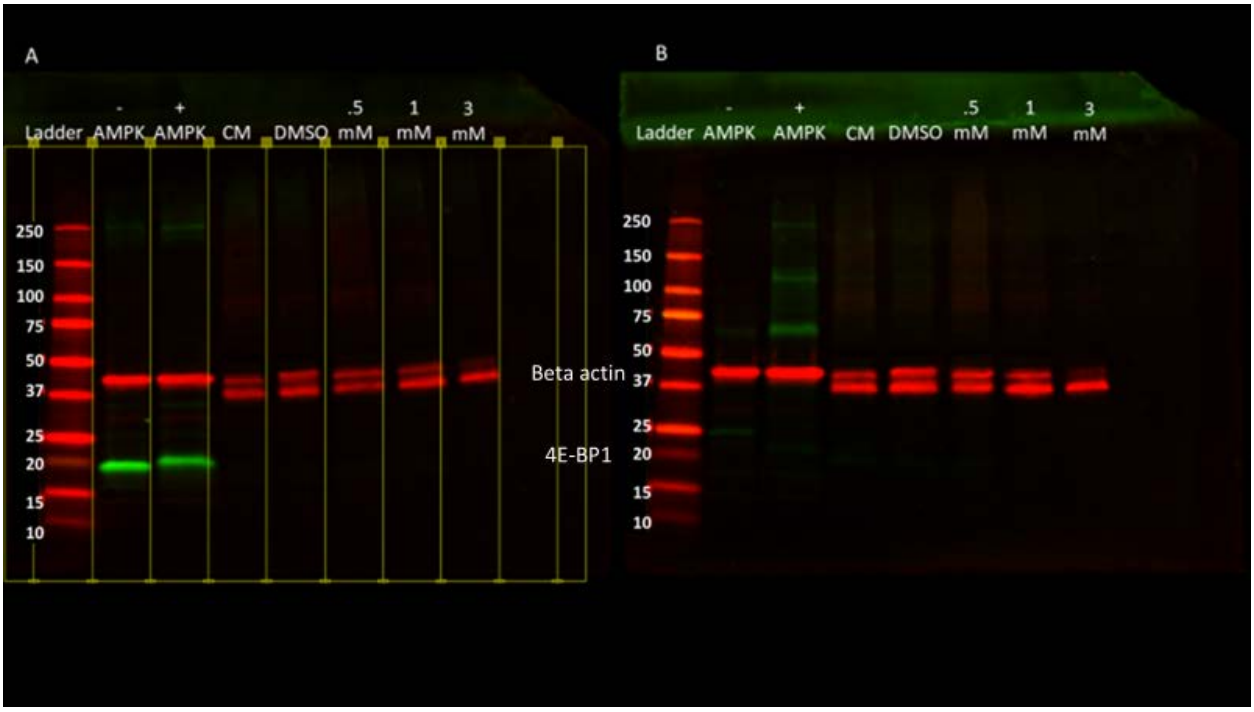

Figure 3: Western blot comparison of the effects of AICAR on site-specific phosphorylation of 4E-BP1, AMPK, and ACC (A) and their phosphorylated counterparts PC3 cells (B). Cells were treated with control, .5, 1, or 3 mM of AICAR for 24h. Rabbit anti-human primary antibodies were used to probe PVDF membranes. The membrane labeled A was probed, using rabbit anti-human primary antibodies, for 4E-BP1, AMPKa, and ACC (A). The membrane labeled B was probed for phosphorylated 4E-BP1, AMPKa, and ACC, phosphorylated at T37, T172, and S80, respectively (B). Both membranes were also probed with mouse anti-antihuman B-actin, which serves as a housekeeping control in these experiments. Beta actin should be visualized at approximately 42 kDa (red). AMPK should be visualized at approximately 63 kDa (green). 4E-BP1 has a molecular weight of 15 kDa (green). ACC has a molecular weight of 265 kDa (green).