Altering the ADIPO:LEP Ratio Secreted by Obese Adipose Tissue Affects the Tumor Growth Microenvironment of MCF7 Breast Cancer Cells

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A DISSERTATION SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCE

YORK UNIVERSITY

TORONTO, ONTARIO

NOVEMBER 2016

Abstract

Breast cancer continues to be the number one cause of new cancer cases and second in mortality rate in Canadian women. For almost 50 years now researchers have shown a statistical link between obesity and breast cancer. Although adipose tissue in the past was considered an inert storage depot, we now know it produces and secretes the adipokines adiponectin (ADIPO) and leptin (LEP). Lean individuals have been shown to have higher circulating levels of ADIPO and lower levels of circulating LEP, while the opposite is found in obese individuals, which ultimately alters the ratio between the two adipokines. Adiponectin has been shown to bind to its membrane receptor AdipoR1 and activate the cell signalling pathway AMPK, stabilizing the cell cycle inhibitor protein p27, leading to cell cycle arrest. Leptin on the other hand binds to its membrane receptor Ob-Rb and activates the Akt signaling pathway, increasing the cytoplasmic localization of p27, leading to cell proliferation. The purpose of this thesis was to look at altering this ADIPO:LEP ratio through diet, physical activity and nutraceutical methods to determine if an altered secretome secretion profile could alter the adipose-dependent effects of obesity on breast cancer cell proliferation. The results show that any intervention that led to an alteration of the ratio between ADIPO:LEP ultimately leads to varying degrees of cell cycle arrest in MCF7 breast cancer cells depending on diet, volume of physical activity and nutraceutical intervention. I show that regardless of menopausal status, the ADIPO:LEP ratio remained as a reliable and consistent predictor of the proliferative tumor growth microenvironment created by obese adipose tissue. Additionally, stabilizing ADIPO signaling through AdipoR1 abolishes the proliferative effects of an obese adipose-dependent growth microenvironment on breast cancer cells. The implications of this thesis is that adipose tissue presents a stable and predictable component of a patient's physiology providing possible novel treatment avenues for obesity-linked cancers.

Acknowledgments

I would first like to thank my supervisor Dr. Michael Connor for his support and help throughout my entire graduate degrees. As any student athlete knows it is challenging to balance your academic work with your athletic endeavors and this is only more challenging through a Masters and PhD degrees. Yet throughout the process you have been extremely supportive and have been through my many ups and downs over the last six years not only in the lab and publications but also with my athletic career. For this I thank you as I could not have asked for a better supervisor. I would also like to thank the past and present Connor lab members who help with my project over the years, most notably Mathew Triolo. I would like to thank other labs who helped with my project as well, most notably the Riddell lab. I would also like to thank the members of my defense committee Dr. Michael Riddell, Dr. Christopher Perry, Dr. Samuel Benchimol and Dr. Rebecca MacPherson. I have to thank my parents for their continued support over the years which have made balancing graduate degrees and training much easier. Although you may not understand my research, I still know you are proud of all the work I have accomplished. I would also like to thank Katie Pistor for your help during my thesis as you have acted as an academic and mental spring board for me, always asking questions about my research, forcing me to look at it from different perspectives.

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List of Abbreviations

Abs	Antibodies
ADIPO	Adiponectin
AdipoR1	Adiponectin Receptor 1
AdipoR2	Adiponectin Receptor 2
AICAR	5-Aminoimidazole-4-Carboxyamide 1-D-ribonucleoside
AMEM	Alpha Modification of Eagle's Minimum Essential Medium
AMPK	5'Adenosine Monophosphate-activated Protein Kinase
AP-1	Activator Protein 1
BAT	Brown Adipose Tissue
BMI	Body Mass Index (kg/m^2)
BSA	Bovine Serum Albumin
CC	Compound C
CD	Chow Diet
CD-CM	Chow Diet-Conditioned Media
CD+PA	Chow Diet + Physical Activity
Cdk	Cyclin-dependent Kinases
cDNA	Complementary Deoxyribonucleic Acid
CKI	Cyclin-Dependent Kinase Inhibitors
CM	Conditioned Media
CRM1	Exportin-1 (XPO1)
DCIS	Ductal Carcinoma *in-situ*
DNA	Deoxyribonucleic Acid
E2	Estradiol
ER	Estrogen Receptor
ERE	Estrogen Response Element
FBS	Fetal Bovine Serum
G0	Gap Zero Phase
G1	Gap Phase 1
G2	Gap Phase 2
GRB2	Growth Factor Receptor-Bound Protein 2

GSK-3β	Glycogen Synthase Kinase 3 Beta
HFD	High Fat Diet
HFD-CM	High Fat Diet-Conditioned Media
HFD+HPA	High Fat Diet + High Physical Activity
HFD+LPA	High Fat Diet + LPA Physical Activity
hKIS	Human Kinase Interacting with Stathmin
HPA	High Physical Activity
IL-6	Interleukin 6
INK	Inhibitor of Cdk-4/6
JAK2	Janus Tyrosine Kinase 2
KPC	KIP-1 ubiquitylation Promoting Complex
KIP	Kinase Inhibiting Protein
LPA	Low Physical Activity
LEP	Leptin
LKB1	Liver Kinase B1
MAPK	Mitogen Activated Protein Kinase
MET	Metabolic Equivalent of Task
MNU	1-Methyl-1-Nitrosourea
NES	Nuclear Export Signal
NLS	Nuclear Localizing Signal
NF-κB	Nuclear Factor-κB
Ob-Rb	Long Form Leptin Receptor
PA	Physical Activity
PBS	Phosphate Buffered Saline
PI-3K	Phosphatidylinositol 3-OH-Kinase
PKB/Akt	Protein Kinase B
PP2A	Protein Phosphate 2A
SRC	Non-Receptor Tyrosine Kinase of the Sarcoma Family
Rb	Retinoblastoma
RSV	Resveratrol
ROSI	Rosiglitazone

S	Serine
SCF	Skp1, Cullin, F-box Containing Complex
SHBG	Sex Hormone Binding Globulin
SHC	SHC-transforming protein
SOCS3	Suppressor of Cytokine Signaling 3
SOS	Son of Sevenless Homolog
Src	Proto-Oncogene Tyrosine-Protein Kinase Src
STAT	Signal Transducers and Activators of Transcription
T	Threonine
TGF-β	Transforming Growth Factor-β
TNFα	Tumor Necrosis Factor Alpha
TSP	Tumor Suppressor Protein
TZD	Thiazolidinediones
Ub	Ubiquitin
UT	Untreated
VEGF	Vascular Endothelial Growth Factor
WAT	White Adipose Tissue
WC	Waist Circumference
WHR	Waist-to-Hip Ratio
Y	Tyrosine

1. INTRODUCTION

The burden of cancer in Canada is an intricate and important topic to understand, especially with our ever growing elderly population. Cancer continues to be the leading cause of death in Canada for the past 10 years accounting for roughly 30% of all deaths. While cancer continues to be the leading cause of death in Canada, the overall cancer mortality rate has been decreasing slowly since the late 80s, especially in women and mainly attributed to a decrease in breast cancer deaths. This decreased breast cancer death rate has come with an increased incidence rate which has been attributed to increased early detection, better understanding of breast cancer mechanisms of development and the many factors which influence cancer progression (31). Although this decrease in overall percent mortality has been occurring it is important to note that there has been an actual increase in the absolute number of deaths, further stressing the importance of breast cancer research.

The latest Canadian cancer statistics from 2015 show that there will be an estimated 24,100 new breast cancer cases in women, accounting for 25% of all new cancer cases (31). It is estimated that 1 in 9 women will be diagnosed with breast cancer in Canada in 2015 and of that, 1 in 30 will die from the disease (31), making it the second deadliest cancer in women aside from lung. The need to study cancer continues to be important as the growth and aging population in Canada is expected to contribute to a substantial increase in cancer burden for our healthcare system within the next 20 years.

Cancer, in its simplest form, is a disease of the cell cycle. Typically tissues that must readily repair and turn over in order to maintain the body enter the cell cycle to induce cell replication. The mammalian cell cycle is a precisely regulated series of events with several safety mechanisms in place in order to ensure proper function of events. The cell monitors extracellular signals which

determines when it is necessary and appropriate to enter cell cycle to repair or replace damaged cells. Upon receiving a mitogenic signal the cell is prompted to begin dividing in order to produce two identical daughter cells. Throughout the cell cycle, safety mechanisms in the form of check points are used in order to notice any mistakes within the dividing cell and then either fix the problem or terminate the cell. A tumor cell on the other hand has escaped the normally precise cell cycle control which can lead to the cell undergoing uncontrolled proliferation.

Increasing research has been recently focused on understanding how and why these cancerous cells evade normal cellular function. In order to understand how this transition from controlled to uncontrolled growth occurs, it is first important to understand the events and mechanism which govern the normal mammalian cell cycle and how these mechanisms are altered in order to allow for cancer progression.

1. The Mammalian Cell Cycle

The mammalian cell cycle is a precise and intricate series of tightly regulated events which allow for cell replication of the DNA followed by the division of the nucleus and partitioning the cytoplasm to yield two identical daughter cells. The cell division was originally divided into two stages termed mitosis (M-phase), where nuclear division occurs and interphase, the interlude between two M-phases (241). Although M-phase is where most scientists originally focused their work following chromosome separation, interphase has since become more than just a rest stage for cell growth. Different techniques have allowed interphase to be revealed as actually broken into three additional stages of gap phase one (G1), DNA replication (S-phase) and gap phase two (G2; Figure 1.1) (164). S phase is preceded by G1 and followed by G2, where a cell must prepare for DNA synthesis or prepare for mitosis, respectively (146). During the gap phases, information

is integrated in order to determine the readiness of the cell to enter either S or M-phase and allow for the cell to repair DNA damage or replication errors. If DNA damage is found which is unable to be repaired, the cell will induce the process of cell death or apoptosis under normal circumstances (28). Cells in G1 can also enter a biochemically distinct quiescence state before commitment to DNA replication, called G0. Cells in G0 account for the majority of cells within the human body that are non-growing and non-proliferating (241). Cells can exit this G0 state when stimulated by an external mitogenic signal to divide.

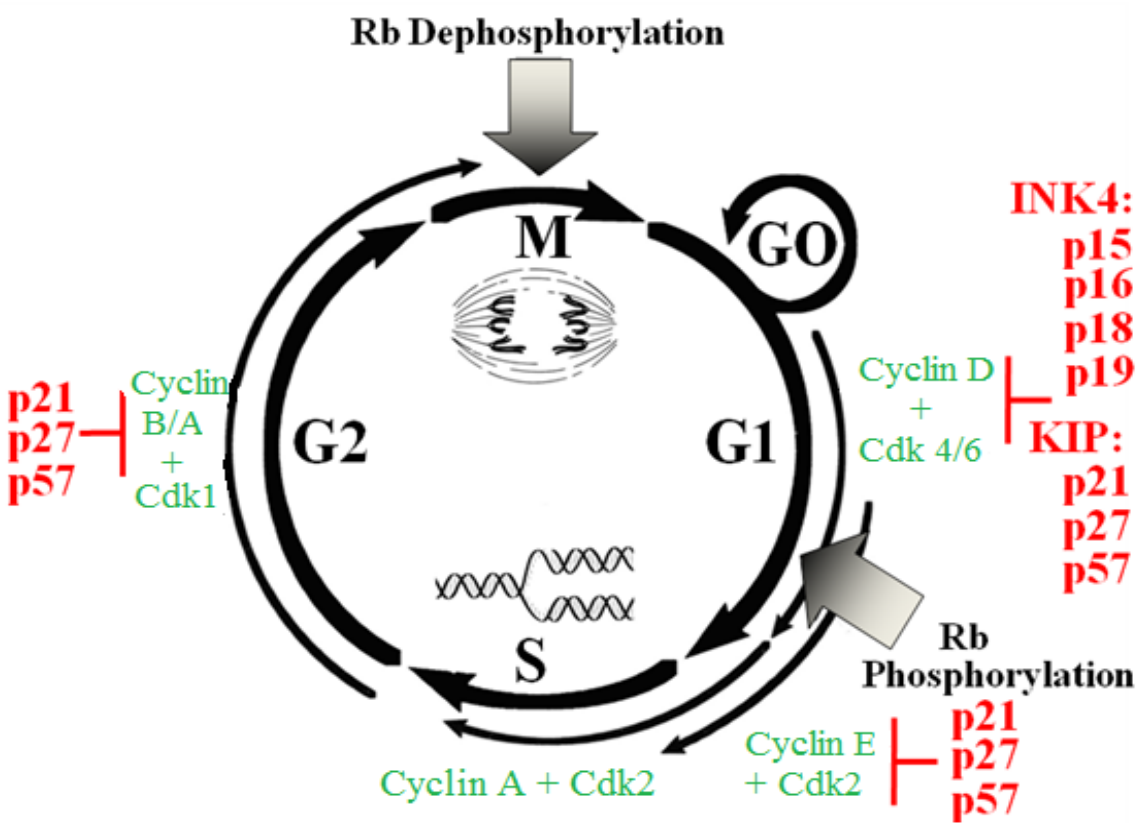

Figure 1.1: A simplified diagram of the mammalian cell cycle showing the cyclin/cdk complexes and the inhibitor proteins (INK4 and KIP) which affect them. Modified from Slingerland and Pagano, *J Cell Physiology*, 2000

One of the most important phases has been considered the G1 phase as this is the point in cell division where a cell must commit to replicating its DNA and undergoing cell division (146).

During G1, a cell must evaluate its extracellular and intracellular conditions and determine whether to divide or regress back into G0. Several signals have been shown to intervene in G1 in order to influence cell division and the deployment of the cells developmental programme (146). A restriction point, a “point of no return” exists in each phase including G1, which the cell is committed to enter the cell cycle. Studies have demonstrated that cells starved of serum before the restriction point enter a G0 state while cells starved that have progressed past the restriction point in late G1 are unaffected and continue through mitosis (171).

1.1 Regulation of the Cell Cycle

Compared to S-phase and M-phase, which follow canonical steps varying from cell to cell, the steps controlling entry and progression through G1 are largely dependent on cell surroundings. The transition from one phase to another occurs in an orderly fashion and is regulated by several different cellular proteins. The key or gas pedal to moving through the cell cycle are regulatory enzymes called cyclin-dependent kinases (CDK) which are a family of serine/threonine kinases that are activated at precise points throughout the cell cycle. Several CDKs have been discovered while only five have been shown to be active during the cell cycle including during G1 (cdk4, cdk6 and cdk2), S (cdk2), G2 and M (cdk1; Fig.1) (241). CDKs generally remain at a constant level throughout the cell cycle while the binding partners (cyclin proteins) and posttranslational modifiers (kinases and phosphatases) undergo specific periodic fluctuations in order to regulate cell division (28). The cyclins are upregulated by alternating cycles of transcription and translation to increase protein levels or can be degraded by ubiquitin mediated proteolysis to decrease protein levels. Different cyclins are responsible for different phases of the cell cycle including the D type cyclins (cyclin D1, D2, D3) which bind to cdk4 and cdk6 that are essential for entry into G1 (204).

Unlike the other cyclin subunits, cyclin D expression is not dependent on prior activation by other cyclins, but rather is synthesized as long as a growth factor stimulation is present (6). The other G1 cyclin is cyclin E which associates with cdk2 to regulate progression from G1 into S-phase and is activated after cyclin D (166). Two other cyclin proteins and cdk associations help with the transition from S-phase into G2 and during M-phase (Fig. 1). Cyclin A/cdk2 is required during S-phase, while in late G2 and early M-phase cyclin A/cdk1 is necessary while mitosis is regulated by cyclin B/cdk1 (5). While these cyclin/cdk complexes are important for the transition of later events in the cell cycle, this thesis focuses primarily on the transition from G1 to S-phase and its thought that being able to establish control of this transition and its respected complexes may stop cancer progression.

In order to continue to progress through G1 into S-phase, cdk activity is regulated not only by cyclin binding but also by phosphorylation. Full activation of G1 cdks is brought on by phosphorylation (cdk4/cdk6 on T160, cdk2 on T172) carried out by the cyclin activating kinase (CAK) which induces conformational changes, allowing for enhanced binding of cyclins (172). The Wee1 kinase phosphorylates these G1 cdks on tyrosine (Y) 15 which inactivates the kinases (130). Dephosphorylation at Y15 by the phosphatase enzyme Cdc25 is necessary for the activation of G1/S-phase cdks in order to progress further through the cell cycle (130). The cdk inactivating kinase Wee1 helps to protect the cell from premature mitosis, limiting the chances of error in the cell division process.

Like most systems in our body, the cdks can be counteracted in order to regulate the control of these proteins to prevent premature cell cycle initiation. Cell cycle inhibitor proteins, called cdk inhibitors (CKI) bind to cyclin-cdk complex in order to regulate cdk activity (Fig. 1). Two families of inhibitors have been discovered consisting of the inhibitors of cyclin dependent kinase 4 (INK4)

family and the kinase inhibitor proteins (Kip) family (204). The INK4 family includes $p15^{INK4}$, $p16^{INK4}$, $p18^{INK4}$ and $p19^{INK4}$ which only specifically inactivate G1 cdk4 and cdk6 complexes. These INK4 CKIs form stable complexes with cdk enzymes before binding to cyclin D preventing the cyclin-cdk association and subsequent activation (34). The KIP family consists of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP1}$ which bind and inhibit all cyclin/cdk complexes besides the cyclin D complexes (48), primarily inhibiting the G1 cyclin/cdk complexes (100). These CKIs have been shown to be regulated by both internal and external signals (34), displaying the complexity of their activation.

In terms of cell cycle regulation, the sole target phosphorylated by cyclin D/cdk4/6 is the retinoblastoma tumor suppressor (Rb; Fig. 1). When a cell is in the G0 quiescent state, Rb is in a hypophosphorylated state and bound to E2F transcription factors. During early G1, Rb becomes hyperphosphorylated and inactivated by cyclin D/cdk4/6 complexes, subsequently releasing the transcription factor E2F-1. This positively regulates the transcription of genes whose products are required for the progression into S phase such as cyclin E and cdc25 (22). The limiting factor for progressing through the restriction point in late G1 is the amount of cyclin E available keeping cells inactive until a mitogenic signal intervenes. Cyclin E expression is dependent on E2F transcription factors once they have been released from Rb.

Initially, mitogenic signals induce the expression of cyclin D that binds to cdk 4/6 to then form an active kinase state and assembly with p27 in order to phosphorylate Rb. This phosphorylation causes the dissociation of E2F from Rb which then transcribes cyclin E allowing for the formation of the active kinase cyclin E/cdk2 (Fig. 2). This newly formed cyclin E/cdk2 then works through a positive feedback loop to enhance the hyperphosphorylation of Rb, thus causing E2F to fully activate its target genes and induce the transition into S phase and DNA

replication. Therefore, the regulation and localization of p27 plays an intricate role in the G1 to S-phase transition of the cell cycle.

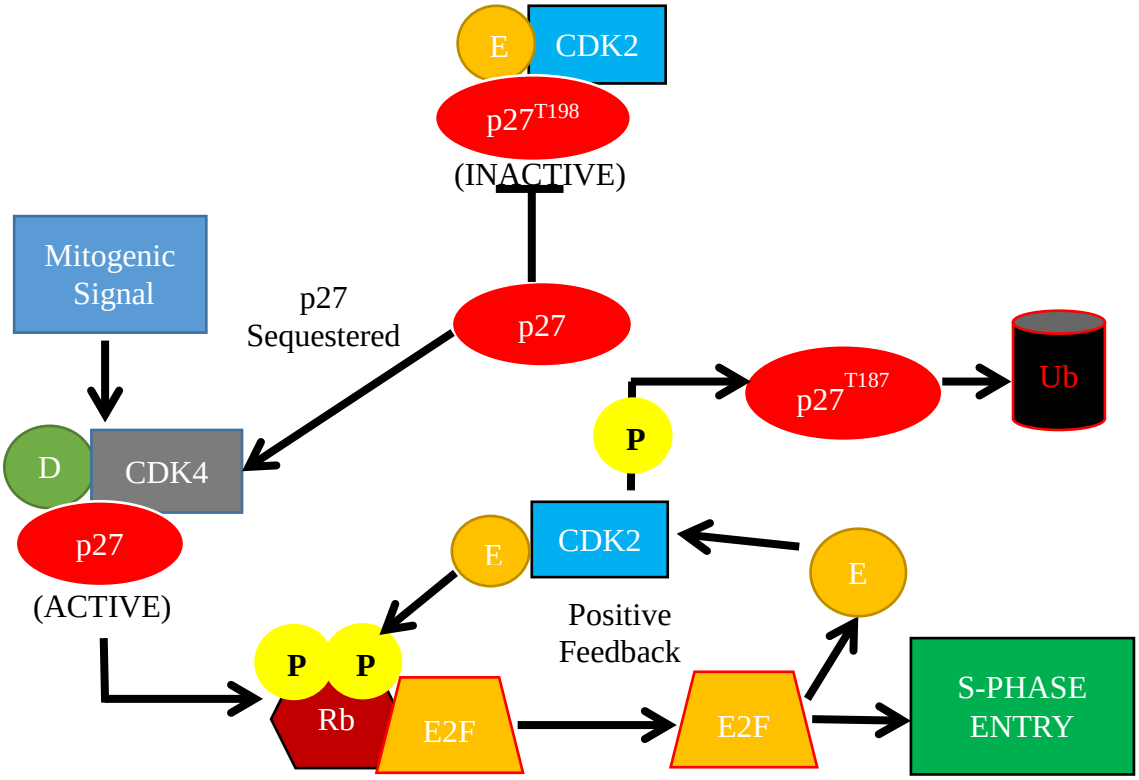

Figure 1.2: Summary of G1 to S-phase transition and the cyclin/cdk complexes which participate in the transition as well as the role p27 plays in inhibiting this transition until proper external and internal cues are present.

1.2 Importance of $p27^{KIP1}$

The importance of p27 lies in its ability to act as a key regulator of the G1-S phase transition. It serves to prevent premature activation of cyclin E/cdk2 in early G1 and helps with the assembly and activation of cyclin D/cdk4/6 in late G1. Therefore p27 can be thought of as a positive regulator of G1 progression by this assembly and nuclear import of cyclin D/cdk4/6 (48). The discovery of p27 was first shown by its inhibition of cyclin E/cdk2 and cyclin A/cdk2 in cells arrested by TGF-β and contact inhibition (122, 180, 213). Specifically, p27 was shown to exhibit

its inhibitory effect on cyclin E/cdk2 by binding to the catalytic cleft of the complex, preventing it from phosphorylating Rb (87). Research has now pointed to the balance between the levels of cyclin D1 and p27 as the main regulating mechanism for controlling proliferation of cells instead of their absolute levels within the cells (84). Mitogenic growth factor signaling such as Ras, a small GTPase that acts as a potent mediator of cell growth and survival signals, and mitogen activated protein kinase (MAPK) pathways have been shown to cause loss of p27 (247), while p27 increases in response to signaling by growth-inhibitory factors such as transforming growth factor- β (TGF-β) (122). Thus, p27 is regulated by proliferative and anti-proliferative signals and acts as a major control in the transition from G1 into S-phase of the cell cycle.

A study by Nakayama *et al.* (1996) found that p27 knockout mice that develop multi-organ hyperplasia and parathyroid tumors supporting the role of p27 in cell proliferation (161). Allelic haplo-insufficiency for p27 is seen in many human tumors (116) and p27 haplo-insufficient mice are more sensitive to malignant tumor induction by radiation and chemical carcinogens (76).

As cells exit G0 and enter G1, p27 that is bound to cyclin E initially must be degraded and newly synthesized p27 held within the cytoplasm. This helps facilitate the assembly and nuclear import of cyclin D/cdk. These effects are regulated by changes in p27 phosphorylation and subsequent activation or inactivation (50, 242). The mRNA levels of p27 have been shown to be constant throughout the cell cycle and p27 protein levels are regulated by translational controls (101, 152) and by ubiquitin-mediated proteolysis (170). p27 is predominately located within the nucleus in quiescent cells where it can act to inhibit cell cycle progression by inhibiting cyclin E/cdk2 (49). Pagano *et al.* (1995) demonstrated that p27 levels and stability are high in quiescent cells and both fall during G1 to allow for activation of cyclin E/cdk2 and the progression into S phase (170). This fall of p27 levels has been observed in the transition between G0 and S-phase

primarily because of the dramatic decrease in p27 half-life that is six to eight times longer in quiescent cells compared to proliferating cells (170). Analysis of human tumors has shown that p27 does not act as a classical tumor suppressor as both alleles are very rarely inactivated as seen in other tumor suppressor such as p53. Instead, early analysis showed in several tumors, especially in breast cancer, the levels of nuclear p27 were low with a corresponding increase in cytoplasmic p27 associated with poor clinical outcomes (42). The balance of p27 is fine-tuned by multiple post-translational modifications that affect p27 function by altering protein-protein interactions, affecting subcellular localization, modulating protein stability and increasing degradation machinery.

1.3 Regulation of $p27^{KIP1}$

Being associated with one of the central interfaces that control the cell cycle means p27 must be highly regulated in order for proper cell function to occur. As eluded to earlier, the mRNA levels of p27 remain constant throughout the cell cycle. Protein content of p27 is regulated by stabilizing phosphorylation in G0/early G1 and inactivating phosphorylation in late G1/early S-phase (152) shown in figure 1.3.

As described, p27 acts on the catalytic cleft of cyclin E inhibiting its interaction with Rb in early G1. Recently, three tyrosine residues (Y74,Y88 & Y89) have been identified within the middle of the p27 amino acid sequence which are vital for its binding with cyclin E/cdk2 via hydrophobic interactions (Fig. 3) (87). The conserved Y residues located within the cdk-binding domain of p27 can be phosphorylated by the SRC-family kinases and the oncogene product BCR-ABL early in G1 (87). If any of the Y residues are phosphorylated, the inhibitory helix of p27 is ejected from the cdk2 active sites, allowing partial cyclin E/cdk2 activation. The ejected p27 is

then subject to modifications which promote the cytoplasmic localization, sequestration and/or proteolytic degradation, as will be discussed below.

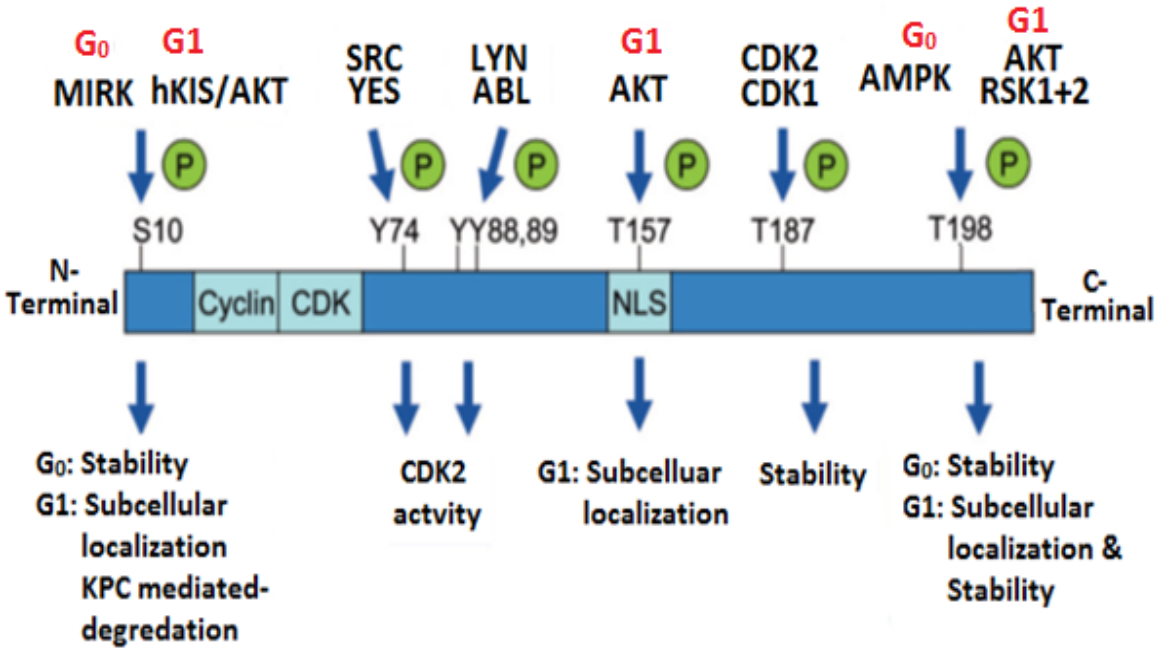

Figure 1.3: Summary of post translational phosphorylation sites observed in p27 with known kinases that modify the individual sites as well as functions associated with these post-translational modifications. Modified from Vervoorts *et al.*, *Cell. Mol. Life Sci.* 2008

After ejection from cdk2, one of the initial modifications of the nuclear pool of p27 is due to mitogenic signals, resulting in p27 to undergo nuclear export that is dependent on phosphorylation at serine 10 (S10) (54, 190). As shown in figure 1.3, human kinase interacting stathmin (hKIS) as well as protein kinase B (Akt) have been shown to phosphorylate p27 at S10 leading to its translocation from the nucleus to the cytoplasm (3). Once p27 is phosphorylated at S10, it facilitates the binding of p27 to chromosome region maintenance 1 (CRM1) for nuclear export (Fig. 3) (108), allowing for further G1 progression. *Connor et al.* (2003) found that the amino acid sequence of p27 contains a nuclear export signal (NES), which when mutated, causes a decrease in p27-CRM1 binding, nuclear export and p27 degradation (54). p27 that is located in

the cytoplasm in early G1 is ubiquitinated and degraded by members of the KIP1-ubitquitylation promoting complex (KPC 1 and 2) (87). This initial degradation of p27 is necessary in order to promote the initial loss of p27 needed to activate the cyclin E/cdk2 located in the nucleus and promote S phase entry (3).

The proteolysis of p27 is regulated by at least two distinct mechanisms. One previously described is the KPC complex and the second is by the very complex p27 was inhibiting, cyclin E/cdk2. Early in G1, mitogens activate the export-linked degradation of p27 through the KPC complex that is followed in late G1 by the cyclin E/cdk2 dependent degradation of remaining nuclear p27. Cdk2 dependent phosphorylation of p27 on threonine 187 (T187) is required for the majority of p27 ubiquitination and subsequent degradation (Fig. 2) (155). Efficient ubiquitination of p27 requires its stable association with the cyclin E/cdk2 complex (155). For ubiquitination and degradation, the interaction of a ubiquitin-conjugating enzyme with a substrate is facilitated by the ubiquitin protein ligase complex. One family of such ubiquitin complexes are called SCF complexes that are formed by four basic subunits comprised of: Skp1, a cullin subunit (Cul1), Roc1 protein and an F-box protein (165). Each SCF ligase brings a ubiquitin-conjugating enzyme to specific substrates which are required for different F-box proteins. A survey of seven mammalian F-box proteins found that p27 specifically bound to Skp2 only when it was phosphorylated at T187 mediating p27 ubiquitination and degradation by the 26S proteasome (36, 237).

Both of these independent mechanisms of p27 degradation play important roles in order to promote the loss of p27 necessary to progress into S phase. As a whole, the initial mitogen stimulation and subsequent export linked KPC degradation of p27 allows for the incremental activation of cyclin E/cdk2 which is followed by rapid cdk2 activation and T187 phosphorylation

dependent degradation of p27 allowing S phase entry (Figure 1.4). Skp2 has been shown to cause the ubiquitin mediated degradation of approximately 80% of all p27 while the initial degradation of p27 due to KPC accounts for the other 20% needed for cell cycle progression (Figure 1.4) (102).

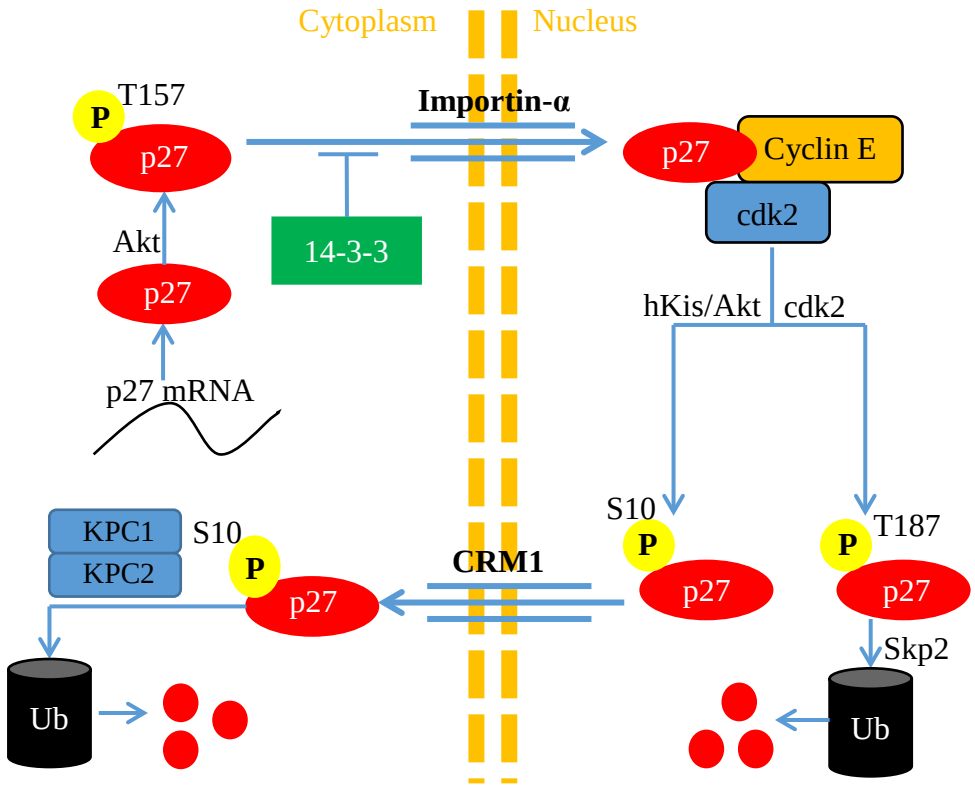

Figure 1.4: Summary of p27 degradation and translocation mechanisms. Phosphorylation sites on p27 with effects of hKis/Akt (S10), cdk2 (T187) and Akt (T157).

Along with the two described degradation mechanism of p27, newly translated p27 can also be phosphorylated on T157 by Akt (Fig. 3) (134). As displayed in figure 1.3, within the nuclear localizing signal (NLS) of p27 an Akt consensus binding site exists at T157 which leads to the interaction with the 14-3-3 protein, a family of conserved regulatory molecules that sequester T157 phosphorylated p27 in the cytoplasm (205). Shin *et al.* (2005) discovered that S10 phosphorylation and CRM1 export can lead to Akt further phosphorylating the cytoplasmic pool

of p27 at T157 and the 14-3-3 protein family then impairs its interaction with importin-α (Fig. 4) (205).

Although all previously described phosphorylation sites on p27 have to do with degradation or impairment of p27 function, another phosphorylation site facilitates p27 effects on cell cycle arrest. The most significant stabilizing phosphorylation to p27 is caused by AMP-activated protein kinase (AMPK) on T198 (92, 133). This stabilization event is vital in quiescent cells and early G1 cells as the $p27^{T198}$ inhibits cyclin E/cdk2 causing cell cycle arrest. Research has shown that AICAR, an AMP analog, up-regulates liver kinase B1 (LKB1) which activate AMPK which in turn phosphorylates p27 at T198, increasing the proteins half-life leading to cell cycle arrest (186)(133). AICAR also inhibited the Akt pathway through its upstream activator phosphatidylinositol-3-OH kinase (PI3K), which when taken together, caused 70-80% of breast cancer cells to undergo growth arrest in S-phase (186). These results proved the importance of this stabilizing phosphorylation on p27 to assist in cell cycle control. Any alterations to one or more of the previously outlined p27 phosphorylation sites can lead to a cell that is no longer under proper cell cycle control, ultimately leading to uncontrolled proliferation and cancer.

1.4 Deregulation of the G1 Phase of the Cell Cycle in Cancer

In any cancer, there are fundamental alterations in the genetic control of cell division which results in unrestricted cell proliferation. Mutations can occur primarily in two classes of genes known a proto-oncogenes (i.e. Ras, MAPK, cyclin D, cyclin E) and tumor suppressor genes (p53, pRb). In normal proliferating cells, proto-oncogenes are necessary to stimulate normal cell growth but if mutated, versions of proto-oncogenes can promote tumor growth. Inactivation of tumor suppressor genes can also result in the dysfunction of proteins that normally inhibit cell cycle

progression. The orchestration of the G1/S-phase transition is an elaborate mechanism with several key tumor suppressors having multiple isoforms in the event others become mutated. This G1/S-phase transition does not constitute the only cell cycle transition that has been implicated in the development of cancer but many of the cell cycle events which are necessary for controlled proliferation have been found to be altered, specifically in breast cancer (28).

Cyclin D1 has been discovered to be overexpressed with or without gene amplification, in over 50% of breast cancers and is more prominent in estrogen receptor positive (ER+) breast cancer (26). Cyclin D1 has been shown to be required in order for mammary tumor formation by the oncogenic Ras pathway (263). As mitogenic signals begin to cause a rise in Ras occurring early/mid G1, this increase causes an increased production of cyclin D1 which is necessary for the sequestration of p27 and phosphorylation of Rb. These mitogenic signals, acting through different receptor tyrosine kinases or G-protein coupled receptors not only activate Ras but also downstream effector proteins such as the PI3K pathway in order to stimulate cell proliferation and survival.

PI3K promotes cell survival by upregulating Akt which in turn assists with cdk activation by relieving two arresting constraints of the cell cycle. As previously described, Akt can phosphorylate p27 at both T157 and S10, which can promote cytoplasmic localization and increase ubiquitin mediated degradation of p27. The second method is achieved by Akt phosphorylating and inactivating glycogen synthase kinase 3β (GSK-3β) at S9, which acts to both directly and indirectly inhibit cyclin D (199). GSK-3β has been shown to play an intricate role in keeping cyclin D1 levels in check by directly phosphorylating it at T286, causing cyclin D nuclear export and degradation (60). GSK-3β can indirectly inhibit cyclin D by phosphorylating cyclin Ds transcription factor c-Myc at T58, leading to c-Myc destabilization (261). GSK-3β also plays a role in destabilizing c-Myc by indirectly inhibiting cyclin E transcription as c-Myc acts to

transcribe both G1 cyclins (86). C-Myc plays an integral role in breast cancer development as it has been found to be overexpressed in over 50% of breast tumors regardless of ER status (160). Without a mitogenic signal, the half-life of c-Myc is very short due to degradation by the 26S proteasome (198).Thus, any amplification of c-Myc appears to lead to breast cancer development and as shown in figure 1.5, several upstream oncogenes regulate the expression of c-Myc. One such upstream protein, MAPK, can phosphorylate c-Myc on S62 leading to increased stability against GSK-3β mediated degradation (198).

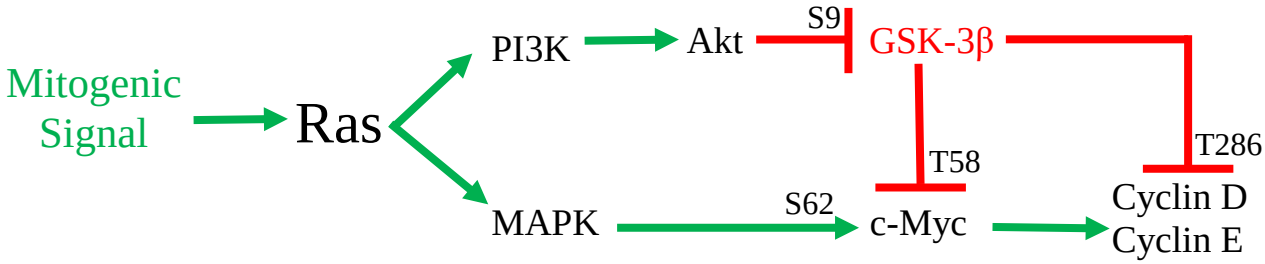

Figure 1.5: Summary of Ras and c-Myc activation and inactivation due to a mitogenic signal. Constituently active Ras or c-Myc can cause increased expression of cyclin D/E, leading to increased proliferation and tumor development.

Along with cyclin D1, cyclin E1 has also been found to be frequently overexpressed in breast cancers, particularly in ER- (219). The overexpression of cyclin E therefore may be an important indicator of breast cancer prognosis, especially when p27 expression if reduced and cdk2 activation is increased. The levels of cyclin E as previously stated fluctuate and are kept in check by ubiquitin mediated degradation. The F-box protein Fbw7 is part of the SCF-ubiquitin ligase complex responsible for the ubiquitin mediated degradation of Ras, c-Myc and cyclin E (248). A loss of Fbw7 in many breast cancers leads to c-Myc activation and cyclin E stabilization resulting in cell proliferation. The progression of breast cancer is a complex series of overexpression, under expression or loss of function of key proto-oncogenic and tumor suppressor proteins. Many

cancers display an overexpression and/or amplification of either Ras or c-Myc leading to increased proliferation and cell cycle entry by means of stabilizing downstream cell cycle proteins. Although several proteins attempt to stop this proliferative transition, many become deregulated and subsequently unable to perform their regular duties, including p27.

1.5 Deregulation of $p27^{KIP1}$ in Cancer

As previously demonstrated, p27 is regulated by several phosphorylation events which determine its location within the cell as well as it rate of degradation. Although uncommon loss of a single p27 allele occurs in some human malignancies. However, silencing of the remaining allele is rare (116). Increased Ras expression in many breast cancers can lead to an increase in Akt activity which in turn can increase p27 proteolysis and cytoplasmic localization. This reduction in p27 protein levels has been shown in up to 60% of all cancers including breast (212). This highlights the importance of completely unraveling the complexities of the regulation of p27 protein expression.

p27 protein levels and localization appear to have prognostic significance in breast cancer. Strong p27 immunostaining has been shown in the nuclei of normal human mammary duct epithelial cells while breast cancers often exhibit reduced p27 expression and/or mislocalization to the cytosol (3, 33). Three studies of differing cohort compositions have identified p27 as an independent prognostic factor in primary breast cancer s (42, 181, 223). Tan *et al.* (1997) demonstrated that patients with breast cancer and less than 50% of p27 within the nucleus using immunohistochemical staining, was an independent prognostic factor with a 3.4 fold increased risk of death (223). This study was using older women with the mean age of 60. This trend of decreased nuclear p27 resulting in a lower survival rate also has been shown in younger women. In a study

by Porter *et al.* (1997), it was observed that in breast cancer patients under the age of 45, decreased p27 is a predictor of poor overall survival using multivariate analysis (181). The group also looked at cyclin E and found that patients with tumors that displayed both low p27 protein and elevated cyclin E protein had the highest mortality (181). A reduction in p27 has been found to precede the development of breast cancer invasion and progression as decreased p27 has been found in premalignant and noninvasive ductal carcinoma *in-situ* (DCIS) (158). High expression of Skp2 in breast cancers also correlates with low p27 levels, but a large number of cancers with low p27 expression actually do not overexpress Skp2, indicating that other mechanisms must account for p27 degradation (207).

The localization of p27 is extremely important in determining cancer progression and overall possible survival. Tumors that retain abundant p27 are found to often show p27 mislocalization within the cytoplasm away from its nuclear cyclin/cdk targets. Studies of primary breast cancer specimens found that the presence of cytosolic p27 was associated with its phosphorylation on T157 by active Akt which led to lower disease-free and overall survival ratio (3). It has been reported that p27 has been found to be mislocalized within the cytoplasm in up to 40% of primary human breast cancers (134, 206). Women with reduced levels of nuclear p27 and node negative breast cancers, meaning the cancer has not spread of any lymph nodes, were found to have a 10-fold increase in the risk of cancer relapse compared to women with higher levels of nuclear p27 (45). The oncogenic activation of the PI3K/Akt pathway causes the Akt dependent phosphorylation of p27 and represents an important mechanism underlying the cytoplasmic mislocalization in human cancers.

While low and mislocalized levels of p27 have been shown to have a negative impact on breast cancer progression, high levels of p27 have also been shown to have a prognostic impact.

Gillett *et al.* (1999) discovered that patients with higher nuclear p27 expressing tumors had a significant longer relapse period and overall survival compared to those who had low nuclear expressing p27 tumors (84). The same group found that after 5 years, 80% of patients with high p27 expressing tumors were surviving compared to only 50% of patients with low p27 expressing tumors. Thus p27 presents a strong predictor, in tandem with other proteins, as a method of determining the severity of a breast tumor as well as possible mechanisms in order to try and regain control and proper function/localization in order to slow and even stop breast cancer progression.

2. Obesity

The prevalence of obesity has more than doubled since 1980. More than 1.9 billion people worldwide are considered overweight, of which 600 million are considered obese (254). This represents an increase of 100 million in just the last 4 years. Currently in Canada, over 6 million women are considered overweight or obese, translating to roughly 35% of women in Canada (216). In Canada and throughout the world, obesity has been designated a serious health problem for women, associated with a variety of metabolic disorders and conditions.

The term overweight and obese are used extensively to describe an individual's weight status. A worldwide index known as the body mass index (BMI) is often used in order to classify adults as overweight or obese. The BMI is a simple weight to height calculation (kg/m^2) in which an individual with a BMI>25 is considered overweight, while a BMI>30 is considered obese. BMI provides the most useful population level measure of overweight and obesity as it is the same for both sexes and all ages of adults. It is important to note that the use of the BMI index is a rough guideline and does not equate the same level of "fatness" in all individuals of the same BMI. One fundamental flaw of BMI index is it does not tell you the location of the fatness. Increased

adiposity, particularly in the visceral (intra-abdominal) compartment, has been found to be associated with several negative health issues such as insulin resistance, hyperglycemia, dyslipidemia and hypertension (90).

Obesity can be thought as an energy balance or scale, where an individual is in a prolonged state of positive energy balance. This can be from excess dietary caloric consumption and/or insufficient caloric expenditure due to physical inactivity. Due to this net positive balance, energy in the form of triglycerides is stored inside specialized fat cells, known as adipocytes, until the body requires increased substrate for energy production. Obesity is characterized by an excess accumulation of white adipose tissue (WAT) which is primarily composed of adipocytes, fibroblast, macrophages, stromal cells and endothelial cells (255). Obesity in females has been found to be associated with certain types of cancers such as ovarian, cervical, endometrial and breast (11). Understanding this association between obesity and cancer is becoming increasingly important as obesity rates continue to rise and the growing elderly population here in Canada begins to peak at an age of increased cancer incidence.

2.1 Obesity and Breast Cancer

For almost 50 years now researchers have found a statistical association that an increase in adiposity is correlated with an increased risk of cancer (214). In 2007 reports from the American Institute for Cancer Research and the World Cancer Research Fund predicted that obesity will overtake smoking as the leading cause of cancer (253). Although this trend of obesity overtaking smoking has yet to occur, the newest statistics from the World Cancer Research Fund show every year the gap between smoking and obesity related cancers continues to shrink.

Several studies over the years have shown a strong association between obesity and postmenopausal breast cancer. Obesity causes an increased risk of breast cancer in postmenopausal women that is 30-50% greater than their lean counterparts (252). The effect of increased BMI on breast cancer risk is particularly observed in breast tumors with positive estrogen receptors (ER+) and progesterone receptors (PR+) (178). Not only is the incidence higher in obese postmenopausal women but the death rate is also higher. A study by Calle *et al.* (2003), discovered that obese postmenopausal women in the highest quintile of BMI had double the death rate (relative risk, 2.12) from breast cancer compared to postmenopausal women in the lowest quintile of BMI (29). Obese postmenopausal breast cancer patients seem to have a higher risk for lymph node metastasis, larger tumors and death when compared to non-obese breast cancer patients (13). A meta-analysis by Harvie *et al.,* (2003), calculated a 34% lower risk of breast cancer in postmenopausal women with the smallest waist-to-hip ratio (WHR) compared with those who had the highest WHR (96). Although menopausal status appears to play a role in the obesity breast cancer link, studies have shown that regardless of menopausal status, overweight/obese and physically inactive patients appear to be at an increased risk for breast cancer progression and breast cancer -related mortality (70, 178, 187).

2.2 Menopausal Status and Breast Cancer

A large body of research has repeatedly shown that obesity increases the risk of breast cancer in postmenopausal women. Yet this link between obesity and breast cancer has been far from clear in premenopausal women. The majority of evidence available has demonstrated a non-linear positive relationship between obesity and breast cancer in postmenopausal women (258). While this relationship between obesity and breast cancer in premenopausal women is sparse and

inconsistent. Conflicting studies have suggested that obesity in premenopausal women is inversely associated with breast cancer (21, 151, 239), shows no association (112, 126), or like in postmenopausal women shows a positive association (43). While these results conflict with one another it has been hypothesized that the discrepancy between each study could be attributed to different parameters, age for defining premenopausal status as well as the effect of obesity on ER+ and ER- breast cancers. One study found a positive association between obesity and breast cancer only in ER+ cancers compared to ER- (43). While others have shown the opposite, with a direct association between abdominal adiposity and ER- breast cancer only (95). The inverse association between BMI and breast cancer in premenopausal women can not be explained by menstrual cycle characteristics, possibly highlighting an indirect effect of circulating estrogen on breast cancer reduction (151). It has been suggested that the possible inverse effect of obesity on premenopausal breast cancer risk is only present among women 35 years and younger (174), an age where estrogen levels are increased and therefore this proposed protective effect of estrogen may be lost as a woman ages and estrogen decreases. Also of note, in a study that showed a positive association between BMI and breast cancer examined women who were considered "high risk" with a Gail score $\geq$1.66. This is a scale that gathers information about the participants age, race, age at menarche, age at first live birth, number of previous biopsies and number of first degree relatives with a history of breast cancer (43). Other studies have shown using high risk premenopausal women who have a family history of breast cancer attenuates the inverse association between obesity and premenopausal breast cancer (21, 51). Premenopausal women with invasive ductal breast cancer have been shown to have a higher mortality rate (relative risk, 2.50) if they were in the highest quartile of BMI compared to women in the lowest quartile (64). Consequently, there may be an underlying difference in high risk women that influences the effect of BMI and adiposity

on breast cancer risk. Therefore, these observations between pre and postmenopausal women cannot be fully explained by alterations in sex hormone levels (estrogen) alone and therefore must be mediated by other mechanisms.

2.3 Link between Obesity and Breast Cancer

The molecular mechanism connecting obesity with breast cancer has yet to be completely elucidated. In postmenopausal women, adipose tissue is the primary site of peripheral aromatization and estrogen production from testosterone, which has been suggested to accelerate mammary epithelial cell growth (61). Increased adiposity, in particular visceral fat, may cause hyperinsulinemia, insulin resistance and dyslipidemia which in turn leads to higher insulin-like growth factor-I (IGF-I) which could exert mitogenic effects on both normal and neoplastic epithelial cells (243). Both of these theories have tangible effects on the obesity/ breast cancer link but no one theory has yet to fully explain the link. A newer hypothesis places adipocytes and their autocrine, endocrine and paracrine functions at the forefront of breast tumorigenesis (135). Initially adipocytes were believed to be inert storage depots, but we now know that they are highly active endocrine cells that secrete many factors including growth factors, cytokines, extracellular matrix proteins and hormone like molecules known as adipokines (14, 154, 196). These adipokines can act in an endocrine fashion via the peripheral circulation or in a more direct paracrine method, binding to their respected membrane receptors on the surrounding epithelial cells. As shown in figure 2.1, adipokines which are produced and secreted by the adipose tissue can enter the interstitial space and follow either two paths.

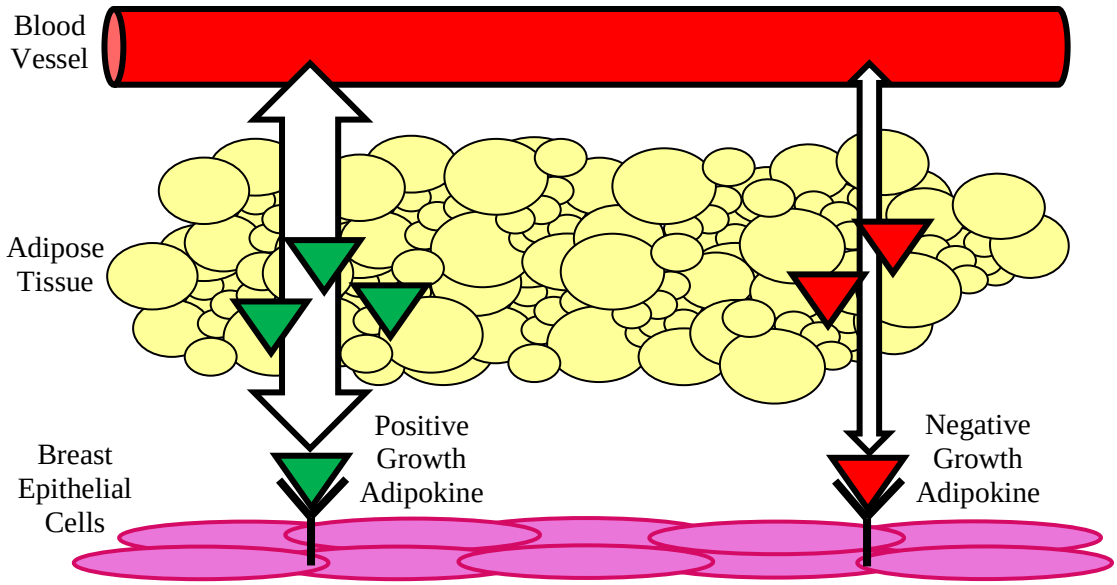

Figure 2.1: Example of how adipokines can be produced and secreted into the interstitial space where they can either act on a total body wide scale through endocrine signalling (blood vessel) or can act locally on surrounding microenvironment though paracrine signalling (breast epithelial cells).

Adipokines produced by the adipose tissue, both subcutaneous and visceral, can enter the systemic circulation and act on distant targets in the body as an endocrine factor. Adipokines can also play a more direct role in signalling locally, especially in subcutaneous depots, by binding to their respective membrane receptor or the plasma membrane of the immediate surrounding tissue acting as a paracrine factor. This paracrine signaling may play an important role in the breast as adipocytes make up the bulk of breast tissue and represent one of the most abundant cell types surrounding mammary epithelium with epithelial cells only accounting for 10% of breast volume (135). This could therefore play a large part in the stromal-ductal epithelial cell interactions within the mammary microenvironment (110). This is important as many primary breast tumors originate from ductal or intra-ductal epithelial cells. Obesity-mediated breast carcinogenesis is represented by the interaction between tumor cells and the surrounding microenvironment comprised of stromal cells, soluble factors, signaling molecules and the extracellular matrix which can all

promote tumorigenesis. It is still not entirely clear how adipocytes influence breast tumor cell behaviour but a growing amount of research is now suggesting the possibility of the paracrine factors (adipokines) secreted by the adipocytes may change the phenotypic behaviour of malignant cells.

2.4 Adipokines as Circulating Hormones in Breast Cancer

So far over 400 different adipokines have been characterized and this number continues to grow every year (267). These adipokines are involved in metabolic functions including insulin sensitivity, glucose metabolism and fatty acid oxidation as well as contributing to protein synthesis and cell proliferation in target cells (135, 195, 235). The deregulated expression of adipokines may thus be involved in the association between obesity and breast cancer. Adipokines also encompass the growing number of pro-inflammatory cytokines produced by the adipose tissue and give credence to the growing notion that obesity is a prolonged chronic state of inflammation. These inflammatory molecules have been suggested to play a role in breast cancer as several, including interleukin-6 (IL-6), transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α), have been shown to cause breast cancer cell proliferation (66, 128, 211). While these adipokines have been shown to play a role in breast cancer progression, two adipokines in particular, adiponectin (ADIPO) and leptin (LEP) have come to the forefront of the adipose tissue-breast cancer link.

One reason for this focus is that both ADIPO and LEP are among the most abundant adipocytokines produced almost exclusively by adipocytes and have also both been shown to affect the growth status of cells. Specifically, both ADIPO and LEP have been shown to be involved in regulating the proliferation of breast cancer cells, both in cell culture and *in-vivo* (110). The effects

of ADIPO and LEP both on metabolism and cell proliferation act as antagonists to one another (110, 111, 163). LEP synthesis and plasma levels are increased with obesity and higher LEP levels and have been significantly associated with increases in breast cancer incidence (56, 256). Paradoxically, circulating levels of ADIPO are decreased in obese subjects and an inverse relationship between serum ADIPO levels and breast cancer risk has been reported (147, 243). Figure 2.2 illustrates how the ratio between ADIPO and LEP shifts as an individual increases or decreases the size of adipose depots within the body.

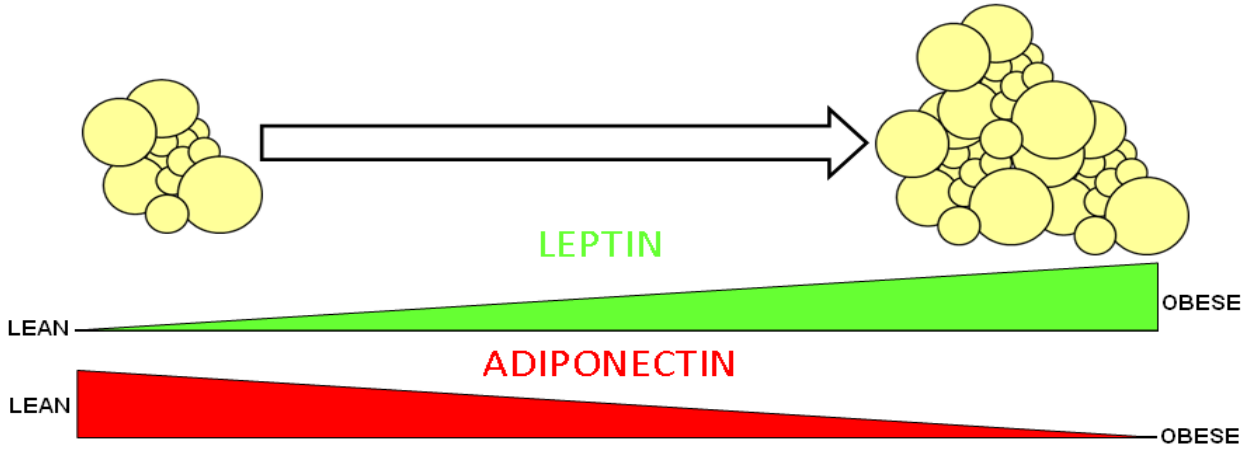

Figure 2.2: Relationship between ADIPO:LEP ratio in lean and obese individuals. A lean individual has a higher ratio of ADIPO:LEP. As this individual becomes obese (size and number of adipocytes increases) the ratio of ADIPO:LEP decreases. The ratio between the two adipokines rather than the absolute values may be a greater prognostic significance.

In a study observing Taiwanese breast cancer patients, Chen *et al.* (2006) discovered that the serum ADIPO:LEP ratio was significantly decreased in breast cancer patients when compared to patients without breast cancer (46). Independent of adiposity, serum ADIPO has also been found to be reduced while LEP is increased in women with breast cancer compared to women without the disease (153, 195). Microdialysis showed that breast cancer tissue had lower ADIPO and higher LEP compared to normal adjacent breast tissue in the same patient (157). The ratio between

ADIPO:LEP becomes altered as a women becomes obese. Lean women have been shown to have an ADIPO:LEP ratio of 1588:1 while obese women showed a significantly lower ratio of 163:1 (208). The status of the estrogen receptor (ER), HER2/neu or lymph node metastasis do not affect serum ADIPO or LEP levels in breast cancer patients and after adjusting for confounding factors, serum ADIPO and LEP levels were negatively ($r=-0.333$, $P=0.001$) and positively ($r=0.323$, $P=0.001$) correlated to BMI, respectively (46). Aside from the changes in the ratio between ADIPO:LEP both with obesity and breast cancer, both adipokines have antagonistic effects on the growth of breast cancer cells.

2.5 Leptin (LEP)

LEP is a 16 kDa protein produced from the obese (ob) gene in WAT and in the pituitary gland, acting through the hypothalamus as a regulator of body weight and energy balance by inhibiting food intake and increasing energy expenditure (265). In animals which have mutations in the gene encoding LEP (ob/ob) or the ob receptor (ZDF), morbid obesity is observed. However, in humans these mutations very rarely occur and obesity is related to LEP resistance rather than LEP deficiency (18, 107, 144). A rise in circulating LEP was thought to prevent obesity by decreasing appetite and increasing thermogenesis. However, most cases of human obesity occur with increased levels of LEP (56). Circulating LEP is directly proportional to the amount of body fat an individual has and fluctuates with acute changes in caloric intake which signals the amount of energy stored in adipose tissue (62). Circulating levels of LEP have shown a strong positive correlation with total body fat and a lesser degree with BMI (56, 140). Women tend to have higher LEP levels than men, even after correction for differences in body fat composition (62). Also subcutaneous fat has been associated with higher levels of LEP mRNA expression compared to

visceral fat (62). One possible explanation for this is that LEP may be upregulated in response to estrogen (38). The synthesis of LEP in adipocytes is influenced by different humoral factors such as insulin (129), TNFα (264), glucocorticoids (59) and estrogen (137). What is important about all these influencing factors is many have been shown to be associated with breast cancer progression.

2.6 Leptin Signaling and Cancer

LEP circulates while bound to a soluble form of its receptor and exerts its effects through binding to the LEP receptor (OB-R), which is a member of the cytokine family of transmembrane receptors (135). Five isoforms of the LEP receptor have been identified, the best-characterized being the long isoform (Ob-Rb), that activates the Janus kinase/signal transduction and activators of transcription (JAK/STAT) signaling pathway (224, 250). Only the full length Ob-Rb has full signaling capabilities, whereas the short isoforms such as Ob-Ra lack major transmembrane domains that are needed to recruit downstream effectors. Both the long and short isoforms of LEP receptors have been discovered in the human breast cancer cell lines MCF7, T47D and MDA-MB-435 (69, 82). LEP receptors were undetectable in normal mammary epithelial cells by immunohistochemistry whereas breast cancer cells showed a positive staining for Ob-R in 83% of cases (109). Interestingly, distant metastasis was present in 34% of the cases of Ob-R positive tumors with LEP overexpression, yet no metastasis was found in any of the cases where tumors lacked Ob-R expression or LEP overexpression (109). Thus, breast cancer patients that present with LEP receptor positive tumors show significantly lower survival then those without LEP overexpression or LEP receptor expression.

Upon LEP stimulation, intracellular JAK2 are activated through transphosphorylation, phosphorylate tyrosine residues on Ob-Rb and on STAT3 proteins (8). These phosphorylated

STAT proteins can then dimerize and translocate to the nucleus where they activate numerous genes including several involved in cell proliferation such as *c-fos*, *c-jun*, suppressor of cytokine signaling (SOCS3) and upregulate the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (220). LEP signaling through Ob-Rb has also been shown to activate the Ras/MAPK pathway as well as the PI3K/Akt pathway (8, 83) and both pathways increase cell cycle proliferation by affecting p27 localization or stability. LEP signaling has been shown to crosstalk with both polypeptide growth factor signaling and with steroid receptor function. For example, insulin can cause an increase in LEP expression but an increase in insulin can also lead to LEP resistance by inhibiting LEP signaling through JAK2, by SOCS3 (118, 129). LEP can accumulate in the serum in obese individuals, binding to its receptor and activating the JAK2/STAT3 pathway. STAT3 can then dimerize and translocate to the nucleus where it upregulates the negative feedback gene SOCS3 which inhibits JAK2 (118, 129). One classic criticism for the notion that increased LEP concentrations increase breast cancer cell growth in obese women is LEP resistance through SOCS3 negative feedback would inhibit JAK2/STAT3 signaling pathway. Yet previous unpublished work from our lab has shown that LEP signaling can continue to affect downstream cell cycle proteins such as p27 even when the JAK2/STAT3 pathway is pharmacologically inhibited (245). Therefore LEP appears to exhibit its cell cycle effects through another signaling pathway other than JAK2/STAT3, possibly the PI3K/Akt pathway. This may be supported by the fact that stimulation of Akt occurs several hours after LEP addition, suggesting the involvement of intermediate signalling mechanisms, possibly other than the JAK2/STAT3 pathway (82). Figure 2.3 displays the LEP signaling pathway and the downstream signalling when bound to its transmembrane receptor Ob-Rb.

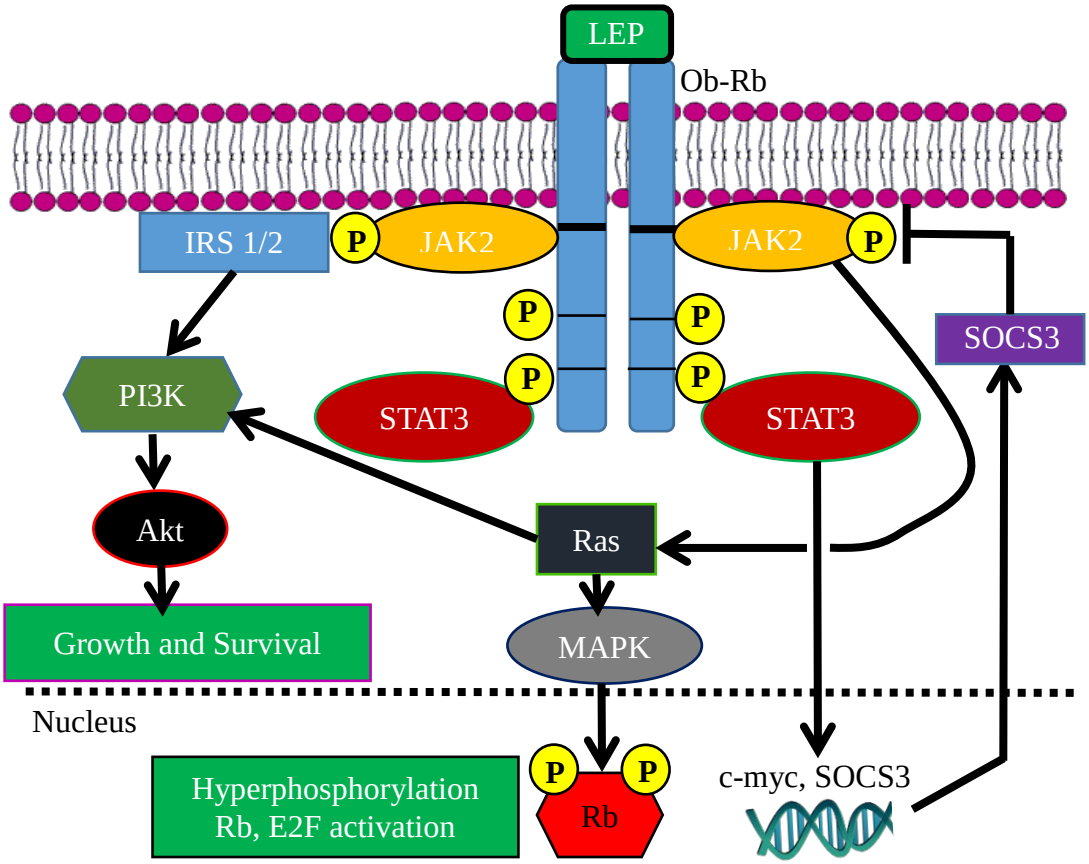

Figure 2.3: LEP receptor (Ob-Rb) signaling. LEP stimulates Akt and Ras/MAPK, both of which lead to cell growth and proliferation by inhibiting cell cycle inhibitors and tumor suppressors $p27^{KIP1}$, Rb and p53. STAT3 can translocate into the nucleus and induce the transcription of genes including SOCS3 which acts as a negative feedback mechanism for

LEP has been demonstrated to act as a mitogen, a transforming factor or a migration factor for many different cell types including both normal and malignant mammary epithelial cells (69). Interestingly, LEP activity is reinforced through a woven crosstalk with insulin, multiple oncogenes, other cytokines and growth factors. As shown in figure 2.3, insulin via IRS1/2 and the PI3K can induced LEP and Ob-Rb overexpression in human breast cancer cells contributing to an autocrine stimulation of breast cancer cells (244). Kim *et al.*, (2009) found a significant association between the expression of leptin and proliferation marker Ki-67 in tumors (120). LEP has been discovered to induce cell cycle progression by upregulating cdk2 and cyclin D1 levels, both of

which have been found to be upregulated in many breast cancers and force the G1 to S phase transition (167, 175). Yin *et al.* (2004), found that LEP increases both cyclin D1/E by stimulating the expression of c-Myc in MCF7 breast cancer cells (262). Saxena *et al.* (2007) showed that treating MCF7 cells with LEP causes an increase in the number of cells which had entered S-phase and a decrease in the number of cells still in G0/G1 while also showing that LEP stimulated the growth of MCF7 cells in both a time and dose dependent manner (194). Previous work from my masters thesis found that LEP causes a dose-dependent decrease in $pAMPK^{T172}$, p27 and $p27^{T198}$ while increasing $pAkt^{T308}$ in MCF7 cells leading to an increased number of proliferating cells (226). All these studies make the case that LEP causes an increase in several key regulatory components of the cell cycle, promoting a positive growth environment in breast cancer cells possibly explaining why obese individuals have a higher incidence of breast cancer.

In contrast to many *in-vitro* studies, epidemiological studies have shown inconsistent and conflicting associations between circulating LEP levels and risk of breast cancer (156). Several studies have shown an association of hyperleptinemia with the risk of breast cancer (94, 256) and an advanced disease state (58). In a retrospective study by Ollberding *et al.,* (2013), elevated prediagnostic LEP levels were associated with an increased risk of postmenopausal breast cancer independently from BMI (168). While other studies have shown no association of LEP levels with pre or postmenopausal breast cancer (193). Serum LEP levels have also been shown to not increase the risk of premenopausal breast cancer *in-situ* and invasive pre and postmenopausal breast cancer (156). Interestingly if LEP is taken out of the equation, there is a clear decrease in cancer development. Cleary *et al.* (2004) showed that obese mice genetically deficient in the long isoform of the LEP receptor Ob-Rb ($Lepr^{db}Lepr^{db}$) and overexpressing the oncogene TGF-α actually do not develop oncogene induced mammary tumors (52). This suggests that in the absence of LEP

obese mice have a reduced risk of developing oncogene induced mammary tumors, further stressing the importance of LEP in cancer development and progression. Therefore, so far the utility of LEP as a breast cancer biomarker is unclear by itself.

2.7 Adiponectin (ADIPO)

ADIPO (also known as ACRP30, ADIPOQ and ampM1) is a 30 kDa adipocytokine secreted predominately by WAT and is abundantly present circulating in human plasma at concentrations between 2-20 µg/ml (256). The physiological function of ADIPO is still not fully understood, but it has been shown to have the ability to decrease glucose, lipids and triglycerides as well as play an important role in metabolic syndrome (159). One of the most well-known physiological functions of ADIPO is its effect on food intake. ADIPO enhances AMPK activity in the arcuate hypothalamus via its receptor, ADIPO receptor 1 (AdipoR1) to stimulate food intake (125). ADIPO has also been found to have anti-inflammatory effects as a negative regulator of pro-inflammatory cytokines such as IL-6 and TNF-α (125) as well as affect cell proliferation (111). Serum levels of ADIPO have been shown to have a strong inverse correlation with waist circumference and visceral fat, even more so than BMI (217). One hypothesis as to why ADIPO is decreased in obese individuals is that both TNFα and IL-6 are increased in obesity and both have been shown to repress genes of transcription factors involved in ADIPO synthesis (268). While another study found in 3T3-L1 adipocytes, TNF-α caused a dose-dependent reduction in the expression of ADIPO by suppressing its promoter activity (139).

Women with breast cancer have been shown to have low ADIPO levels in their plasma serum, as observed in other cancers (117). Macis *et al.* showed that lower circulating plasma ADIPO levels in premenopausal women is a risk factor for progression from intraepithelial

neoplasia to invasive cancer which was independent of age, BMI and treatment group (138). Mantroros *et al.* (2004) found an inverse relationship between circulating ADIPO levels and breast cancer incidence in postmenopausal women that was independent of insulin growth factor (IGF-1), LEP, BMI and other parameters (142). Low ADIPO levels have also been associated with breast cancer metastasis and increased mortality in breast cancer survivors, even after adjusting for obesity (63). Therefore, ADIPO could play a role in breast cancer etiopathogenesis, specifically in low-estrogen environment seen in postmenopausal women (63). As previously stated, adipocytes constitute a large part of the breast stromal composition and ADIPO therefore may exert a major paracrine and autocrine effect on mammary epithelium.

ADIPO exists in both a proteolytic and full-length form within the plasma. Full length ADIPO, upon proteolytic cleavage, forms globular ADIPO (gADIPO) which interacts with ADIPO receptors. ADIPOs four isoforms consist of homotrimers (90 kDa), a hexamer (complex of two trimers), a 180 kDa low molecular weight (LMW) and a 360-400 kDa high molecular weight (HMW) form (91). The different configurations present different biological effects as non-HMW ADIPO show stronger anti-inflammatory actions, HMW which constitutes 70% of circulating ADIPO is related to insulin sensitivity (234). The different free forms of ADIPO have been detected within human plasma and have been found to bind to the two ADIPO receptors with differing affinities, which then initiate the downstream effects observed by ADIPO stimulation.

2.8 Adiponectin Signaling and Cancer

ADIPO mediated affects are the result of the adipokine binding to one of its two membrane receptors. The ADIPO receptors AdipoR1 and AdipoR2 are integral membrane proteins with seven transmembrane domains with each shown to have unique distributions throughout the body and

altered affinities for the different forms of circulating ADIPO (259). The receptors have an internal N-terminus and an external C-terminus (259), an orientation opposite the topology observed in G protein-coupled receptors. AdipoR1 exhibits high affinity for gADIPO (259), while AdipoR2 has been shown to have intermediate affinity for both globular and full length ADIPO (85). ADIPOR1 has been found to be most abundantly located in skeletal muscle (85), but has also been found present in healthy breast epithelial cells (123), in human adipocytes (185) and in both invasive and pre-invasive breast cancer tissue (176). AdipoR2 has been located primarily in the liver with low levels found in other tissue locations (85). AdipoR1 mRNA and protein have been detected in many primary breast cancer cell lines including MCF7, T47 and the MDA-MB cell lines while AdipoR2 expression was found to be much lower (68, 222). Along with breast cancer cells, AdipoR1 mRNA expression is 10-15 times higher than AdipoR2 in human isolated adipocytes (185). Since AdipoR1 is expressed to a much higher level than AdipoR2 in breast cancer lines and adipose tissue samples, this thesis focuses on AdipoR1 exclusively and the interaction ADIPO through AdipoR1 plays on downstream cell cycle signaling pathways.

AdipoR1 mediates fatty-acid oxidation and glucose uptake once stimulated by ADIPO by activating the downstream phosphorylation-dependent activation of AMPK (260). New research has shown that ADIPO mediated activation of AMPK through AdipoR1 is dependent on the adaptor protein containing pleckstrin homology domain, phosphortyrosine binding domain and leucine zipper motif (APPL1) (65, 143). APPL1 interacts with AdipoR1 in mammalian cells and the interaction is stimulated by ADIPO. APPL1 is also essential for mediating the ADIPO signal to induce the cytosolic translocation of the Serine/Threonine liver kinase B (LKB1), an essential step for the activation of AMPK (65). AMPK is a central metabolic switch found in all eukaryotes that governs glucose and lipid metabolism in response to alterations in nutrients and intracellular

energy levels (202). Taken together, LKB1 and AMPK control cell growth in response to environmental nutrient changes. AMPK is a heterotrimer composed of a catalytic AMPKα subunit and two regulatory subunits (AMPKβ and AMPKγ) (202). The activation of AMPK occurs when there is an increase in the AMP:ATP ratio. This increase then allows for AMP to bind to the AMPKγ Bateman domain causing a conformational change in AMPK exposing its catalytic domain on the α subunit (98). With its catalytic domain exposed, LKB1 can directly phosphorylate AMPK at T172 causing its activation (203). Our lab has previously shown in MCF7 breast cancer cells that AMPK activation, regardless if by ADIPO, metformin or AICAR, leads to AMPK directly stabilizing AdipoR1 and increasing receptor half-life (226). ADIPO is able to not only activate AMPK and elicit its downstream metabolic effects, but this activation of AMPK also has been found to have cell cycle effects.

ADIPO treatment leads to the activation of AMPK in several breast cancer cell lines (MCF7, T47D and MDA-MB-231), all differing in the status of their ER and presence of a p53 mutation (68, 121, 123, 162). MCF7 cells treated with ADIPO were found to undergo both a time (4, 121) and dose-dependent inhibition of proliferation (89). Work from my undergraduate project found that increasing concentrations of ADIPO leads to an increase in both total p27 and stabilized $p27^{T198}$ (226). ADIPO treatment in MCF7 cells was also found to decrease the amount of active Akt ($pAkt^{308}$), which has been shown to cause breast cancer proliferation by inactivating downstream anti-proliferative proteins such as p27 (226). This ADIPO mediated increase in $pAMPK^{T172}$ and decrease in $pAkt^{T308}$ works in a dose-dependent manner, leading to cell cycle arrest in S-phase in MCF7 cells and up-regulates the cell cycle regulator proteins p27 and p53 (186, 226). Along with inhibiting the PI3K/Akt pathway, ADIPO has also been shown to decrease the activation of MAPK as well as decrease the mRNA expression of cyclin D1 and c-Myc in

MCF7 breast cancer cells (68). From my masters work I also found ADIPO dose-dependent inhibitory effects on Akt activation were identical to those seen when using a pharmacological Akt inhibitor (226). Figure 2.4 shows a simplified depiction of the ADIPO and AMPK activation and subsequent downstream cell cycle effects.

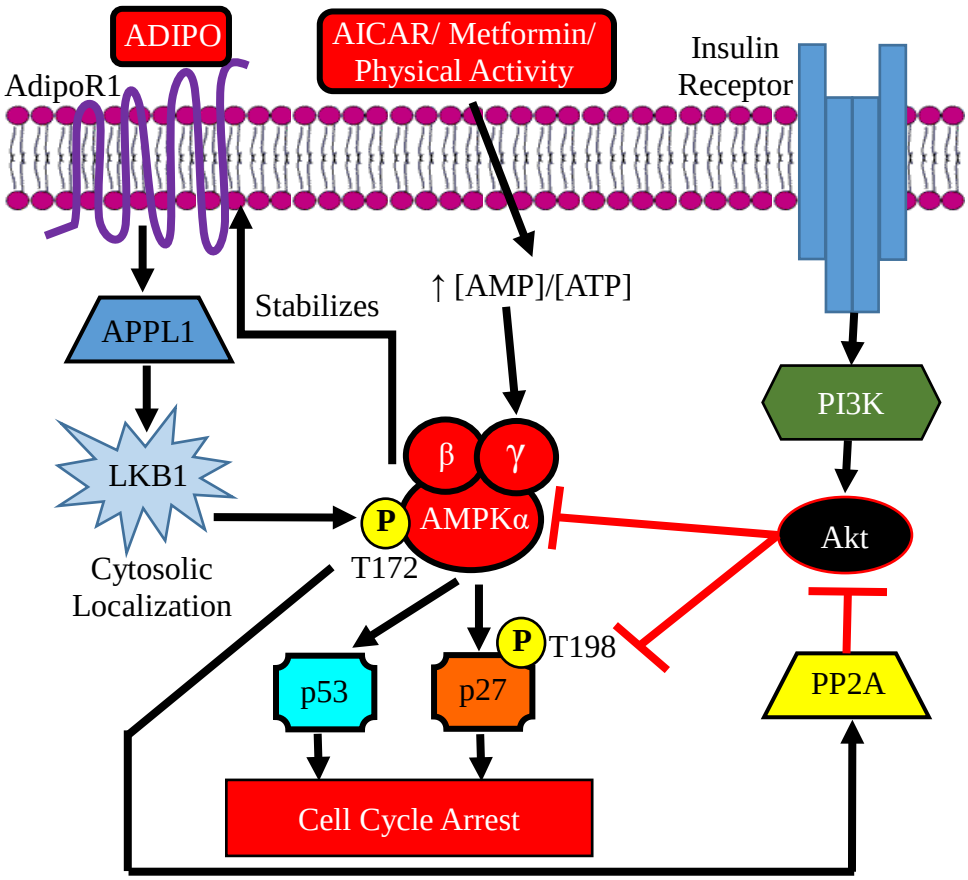

Figure 2.4: ADIPO and AMPK signaling. The energy sensing kinase AMPK is activated by ADIPO as well as by exercise. Once activated, AMPK phosphorylates and stabilizes p27 leading to cell cycle arrest in both normal and malignant cells.

From these studies we can observe that ADIPO causes an increase in pAMPK and a decrease in pAkt yet the mechanisms are unclear. Kim *et al.* (2009) uncovered that ADIPO treatment increased pAMPK (Thr 172), which caused the dephosphorylation of Akt (Thr 308 and Ser 473) in MDA-MB-231 breast cancer cells by increasing protein phosphatase 2A (PP2A) activity (121). My masters thesis also confirmed that ADIPO, signaling through AMPK, caused a

decrease in pAkt^{308} in MCF7 cells (226). Specifically, the group found that PP2A activity was due to activation from AMPK, via ADIPO signaling through AdipoR1. The dephosphorylation of Akt by ADIPO was blocked once the AMPK inhibitor Compound C was added to the ADIPO treatment, while the use of the PP2A inhibitor okadaic acid caused all pAMPK effects on pAkt to be abolished (121). This indicates that AMPK, working through PP2A, could be causing the decrease in pAkt in MDA-MB-231 cells. Although my thesis did not confirm this PP2A method of inactivating pAkt, it still remains a plausible mechanism by which ADIPO can inactive the Akt proliferation pathway under situations of energy limitation or a method of "tricking" a growing tumor to slow its growth.

2.9 Importance of AdipoR1 in Breast Cancer

From the previous section, we observed how ADIPO can play an important role in slowing the growth of breast cancer by upregulating and stabilizing downstream tumor suppressors. Therefore, any alterations in AdipoR1 expression can compromise ADIPOs ability to properly elicit its downstream cell cycle effects. AdipoR1 gene expression was also found to be 62% lower in obese women compared to lean women in omental adipose tissue and 60% lower in subcutaneous adipose tissue (185). Interestingly, these alterations in AdipoR1 expression in adipose tissue can be reversed. The consumption of very low calorie diets sustained for 8 weeks with an average weight loss of 12.1kg was shown to increase expression of AdipoR1 in subcutaneous adipose tissue by 80% and increase ADIPO expression in adipose tissue by 65% in women (185). In support of increasing the stability of AdipoR1 as a target for breast cancer, a study by Pfeiler *et al*., (2010) found no correlation between menopausal status and AdipoR1 expression in both invasive and preinvasive lesions (176). This is important, as regardless of

menopausal status AdipoR1 levels appear to react similar in regards to neoplastic formation. In the same study, the group found an inverse correlation between AdipoR1 expression and tumor size of pre-invasive ductal carcinomas *in-situ* (176). Work from my Master's thesis showed that MCF7 cells transfected with an AdipoR1 expression plasmid which produced a 2-fold increase in AdipoR1 protein, grew at a much slower rate than mock transfected (MockT) cells (226). Also, the transfected MCF7 cells were able to abolish the proliferative effects of LEP supplementation indicating that just increasing the potential binding sites for ADIPO without altering the levels of ADIPO in any way can overcome the proliferative microenvironment in an obese individual (226). As stated, one of the easiest methods of increasing AdipoR1 expression is simply through physical activity (185). Therefore, it can be theorized that a decrease in adiposity can increase the amount of AdipoR1 leading to better utilization of existing ADIPO both in the circulation and the interstitial fluid surrounding the tumor. This is important for obese women as there will be a decrease in ADIPO and a decrease in AdipoR1 coupled with an increase in LEP, all of which have been shown to produce a positive tumor growth environment. Maintaining AdipoR1 levels within a tumor may lead to better utilization of existing ADIPO and a subsequent decrease in breast cancer growth and proliferation despite the make-up of the external tumor growth microenvironment.

2.10 Estrogen

In premenopausal women, estrogen, a C_{18} steroid has been shown to exhibit many physiological functions as an endocrine factor such as development of secondary sexual characteristics, preparation of tissues for progesterone response and regulation of insulin responsiveness (27). Three major naturally circulating isoforms of estrogen have been found and termed estrone (E1), estradiol (E2) and estriol (E3) (106). E2 has been shown in high levels in pre

and postmenopausal women and has been shown to be the most potent of all estrogens (79). Estrogens are transported in the blood bound to a glycoprotein termed sex hormone-binding globulin (SHBG), which regulates estrogen access to tissues and functional bioavailability (93). E2 has the highest binding affinity for SHBG out of all estrogens and circulates in the blood primarily bound with only about 1-2% unbound and biologically active (215). Obesity has been shown to lower the levels of SHBG in women possibly because insulin, IGF-I and TNFα which are all increased with obesity, have been shown to downregulate SHBG (184, 200). In one study, obese women had an average SHBG concentration that was half that of normal weight (BMI $<25 kg/m^2$) women (149). Therefore in obese women, less SHBG leads to more circulating bioactive E2.

Estrogen biosynthesis is catalyzed by the enzyme aromatase (cytochrome P450 aromatase) in the endoplasmic reticulum of cells and is responsible for catalyzing the conversion of androgens, mainly testosterone and androstenedione to estrogen (Fig. 2.5). The primary site of aromatase expression in premenopausal women is within the ovarian granulosa cells while the peripheral tissues (adipose tissue) are the primary site for estrogen production in postmenopausal women (88, 210). In postmenopausal women, the conversion of androgens to estrogen is elevated in obese women (23, 47). Interestingly, both aromatase activity and mRNA in adipose tissue primarily reside in adipose fibroblasts and not in mature adipocytes (2). Other researchers have proposed that cytokines may also play a role in regulating aromatase gene expression in obesity. Increased circulating levels of proinflammatory cytokines such as TNFα, IL-6 and LEP are have been shown to induce aromatase expression in human adipose tissue (266). This increased aromatase activity by the adipose tissue has been suggested in the pathogenesis of breast cancer, especially in obese postmenopausal women.

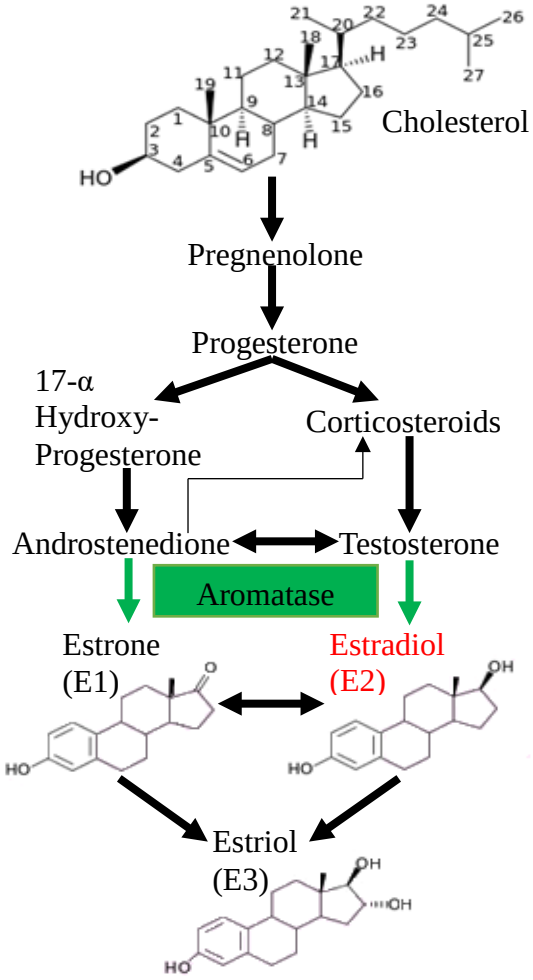

Figure 2.5: Estrogen biosynthesis. Beginning with cholesterol, all three isoforms of estrogen are produced through different means but all must be produced from the conversion enzyme aromatase from either androstenedione or testosterone. E2 shown in red is one of the most abundant estrogens in women and shown to alter breast cancer cell growth.

2.11 Estrogen Signaling and Cancer

E2 or more precisely, 17β-estradiol, that is free from SHBG can readily pass through the cell membrane as a steroid hormone and bind to one of its two cytoplasmic receptors ERα or ERβ, members of a large superfamily of nuclear receptors that function as ligand-activated transcription factors (115). Hormone-activated estrogen receptors form dimers in the form of ERα (αα), ERβ (ββ) homodimers or ERαβ (αβ) heterodimers (131). E2 has been demonstrated to bind with equal

affinity for both ERs (269). Once activated by E2, the ER is able to translocate into the nucleus and bind to DNA to regulate the activity of different genes via estrogen response elements (ERE) located within the promoters, recruitment of coactivators or corepressors to the promoter, resulting in increased or decreased target mRNA levels and associated protein production. This mechanism of action is thought to be the typical canonical steroid-receptor interaction (genomic effects). New evidence now suggests that the ERα participates in extra-nuclear signaling through a membrane bound receptor (mERα) signalling through coupling, directly or indirectly, to G-proteins (257). The primary G-protein signaling has been shown to be through GPR-30, which in addition to the mER, can activate MAPK and PI3K signaling (77). Membrane-bound ERα reportedly interacts with growth factor receptors such as IGF-1R and epididymal growth factor receptor (EGFR) (78, 113), which promotes stimulation of Src kinase, MAPK and PI3K pathways in the cytosol (44), which have all been implicated in breast cancer progression (Fig. 2.6). E2, via ERs and GPR-30 (GPCR) can increase proliferation of mammary tissue, possibly in part by the induction of growth factors (67). It is believed that this proliferation of mammary cells may result in an increased cell division and DNA replication leading to an increased risk of mutations and ultimately cancer.

Circulating levels of estrogen have a strong linear association to adiposity in postmenopausal women with obese postmenopausal women more often having ER+ breast cancers (191). Both ERs have been shown in varying levels in several ER+ breast cancer cell lines, with ERα being the major ER subtype in the mammary epithelium which plays a critical role in breast cancer progression (57). E2 has been shown to stimulate breast cancer cell proliferation in estrogen receptor positive (ER+) cells by activating ER transcriptional activity and also by directly activating intracellular signaling pathways such as MAPK and PI3K (Fig. 11) (127). ER can bind to PI3K in a ligand dependent manner (209) and PI3K has been demonstrated to be necessary for

E2/ERα signaling (30). Estrogen induces mitogenic effects on breast epithelial cells by stimulating G0/G1 quiescent cells to re-enter the cell cycle mediated by increased expression of c-Myc and cyclin D1 genes (37, 68). The expression of c-Myc is rapidly induced following E2 stimulation and downregulated following antiestrogen treatment (37). The use of antiestrogens such as ICI 182,780 among others, have been shown to induce rapid ERα degradation through ubitquitin-mediated pathways (25, 75, 145), inhibit nuclear ERα expression and reduce ERα-dependent transcription from ERE, resulting in loss of E2 proliferation in breast cancer cells (82). Similar to LEP, E2 can act through the MAPK and PI3K/Akt pathway to promote the cell survival and proliferation. E2 induced PI3K/Akt activation has been shown to occur through the ERα and not the ERβ in ER+ MCF7 cells (127). Interestingly, in ER- MDA-MB-231 breast cancer cells, E2 can stimulate Akt activation in a time and dose-dependent manner which is blocked by inhibitors of PI3K and Src kinase but not estrogen antagonists (236), which suggests a receptor-independent function of E2 in Akt activation. The cell cycle inhibitor p27 also plays a role in E2 mediated cell proliferation. *In-vitro* treatment of E2 with several female cancers have shown that E2 can cause a loss of nuclear p27 by inducing MAPK mediated phosphorylation of p27 on T187 leading to Skp2-dependent degradation of p27 (105, 173).

While this direct E2-ER modulation has been shown to increase the transcription of cell proliferation and survival proteins, other cell signaling pathways have also been shown to modulate the transcriptional activity of the ER (115). Research has also shown a role of adipokines in abolishing or supporting these E2-depedent effects as well as E2 altering adipokine secretion profiles. Higher plasma E2 concentrations have been shown to be correlated with lower local extracellular ADIPO and ADIPO:LEP ratio and positively correlated with LEP in breast adipose tissue using microdialysis in women regardless of menopausal status (157). A study by *Dieudonne*

et al.,(2006) found that simultaneous exposure of ADIPO and E2 supressed the mitogenic effect that E2 had alone on ER+ MCF7 cells (68). Another study by *Pfeiler et al.*,(2008) demonstrated that MCF7 cells treated with E2 caused a 50% decrease in AdipoR1 mRNA and this was completely abolished with the addition of ADIPO (177). No effect of E2 was shown on AdipoR2 mRNA. The E2 effects shown in MCF7 cells on AdipoR1 were not seen in the ER- MDA-MB-231 breast cancer cell line. Treatment of MCF7 cells with E2 alone was found to increase proliferation while the addition of ADIPO increased the apoptotic rate (177). Higher concentrations of ADIPO are needed to inhibit proliferation of ER- breast cancer cells compared to ER+ cells which may provide a reason why obese women (low ADIPO) with ER- cancer tend to have a worse prognosis than those with ER+ tumors (89). A mutation of LKB1 is associated with aromatase overexpressing tumors, suggesting a role for AMPK in aromatase regulation and may partly explain why in individuals with low ADIPO we see higher aromatase expression (24, 104).

While ADIPO appears to counteract the proliferative effects of E2 on ER+ breast cancer cells, the opposite has been found in regards to LEP. LEP has been shown to enhance aromatase mRNA expression, aromatase content and its enzymatic activity in MCF7 cells via transcription factor activator protein 1 (AP-1), enhancing *in-situ* E2 production and promoting estrogen-dependent breast cancer progression. (40). ERα plays an important role in breast cancer development and can be transcriptionally activated in a ligand-independent manner. Free ERα is an effector of MAPK signaling and LEP via JAK can activate the Ras-dependent MAPK pathway, demonstrating how LEP can transactivate ERα through the MAPK pathway (41). Reciprocally, E2 has been shown to up-regulate LEP mRNA and protein synthesis in adipocytes (137) and modulate Ob-Rb expression (12). LEP is also able to interfere with the action of ICI 182,780 by stabilizing

nuclear and cytoplasmic ERα, independent of E2 (82). One reason for this is MAPKs and PI3K, both activated by LEP, have been shown to impede proteasome-mediated degradation of ligand free ERα and inhibit the degradation of ER species (145). While the effects of E2, ADIPO and LEP on one another, both in growing breast tumors and surrounding adipose tissue, is complicated with several intersecting signaling pathways, the contribution of obesity on the development of breast cancer cannot be explained by estrogen levels only.

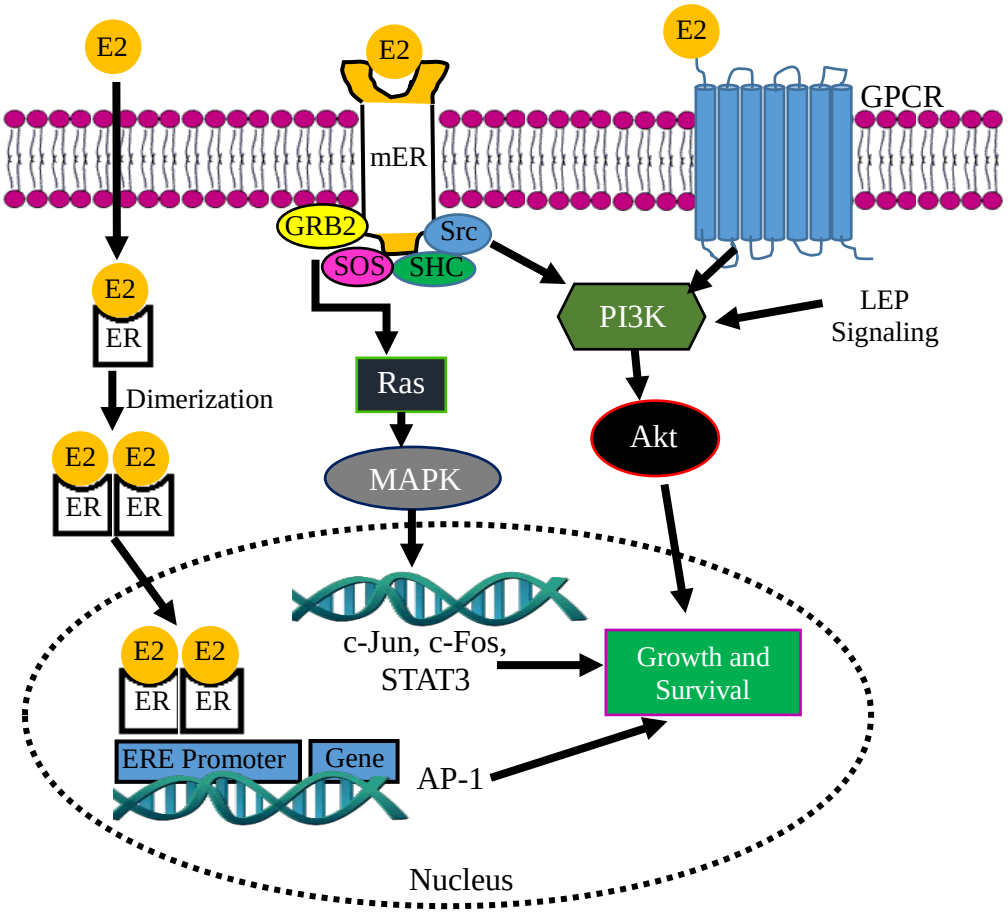

Figure 2.6: Estrogen receptor (ERα) signaling. Estradiol (E2) can activate several methods of cell signalling from ER dimerization, membrane ER, GPCR as well as integrate cell signaling form several growth factors (LEP, EGF, insulin) through PI3K/Akt and MAPK. Estrogens effects lead to upregulation of genes which promote cell survival and proliferation.

3. Methods of Altering Tumor Growth Microenvironment and Breast Cancer

Breast cancer is a dynamic, multi-factorial, inherently complex disease. Each patient likely possesses a unique and specific carcinoma making developing global cancer therapies difficult, as tumors with different underlying mechanisms may be resistant to similar therapies. Unlike the heterogeneity of breast cancers, the growth environment that exists within each patient is more stable and uniform, since the majority of factors that contribute to this environment are produced by predictable components of the patient's physiological systems rather than by the unreliable tumor itself. Thus, targeting this growth microenvironment therapeutically may result in more predictive and reliable treatment outcomes. Since the vast majority of all tumors are surrounded by adipocytes and adipocytes serve as active endocrine cells, there may be important paracrine/endocrine effects of adipose derived factors on tumor proliferation and progression, potentially representing the development of a viable target tissue for novel cancer therapies. If research can find methods of altering the ADIPO:LEP ratio in this growth microenvironment we may be able to slow and even stop the progression of breast cancer in obese women. One possible route researchers are considering for breast cancer therapy in obese women is reactivating the diminished signaling of ADIPO or activation of AMPK signaling (218). The therapies proposed are simple such as physical activity/exercise, diet as well as pharmacological agents, which have been shown to alter the ADIPO:LEP ratio and could therefore be a reason for the decreased incidence and severity of breast cancer in these patients.

3.1 Effect of Diet and Physical Activity on Adipokine Profile

As previously described, obesity has a profound effect on promoting breast cancer progression, especially in postmenopausal women. Several methods of combating obesity have

also been shown to have an effect on circulating ADIPO and LEP. The most studied and simplest of which are weight loss induced by low fat or low calorie diets and through exercise/physical activity (PA). The effects of dietary weight loss and PA on circulating ADIPO and LEP have been shown with conflicting results. An exercise intervention program for obese women, both pre and postmenopausal, found that exercising at 50% max VO_2 for 45 min 5 days a week caused a 25.7% decrease in plasma LEP but no change in ADIPO (179). Despite the fact that in the subcutaneous adipose tissue LEP mRNA was decreased by 1.9-fold and ADIPO was increased by 1.6-fold (179). Diet has also been shown to affect adipokines in overweight/obese postmenopausal breast cancer survivors. A recent study by Thompson *et al.,* (2015) found that a low fat diet for 6 months led to an 81% decrease in circulating LEP while no change in ADIPO was found (229). Although circulating levels of ADIPO were not found to be changed in these studies, the overall ratio between ADIPO:LEP was increased. Other studies have shown that both a low calorie diet (1000 calories/day) and exercise program in obese women increases circulating levels of ADIPO and decreases circulating levels of LEP (97). It is uncertain why some studies of diet and PA appear to have conflicting results in regards to changes in circulating ADIPO. One reason may be that other cytokines affect ADIPO transcription (i.e. IL-6 and TNF-α) and potentially others may not be changing enough to produce a marked increase in ADIPO. Regardless, a decrease in LEP and constant ADIPO levels led to an overall increase in the ratio between the two which may be of more significant chemical/functional importance.

A study by Fabian *et al.*, (2013) looked at overweight and obese women at an increased risk of breast cancer and the effect of a 6 month intervention of a low calorie diet (1000 cal/day) and PA protocol (>300 min/wk) on specific tissue and serum biomarkers for breast cancer progression (74). The group found using random peri-areolar fine needle aspiration that a

significant tissue modulation was noted as indicated by a decrease in the proliferation marker Ki-67, an increased ADIPO:LEP ratio and a decrease in phosphorylated Rb proteins after the 6 week protocol (74). Favourable modulation of serum markers were also found such as an increase in SHBG, ADIPO and ADIPO:LEP ratio while a decrease in bioavailable E2 and LEP (74). Most importantly, the group found that both serum and tissue biomarker modulation only occurred in women who had >10% weight loss over the 6 month protocol. Changes in breast cancer rate and severity may, in postmenopausal women, be due to PA altering the adipokine secretion profile and tumor growth microenvironment created by the adipose tissue. Postmenopausal women consuming a calorie restricted diet and engaging in moderate PA for 12 months were found to cause an increase (9.5%) in plasma ADIPO and a decrease (40.1%) in LEP (1). Thus, there are clear positive dose-dependent effects of diet and the amount of physical activity and its benefits to breast cancer patient prognosis in regards to altering the tumor growth microenvironment and may partly explain why research has shown a profound effect of diet and PA on breast cancer.

3.2 Effect of Weight Reduction on Estrogen

Weight loss, either through caloric restriction or PA, has been shown to lead to a reduction in circulating estrogen yet the relationship between the amount of weight loss and reduction in circulating estrogen is not always proportional. A diet intake of 1200 kcal/day for an average of 14 months resulted in an average weight loss of 14.5 kg (15.6% decrease from initial body weight) in postmenopausal women which resulted in an average serum E2 reduction from 25.5 to 17.9 pg/ml (225). Besides a reduction in estrogen as a result of caloric restriction, studies have also shown weight reduction of 4% in postmenopausal women to elicit an 18% decrease in serum E2 and also produce a significant increase in SHBG (16). In premenopausal obese women (average

age 34.7 years) who underwent weight reduction after 12 months with an average of 59 kg weight loss, serum levels of E2 decreased from 94.9 to 73.6 pg/ml (9). A recent systematic review and meta-analysis of randomized control trials found that the overall effect of PA produced a significant decrease in both total E2 (SMD -0.12, p= 0.01) and free E2 (SMD -0.20, p=0.00005) (72). Interestingly, the group found that this PA effect was independent of menopausal status and is more noticeable for non-obese women. PA was also found to cause a significant increase in SHBG (72). Due to the fact that many breast tumors, at least in postmenopausal women, are partly reliant on E2 for growth, it seems likely that weight loss and the concomitant reduction in E2 levels should lead to a reduction in breast tumor growth.

3.2 Effect of Physical Activity on Breast Cancer

Research has begun over the last decade to show an association between PA and breast cancer risk. A sedentary lifestyle has now been widely accepted as a major contributor to the increase in obesity and its associated disorders (197). This highlights PA as a potential prevention/intervention for the development of obesity and its associated effects on breast cancer (20, 56, 71). Several epidemiological studies have provided strong evidence that women who are in the highest quartile of PA have a lower incidence of breast cancer than those who are in the lowest quartile (148, 232, 233). The magnitude of this protection has been shown to be greater in individuals in the lowest quartile of BMI and the highest quartile of PA, which suggests that PA may exhibit effects on breast cancer risk independent of body size (232, 233). These results have also shown by *Thune et al*., (1997) who observed greater leisure-time activity was associated with a reduced risk of breast cancer even after adjustment for age, BMI, height and country of residence compared to sedentary women (232). Moderate physical activity (>0.64 MET-hours/day) reduces the incidence of breast cancer, with women who are physically active exhibiting a 20-30%

reduction in the relative risk of developing breast cancer compared to their sedentary counterparts (114, 169, 189, 201, 233). The effect of physical activity is also important in improving patient survival in breast cancer as seen by an up to 40% reduction in cancer-related death and cancer recurrence in physically active women (103).

There are established dose-dependent (intensity and duration) relationships among physical activity, cancer risk and overall survival in breast cancer patients (251). Breast cancer patients participating in physical activity consisting of walking as little as one hour/week was associated with improved survival compared to sedentary women (103). These effects were more pronounced in women who engaged in moderately intense exercise between 3-5 hours per week (103). When exercise intensity was increased further (running >1.8 MET-hours/day) breast cancer patients had an almost 90% lower risk of cancer mortality compared to women who walked (<1.07 MET-hours/day) (251). Other studies have confirmed this duration effect as women who engaged in equivalent of 1.25-2.5 hrs/wk of brisk walking had an 18% decrease in breast cancer risk compared to sedentary women (148). A greater reduction in risk was observed in women who engaged in equivalent of ≥10 hrs/wk of brisk walking (148). Clearly PA has an effect on breast cancer development both as a protective mechanism and also mitigating the effects of women who already have the disease. Understanding the mechanism of why PA appears to have this effect is important in understanding how to prescribe PA to patients.

3.3 Effect of Menopausal Status on Physical Activity and Breast Cancer

Although there are some discrepancies for the role of obesity and breast cancer risk in pre vs. postmenopausal women, research shows that PA lowers the risk of breast cancer for both menopausal status (15, 35). The effect of PA has been shown in both menopausal status women

but a large body of research now points to the greatest breast cancer risk reduction effects of PA in premenopausal women. In regularly exercising women, the risk reduction of breast cancer is greater in premenopausal women compared to postmenopausal women, defined as women >45 years of age (232). The effects of PA have been shown to be attenuated with age as women ≤35 years of age who engage in regular PA have a 14% decreased risk of breast cancer which is attenuated at >50 years of age (148). Higher recreational PA (>17.6 MET hrs/wk vs. sedentary) has been shown to associated with a 30-60% reduction of all ER/PR subtypes in premenopausal women compared to sedentary women (71), whereas moderate PA (>0.64 MET-hours/day) causes a similar 30% reduction in postmenopausal women (114, 169, 189, 201, 233). A review article on the findings from the National Action Plan on Breast Cancer's Workshop on PA and Breast Cancer, found that the breast cancer risk reduction associated with PA may be greatest among women who are lean and premenopausal (80). Other studies have also shown that risk reduction of breast cancer due to regular PA is greater among premenopausal women <45 years of age compared to postmenopausal women (148, 232).

3.4 Physical Activity and Breast Cancer Link (Animal Model)

Rodents are the main animal species currently used in breast cancer experimental studies as their mammary glands have the most similar structure and function to that of human glands (32). The similar basic structures between humans and rodents mammary glands makes it possible to conduct experiments that compare the two in the process of carcinogenesis and breast cancer development (246). In an animal model of mammary carcinogenesis, several studies have looked at the effect of PA on carcinogenic response in 1-Methyl-1-Nitrosourea (MNU) induced female rat models of breast cancer. The findings clearly show that voluntary or motorized wheel running

lower the incidence and multiplicity of cancer compared to sedentary animals (228, 231, 249, 270, 271). The difference between relative risk for incident cancer using a motorized wheel (similar to a treadmill) or a non-motorized wheel were almost identical (0.68 vs. 0.69, respectively) relative to sedentary animals (231). This suggests that voluntary wheel running PA causes the same cancer preventing effects as motorized and can be seen as a valid rodent model for PA. The levels of several adipokines, inflammatory factors and other biomarkers were probed in the serum of breast cancer induced MNU animals between lean sedentary and wheel running rats. Several studies have shown that in the wheel running PA group with lower cancer incidence, the levels of ADIPO are increased (271), LEP is decreased (231, 270, 271), but no change in estradiol levels (231, 270, 271) compared to sedentary animals were evident. Voluntary PA has also been shown to alter the production of both ADIPO and LEP in rats fed a high fat diet (HFD), lowering the levels of LEP and increasing the levels of ADIPO in the circulation compared to sedentary HFD fed animals (20, 124, 231, 270, 271). PA decreased $pAkt^{T473}$ and cyclin D1 and increased $pAMPK^{T172}$ and p27 within mammary carcinomas of those same animals (270).

Although the administration of PA has been shown to decrease the promotion and progression of MNU induced mammary tumors, several studies have also attempted to demonstrate the influence of PA on carcinogenesis with respect to the intensity of physical training. A study by *Cohen et al.,* (1991), demonstrated that moderate PA in rats had a preventative and inhibitory effect on the progression of carcinogenesis (53). While high-intensity PA has been shown to cause a 37% decrease in breast cancer growth and a 60% decrease in tumor multiplicity compared to sedentary controls (230). The same study found that the degree of protection against cancer was proportional to the intensity but not to the duration of exercise. These results have also been shown in a study by *Malicka et al.,* (2015), where they looked at the effect of low,

moderate and high intensity PA (treadmill) on MNU induced mammary carcinogenesis (141). They showed a decrease in tumor number and number of affected rats between all training groups vs sedentary. Interestingly, there was no difference between moderate and high intensity PA as both showed similar reduction in affected rats and tumor number in those rats affected (141), suggesting a possible celling or maximum effect of PA at moderate PA on MNU induced breast carcinogenesis that has no further benefit at high intensity PA. It has also been shown that moderate had no effect on the development of MNU induced mammary tumors (227). Another study even showed that low intensity short duration PA enhanced the rate of occurrence of mammary tumors, while increasing the intensity and duration of exercise resulted in a protective effect against mammary cancer development (227). Therefore, PA appears to have a clear effect on both protecting against breast cancer development but more strikingly, PA can also counteract the proliferative nature of developing breast tumors possibly by altering the composition of the tumor growth microenvironment.

3.5 Resveratrol and Breast Cancer

Aside from diet and exercise, other nutraceutical agents have been shown to affect breast cancer cell growth possibly by altering the ratio of ADIPO:LEP, and in turn altering the tumor growth microenvironment. Resveratrol (RSV; *trans*-3-4',5-trihydroxystilbene), a well-known polyphenolic compound which is produced by plants and is contained within the skin of red grapes, is known to have several beneficial effects including anti-cancer properties (240). The multiple effects of RSV may be mediated in part by its proposed effect on obesity. Research has shown that RSV exerts beneficial effects in rodents that are fed a HFD, substantially reducing visceral fat as well as whole body weight gain (7, 136). Several mechanisms have been proposed to underlie

these RSV affects including possibly altering the secretion and plasma concentrations of several key adipokines, such as ADIPO and LEP. RSV has been found to increase the circulating levels of ADIPO (10, 188) while concomitantly lowering the circulating levels of LEP in rodents (10, 221). The metabolic effects of RSV appear to be mediated, in part, by AMPK within the adipose tissue, possibly by an increased production of ADIPO by the adipocytes (188, 238). This alteration in the ADIPO:LEP ratio caused by RSV in the adipose tissue and subsequently the microenvironment, may explain why RSV acts as an anti-cancer agent in obese individuals.

RSV is considered a phytoestrogen that appears to exert both antagonistic and agonistic effects on estrogen (17, 19). Due to this, research conducted on RSV and estrogen related cancers has found conflicting results. An initial study by *Bhat et al. (2001),* found that RSV administered to MCF7 cells, caused a weak estrogenic response but if RSV was combined with E2 (1 nM), there was a dose-dependent antagonism (17). In contrast, RSV functioned as a pure antagonist with T47D cells (17). Despite the mixed results in two ER+ cell lines, a large body of research has shown RSV to display anti-proliferative and pro-apoptotic effects on several other cancer cell lines. RSV has been shown to inhibit the proliferation of MCF7 cells by interfering with the ERα associated PI3K/Akt pathway, increasing p27 and p53, leading to apoptosis induction (132, 182, 183). In an *in-vivo* model, RSV inhibited the formation of estrogen-dependent pre-neoplastic ductal lesions induced by 7,12-dimethylbenz(a)anthracene (DMBA) and reduced MNU induced mammary tumorigenesis when administered to female Sprague Dawley rats (17). A common combination of RSV with other dietary polyphenols (quercetin and catechin), reduced tumor growth of MDA-MB-231 breast cancer xenografts in nude mice and reduced lung and bone metastasis (39). Therefore, it appears in the absence of E2, RSV exerts mixed estrogen agonist/antagonist activities in some mammary cancer cell lines, but in the presence of E2, RSV

functions as an antiestrogen. RSV has also been shown to display chemosensitization effects both *in-vitro* and *in-vivo* on several chemo resistant cancers including breast. Many reports show that RSV sensitizes tumor cells to chemo agents by modulating cell survival proteins. RSV was found to enhance the effects of doxorubicin on MCF7 cells by inducing cell cycle arrest in S-phase, downregulating survivin expression and increasing apoptosis preferentially out of S-phase (81). All of this information as a whole provides a clear notion that RSV supplementation has an effect both directly on breast cancer cells by modulating cell growth/survival but possibly more important is indirectly by altering the tumor growth environment in obese patients via altered adipokine secretion profile.

3.6 Role of Race in Breast Cancer and Adipokine Profile

The vast majority of breast cancer research has been done on North American and European white females. Scientific evidence now suggests the existence of a variability in molecular signature between breast cancers from patients of different ethnic groups. While understanding the development and gene associations is important, growing evidence now suggests that racial differences in breast cancer incidence, age of development, effect of obesity on breast cancer severity and mortality. Specifically, Southeast Asian (SEA) women have been suggested to present with an altered rate of breast cancer incidence and mortality. According to the National Cancer Registry in Saudi Arabia, where many studies on SEA women have been conducted, the median age at presentation is around 48 years compared to the United States and Western Europe where its 63 years (150, 192). The majority of breast cancer cases in Saudi Arabia (62%) have been shown in premenopausal women compared to post, which is opposite to the pattern seen in Western countries (73). Significant risk factors of breast cancer in Western

countries such as first pregnancy at late age, history breast of feeding and low parity are not normally practiced in SEA society. However, breast cancer incidence is still high among women (73). Interestingly, in premenopausal women 73.3% of breast cancer patients were obese or overweight, suggesting a possible racial difference in adipose created growth microenvironment, an association that is still controversial in Caucasian (CA) premenopausal women (150). A stronger association has been found in SEA women between BMI and both pre and postmenopausal breast cancer compared to CA women (187).

One proposed difference in breast cancer rate in SEA and African-American women compared to CA women is an altered adipokine profile at similar BMIs, especially evident in the obese subgroup of women. The study grouped Asian women together and found that they had lower levels of both circulating total and HMW ADIPO and higher LEP levels compared to CA women after adjusting for total adiposity (119). The study showed that significant race-ethnic differences exist in circulating adipokines and the phenotype in Asian women is indicative of a more proliferative tumor growth microenvironment. Other studies have also mirrored these racial differences in Asian population as routinely these women present with lower circulating ADIPO compared to CA women of similar BMI (55). Some studies have also shown Asian women to have lower circulating LEP compared to CA women but the lower level of ADIPO is more pronounced, causing an overall decrease in the circulating ADIPO:LEP ratio (55). Functional analysis of breast cancer-associated genes uncovered that the genes involved in cell cycle progression, DNA repair and tumor morphology in SEA women were similar to that in CA women, but there were several distinct differences as well. Of the several genes dysregulated in Saudi breast cancer patients, ADIPO was found to be downregulated and LEP upregulated to a greater extent than in CA breast cancer patients (99, 150). Because of this, certain scales such as the GAIL score, which determines

chance of developing breast cancer in next 5 years may need to take into account racial differences in adipose tissue growth microenvironment that could increase the risk of developing breast cancer compared to CA counterparts.

4. Study Objectives

1. To prove that the adipocytes are the major contributing factor to the obesity-breast cancer link.
2. To show that the ADIPO:LEP ratio serves as a consistent predictor of tumor growth microenvironment regardless of internal and external conditions.
3. To determine whether PA can counteract the effects of obese adipose tissue on tumor growth microenvironment and whether a volume-dependent effect of PA is evident.
4. To determine what effect estrogen plays on adipose tissue tumor growth microenvironment.
5. To show that stabilizing ADIPO signaling can overcome the effects of obesity on breast cancer.

5. Hypothesis

1. In obese individuals, the adipose tissue creates a tumor growth promoting environment and this can be blunted by altering adiposity.
2. Altering the ADIPO:LEP ratio through diet, PA or RSV supplementation will blunt the proliferative effects of obesity on breast cancer growth.
3. Voluntary PA will have a volume-dependent effect on ADIPO:LEP ratio counteracting the effects of obesity on breast cancer cell proliferation.
4. Circulating estrogen will have an effect on adipose tissue that blunts the effects of obesity on breast cancer cell proliferation.
5. Upregulating/stabilizing AdipoR1 protein expression in breast tumors will ameliorate the proliferative effects of obesity, increasing the cell cycle inhibitory effects of ADIPO.

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7. Academic Research Paper #1

Voluntary Physical Activity Abolishes the Proliferative Tumor Growth Microenvironment Created by Adipose Tissue in Animals Fed a High Fat Diet[1]

The molecular mechanisms behind the obesity-breast cancer association may be regulated via adipokine secretion by white adipose tissue. Specifically, adiponectin (ADIPO) and leptin (LEP) are altered with adiposity and exert antagonistic effects on cancer cell proliferation. We set out to determine whether altering adiposity *in-vivo* via high fat diet (HFD) feeding changed the tumor growth supporting nature of adipose tissue and if voluntary physical activity (PA) could ameliorate these HFD-dependent effects. We show that conditioned media (CM) created from the adipose tissue of HFD fed animals caused an increase in the proliferation of MCF7 cells compared to cells exposed to CM prepared from the adipose of lean chow diet fed counterparts. This increased proliferation was driven within the MCF7 cells by an HFD-dependent antagonism between AMPK and Akt signaling pathways, decreasing p27 protein levels via reduced phosphorylation at T198 and downregulation of AdiporR1. PA can ameliorate these proliferative effects of HFD-CM on MCF7 cells, increasing $p27^{T198}$ by AMPK, reducing $pAkt^{T308}$ and increasing AdipoR1, resulting in cell cycle withdrawal in a manner that depends on the PA

[1]**Christopher F. Theriau, Yaniv Shpilberg, Michael C. Riddell and Michael K. Connor**
STATEMENT OF LABOUR:
Christopher Theriau:
Wrote thesis manuscript and created all figures/table, Collected all anthropometric data and daily running distances on animals throughout experiments and at time of sacrifice, Performed all animal tissue extractions (adipose/muscle) with exception of 4 HFD (adipose tissue) and 4 CD (adipose tissue) animals, Created all conditioned media using epididymal adipose tissue with exception of 3 HFD and 3 CD animals, Performed and analysed all western blot (MCF7), FACS (MCF7), CYTOX (gastrocnemius) and ELISA (conditioned media) experiments, Created stability transfected AdipoR1-T MCF7 cells
Yaniv Shpilberg:
Performed epididymal adipose tissue extraction on 4 HFD and 4 CD animals, Created conditioned media from adipose tissue from 4 HFD and 4 CD animals

intensity. High physical activity (>3 km/day) completely abolished the effects of HFD feeding. In addition, AdipoR1 overexpression mimics the effects of exercise, abolishing the proliferative effects of the HFD-CM on MCF7 cells and further enhancing the anti-proliferative effects physical activity on the HFD-CM. Thus, VPA represents a means to counteract the proliferative effects of adipose tissue on breast cancers in obese patients.

Keywords: Physical Activity, obesity, adipokines, breast cancer

New & Noteworthy

We hypothesized that voluntary physical activity (PA) would counteract the deleterious adipose-dependent growth microenvironment that a breast cancer is exposed to. We show that PA altered the adipokine secretion profile of adipose in a volume-dependent manner. This alteration resulted in growth inhibition of estrogen receptor positive breast cancer cells in culture. Furthermore, stabilizing adiponectin receptor 1 expression in the cancer cells made them resistant to the cell cycle entry effects that accompany obesity.

INTRODUCTION

Obesity continues to be a growing concern not only within North America but around the world. It has been linked with several detrimental health issues such as insulin resistance, hyperglycemia, dyslipidemia and hypertension (16). Growing evidence now supports the notion of several cancers being associated with obesity including breast, renal, esophageal, gastrointestinal and reproductive cancers. Numerous clinical and preclinical studies have demonstrated that increased adiposity increases the risk of cancer incidence, morbidity and mortality while imparting a poorer response to therapy (5, 31, 42). Specifically, obese breast cancer patients in the highest quintile of BMI have a more than two-fold higher mortality rate compared to their lean counterparts (5).

To elucidate the mechanism behind the association between obesity and breast cancer investigations have focused on the role of adipose-derived cytokines, termed adipokines. There are more than 400 adipokines released from adipocytes which are also dysregulated in obese individuals and exert endocrine effects on numerous different body tissues (48). Given that the human breast is composed primarily of epithelial cells surrounded by adipose tissue, the potential exists for these adipokines to exert their effects directly on breast cancers. These adipokines can play a crucial role in shaping the growth microenvironment that a breast cancer is exposed to within the body. In addition to the adipocytes, the stromal compartments of adipose tissue have been shown to elicit effects on breast cancer proliferation (32). Of the adipokines identified to date, adiponectin (ADIPO) and leptin (LEP) represent major potential contributors to adipose-dependent effects. They are among the most abundant adipokines produced/secreted, both are altered by obesity and they exert antagonistic cell cycle regulatory effects on breast cancer cells (10, 11, 20). ADIPO is a 30 kDa protein whose production/secretion decreases with obesity and induces cell cycle exit by activating AMPK, which directly phosphorylates p27 at T198, increasing

p27 stability and inducing G1 arrest (10, 15, 26). LEP (16 kDa) production increases with adiposity and its cell cycle effects directly oppose those of ADIPO (10, 11). LEP induces cell cycle entry by activating Akt, which phosphorylates p27 at T157, denying p27 entry into the nucleus, thereby preventing it from inhibiting cyclin E/cdk2 and inducing cell cycle entry (11, 14, 27). The decreased ADIPO and increased LEP in the circulation of obese individuals creates a microenvironment that promotes tumor growth by accelerating cell cycle entry, causing a greater incidence of detectable tumor formation and more advanced tumors in obese women than in lean women (6, 28). Independent of adiposity, serum ADIPO has also been found to be reduced while LEP is increased in women with breast cancer compared to women without the disease (23, 29, 37). Decreased ADIPO signaling through AdipoR1 has been shown to be associated with higher tumor grade and poorer patient outcomes (33). We have previously demonstrated in cell culture and *in-vivo* that increasing AdipoR1 levels increases the cell cycle effects of ADIPO via AMPK signaling and can counteract the antagonism of ADIPO by LEP (51), a condition that exists in obese breast cancer patients and may underlie the association with poor prognosis and a less favorable response to therapy.

An increasingly sedentary lifestyle is a major contributor to the increase in obesity and its associated disorders (38). This highlights increased physical activity as a potential prevention/intervention for the development of obesity and its associated effects on breast cancer (4, 9). Moderate physical activity (>0.64 MET-hours/day) reduces the incidence of breast cancer, with women who are physically active exhibiting a 20-30% reduction in the relative risk of developing breast cancer compared to their sedentary counterparts (22, 30, 35, 39, 45). The effect of physical activity is also important in improving patient survival in breast cancer as seen by an up to 40% reduction in cancer-related death and cancer recurrence in physically active women

(17). In addition, there are dose-dependent (intensity and duration) relationships among physical activity, cancer risk and overall survival in breast cancer patients (46). Breast cancer patients participating in physical activity consisting of walking as little as one hour/week was associated with improved survival compared to sedentary women (17). These effects were more pronounced in women who engaged in moderately intense exercise between 3-5 hours per week (17). When exercise intensity was increased further (running >1.8 MET-hours/day) breast cancer patients had an almost 90% lower risk of cancer mortality compared to women who walked (<1.07 MET-hours/day) (46). Voluntary physical activity alters the production of both ADIPO and LEP in rats fed a high fat diet, lowering the levels of LEP and increasing the levels of ADIPO in the circulation compared to sedentary high fat diet fed animals (4, 24, 45, 50). Physical activity decreased $pAkt^{473}$ and cyclin D1 and increased $pAMPK^{172}$ and p27 within mammary carcinomas of those same animals (49). Furthermore, postmenopausal women exposed to 12 months of consuming a calorie restricted diet and moderate physical activity caused an increase (9.5%) in plasma ADIPO and a decrease (40.1%) in LEP (1). Thus, there are clear positive dose-dependent effects of diet and the amount of physical activity and its benefits to breast cancer patient prognosis.

The exact mechanisms behind the effects of physical activity on regulating adipose-dependent tumor growth microenvironment remain unclear. We set out to determine whether a dose-dependent relationship between physical activity and the production/secretion of adipokines exists and whether these effects can alter the deleterious adipose-dependent tumour growth microenvironment created in animals fed a high fat diet (HFD). We show that HFD fed animals decreased the ADIPO:LEP ratio secreted by isolated adipose tissue into culture media. We also found a decrease the levels of $pAMPK^{T172}$, $p27^{T198}$, p27, AdipoR1 and an increase $pAkt^{T308}$ in MCF7 cells grown in the conditioned media prepared from the adipose of HFD animals resulting

in cell cycle entry. A dose-dependent effect of physical activity was observed on the adipokine profile by increasing the ratio of ADIPO:LEP. Physical activity counteracted the effects of the HFD with high physical completely abolishing the effects of the HFD conditioned media on MCF7 cell cycle regulation. In addition to physical activity, we show that over expressing AdipoR1 in the MCF7 cells also counteracts the effects of HFD, highlighting the importance of AdipoR1 signaling on overcoming the positive growth promoting microenvironment that is present in obese breast cancer patients.

METHODS

Animals. All animal experiments were approved by York University Animal Care Committee in accordance with Canadian Council for Animal Care guidelines. Forty four male Sprague-Dawley rats (7 weeks of age) were purchased from Charles River Laboratories (Montreal, QC, Canada) and were singly housed in standard clear, plastic cages. Male rats were used in order to create an estrogen free environment and allow for the delineation of the effects of diet and exercise on the microenvironment created by the adipose tissue adipokine secretion profile from additional estrogen-dependent effects. All animals had a 7 day habituation period to a 12 hour light-dark cycle (lights on at 0600) in a temperature (22°C) and humidity (50-60%) controlled room.

After the habituation period, animals were randomly selected and given free access to a running wheel (wheel circumference, 106 cm; Harvard Apparatus, Holliston, MA) in their cage. A magnetic counter was mounted to each wheel which detected the revolutions after 24 hours of use. The animals were given a 7 day acclimation period to the wheels. After this period, animals were then divided into two groups: chow diet (CD; n=19) and high fat diet (HFD; n=25) with both

groups given access to food and water (*ad libitum*). The CD (no. 5012 Lab Chows, Ralston Purina, St. Louis, MO) had caloric make-up of 14% fat, 54% carbohydrate, 32% protein (3.02 calories/g). The HFD (Harlan Laboratories, Madison, WI) had a caloric breakdown of 60% fat, 21% carbohydrate, 18% protein (5.1 calories/g). HFD and CD fed animals were further subdivided into sedentary and physical activity (PA) groups designated as chow diet-sedentary (CD; n=11), chow diet-low physical activity (CD+LPA; <3 km/day; n=4), HFD-sedentary (HFD; n=13), and HFD-physical activity (HFD+PA; n=12) for 6 weeks. Animals in the HFD-PA group were further sub-divided depending on average wheel running distances into animals that ran less than 3km/day (n=6) classified as "low physical activity" group (HFD+LPA) while animals that ran more than 3km/day (n=6) were classed as "high physical activity" (HFD+HPA) to determine if a dose response to physical activity was evident. Our physical activity cutoff of 3km/day has also been used previously with Sprague-Dawley rats and wheel running to create a low and high PA level (21). Food intake and running distances were measured each day, and body weight was measured three times per week.

Tissue Collection and Conditioned Media. After the 6 week protocol, visceral (epididymal) adipose tissue was quickly removed from CD, CD+PA, HFD, HFD+LPA and HFD+HPA animals and cultured as previously described (38). Briefly, the epididymal fat was weighed, minced into ~5-10mg pieces and immediately placed in 50 ml vented conical tubes containing AMEM (7.5 ml/g tissue; Wisent, St.Bruno, QC) supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific, Whitby, ON), 2% antimicotic/antibiotic (Wisent), 1 mM sodium pyruvate (Sigma, Oakville, ON), non-essential amino acids (Sigma), and 10 µg/ml insulin from human pancreas (Wisent) under sterile conditions and incubated for 24 hours at 37°C with 5% CO_2. After 24 hours, conditioned media (CM) was then collected and stored at -84°C. We have conducted

numerous experiments using this type of model to establish the efficacy of combining rat adipose tissue and human epithelial cells. In addition, many xenograft models grow human cells in mice, further illustrating the benefits of using a rodent/human tumor growth model. In order to ensure that our mass:volume preparation of CM was not skewed by the presence of vast differences in adipose cellular content, we diluted 10-15 mg sections of adipose 30:1 in RIPA buffer for protein extraction. Equal volumes of lysate (25 μl) were subjected to SDS-PAGE using 12% gels and membranes were probed for total Akt and β-actin to evaluate equivalency of specific protein content between groups. Weights of all tissues collected were measured and normalized per 100 g of body weight. The sequence in which rats were sacrificed was randomized across groups so as to minimize the likelihood that order effects would masquerade as treatment-associated effects. At time of sacrifice the gastrocnemius, soleus and tibialis anterior were immediately excised, weighed, frozen and stored at -84°C for future analyses.

Cytochrome-c Oxidase Activity Assay. In order to confirm that the physical activity protocol elicited a training effect, we measured cytochrome-c oxidase (COX) activity in the gastrocnemius muscles. COX activity was determined according to a modification of a method previously described (8). Briefly, cross-sections of mixed gastrocnemius muscles (from the mid-section of the muscle belly) weighing roughly 20-30 mg were diluted 80-fold (sedentary) or 160-fold (PA) in extraction buffer (100mM Na-K-Phosphate, 2 mM EDTA, pH 7.2). Muscle extracts were prepared by homogenization with metal beads (2X30 sec) at a frequency of 30 Hz in a magnetic homogenizer (Mixer Mill MM 400, Retsch, Germany). These homogenates were then used for the analysis of the maximum rate of oxidation of fully reduced cytochrome c at 30°C as indicated by changes in absorbance (550 nm).

Co-Culture Adipokine Measurement. The levels of ADIPO and LEP produced and secreted into the co-culture media by adipocytes was determined using a rat adiponectin sandwich ELISA kit (BioVision, Milpitas, CA) and a mouse/rat leptin quantikine sandwich ELISA kit (R&D Systems, Minneapolis, MN), respectively, as per manufacturer instructions. For ADIPO aliquots of conditioned media were diluted 50-fold (HFD) and 100-fold (PA and CD; 100 µl total), while LEP ELISAs used a 5-fold (HFD) or undiluted (PA and CD) conditioned media (50 µl total) and were analysed against standard curves. The levels of each adipokine were calculated in ng/ml values and converted to nM values for stoichiometric comparison.

Cell Culture. MCF7 cells were purchased from the American Tissue type Culture Collection (ATCC, Manassas, VA) and were maintained in AMEM, 10% FBS, 2% antimicotic/antibiotic, 1 mM sodium pyruvate, non-essential amino acids, and 10 µg/ml insulin from human pancreas at 37°C and 5% CO_2.

Transformed DH5α *E.coli* bacterial cells containing AdipoR1 plasmid vectors (OriGene, Rockville, MD) were obtained in glycerol stock. Plasmid vectors were driven by a CMV promoter with an N-terminal FLAG-tag and contained neomycin and kanamycin resistance regions. AdipoR1 cDNAs (5 µg) were transfected into MCF7 cells using ExGen 500 *in-vitro* transfection reagent according to manufacturer instructions (Fermentas, Burlington, ON). Transfected MCF7 cells were treated with G418 sulfate (400 µg/ml, Wisent) and G418 resistant colonies were transferred to a 24 well plate in AMEM. After 24 hrs G418 was reduced to 200 µg/ml, allowing for growth but maintaining selection pressure. Viable colonies were subsequently tested for FLAG-tag expression and ADIPOR1 protein levels.

Mock transfected (MockT) MCF7 cells and stably transfected AdipoR1 over-expressing and FLAG-expressing cell (p31-4-2-2) were seeded in 6 well plates with AMEM for 24 hrs. At

70% confluence, cells were washed with PBS and incubated with CM produced from adipocytes for 24 hours. MCF7 cells grown in AMEM supplemented with 10% FBS served as untreated controls (UT). For initial experiments, CD-CM and HFD-CM were further supplemented with either 18 nM human globular adiponectin (Peprotech, Rocky Hill, NJ) or 300 nM human recombinant LEP (Peprotech) for 24 hrs.

Immunoblotting. The effects of adipokines on specific cellular proteins were measured using standard SDS-PAGE protocols using 12% polyacrylamide gels. Proteins (25 µg) were transferred to PVDF membranes (Bio-rad, Mississauga, ON, CAN), blocked for 2 hours in 10% skim milk and subsequently incubated overnight with primary antibodies: $p27^{Kip1}$ (BD Biosciences); $p27^{T198}$ (R&D Systems); $pAkt^{T308}$, Akt, $pAMPK^{T172}$ and AMPK (Cell Signaling); AdipoR1 (Santa Cruz Biotech, Santa Cruz, CA) and β-actin (Abcam, Cambridge, MA). Anti-mouse and anti-rabbit (Promega, Madison, WI) and anti-goat (Santa Cruz) horseradish peroxidase secondary antibodies were used to visualize proteins using Immobilon enhanced chemiluminescence substrate (Millipore, Whitby, ON, CAN) and detected/quantified on a Kodak In Vivo Pro imaging system (Marketlink Scientific, Burlington, ON, CAN).

Cell cycle analyses. MCF7 cells isolated from 6-well plates following trypsinization were washed in cold PBS and fixed by drop wise addition of ice-cold 70% ethanol. Cells were washed twice in PBS, re-suspended in a propidium iodide/RNAse solution and subjected to FACS analyses (Gallios Flow Cytometer, Beckman Coulter Mississauga, Canada). Cell cycle profiles were determined using Mod-fit software (Verity Software House, Topsham, ME), by fitting curves to profiles and measuring the areas under the curve to determine relative numbers of cells in G1, S and G2/M phases.

Statistical Analyses. All values are expressed as means ± SEM of five to thirteen separate experiments (as indicated) and statistical analyses were performed using a one-way ANOVA with Tukey's post-hock tests conducted when significant main effects were found. A two-way ANOVA with Bonferroni post-test comparisons was used for the CM plus adipokine experiments. Individual t-tests were used to identify differences in FACS analysis between groups. Group means value $p \leq 0.05$ were considered to be significantly different.

RESULTS

HFD increases adiposity which is prevented by physical activity. Animals were fed either a high-fat diet (HFD) or standard chow diet (CD) and a given access to running wheels as voluntary physical activity. Animals were placed in either a low physical activity (HFD+LPA, CD+LPA) or high physical activity (HFD+HPA) groups. The CD+LPA and HFD+LPA animals ran similar distances (2.2±0.3 vs. 2.5 ±0.6 km/day) but both ran less than the HFD+HPA (7.21 ±3.1 km/day). HFD-fed animals showed no difference in total body mass compared to sedentary CD fed animals (Fig. 7.1A). Despite the lack of difference in total body mass there was a 2.31-fold higher relative epididymal fat mass (2.22 ±0.26 vs. 0.96 ±0.23 g/100 g body weight; Fig. 7.1B) in HFD fed sedentary animals compared to CD fed sedentary animals. There was no difference in overall weekly calorie intake between the CD and HFD sedentary animals, indicating that the increased epididymal adiposity in HFD animals was a result of increased fat content and not increased caloric intake.

High intensity voluntary physical activity (HPA) counteracted the HFD-dependent increase in epididymal adiposity as demonstrated by the HFD+HPA animals being 16% lighter than their sedentary counterparts (452.9 ±19.1 g vs. 525.2 ±12.0 g; Fig. 1A). Consistent with these

results we observed a 52% decrease in epididymal fat mass between HFD+HPA and HFD sedentary animals (Fig. 7.1B). In contrast, LPA-HFD animals had no difference in total body weight or body weight adjusted epididymal fat mass compared to their sedentary counterparts (Figs. 7.1A,B). The LPA-CD animals were 17% lighter and had 50% of the epididymal fat mass compared to HFD sedentary animals (Figs. 1A,B).

In order to determine if PA induced a training effect, we measured the weights of the gastrocnemius, soleus and tibialis anterior muscles. PA increased gastrocnemius, soleus and tibialis relative weights in both HFD+LPA and HFD+HPA animals compared to their sedentary counterparts (Figs. 7.1C,D,E). LPA and HPA resulted in an overall increase of 27±3% and 38±4% in muscle mass above HFD animals, respectively. This volume-dependent effect of training was further evident from the changes in oxidative capacity of gastrocnemius muscles (Fig. 7.1F). LPA increased mixed gastrocnemius COX activity by 2.1-fold in HFD-fed animals while HPA further increased COX activity to levels that were 2.8-fold above those in sedentary HFD-fed animals.

Adipose tissue was excised from animals in each of the experimental groups and used to prepare conditioned media (CM). We measured the levels of ADIPO and LEP within the CM created from the secretome of the adipose tissue. In agreement with epididymal fat mass, the ratio of ADIPO:LEP was higher in the CD-CM compared to the HFD-CM (566.5 ± 197.2 vs.122.1 ± 52.1 ng/ml; Table 7.1). HPA prevented this HFD-dependent decrease in the ratio of ADIPO:LEP. The HFD+HPA had higher levels of ADIPO (1052.0 ± 246.9 ng/ml) and lower levels of LEP (1.17 ± 0.3 ng/ml) then both the HFD sedentary (ADIPO: 558.9 ± 99.4 ng/ml, LEP: 2.69 ± 0.8 ng/ml) and the HFD+LPA (ADIPO: 622.0 ± 141.0 ng/ml, LEP: 1.71 ± 0.4 ng/ml) groups, respectively. The overall stoichiometric ratio between ADIPO:LEP within the CM was increased in the HFD+HPA (529.8 ± 105.3:1) compared to both HFD (122.1 ± 52.1:1) and HFD+LPA (199.7 ±

57.7:1) animals. The ratio of ADIPO:LEP in the CM prepared from HFD+HPA animals was not different than those of both CD sedentary and CD+LPA. A linear relationship between the distance ran and the ratio of ADIPO:LEP in the CM was evident (m= 85.94 ± 21.83, R=0.795, p= 0.0034; Fig 7.1G).

Conditioned media induces effects on cell signaling and cell cycle proteins in MCF7 cells. In order to determine whether these changes in ADIPO and LEP elicit any effects on the breast cancer tumor growth environment, we treated MCF7 cells with CM created from the adipose tissue of HFD and CD fed animals. Additionally, we also wanted to determine if adding additional ADIPO or LEP to CM would elicit any further effects on MCF7 cell cycle regulation. CM was created using equivalent dilutions of adipose tissue across treatment groups (7.5 ml of media/g of tissue). In order to determine whether approximately equal amounts of protein were being used in our CM preparation, we conducted western blot analyses using proteins isolated from the adipose tissue used in our CM treatments and measured the levels of Akt and β-actin proteins that are often used as loading controls in various experimental treatments (Fig 7.1H). We found that there was no specific pattern that suggested inequivalence of proteins loaded for any specific treatment (i.e. HFD had more protein than HFD+HPA), giving us confidence that the CM was not subjected to any preparation artifacts. However, we did see that CM treatment caused profound differences in MCF7 cells (Fig. 7.2). CD-CM increased pAMPK^{T172}, p27^{T198}, p27 and AdipoR1 while lowering pAkt^{T308} levels compared to HFD-CM treated MCF7 cells (Fig. 7.2A-F). The addition of 18 nM gADIPO to CD-CM caused no further increases in pAMPK^{T172} (Fig. 7.2A,B), p27 (Fig. 7.2A,D), p27^{T198} (Fig. 7.2A,E), AdipoR1 (Fig. 7.2A,F) or decrease to pAkt^{T308} (Fig. 7.2A,C). Similarly, the addition of exogenous 300 nM LEP to CD-CM caused no effects on measured proteins. As was the case in CD-CM treated cells, LEP caused no further decrease in pAMPK^{T172}, p27^{T198}, p27 and

AdipoR1 or increase in pAkt^{T308} while ADIPO could not rescue the levels of the proteins measured in HFD-CM treated MCF7 cells. Thus, the CM created from adipose tissue of HFD and CD fed animals was the driving force in creating the growth environment for the MCF7 cells. No changes in total AMPK and Akt were evident.

AdipoR1 overexpression ameliorates the effect of HFD-CM. We next determined whether AdipoR1 overexpression would ameliorate the effects of HFD-CM (Fig 7.3A,B). Antibiotic-resistant colonies were expanded (19 total) and 2 cell lines were chosen for their expression of the FLAG-tag and increased AdipoR1 expression compared to MockT cells (Fig. 7.3B). The level of overexpression of AdipoR1 in these cell lines amounted to 2.70±0.46 (p31-3-2) and 2.64±0.51 (p31-4-2-2) fold above MockT cells. Pilot experiments showed that each cell line elicited identical results so one cell line (p31-3-2) was used for the AdipoR1 overexpression experiments. The CD-CM caused an increase in pAMPK^{T172} (Fig. 7.3A,B), p27 (Fig. 7.3A,D), p27^{T198} (Fig. 7.3A,E) and AdipoR1 (Fig. 7.3A,F) compared to both UT and HFD-CM cells. This result was similar to what was seen in MockT cells (Fig. 7.2). Unlike what was observed in MockT cells, CD-CM was found to cause no decrease in pAkt^{T308} compared to either UT or HFD-CM treated cells (Fig. 7.3A,C) . Unlike MockT cells, HFD-CM caused no effects on MCF7 cells compared to UT cells in all proteins except for p27 (Fig. 7.3A,D). Most notably, HFD-CM treated cells caused no increase in pAkt^{T308} compared to CD-CM treated cells, again different than what was observed in MockT cells (Fig. 7.2A,C vs. Fig. 7.3A,C). Overall, unlike what was observed in MockT cells, the HFD-CM did not seem to cause the same effects compared to UT cells, seemingly eliminating the dominant effects of HFD-CM on MCF7 cell cycle regulation, compared to UT cells that were apparent in MockT cells. As observed in MockT cells, addition of 18 nM gADIPO (lane 2, 5 & 11) or 300 nM recombinant LEP (lane 3, 6 & 12) to either CD-CM or HFD-CM caused no additional effects

in MCF7 cells. Unlike what was observed in MockT cells, addition of recombinant LEP had no effects compared to UT cells. This suggests that AdipoR1 overexpression can overcome any cell cycle entry effects of increasing LEP, as is seen with increased adiposity, in MCF7 cells. No changes in total AMPK and Akt were evident. These results highlight the importance of available AdipoR1 and show that increasing the available binding sites for ADIPO can override the cell cycle control regardless of the external growth environment.

Voluntary physical activity ameliorates HFD induced effects in a dose-dependent manner. PA has been shown to counteract obesity and we wanted to observe whether the effects of voluntary PA which altered the adipokine profile (ADIPO:LEP ratio) that was created within the CM led to any changes within co-cultured MCF7 cells. Voluntary PA elicited a dose-dependent response counteracting the effects of HFD on MCF7 cell cycle regulation (Fig. 7.4A lane 3 vs 5 & 6). Specifically, HFD+HPA-CM increased pAMPK^{T172}, p27 and AdpoR1 by 93%, 67% and 58%, respectfully, compared to HFD-CM treated cells (Figs. 7.4A,B,D,F). In addition, pAkt^{T308} was decreased by 40% (Figs. 7.4A,C). Furthermore, HFD+HPA-CM elicited the same effects on MCF7 cells as CD-CM (Fig. 7.4A lane 2 vs. 5). HFD+LPA-CM caused effects that were intermediate to those observed in HFD and HFD+HPA CM treated cells. This indicates dose-dependent effects of PA on MCF7 cell growth displayed by the HFD+LPA-CM, increasing pAMPK^{T172} and AdipoR1 while decreasing pAkt^{T308} compared to the HFD-CM (Fig. 7.4A,B,C,F). Surprisingly, the effects of HFD+HPA observed were also similar to those elicited by CD+LPA-CM on MCF7 cell cycle regulation (Fig. 7.4A, lane 2 vs. lane 7). No changes in total AMPK and Akt were evident.

AdipoR1 overexpression enhances the effects of PA. Given that the CM elicits regulatory effects on AdipoR1, we overexpressed AdipoR1 to determine any absolute/synergistic effects of

augmented AdipoR1 signaling in MCF7 cell cycle regulation. AdipoR1 overexpression increased the levels of pAMPK^{T172}, p27, p27^{T198} while pAkt^{T308} levels were decreased when compared to MockT cells (Fig. 7.4 vs 7.5). While a dose-response of PA was observed in MockT cells, the effects of the HFD+LPA-CM were further amplified with AdipoR1 overexpression such that the effects were no different from those elicited by CD-CM or HFD+HPA-CM. The HFD+HPA-CM was found to cause an increase in pAMPK^{T172} (Figs. 7.5A,B), p27 (Figs. 7.5A,D) and AdipoR1 (Figs. 7.5A,F) by 58%, 27% and 19%, respectively compared to HFD-CM treated cells. Additionally, pAkt^{T308} was decreased by 25% (Figs. 7.5A,C) compared to HFD-CM treatment. CD+LPA-CM increased pAMPK^{T172} by 37% above CD-CM (Fig. 7.5A,B).

AdipoR1 overexpression abolishes effect of HFD and accentuates PA cell cycle effects in MCF7 cells. Despite the observed changes in cellular protein levels we set out to determine whether HFD and PA caused corresponding overall cell cycle changes in the cultured MCF7 cells. Cell cycle status was determined using propidium iodide staining and computational analyses (Figs. 7.6A,B). When cells were exposed to HFD-CM there was a 27% decrease in the number of cells in G1/G0 (53% vs. 42%) and a 34% increase in the number of cells in S-phase (20% vs. 28%) compared to CD-CM in MockT MCF7 cells (Fig. 7.6C). Elevating AdipoR1 expression increased the percentage of cells in G0/G1 in both CD-CM and HFD-CM while decreasing the number of cells in S-phase in the HFD-CM when compared to MockT HFD-CM treated cells (Figs. 7.6C vs. D). The HFD-CM decreased the number of cells in G1/G0 by 8% and increased the number of cells in S-phase by 16% compared to CD-CM treated cells (Fig. 6D). Strikingly, AdipoR1 overexpression increased the number of cells in G0/G1 by 24% (52% vs. 41%) compared to MockT cells when exposed to HFD-CM (Figs. 7.6C vs. D). This highlights the overall proliferative effects of the microenvironment created by the adipose tissue from HFD-fed animals

and the powerful inhibitory effects that maintaining AdipoR1 protein expression elicits on the HFD-dependent effects of adipose tissue on cell cycle regulation.

In addition, we evaluated whether voluntary PA was able to counteract the HFD-dependent overall cell cycle effects on MCF7 cells. As observed with individual proteins there were dose-dependent effects evident depending on the intensity of PA. The HFD+HPA-CM caused a 17% increase in G1/G0 cells (49% vs. 41%) and a 15% decrease in the number of S-phase cells (24% vs. 28%) compared to HFD-CM (Fig. 7.6E). LPA caused intermediate effects with HFD+LPA-CM increasing the percentage of G1/G0 cells by 9% (46% vs. 41%) and decreasing the number of S-phase cells by 17% (23% vs. 28%) compared to HFD-CM. The CD+PA elicited the same cell cycle effects compared to CD-CM. By overexpressing AdipoR1 within the MCF7 cells, there again was a dose-response to PA but to a much lesser extent (Fig. 7.6F).

DISCUSSION

It is clear that adipose tissue elicits proliferative effects on breast cancer cells, in large part due to the production/secretion of over 400 adipose-derived proteins, with the most abundant being adiponectin (ADIPO) and leptin (LEP; 48). While each of these adipokines elicit effects individually, emerging evidence suggests that the ADIPO:LEP ratio may be a more reliable predictive indicator of the adipose-dependent proliferative effects on breast cancer cells (2, 6). Given the inherent genetic variability and instability that are characteristic of cancers in general, it is likely that individual breast cancer patients likely possess unique and specific carcinomas making tumor-directed therapies an imposingly difficult therapeutic avenue. However, the overall tumor growth microenvironment that a tumor is exposed to and that exists among patients is regulated by far more stable and predictable physiological mechanisms. Adipose is one of the most

important tissues that contributes to this growth microenvironment and alterations in adipokine secretion profile that accompany obesity may represent the molecular link between obesity and cancer. ADIPO and LEP have emerged as prime candidates as master regulators of this phenomenon because of their relatively high abundance, their levels are altered with obesity and they have been shown to elicit numerous effects on breast cancer cell cycle regulation (10, 11, 19). In addition, ADIPO and LEP activate unique intracellular signaling pathways (AMKP vs. Akt) which directly antagonize each other and elicit opposite effects on proliferation, with AMPK promoting cell cycle exit and AKT leading to cell cycle entry (51). This suggests that the ADIPO:LEP ratio may represent a more reliable indicator of tumor growth microenvironment and better predictor of cancer aggressiveness and patient outcome in breast cancer patients than either adipokine alone (6). In support of this, in obese breast cancer patients the serum ADIPO:LEP ratio is decreased and this is associated with more aggressive tumors and a poorer prognosis (9).

In order to induce adipose expansion we employed high-fat diet (HFD, 60% calories from fat) feeding as a means to induce obesity and observe the resultant effects on ADIPO and LEP production/secretion. Given that physical activity is a definitive means to manage body fatness, we also determined whether there were combinatory effects of HFD and physical activity (PA) on adipose physiology. Since the association between obesity appears stronger in post-menopausal women and studies have shown that HFD feeding can promote mammary tumor progression in ovariectomized mice, we conducted our interventions in male animals to simulate an estrogen-free environment without the potential surgical complications associated with ovariectomizing mice (7). Adipose tissue from HFD fed animals demonstrated alterations in adipokine secretion profile with lower levels of ADIPO and higher levels of LEP compared to their lean chow diet (CD) fed counterparts (Table 7.1), a result similar to that seen in humans (41). Surprisingly, we observed no

measureable difference in total body weight between the HFD- and CD-fed animals. This may be due to the fact that the CD and HFD animals had identical specific daily caloric intake. Despite this lack of difference in total body weight, there was an evident 2.3±0.3-fold HFD-dependent increase in visceral fat mass and a 17±3% decrease in measured muscle mass (gastrocnemius, soleus and tibialis anterior; Fig. 7.1). Thus, although body weight did not change, there was a definitive redistribution of body mass within the HFD-fed animals. Furthermore, given that these animals were in a rapid growth phase, adipose mass difference between our CD and HFD-fed animals may have been masked by rapid overall increases in body size (Fig 7.1A). The decrease in ADIPO:LEP ratio that was induced by HFD feeding resulted in a reduction in protein levels of certain cell cycle inhibitory regulators (Fig. 7.2) and caused S-phase entry in MCF7 cells exposed to conditioned media prepared from the adipose of HFD-fed animals (Fig. 7.6C). Our results point to HFD-dependent effects being completely abolished by high levels of physical activity, illustrating exercise as a powerful intervention/prevention strategy for obesity-linked cancers. However, given that cancer patients often suffer from other co-morbidities, implementing higher intensity exercise may not be an ideal option. Importantly, we demonstrate that lower intensity physical activity interventions, ones that do not induce weight loss, are still effective in counteracting the adipose-dependent deleterious growth microenvironment that a breast cancer is exposed to. This also suggests that the effects of diet and exercise that are protective against breast cancer depend on the alterations in the adipokine secretion profile from adipose tissue rather than the loss of fat mass itself.

Previous work in our lab has shown that increasing the ratio of ADIPO:LEP using recombinant proteins in cell culture subsequently increases the levels of $pAMPK^{T172}$ and decreases $pAkt^{T308}$, causing MCF7 breast cancer cells to arrest (51). In corroboration with these results,

altering these ratios by using conditioned media (CM) prepared from adipocytes isolated from CD fed animals *in-vivo* elicited identical effects when compared to cells treated with HFD-CM (Fig. 7.2). In addition, the growth environment created by the CM basically rendered the MCF7 cells unresponsive to the addition of exogenous ADIPO and LEP, which highlights the powerful nature of the control on MCF7 cell cycle regulation exerted by the adipose-created growth microenvironment. HFD feeding decreased the ADIPO:LEP ratio in the CM which increased MCF7 cell proliferation by activating AKT and inhibiting AMPK, ultimately reducing the cell cycle inhibitory effects of p27 and fostering S-phase entry (Fig. 7.6C). We have previously shown that stabilizing AdipoR1 by constitutively overexpressing the receptor we enhance the effects of ADIPO present in the media and counteract the effects of HFD on adipose-dependent alteration in tumor growth environment (51). AdipoR1 overexpression is also able to overcome the effects of addition of recombinant LEP (300 nM) to HFD-CM (Fig. 7.3), further highlighting the importance of maintaining ADIPO signaling in obese cancer patients. This observation has clinical relevance, since AdipoR1 protein levels are decreased in subcutaneous and visceral adipose tissue of obese women (34) and also are down regulated in pre-invasive DCIS (33). Thus, we feel that de-stabilizing the level of AdipoR1 within mammary carcinomas and in healthy breast tissue may be one of the important factors driving the increased rate and aggressiveness of breast cancers in obese women compared to lean women, in conjunction with the decreased levels of ADIPO. Increasing AdipoR1 protein levels increases the possible binding sites available for ADIPO and the cell cycle inhibitory effects of ADIPO can then be enhanced, thereby supressing tumor growth without specifically altering individual components within the growth microenvironment. This also highlights AdipoR1 stabilization as a target for novel breast cancer pharmacological therapeutics.

Given the strong association between obesity and breast cancer development/progression, interventions directed to counteract the effects of increased fat mass on adipokine secretion profile and the accompanying promotion of breast cancer cell proliferation is an important observation to establish. The effects of PA appear to be dose-dependent and do not follow threshold characteristics, meaning that there seems to be an effect of increasing the volume of exercise/day rather than a simple *"response or no response"* effect. With increased numbers we may have been able to establish a linearity of overall endocrine effects of adipose tissue on MCF7 cell cycle regulation response relative to daily km run. By plotting the effects of km run/day and effects on adipokine secretion we show linear correlations for the levels of ADIPO and LEP secreted into the culture media (Fig. 7.1G). While we are unable to specifically categorize the precise exercise performed (i.e. run, jog, walk) we do show that HFD-fed animals that completed PA of over 3 km/day were lighter and had smaller visceral fat depots compared to their sedentary counterparts which was accompanied by a higher ADIPO:LEP ratio secreted into the CM (Fig. 7.1, Table 7.1). Volume dependency was illustrated by the fact that HFD fed animals who performed PA that was less than 3 km/day had similar body and visceral fat masses as their sedentary counterparts, despite clear evidence of training adaptations in their hind limb muscles (Fig. 7.1). Despite this lack of difference in fat mass, HFD+LPA-CM had lower levels of LEP compared to HFD-CM with the ADIPO:LEP ratios being similar (Table 7.1). This altered LEP level may explain why we see an intermediate effect of the HFD+LPA-CM on MCF7 cells. The HFD+LPA-CM treated cells were found to have higher levels of $pAMPK^{T172}$, AdipoR1 and lower levels of $pAkt^{T308}$ (Fig. 7.4). In addition, HFD+LPA-CM increased and decreased the percentage of MCF7 cells in G0/G1 an S-phase, respectively, with respect to HFD-CM treated cells, but failed to completely abolish all of the effects of the HFD-CM treated cells (Fig. 7.6). Taken together these results suggest that any

interventions designed to counteract the effects of obesity on breast cancers do not necessarily have to alter absolute adiposity, but do need to elicit effects on adipokine production/secretion from adipose tissue, as this appears to be the major underlying contributor to adipose-dependent control of tumor growth microenvironment. Although our work used ADIPO and LEP as markers of adipokine secretion profile, we are in no way suggesting that these are the only adipokines of the more than 400 produced by adipocytes that underlie adipose-dependent effects. However, it is clear their levels, relative to each other, are likely candidates for accurately predicting/estimating the growth microenvironment that a breast cancer in an obese patient is exposed to. Furthermore, therapies that alter the levels/ratio of these adipokines may represent interventions with a higher chance of success in obese breast cancer patients.

In the current study we demonstrate the increasing the volume of exercise (km/day) elicits greater protection against the deleterious effects on high-fat diet in cancer cell cycle regulation. Our voluntary exercise wheel model of physical activity does not allow for specific work rates (i.e. $\%VO_2$ max) to be determined, only the overall amount of work performed per day. This can be problematic when trying to prescribe a specific dose of exercise for therapeutic intervention. In order to give a gross approximation of the relative workload that the daily km completed by the animals in our study would equate to, we have tried to use literature values for an exercise protocol that would elicit adaptations in mixed gastrocnemius oxidative enzyme activities akin to what we observed. Evaluation of studies which used motorized wheel running (40) showed that despite our animals exercising at a much lower intensity (km/hour), the longer duration in our study resulted in a higher total daily distance (1.71 km/day vs. LPA 2.47 km/day & HPA 7.21 km/day). Our daily distance PA division was based on where the gap in distance covered appeared to lie and is in line with previously published data for creating low and high PA groups (21). The COX activity in

mixed gastrocnemius muscle for the HFD+LPA group was 2.1-fold higher than HFD sedentary animals (Fig. 7.1F). A paper by *Samelman et al.* (2000) found that rats exercising at a treadmill exercise dose of 15m/min at a 10% gradient for 1hr/day, 5 days/wk displayed a 1.8-fold increase in COX activity of the mixed gastrocnemius compared to their sedentary counterparts (36). *LeBlanc et al.* (2008) showed that obese rats exercising at 20m/min at a 10% gradient for 1hr/day, 5 days/week had a gastrocnemius citrate synthase activity (CS) that was 1.5-fold higher than their sedentary obese counterparts (25). Previous work has shown that in rats changes in gastrocnemius COX activity in response to increased physical activity is approximately 1.5-fold higher compared to CS in the same exercising muscle (18). Using this we estimate that the 1.5-fold CS activity in Leblanc et al. would likely be accompanied by a 2.3-fold increase in COX activity. Using these changes as a guide, we estimate that the 2.8-fold increase in COX activity exhibited by our HPA group was suggestive of workload greater than the 20m/min at a 10% gradient used in this study (25). Using these two workload approximations, we can estimate a dose of exercise using a compilation of curves that approximate VO_2max from speed and grade of running (3). Using figures contained in *Brooks and White* (1978) we estimate that our LPA group exercised at a rate roughly equivalent to a VO_2 of 54 ml/kg/min. Using similar analyses, we estimate that the volume of exercise that our HPA group completed caused changes greater than if they were exercised at a VO_2 of 60 ml/kg/min. Although the initial intent of our experiments were not intended to prescribe specific doses of exercise and we can't prescribe specific dose rates of exercise for human patients from our study due to species and age differences and the lack of a clear absolute work rate (i.e. %VO_2max), it is evident that increasing the volume of exercise, whether it be walking, jogging or running, provides a protective effect on the endocrine tumor growth microenvironment created by adipose tissue. It may also be suggestive of volume of exercise being as important as intensity of

exercise. This has important ramifications clinically as older cancer patients may not be capable of performing high-intensity (shorter duration) exercise due to numerous disease-related pathologies.

Previous work in our lab has shown that activation of AMPK signaling decreases AdipoR1 degradation, increasing AdipoR1 protein levels in a positive feedback manner (51). This is an important observation as it highlights the potential of the tumor growth microenvironment that exists within obese breast cancer patients acting through a two-pronged mechanism. Obese patients will have lower levels of circulating ADIPO which will reduce the direct growth inhibitory effects on breast cancer cells. In addition, this reduced ADIPO content will result in decreased activation of AMPK within the cancer cells, which will subsequently result in a secondary destabilization/reduction in AdipoR1 protein levels. This will reduce the number of available receptor sites for ADIPO binding at the cell surface. This mechanism may explain, in part, why obese breast cancer patients have more aggressive tumors and express lower levels of AdipoR1. This AMPK-dependent mechanism may explain why anti-diabetic medications that activate AMPK (i.e. metformin) have been associated with improvements in cancer patient prognosis (12, 13, 47). Furthermore, it may highlight the potential of nutritional supplements that activate AMPK (i.e. resveratrol) as possible augmentations to existing cancer therapies, potentially eliciting few harmful side-effects but at the same time counteracting the deleterious effects of the tumor growth microenvironment that exists in obese breast cancer patients.

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Table 7.1. *ADIPO:LEP ratio for adipose-derived conditioned media*

Group	ADIPO (ng/ml)	LEP (ng/ml)	ADIPO:LEP
HFD	558.9 ± 99.4	2.69 ± 0.8	122.1 ± 52.1
CD	1289.0 ± 348.9*'†	1.26 ± 0.3*'†	566.5 ± 197.2*'†
HFD+LPA	622.0 ± 141.0	1.71 ± 0.4*	199.7 ± 57.7
HFD+HPA	1052.0 ± 246.9*'†	1.17 ± 0.3*'†	529.8 ± 105.3*'†
CD+LPA	1618.2 ± 873.3*'†	1.20 ± 0.6*'†	704.2 ± 258.9*'†

HFD, high fat diet; CD, chow diet; HFD+LPA, high fat diet + low physical activity; HFD+HPA, high fat diet + high physical activity; CD+LPA, chow diet + low physical activity; * indicates significantly different from HFD, † indicates significantly different from HFD+LPA ($p<0.05$, n=6/group).

FIGURE LEGENDS

Fig 7.1. HFD increases epididymal fat and is ameliorated with PA. Body mass changes over the 6 week protocol *(A)*. Body weight adjusted epididymal fat mass in CD (open bar), HFD (black bar), HFD+LPA (light grey bar), HFD+HPA (dark grey bar) and CD+LPA (hatched bar) animals*(B)*. Body weight adjusted muscle mass of the gastrocnemius *(C)*, soleus *(D)* and tibialis anterior *(E)* muscles. Physical activity alters cytochrome C oxidase (COX) enzyme activity in the gastrocnemius muscles of CD, HFD, HFD+LPA, HFD+HPA and CD+LPA animals *(F)*. Plotting of ADIPO:LEP ratio in CM prepared from HPA and LPA animals vs. daily km run *(G)*. Dotted line indicates divider between HPA and LPA groups. Western blots showing levels of Akt and β-actin in adipose tissues from the indicated groups *(H). Fig. A* * indicates different from HFD, CD and HFD+LPA animals ($p<0.05$). Different letters *(Figs. B-F)* indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 7.2. HFD-CM antagonizes the effects of CD-CM. Representative western blots for selected proteins showing the effects of treatment with CM (+/- ADIPO or LEP) prepared from FBS (hatched bar), or CD (open bar) or HFD (black bars) animals on MockT MCF7 cells *(A)*. Graphical representations of multiple experiments showing the effects of CM or CM plus ADIPO or LEP on $pAMPK^{T172}$ *(B)*, $pAKT^{T308}$ *(C)*, p27 *(D)*, $p27^{T198}$ *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 7.3. Overexpression of AdipoR1 ameliorates the effects of the HFD-CM and LEP. Representative western blots for selected proteins showing the effects of treatment with CM (+/-

ADIPO or LEP) prepared from FBS (hatched bars), or CD (open bars), HFD (black bars) animals on AdipoR1 stably transfected MCF7 cells *(A)*. Western blots showing the expression of the FLAG-tag and AdipoR1 in stably transfected cell lines *(B)*. Graphical representations of multiple experiments showing the effects of CM or CM plus ADIPO or LEP on $pAMPK^{T172}$ *(C)*, $pAKT^{T308}$ *(D)*, p27 *(E)*, $p27^{T198}$ *(F)* and AdipoR1 *(G)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 7.4. Physical activity can abolish effects of a HFD on adipose-dependent tumor growth microenvironment. Representative western blots for selected proteins showing the effects of treatment with CM prepared from CD (open bar), HFD (black bar), HFD+HPA (dark grey bar), HFD+LPA (light grey bar) and CD+LPA (hatched bar) animals on MockT MCF7 cells *(A)*. Graphical representations of multiple experiments showing the effects of CM on $pAMPK^{T172}$ *(B)*, $pAKT^{T308}$ *(C)*, p27 *(D)*, $p27^{T198}$ *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 7.5. Overexpression of AdipoR1 can counteract the effects of HFD. Representative western blots for selected proteins showing the effects of treatment with CM prepared from CD (open bar), HFD (black bar), HFD+HPA (dark grey bar), HFD+LPA (light grey bar) and CD+LPA (hatched bar) animals on AdipoR1 transfected (p31-4-2-2) MCF7 cells *(A)*. Graphical representations of multiple experiments showing the effects of CM on $pAMPK^{T172}$ *(B)*, $pAKT^{T308}$ *(C)*, p27 *(D)*,

$p27^{T198}$ *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 7.6. Adipose-dependent growth environment causes cell cycle changes in CM experiments. Typical cell cycle profiles in MockT MCF7 cells *(A)* and stably transfected AdipoR1 overexpressing MCF7 cells *(B)*. Graphical representation of multiple cell cycle profile experiments observing effects of diet on CM effects in CD (open bar), HFD (black bar) animals in MockT MCF7 cells *(C)* and in MCF7 cells stably overexpressing AdipoR1 *(D)*. Graphical representation of multiple cell cycle profiles showing the effects of exercise and diet CD+LPA (hatched bar), HFD+HPA (dark grey bar) and HFD+LPA (light grey bar) on MockT MCF7 cells *(E)* and MCF7 cells stably overexpressing AdipoR1 *(F)*. * indicate groups that are significantly different from HFD treated cells ($p<0.05$, n=6/group).

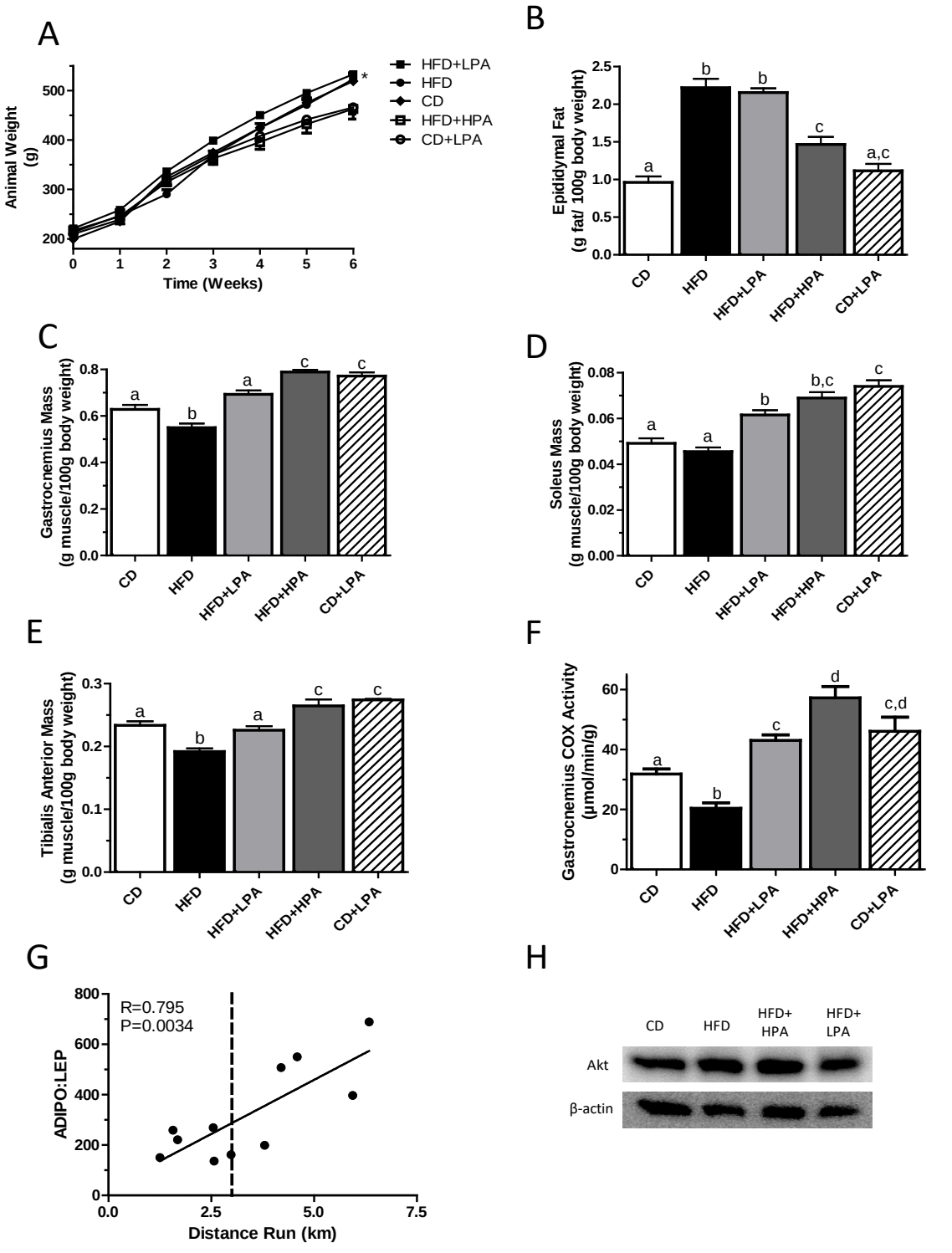
A
Animal Weight (g)
Time (Weeks)
HFD+LPA
HFD
CD
HFD+HPA
CD+LPA
B
Epididymal Fat (g fat/ 100g body weight)
C
Gastrocnemius Mass (g muscle/100g body weight)
D
Soleus Mass (g muscle/100g body weight)
E
Tibialis Anterior Mass (g muscle/100g body weight)
F
Gastrocnemius COX Activity (µmol/min/g)
G
R=0.795
P=0.0034
ADIPO:LEP
Distance Run (km)
H
CD
HFD
HFD+ HPA
HFD+ LPA
Akt
β-actin

Figure 7.1

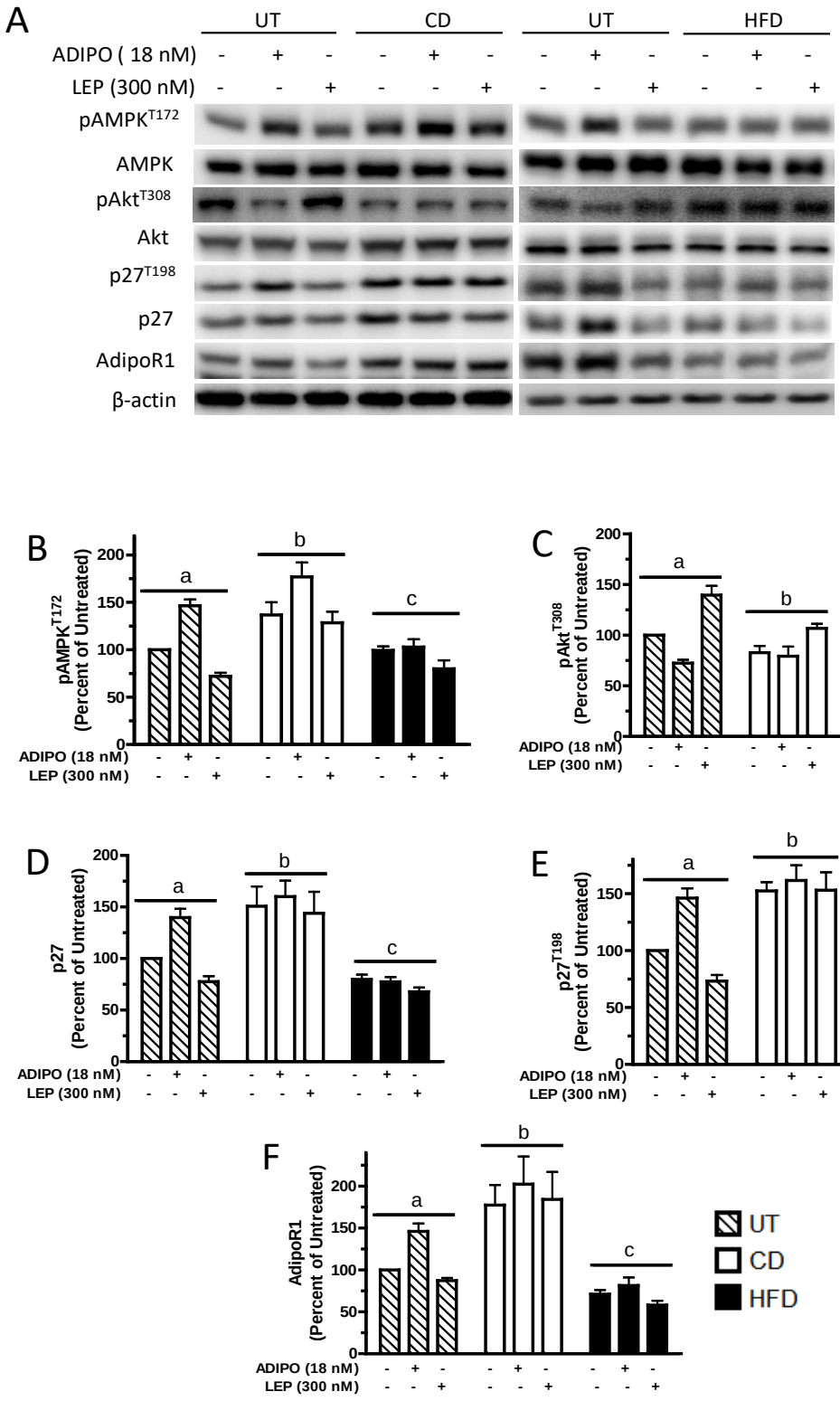
A
UT
CD
UT
HFD
ADIPO (18 nM)
LEP (300 nM)
pAMPK^{T172}
AMPK
pAkt^{T308}
Akt
p27^{T198}
p27
AdipoR1
β-actin
B
pAMPK^{T172} (Percent of Untreated)
C
pAkt^{T308} (Percent of Untreated)
D
p27 (Percent of Untreated)
E
p27^{T198} (Percent of Untreated)
F
AdipoR1 (Percent of Untreated)
a
b
c
ADIPO (18 nM)
LEP (300 nM)
UT
CD
HFD

Figure 7.2

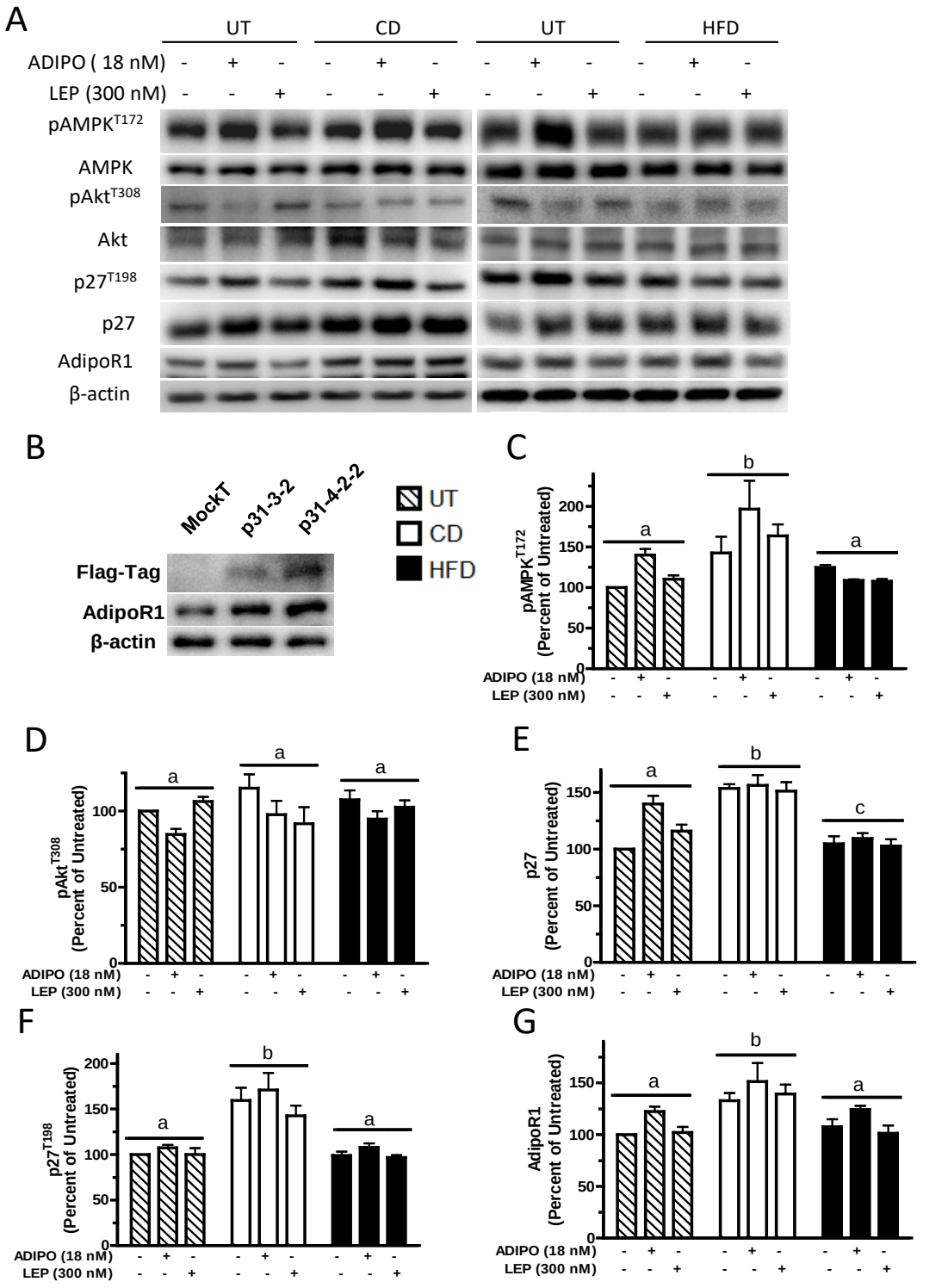
A
UT
CD
UT
HFD
ADIPO (18 nM)
LEP (300 nM)
pAMPK^{T172}
AMPK
pAkt^{T308}
Akt
p27^{T198}
p27
AdipoR1
β-actin
B
MockT
p31-3-2
p31-4-2-2
Flag-Tag
AdipoR1
β-actin
UT
CD
HFD
C
pAMPK^{T172} (Percent of Untreated)
D
pAkt^{T308} (Percent of Untreated)
E
p27 (Percent of Untreated)
F
p27^{T198} (Percent of Untreated)
G
AdipoR1 (Percent of Untreated)

Figure 7.3

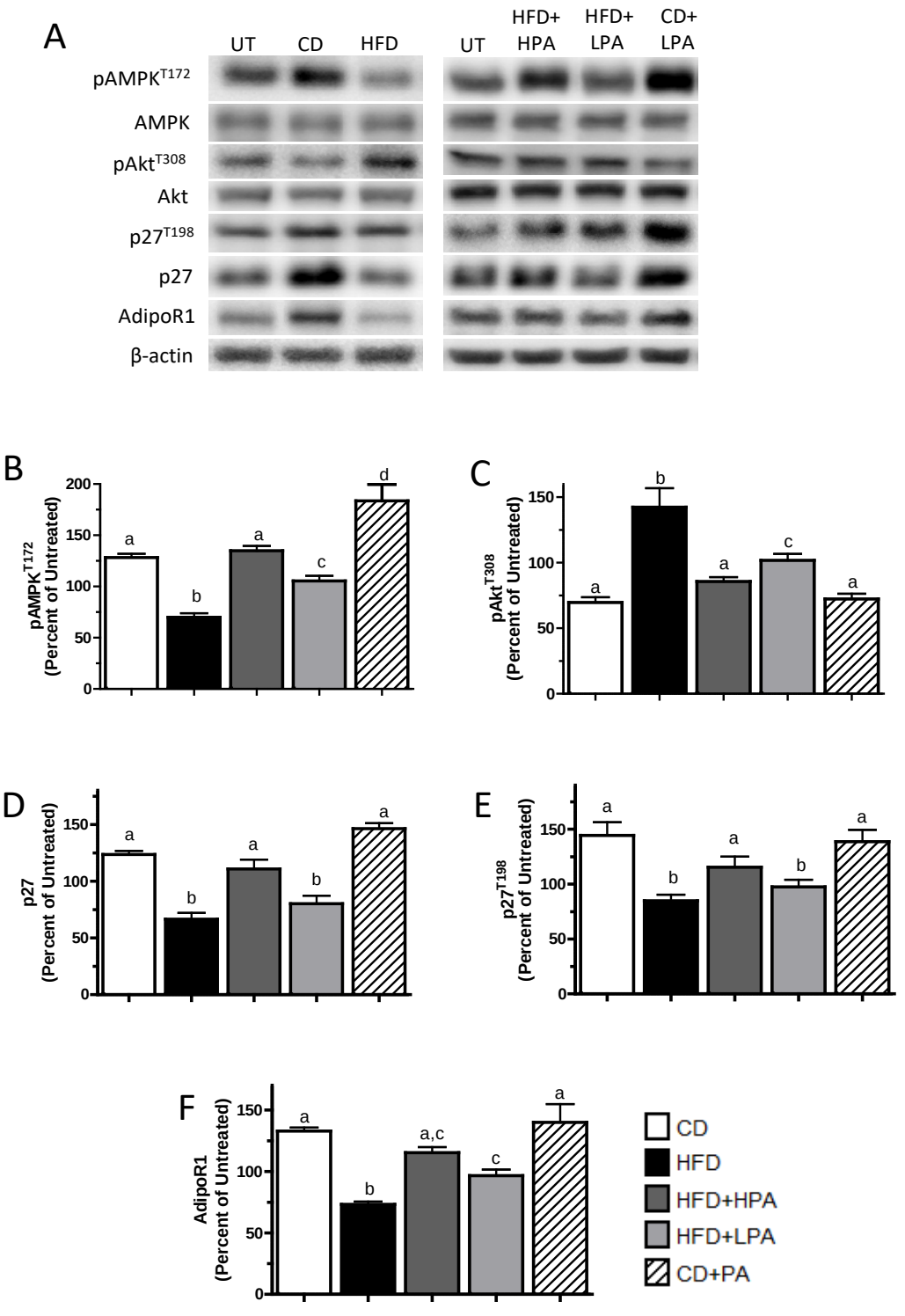
A
UT
CD
HFD
UT
HFD+ HPA
HFD+ LPA
CD+ LPA
pAMPK^{T172}
AMPK
pAkt^{T308}
Akt
p27^{T198}
p27
AdipoR1
β-actin
B
pAMPK^{T172} (Percent of Untreated)
C
pAkt^{T308} (Percent of Untreated)
D
p27 (Percent of Untreated)
E
p27^{T198} (Percent of Untreated)
F
AdipoR1 (Percent of Untreated)
CD
HFD
HFD+HPA
HFD+LPA
CD+PA

Figure 7.4

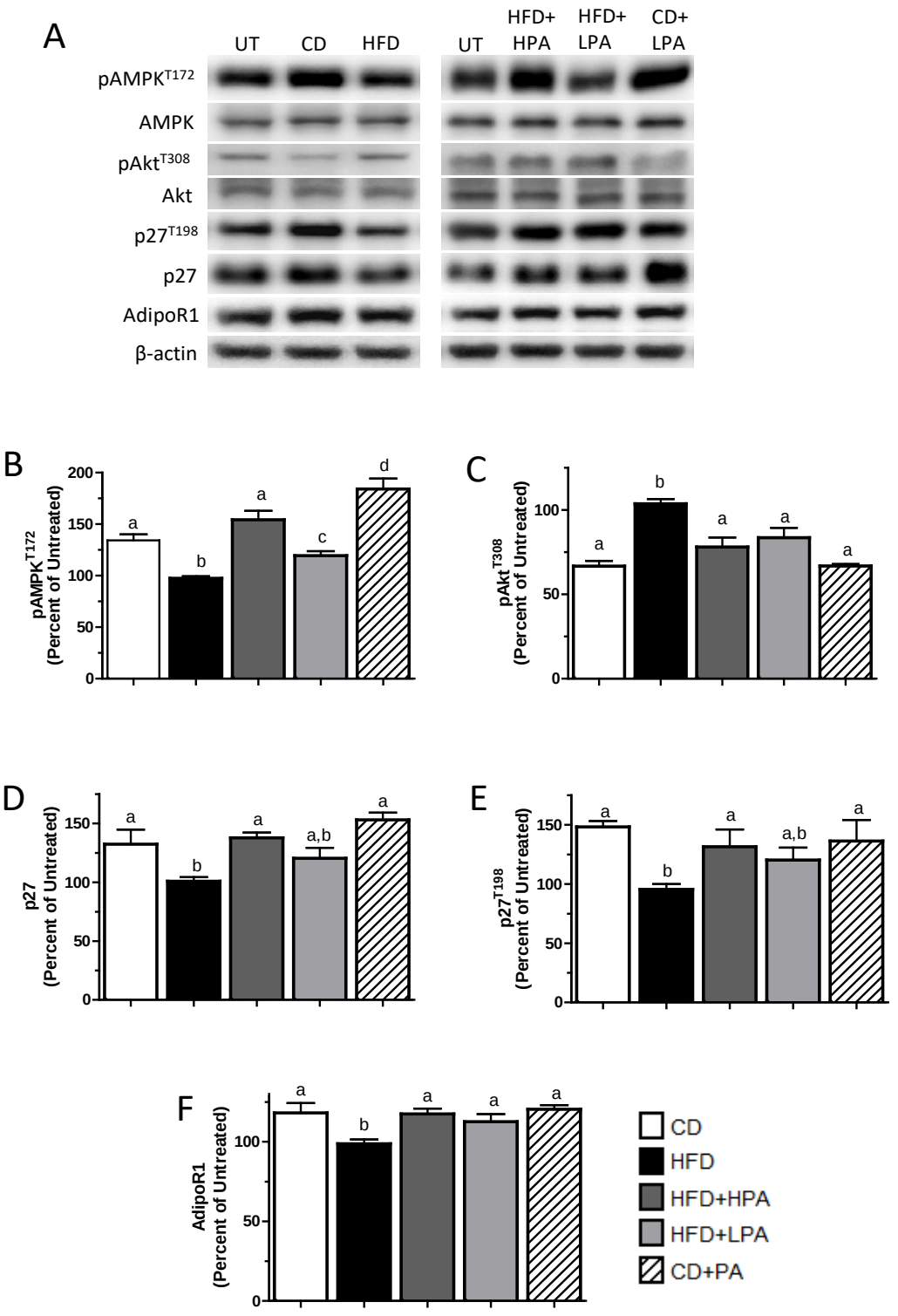
A
UT
CD
HFD
UT
HFD+ HPA
HFD+ LPA
CD+ LPA
pAMPK^{T172}
AMPK
pAkt^{T308}
Akt
p27^{T198}
p27
AdipoR1
β-actin
B
pAMPK^{T172} (Percent of Untreated)
C
pAkt^{T308} (Percent of Untreated)
D
p27 (Percent of Untreated)
E
p27^{T198} (Percent of Untreated)
F
AdipoR1 (Percent of Untreated)
CD
HFD
HFD+HPA
HFD+LPA
CD+PA

Figure 7.5

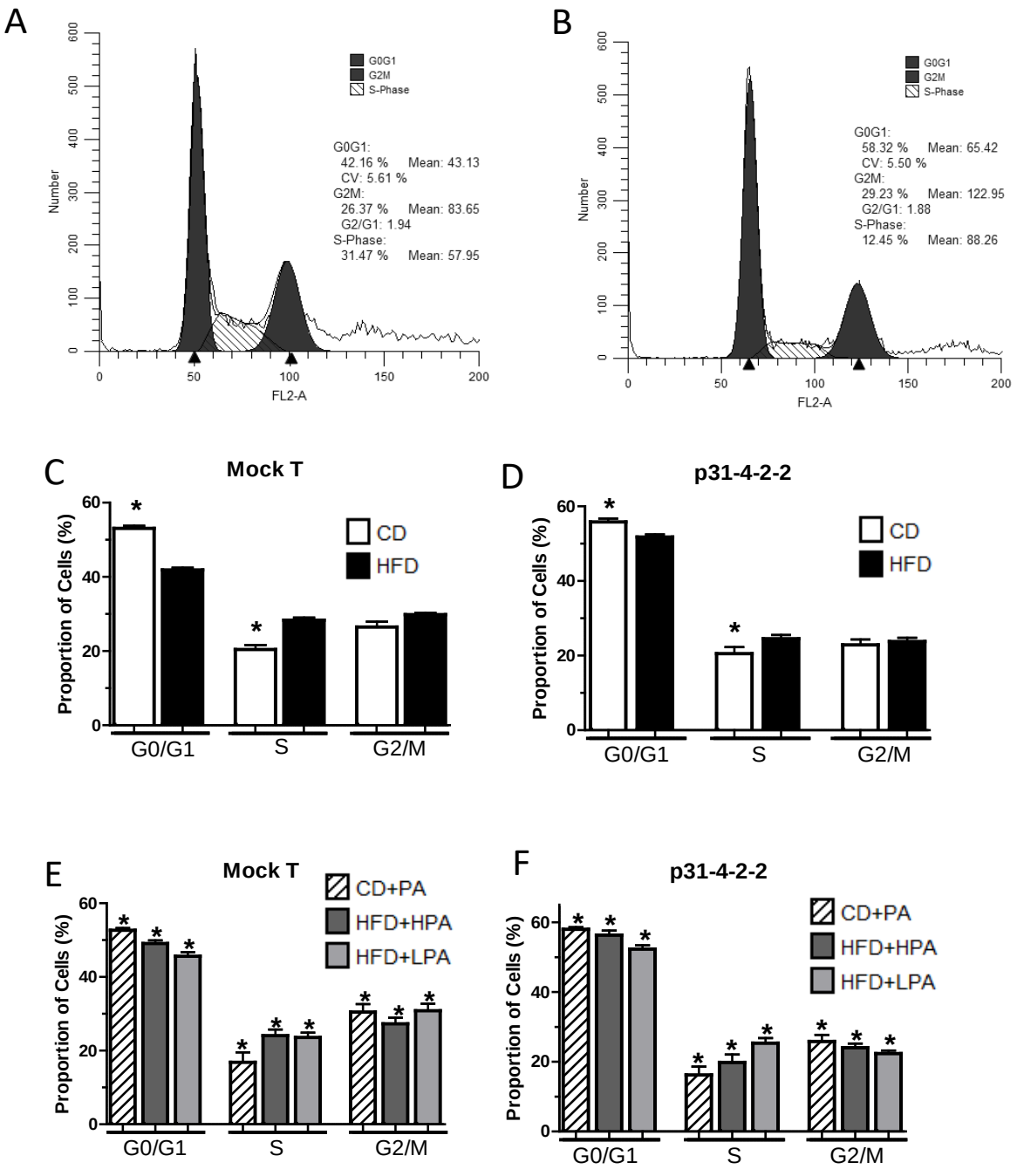
A
G0G1
G2M
S-Phase
G0G1:
42.16 %
Mean: 43.13
CV: 5.61 %
G2M:
26.37 %
Mean: 83.65
G2/G1: 1.94
S-Phase:
31.47 %
Mean: 57.95
Number
FL2-A
B
G0G1
G2M
S-Phase
G0G1:
58.32 %
Mean: 65.42
CV: 5.50 %
G2M:
29.23 %
Mean: 122.95
G2/G1: 1.88
S-Phase:
12.45 %
Mean: 88.26
Number
FL2-A
C
Mock T
CD
HFD
Proportion of Cells (%)
G0/G1
S
G2/M
D
p31-4-2-2
CD
HFD
Proportion of Cells (%)
G0/G1
S
G2/M
E
Mock T
CD+PA
HFD+HPA
HFD+LPA
Proportion of Cells (%)
G0/G1
S
G2/M
F
p31-4-2-2
CD+PA
HFD+HPA
HFD+LPA
Proportion of Cells (%)
G0/G1
S
G2/M

Figure 7.6

8. Academic Research Paper #2

Voluntary Physical Activity Counteracts the Proliferative Tumor Growth Microenvironment Created by Adipose Tissue via High Fat Diet Feeding and Circulating Estrogen[2]

The adipokine secretion profile created from adipose tissue may represent the molecular mechanisms behind the obesity-breast cancer association. Two adipokines, adiponectin (ADIPO) and leptin (LEP), are altered with obesity and exert antagonistic effects on breast cancer proliferation. We set out to determine whether the adipose-dependent tumor promoting growth environment created by a high fat diet (HFD) is affected by estrogen and if voluntary physical activity (PA) ameliorates any HFD-dependent effects. We found that conditioned media (CM) created from the adipose tissue of female HFD-fed rats increased the proliferation of MCF7 cells compared to those cells grown in CM prepared from lean adipose tissue. HFD-CM inhibited AMPK and activated Akt signaling, decreased p27 phosphorylation at T198, reduced total p27 and AdiporR1 protein levels and promoted cell cycle entry. Interestingly, despite greater diet-dependent changes in ADIPO and LEP secretion into the CM, the HFD-dependent effects on MCF7 proliferation were blunted compared to when estrogen was absent. PA reversed the proliferative effects of HFD-CM on MCF7 cells by preventing the effects of HFD on AMPK, Akt, p27 and AdipoR1, ultimately resulting in cell cycle withdrawal. Overexpressing AdipoR1 abolished the proliferative effects of the HFD-CM on MCF7 cells and enhanced the anti-

[2] **Christopher F. Theriau and Michael K. Connor**
STATEMENT OF LABOUR:
Christopher Theriau:
All work both manuscript/figure creation, experimental/animal procedures and data collection/ analyses

proliferative effects PA on the HFD-CM. Thus, PA represents a means to prevent deleterious obesity related alterations in tumor growth environment which are brought about by changes in adipokine secretion profile from adipose tissue, independent of the presence of estrogen.

Keywords: Physical activity, obesity, estrogen, adipokines, breast cancer

INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy among women in the world. Despite some genetic bases, breast cancer arises in a sporadic fashion with a large number of factors identified as increasing the risk of disease development, including a growing evidence which points to obesity as a modifiable risk factor. For almost 50 years there has existed a statistical link between adiposity and an increased risk of breast cancer (55). Several clinical and preclinical studies have demonstrated that increased adiposity is associated with an increased cancer incidence, morbidity, in addition to imparting a poorer response to therapy and higher disease mortality (7, 44, 55). While this association appears to be quite strong in postmenopausal women (64), the relationship between obesity and breast cancer in premenopausal women is far less consistent. Studies have reported that obesity in premenopausal women is inversely associated with breast cancer (6, 39, 62), shows no association (27, 31), or shows a positive association (11). Despite this discrepancy in results, it has been hypothesized that this lack of harmony among the literature may be attributed to variations in experimental design. The possible inverse effect of obesity on premenopausal breast cancer risk has been demonstrated in women 35 years and younger (45), ages that are not typical for breast cancer development and where estrogen levels are high. This increased incidence of breast cancer in obese women has been suggested to be due in part, to the direct effects of estrogen, produced by the peripheral fat depots via aromatization (9, 10). However, recent work suggests disease development occurs independently of circulating estrogen (57).

Adipose tissue has been identified as an active endocrine organ producing adipocyte-derived factors, termed adipokines. These adipokines can act in an autocrine, endocrine and/or paracrine manner, giving multiple potential mechanisms underlying this obesity/cancer link. Thus

far over 400 adipokines have been discovered and several have been shown to become dysregulated in obese individuals (65). Given that the human breast is composed primarily of epithelial cells surrounded by adipose tissue, the potential exists for adipose to contribute to important components of the tumor growth microenvironment surrounding any breast tumor. Of the over 400 adipokines identified to date, adiponectin (ADIPO) and leptin (LEP) represent major potential contributors to the adipose-dependent microenvironment. Both are among the most abundant adipokines produced/secreted, are altered by obesity have documented cell cycle regulatory effects on breast cancer cells (14, 15, 26). LEP, is predominately produced by white adipose tissue and its level in the peripheral circulation is directly proportional to BMI and often higher in women than in men (63). LEP activates several intracellular pathways implicated in breast carcinogenesis, including the phosphoinositide-3/Akt kinase signaling pathway (21, 26). LEP can induce cell cycle entry by activating Akt, which phosphorylates p27 at T157, preventing both its nuclear accumulation and inhibition of cyclin E/cdk2, thereby leading to cell cycle entry (15, 20, 33). Conversely, ADIPO production/secretion decreases with obesity and induces cell cycle exit by activating AMPK, which directly phosphorylates p27 at T198 increasing p27 stability and inducing G1 arrest (14, 23, 32). This is mediated by ADIPO binding to its receptor Adiponectin receptor 1 (AdpoR1) which is also implicated in breast cancer (14, 57). ADIPO-dependent anti-proliferative effects are abolished by siRNA knockdown of AdipoR1 (23, 41) and previous work in our lab has shown that AMPK activation via AdipoR1 increases receptor protein levels in a positive feedback manner (57). Decreased ADIPO signaling through AdipoR1 has been shown to be associated with higher tumor grade and poorer patient outcomes in breast cancer patients (46). Clinical studies in postmenopausal women also suggest that decreased ADIPO:LEP ratios, rather than the levels of individual adipokines, are stronger predictors of breast cancer risk (42). The

decreased ADIPO and increased LEP in the circulation of obese individuals creates a microenvironment that promotes tumor growth by accelerating cell cycle entry (12, 37). In premenopausal women this ADIPO:LEP breast cancer association is less clear, as increased LEP has been shown to be inversely associated with breast cancer risk but possibly independent of BMI (24), while low circulating ADIPO is associated with increased breast cancer risk (35). These results are in contradiction to these adipokines being predictors of breast cancer growth environment.

A recent study by *Morad et al.,* (2014) using microdialysis of breast cancer tissue and adjacent healthy breast tissue discovered that local extracellular LEP was higher, while ADIPO and ADIPO:LEP ratio were lower in tumors compared to normal adjacent breast tissue (40). The group also found that higher plasma estradiol correlated in premenopausal women to lower local extracellular ADIPO and ADIPO:LEP ratio and positively correlated with LEP (40). We have previously demonstrated that increasing AdipoR1 levels in breast cancer cells increases the cell cycle inhibitory effects of ADIPO via AMPK signaling and can counteract the antagonism of ADIPO by LEP (57), something that may be beneficial for obese breast cancer patients by overcoming the adipose-dependent effects that underlie the association between obesity and poor prognosis.

A sedentary lifestyle has now been widely accepted as a major contributor to the increase in obesity and its associated disorders (52). This highlights increased physical activity (PA) as a potential prevention/intervention for the development of obesity and its associated effects on breast cancer (5, 13, 18). Although there are some discrepancies for the role of obesity and breast cancer risk in pre vs. postmenopausal women, research shows that PA can lower the risk of breast cancer regardless of menopausal status (4, 8). Obese and physically inactive breast cancer patients appear

to be at an increased risk for both disease progression and cancer-related mortality, regardless of menopausal status (16, 47, 49). Higher recreational PA has been shown to be associated with a 30-60% reduction of all ER/PR subtypes in premenopausal women compared to sedentary women (18), whereas moderate PA has been shown to exhibit a similar reduction in postmenopausal women (28, 43, 50, 53, 60). The National Action Plan on Breast Cancer's Workshop on PA and Breast Cancer found that the breast cancer risk reduction associated with PA may be greatest among women who are lean and premenopausal (19). Other studies have also shown that risk reduction of breast cancer due to regular PA is greater among premenopausal women <45 years of age compared to postmenopausal women (38, 59). Voluntary PA alters the production of both ADIPO and LEP in female high fat diet (HFD)-fed animals, lowering the levels of LEP and increasing the levels of ADIPO in the circulation compared to sedentary high fat diet fed animals (5, 30, 58, 66, 67). PA was found to decrease chemically induced breast cancer incidence and multiplicity compared to sedentary controls (58, 66, 67). PA decreased $pAkt^{T473}$ and increased $pAMPK^{T172}$ and p27 within mammary carcinomas of those same animals (66). In addition, the reduced cancer incidence was not due to differences in circulating estradiol. In humans, women who consumed a calorie restricted diet coupled with moderate PA experienced a 9.5% increase in plasma ADIPO and a 40.1% decrease in LEP (1). Thus, there are clear positive effects of diet and the volume of physical activity and the benefits to breast cancer patient prognosis. However, the contribution of estrogen in these phenomena remain unclear.

Previous work in our lab found that in the absence of estrogen, visceral fat of obese-fed HFD rats induced cell cycle entry in MCF7 cells by activating Akt and inhibiting AMPK, brought about by a decreased ADIPO:LEP ratio, which elicited cell cycle entry by repressing AdipoR1 and p27 expression (57). Additionally, voluntary PA abolished these HFD-dependent effects as did

increasing the expression of AdipoR1. Given some of the inconsistencies surrounding the role of estrogen in adipose-dependent increases in breast cancer development we wanted to define any direct effects of estrogen on the adipose-dependent tumor growth microenvironment. We show that the ADIPO:LEP ratio is decreased in the circulation of HFD-fed animals which is brought about by a decreased secretion from adipose. This altered secretion caused a decrease in $pAMPK^{T172}$, $p27^{T198}$, AdipoR1 and a decrease in $pAkt^{T308}$, leading to an increase in MCF7 cell growth from conditioned media (CM) prepared from the adipose of HFD animals. Voluntary PA counteracted these HFD effects by increasing the ADIPO:LEP ratio compared to HFD-fed animals. These changes abolish the effects of a HFD on MCF7 cell cycle regulation. Overexpressing AdipoR1 in MCF7 cells was also found to counteract the effects of the HFD-CM. Interestingly, we found that the presence of circulating estrogen altered the composition of the CM compared to when estrogen was absent (57). This altered CM secretome was apparent in HFD-CM as the effect of HFD on the proliferation of MCF7 cells was blunted in comparison (57). These results highlight the importance of PA and stabilizing AdipoR1 signaling in order to overcome the positive tumor growth microenvironment created by obesity even in the presence of circulating estrogen in premenopausal women.

METHODS

Animals. All animal experiments were approved by the York University Animal Care Committee in accordance with Canadian Council for Animal Care guidelines. Thirty female Sprague-Dawley rats (7 weeks of age) were purchased from Charles River Laboratories (Montreal, QC, Canada) and were singly housed in standard clear, plastic cages. All animals had a 7 day

habituation period to a 12 hour light-dark cycle (lights on at 0600) in a temperature (22°C) and humidity (50-60%) controlled room.

After the habituation period, animals were randomly selected and given free access to a running wheel (wheel circumference, 106 cm; Harvard Apparatus, Holliston, MA) in their cage. A magnetic counter was mounted to each wheel which detected the revolutions every 24 hours. The animals were given a 7 day acclimation period to the wheels. After this period, animals were then divided into two groups: chow diet (CD; n=10) and high fat diet (HFD; n=20) with both groups given access to food and water (*ad libitum*). The CD (no. 5012 Lab Chows, Ralston Purina, St. Louis, MO) had caloric make-up of 14% fat, 54% carbohydrate, 32% protein (3.02 calories/g). The HFD (Harlan Laboratories, Madison, WI) had a caloric breakdown of 60% fat, 21% carbohydrate, 18% protein (5.1 calories/g). HFD and CD fed animals were further subdivided into sedentary and physical activity (PA) groups designated as chow diet-sedentary (CD; n=6), chow diet-high physical activity (CD+HPA; >12.5 km/day; n=4), HFD-sedentary (HFD; n=8). HFD animals with access to wheel running were further divided into those that ran more than 12.5 km/day which were designated high fat diet-high physical activity (HFD+HPA; n=8) and those that ran less than 12.5 km/day were put in a high fat diet-low physical activity (HFD+LPA; n=4) group. Previous work with female rats has shown similar cut-off voluntary wheel distances as we utilize for our protocol (22, 61). Food intake and running distances were measured each day, and body weight was measured three times per week.

Tissue Collection and Conditioned Media. After the 6 week protocol, weights of all tissues collected were measured and normalized per 100 g of body weight. The sequence in which rats were sacrificed was randomized across groups so as to minimize the likelihood that order effects would masquerade as treatment-associated effects. At time of sacrifice the gastrocnemius, soleus

and tibialis anterior were immediately excised, weighed, frozen and stored at -84°C for future analyses. Also, 2-3 ml of blood were taken at time of sacrifice, left for 30 min on ice and subsequently centrifuged. The serum was then extracted, frozen and stored at -84°C for future analyses. All visceral adipose tissue was quickly removed from all animals and cultured as previously described (57). Briefly, all visceral fat was weighed, minced into ~5-10 mg pieces and immediately placed in 50 ml vented conical tubes containing AMEM (7.5 ml/g tissue; Wisent, St.Bruno, QC) supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific, Whitby, ON), 2% antimicotic/antibiotic (Wisent), 1 mM sodium pyruvate (Sigma, Oakville, ON), non-essential amino acids (Sigma), and 10 µg/ml insulin (Wisent) under sterile conditions and incubated for 24 hours at 37°C with 5% CO_2. After 24 hours, conditioned media (CM) was then collected and stored at -84°C, as previously described (57). In order to ensure that our mass:volume preparation of CM was consistent among preparations across groups, we diluted 10-15 mg sections of adipose 30:1 in RIPA buffer for protein extraction. Equal volumes of lysate (25 µl) were subjected to SDS-PAGE using 12% gels and membranes were probed for total Akt and β-actin to evaluate equivalency of specific protein content between preparations.

Cytochrome-c Oxidase Activity Assay. In order to confirm that the physical activity protocol elicited a training effect, we measured cytochrome-c oxidase (COX) activity in mixed gastrocnemius muscles. COX activity was determined as previously described (57). Briefly, cross-sections of mixed gastrocnemius muscles (from the mid-section of the muscle belly) weighing roughly 20-30 mg were diluted 80-fold (sedentary) or 160-fold (PA) in extraction buffer (100mM Na-K-Phosphate, 2 mM EDTA, pH 7.2). Muscle extracts were prepared by homogenization with metal beads in a magnetic homogenizer (Mixer Mill MM 400, Retsch, Germany). These

homogenates were then used for the analyses of the maximum rate of oxidation of fully reduced cytochrome c at 30°C as indicated by changes in absorbance (550 nm).

Co-Culture/Serum Adipokine and Estradiol Measurement. The levels of ADIPO, LEP and estradiol (E2) produced and secreted into the co-culture media by adipocytes as well as in the serum at time of sacrifice was determined using rat adiponectin sandwich ELISA kit (BioVision, Milpitas, CA), mouse/rat leptin quantikine sandwich ELISA kit (R&D Systems, Minneapolis, MN), and rat estradiol competitive binding sandwich ELISA kit (Alpco Diagnostic, Salem, NH), as per manufacturer instructions. Aliquots of CM were diluted 50-fold (HFD) and 100-fold (PA and CD) for ADIPO ELISAs, 5-fold (HFD) or undiluted (PA and CD) for LEP ELISAs and undiluted for E2 ELISAs. Aliquots of serum were diluted 1000-fold for all samples for ADIPO ELISAs, 5-fold (HFD and HFD+PA) or undiluted (PA and PA+HPA) for LEP ELISAs and undiluted for E2 ELISAs. The levels of each adipokine were calculated in ng/ml values and ADIPO/LEP converted to nM values for stoichiometric comparison. The levels of E2 were calculated in pg/ml values.

Cell Culture. MCF7 cells were purchased from the American Tissue type Culture Collection (ATCC, Manassas, VA) and were maintained in AMEM, 10% FBS, 2% antimicotic/antibiotic, 1 mM sodium pyruvate, non-essential amino acids, and 10 µg/ml insulin from human pancreas at 37°C and 5% CO_2.

MCF7 cells were transfected with an AdipoR1 overexpressing plasmid vector as previously described (57). Mock transfected (MockT) MCF7 cells and stably transfected AdipoR1 (AdipoR1-T) cells were seeded in 6 well plates with AMEM for 24 hrs. At 70% confluence, cells were washed with PBS and incubated with CM produced from adipocytes for 24 hours. MCF7 cells grown in AMEM supplemented with 10% FBS served as untreated controls (UT).

Immunoblotting. The effects of adipokines on specific cellular proteins were measured using standard SDS-PAGE protocols utilizing 12% polyacrylamide gels. Proteins (25 µg) were transferred to PVDF membranes (Bio-rad, Mississauga, ON, CAN), blocked for 2 hours in 10% skim milk and subsequently incubated overnight with primary antibodies: $p27^{Kip1}$ (BD Biosciences); $p27^{T198}$ (R&D Systems); $pAkt^{T308}$, Akt, $pAMPK^{T172}$ and AMPK (Cell Signaling); AdipoR1 (Santa Cruz Biotech, Santa Cruz, CA) and β-actin (Abcam, Cambridge, MA). Anti-mouse, anti-rabbit (Promega, Madison, WI) and anti-goat (Santa Cruz) horseradish peroxidase secondary antibodies were used to visualize proteins using Immobilon enhanced chemiluminescence substrate (Millipore, Whitby, ON, CAN) and detected/quantified on a Kodak *In Vivo* Pro imaging system (Marketlink Scientific, Burlington, ON, CAN).

Cell cycle analyses. MCF7 cells isolated from 6-well plates were trypsinized, washed in cold PBS and fixed by drop wise addition of ice-cold 70% ethanol. Cells were washed twice in PBS, re-suspended in a propidium iodide/RNAse solution and subjected to FACS analyses (Gallios Flow Cytometer, Beckman Coulter Mississauga, Canada). Cell cycle profiles were determined using Mod-fit software (Verity Software House, Topsham, ME), by fitting curves to profiles and measuring the areas under the curve to determine relative numbers of cells in G1, S and G2/M phases.

Statistical Analyses. All values are expressed as means ± SEM of five to eight separate experiments (as indicated) and statistical analyses were performed using a one-way ANOVA with Tukey's post-hock tests conducted when significant main effects were found. Individual t-tests were used to identify differences in FACS analysis between groups. Group means were considered to be significantly different when $p \leq 0.05$.

RESULTS

HFD-dependent increases in adiposity are prevented by PA. HFD-fed animals demonstrated a 26% higher total body mass compared to CD-fed sedentary animals (328.1 ±21.3 vs. 260.4 ±10.3, respectively; Fig. 8.1A). This increased total body weight was mirrored by an increased total relative visceral fat mass, as HFD animals showed a 4.5-fold increase compared to CD fed sedentary animals (7.61 ±0.97 vs. 1.68 ±0.23 g/100 g body weight; Fig. 8.1B). The HFD sedentary animals also had an increased overall daily calorie intake compared to CD sedentary animals (76.6 ±2.8 vs. 57.1 ±3.2 cal/day), indicating that HFD animals were subjected to both increased fat and increased calorie ingestion. The difference in total body and total relative visceral weights between HFD and CD sedentary animals suggests the HFD induced obesity in the animals.

HPA counteracted the HFD-dependent increase in visceral adiposity as demonstrated by the HFD+HPA animals being 13% lighter than their sedentary counterparts (287.9 ±15.8 g vs. 328.1 ±21.3 g; Fig. 8.1A). However, HPA failed to completely abolish the HFD-dependent increases in total body mass (Fig. 8.1A). Consistent with these results we observed a 44% decrease in total visceral fat mass in HFD+HPA compared to HFD sedentary animals (Fig. 8.1B). In contrast, HFD+LPA animals showed no difference in total body weight or specific visceral fat mass compared to their sedentary counterparts (Figs. 8.1A,B). This may be due to the HFD+LPA group having a 72% higher daily calorie intake compared to HFD sedentary animals (98.5 ±7.5 vs. 57.1 ±3.2 cal/day, respectively). CD+HPA animals were 22% lighter and had 85% less total visceral fat mass compared to HFD sedentary animals (Figs. 8.1A,B). The CD+HPA and HFD+HPA animals ran similar distances (16.3 ±2.3 vs. 18.2 ±2.3 km/day) and both groups were found to run more than the HFD+LPA (9.6 ±1.1 km/day).

In order to confirm that our PA protocol induced a specific aerobic training effect beyond that displayed by the decreases in mass/adiposity, we looked for changes in the weights of the gastrocnemius, soleus and tibialis anterior muscles. PA increased gastrocnemius, soleus and tibialis anterior relative weights in both HFD+LPA and HFD+HPA animals compared to their sedentary counterparts (Figs. 8.1C-E). LPA and HPA also increased muscle mass by an average of 29 ±4% and 47 ±3%, respectively, above HFD animals. This volume-dependent effect of PA was also evidenced by changes in oxidative enzyme capacities of mixed gastrocnemius muscles (Fig. 8.1F). LPA increased mixed gastrocnemius COX activity by 2.0-fold above that in sedentary HFD-fed animals while HPA further increased COX activity to levels that were 3.1-fold above those in sedentary HFD-fed animals. There were no differences in COX activity between HFD+HPA and CD+HPA gastrocnemius (Fig. 8.1F).

ADIPO:LEP ratio is decreased in the serum of HFD-fed animals and this is prevented by PA. Serum ADIPO, LEP and E2 levels were determined at time of sacrifice in order to determine if serum E2 levels correlated with any alterations in ADIPO and LEP due to HFD or PA. Serum E2 and LEP were both elevated by HFD and these increases were mitigated by diet (CD) and HPA but not LPA (Fig. 8.2A). The CD-fed animals were found to have 92% lower LEP (6.90 ±1.61 vs. 0.55 ±0.17 ng/ml), 95% lower E2 (57.2 ±9.2 vs. 2.6 ±2.0 pg/ml) compared to HFD-fed animals (Fig. 8.2A). Although the mean values for E2 and LEP were 29% and 47% lower in HFD+LPA than HFD sedentary animas respectively, they were not different statistically. Serum ADIPO levels showed an opposite expression pattern than E2 and LEP, with ADIPO being lower in HFD animals than CD and HFD+HPA animals (Fig. 8.2A). As a result of these ADIPO and LEP changes, there were corresponding changes in the ADIPO:LEP ratio (Fig. 8.2B). HFD caused a 97% decrease in the ADIPO:LEP ratio compared to CD animals (1854 ±602 vs. 41189 ±15901). LPA increased the

ADIPO:LEP ratio 1.8-fold while HPA increased this ratio by 4.3-fold compared to that in HFD sedentary animals (Fig. 8.2B). In order to determine any association between serum E2 levels and ADIPO and LEP, linear regressions were performed. ADIPO was found to have a negative relationship (m=-2.1, r=0.677, p=0.001; Fig. 8.2B) while LEP showed a positive relationship (m=7.1, r=0.737, p=<0.0001; Fig. 8.2C) with serum E2.

HFD decreases the ADIPO:LEP ratio in adipose derived CM which is prevented by PA. Total visceral adipose tissue was excised from animals in each of the experimental groups. This was used to prepare CM and the levels of ADIPO and LEP were measured within the CM. As was observed in the serum, HFD-CM showed a decreased ADIPO:LEP ratio compared to CD-CM (128±28 vs. 2051±336; Table 9.1). This decreased ratio was brought about by an increased ADIPO and a decreased LEP in HFD-CM. Compared to HFD-CM, HFD+LPA-CM had similar ADIPO and lower LEP levels, resulting in an overall increase in ADIPO:LEP ratio (128.8±28.2 vs. 341.2±91.1; Table 9.1). HFD+HPA-CM had higher levels of ADIPO (889.6±82.4 ng/ml) and lower levels of LEP (0.43±0.15 ng/ml) than the HFD-CM (ADIPO: 438.7±71.5 ng/ml, LEP: 1.85±0.21 ng/ml) and a resultant higher ADIPO:LEP ratio (128.8±28.2 vs. 1250.0±336.3). A linear relationship between the distance ran and the ratio of ADIPO:LEP in the CM was found displaying an effect of volume of PA on adipokine secretion from adipose tissue (m= 96.47±25.47, R=0.769, p=0.0035; Fig. 8.2E). The levels of E2 were found to be no different in the CM among all groups (Table 1). Interestingly, comparing the slopes of ADIPO:LEP and distance ran between this study with circulating estrogen (m= 96.47±25.47) and previously published data in an estrogen free environment (m= 85.94±21.83) (57), we found no difference in the linear regression between the two studies (F=0.033, p=0.86).

Changes in adipokine secretion bring about diet and PA-dependent effects on MCF7 protein expression and signaling. In order to verify that any effects observed between groups were not due to experimental design artefacts, we conducted western blot analyses using proteins isolated from the adipose tissue used in our CM preparations and measured the levels of Akt and β-actin proteins to ensure equal protein contents among groups (Fig. 8.2F). We found no consistent differences between groups which indicated no inequivalence of proteins, supporting the notion that the CM was not subjected to any preparation artefacts. HFD-CM decreased $pAMPK^{T172}$, $p27^{T198}$ and AdipoR1 while increasing $pAkt^{T308}$ levels compared to CD-CM treated MCF7 cells (Fig. 8.3A-F). No difference was seen between HFD-CM and CD-CM in p27 protein levels (Fig. 8.3D). Interestingly, HFD-CM appears to support MCF7 growth eliciting similar protein expresson effects to those seen in UT cells (Fig. 8.3A). No changes were evident in total AMPK and Akt.

Voluntary PA elicited a volume-dependent response counteracting the effects of HFD. HFD+LPA-CM was found to increase $pAMPK^{T172}$ and $p27^{T198}$ while decreasing $pAkt^{T308}$ compared to HFD-CM treated cells (Fig 8.3A,B,C,E). The greatest effect of PA was found in HFD+HPA-CM treated MCF7 cells as illustrated by increases in $pAMPK^{T172}$, $p27^{T198}$ and AdpoR1 by 47%, 46% and 33%, compared to HFD-CM treated cells, respectively (Figs. 8.3A,B,D,F). In addition, $pAkt^{T308}$ was decreased by 40% compared to HFD-CM treated MCF7 cells (Figs. 8.3A,C). Both HFD+LPA-CM and HPA-CM had similar effects on all MCF7 probed proteins compared to CD-CM (Fig. 8.3A-F). This suggests that at any level of voluntary PA, MCF7 protein expression was similar, abolishing the effect of HFD-CM by increasing $pAMPK^{T172}$ and $p27^{T198}$ while decreasing $pAkt^{T308}$. No changes in total AMPK and Akt were evident.

AdipoR1 overexpression ameliorated the effects of the HFD-CM and further enhanced the effects of PA in MCF7 cells. We next determined whether AdipoR1 overexpression could alter the

effects of HFD-CM. We have previously shown our AdipoR1-T MCF7 cells display a 2.7-fold increase in AdipoR1 protein compared to MockT MCF7 cells (57). MCF7 cells overexpressing AdipoR1 were used to determine any absolute/synergistic effects of augmented AdipoR1 signaling in MCF7 cell cycle regulation. HFD-CM decreased pAMPK^{T172}, p27 and p27^{T198} while increasing pAkt^{T308} compared to CD-CM cells (Fig. 8.4A-E). No difference was found between AdipoR1 in HFD-CM and CD-CM treated AdipoR1-T MCF7 cells (Fig. 8.4A,F), suggesting that AdipoR1 was constituently overexpressed across treatment groups. Similar to MockT cells, HFD-CM treated cells showed no difference between all probed proteins compared to UT cells. No changes in total AMPK and Akt were evident.

A volume-dependent effect of PA was also evident in AdpoR1-T cells. HFD+LPA-CM was found to elicit the same effects as HFD-CM on cells for all proteins except for pAkt^{T308} (Fig. 8.4A,C). In contrast, HFD+HPA-CM increased pAMPK^{T172} (Figs. 8.4A,B) and AdipoR1 (Figs. 8.4A,F) by 47% and 50%, respectively compared to HFD-CM treated cells while decreasing pAkt^{T308} by 33% (Figs. 8.4A,C) compared to HFD-CM. Overall, AdipoR1 overexpression increased the levels of pAMPK^{T172}, p27 and p27^{T198} in all treatment groups compared to MockT cells (Fig. 8.3A vs 8.4A). In fact, even though HFD-CM elicited effects on the proteins measured, the expression levels were similar to those in MockT MCF7 cells grown in CD-CM. These results highlight the importance of available AdipoR1 and indicate if we can increase the available binding sites for ADIPO, it is possible to override the obesity-dependent cell cycle control, regardless of the external growth environment.

CM induced changes in MCF7 protein expression leads to overall cell cycle effects. We next wanted to determine whether the diet and PA induced changes in CM-treated MCF7 cell protein expression elicited overall cell cycle effects. Cell cycle status in MockT and AdipoR1-T

was determined using propidium iodide staining and computational analyses (Figs. 8.6A,B). Cells that were exposed to HFD-CM showed a 13% decrease in the number of cells in G1/G0 (55% vs. 48%) and a 26% increase in the number of cells in S-phase (17% vs. 23%) compared MockT MCF7 cells cultured in CD-CM (Fig. 8.5C). AdipoR1 overexpression decreased the HFD-dependent effects observed in MockT cells as HFD-CM was found to cause a 7% decrease in G0/G1 cells and an 11% increase in S-phase cells compared to CD-CM treated cells (Fig. 8.5D).

Increasing the expression of AdipoR1 increased the percentage of cells in G0/G1 and decreased the number of cells in S-phase in both CD-CM and HFD-CM when compared to MockT treated cells (Figs. 8.5C vs. D). AdipoR1 overexpression increased the number of cells in G0/G1 by 12% (54% vs. 48%) and decreased the number of cells in S-phase by 18% (23% vs. 19%) compared to MockT cells when exposed to HFD-CM (Figs. 8.5C vs. D). This result agrees with previously published data in the absence of circulating estrogen (57), as the effect of HFD-CM is blunted in AdipoR1-T MCF7 cells, stressing the potential protective effects of increasing AdipoR1 protein expression in the breast cancers of obese patients.

We also wanted to evaluate whether voluntary PA could counteract the HFD-dependent overall cell cycle effects on MCF7 cells. We found a volume-dependent effect of PA in MockT MCF7 cells. The HFD+HPA-CM caused a 13% increase in G1/G0 cells (55% vs. 48%) and a 13% decrease in the number of S-phase cells (20% vs. 23%) compared to HFD-CM in MockT cells (Fig. 8.5E). Noteworthy, we see a decrease in the number of cells in G2/M in all voluntary PA groups compared to their sedentary counterpart in MockT cells (Fig. 8.5E). As with the protein changes we observed between HFD+LPA-CM and HFD-CM treated AdipoR1-T cells, we found no difference in the number of cells in G0/G1 and S-phase cells (Fig. 8.5F). By overexpressing AdipoR1 within the MCF7 cells, there again was a volume-dependent effect of PA but to a lesser

extent then in MockT cells. Possibly due to the fact that the HFD-CM effects were already blunted upon AdipoR1 overexpression (Fig. 8.5C vs. D).

DISSCUSION

It is now widely accepted that adipose tissue acts not only an inert storage depot but as an active endocrine tissue via the production of adipokines which exert endocrine, paracrine and autocrine effects on the surrounding tissues. Although several hundred adipokines have been discovered to date, several studies have focused on ADIPO and LEP as they are the most abundant, have been shown to directly affect the growth of several cancers including breast and are altered in opposing fashion with obesity (14, 15, 65). Although ADIPO and LEP on their own have been shown to be associated with cancer, emerging evidence now suggests that the ADIPO:LEP ratio may be a more reliable predictor (2, 12, 57). A hallmark characteristic of any cancer is genetic variability and instability, as each cancer patient may possess a unique and specific carcinoma. This makes a broad tumor-directed therapy between patients potentially costly and an imposingly difficult therapeutic avenue. However, there are variables that can affect tumor growth that are more uniform across patients and regulated by stable components of patient physiology. One such characteristic is the overall growth microenvironment created by both the circulation and the adipose tissue surrounding a tumor. Alterations in the adipokine secretion profile in obese individuals may represent the molecular link between obesity and cancer. In breast cancer, this obesity-cancer link has been clearly shown in postmenopausal women but the link is much less evident in premenopausal women.

In order to study the effects of obesity and adipose tissue expansion on breast cancer cell cycle regulation, we induced obesity in female animals using HFD-feeding. We also evaluated the

effects of PA as it has been shown to alter the ADIPO:LEP ratio secreted by adipose in obese animals and has been shown to lower the incidence and severity of breast cancer (36, 57, 58, 66). HFD-feeding induced obesity in the animals (Fig. 8.1), possibly due to either the increased fat content, increased calorie intake or a combination of both. This altered adipose tissue created differing secretomes within the CM, similar to results previously shown in humans and animals (40, 54). PA increased the ADIPO:LEP ratio in both the serum and CM in a volume-dependent manner (Fig. 8.2, Table 9.1) in agreement with previous published data documenting the effects even in the absence of estrogen (57, 58, 67). Serum estrogen levels were positively correlated with serum LEP and inversely correlated with serum ADIPO in agreement with previous studies (Fig. 8.2) (40). These effects of serum E2 did not affect adipose secretion of E2, as we observe no difference in estrogen in the CM between groups (Table 8.1). In the presence of circulating estrogen as in premenopausal women, we found that PA was still able to ameliorate and even abolish the effects created by the HFD-CM on MCF7 proliferation in a volume-dependent manner, in a similar fashion as was the case in the absence of circulating estrogen (57). These volume dependent effects were evident in alterations in total body weight, body weight adjusted visceral fat mass (Fig. 8.1) and the subsequent ADIPO:LEP ratio both in serum and CM (Fig. 8.2; Table 8.1). These alterations led to changes in the tumor growth microenvironment that produced changes MCF7 cell cycle proteins (increased $pAMPK^{T172}$, p27, $p27^{T198}$ and AdipoR1, decreased $pAkt^{T308}$; Fig. 8.3) which lead to overall changes in cell cycle status (increased number of cells in G0/G1, decreased number of cells in G2; Fig. 8.5). These effects of both diet and voluntary PA were similar to those previously shown in the absence of circulating estrogen (57). Despite the presence of circulating estrogen, PA was able to ameliorate or abolish the effects of HFD-CM depending on the volume of PA the animal engaged in, supporting the notion of PA as a

preventative and protective intervention for obese premenopausal women to reduce breast cancer proliferation.

There has been mounting evidence for the strong association between obesity and breast cancer in postmenopausal women, but research is now suggesting that the greatest risk reduction of breast cancer due to regular PA occurs among premenopausal women compared to postmenopausal women (19, 38, 59). Unlike in the absence of estrogen (57), animals in the current study ran a greater volume (1.9-fold increase km/day comparing HPA groups) which agrees with previously published data that female rats run more than males (17, 61). Although the animals ran differing volumes and resultant higher PA cutoffs, we observed similar clear volume-dependent effects of PA on altering the ADIPO:LEP ratio secreted by the adipose of HFD-fed animals to those previously shown (Table 9.1). While we are unable to categorize the precise exercise performed (i.e. run, jog, walk) we show a linear effect of PA (distance run) on the adipose derived CM ADIPO:LEP ratio (Fig. 8.2E) and a volume-dependent effect of PA on visceral fat and body mass in animals consuming a HFD. Despite the seemingly greater effect of PA and HFD in the presence of estrogen on anthropometric variables, the effect on adipose secretome did not translate into similar relative effects on MCF7 growth, compared to when estrogen is absent (57). Interestingly, we found that there was no difference between the current study and that of preciously published data (57) comparing the ADIPO:LEP ratio in relation to daily distance the animal ran. This indicates that the driving factor in altering the ADIPO:LEP ratio appears to be the volume or amount of PA an animal engages in and not as reliant on circulating estrogen or diet the animal consumed. Strengthening the importance of PA in obese women in order to alter their ADIPO:LEP ratio and subsequent tumor growth microenvironment.

Although the overall trend was similar to that seen in the absence of circulating estrogen (57), there were some distinct differences on the adipose-dependent effects in the CM brought about by the presence of estrogen. We found the ADIPO:LEP ratio in the HFD-CM similar between studies (128 vs. 122, respectfully), however the ratio was 3.6-fold higher in CD-CM in the presence of estrogen (2398 vs. 704, respectfully). Therefore, if ADIPO and LEP are the primary contributors to the adipose-derived tumor growth microenvironment as previously shown in the absence of estrogen (57), we would expect a similar effect of CM on cell cycle proteins and FACS profiles. Using FACS as our gauge of how the CM is affecting the proliferation of the MCF7 cells, we found that estrogen lessened the effects of HFD-CM compared CD-CM. Although the ADIPO:LEP ratios were similar comparing HFD-CM between studies, we found a 15% increase in the number of cells in G0/G1 and an 18% decrease in S-phase when estrogen was present. This lower proliferative effect of HFD-CM on MCF7 growth, maybe due to estrogen effects on adipose adipokine profile. This effect was also evident in all cell cycle proteins probed. The HFD-CM produced similar effects on all probed proteins compared to UT cell when estrogen was present (Fig. 8.3). While in the absence of estrogen, HFD-CM further decreased pAMPK^{T172}, p27, p27^{T198}, AdipoR1 and further increased pAkt^{T308}, compared to UT cells (57). This same discrepancy in the ADIPO:LEP ratio and subsequent MCF7 proliferation was also evident when comparing the effects of PA. In an estrogen free environment, we found a 4.3-fold increase in the ADIPO:LEP ratio between HFD+HPA-CM and HFD+CM (57). While in the presence of circulating estrogen we found the adipose-derived ADIPO:LEP ratio to be 9.8-fold higher in HFD+HPA-CM and HFD-CM. This translates to a 2.3-fold increase in the ADIPO:LEP ratio between HFD+HPA-CM and HFD+CM when estrogen is present compared to when absent but this increased ADIPO:LEP ratio did not translate into greater cell cycle arrest with PA. We found similar alterations in cell cycle

status comparing cells in G0/G1 (13% vs. 17% increase) and S-phase (13% vs. 15% decrease) between HFD+HPA-CM and HFD-CM comparing studies when estrogen was present and absent, respectively. Despite these observed effects of estrogen on adipokine secretion it remains clear that PA is an extremely effective intervention/prevention strategy for obesity-linked cancers, regardless of whether estrogen is present or not. The ADIPO:LEP ratio was still found to be a predictor of the proliferative tumor growth microenvironment created by the adipose tissue yet not as strong in the presence of circulating estrogen. These cell cycle effects of both diet and PA were lower than expected by the changes observed in the ADIPO:LEP ratio, suggesting other effects of estrogen on the adipose-derived CM.

The results of this study show the primary effect of adipose tissue and obesity to be blunted by estrogen despite much larger changes in ADIPO:LEP ratio. This may be part of the reason why the link between obesity and breast cancer in premenopausal women is less consistent compared to in postmenopausal women. Estrogen may alter other adipose dependent proteins other than ADIPO and LEP as previously shown (34, 40), which may help blunt the adipose-dependent effects on tumor growth microenvironment. Several other adipokines and inflammatory cytokines have been hypothesized to affect the tumor growth microenvironment. Of these a few have been shown to have an altered production within the adipose tissue due to the presence of estrogen, which may explain the different proliferative effects elicited by HFD-CM between studies. One such adipokine has been shown to be increased in the serum of obese animals (56) and in obese women (51). The insulin-sensing adipokine resistin is higher in postmenopausal breast cancer patients compared to aged matched controls (3). This increased level of resistin was not found in premenopausal breast cancer patients compared to non-breast cancer patients (3). It has been shown that resistin induces cancer cell proliferation through the PI3K/Akt pathway similar to what

we have shown with LEP (29, 57). Research has shown in ovariecotomized rats injected with subcutaneous E2, resistin mRNA and protein content from isolated visceral adipocyte depots was reduced compared to estrogen free animals (25). These results suggest that estrogen plays a role as a negative regulator of resistin gene expression and possibly explains why in the absence of estrogen HFD-CM elicited greater proliferative effect on MCF7 cells compared to the current results where the experimental animals had estrogen in the circulation. There would be greater additive effects of resistin promoting proliferation in the absence of estrogen than when resistin production is suppressed in the presence of estrogen, as in the current study. Overall this may possibly explain why the effect of obesity on breast cancer progression in premenopausal women is not as concrete as compared to postmenopausal women. Our results suggest that while the ratio of ADIPO:LEP represent a possible important indicator of the proliferative tumor microenvironment in both pre and postmenopausal women, estrogen may play a role in altering the production of other pro tumor growth factors in addition to the observed effect on ADIPO and LEP. Nonetheless, it seems clear that there is a major contribution of diet and PA on controlling breast cancer tumor growth microenvironment, regardless of whether estrogen is present.

Although estrogen affects the adipose contribution to breast cancer growth there is still a distinct benefit of increasing or stabilizing AdipoR1 in breast cancer cells. Previous work in our lab has shown constitutively overexpressing AdipoR1 can enhance the effects of ADIPO by presenting more binding sites for ADIPO downstream signaling, regardless of the levels of external ADIPO and counteracting the effects of HFD on adipose-dependent alterations in tumor growth environment (57). This is important as research has shown AdipoR1 protein levels are decreased in the visceral adipose tissue of obese women (48), down regulated in pre-invasive ductal carcinoma *in-situ* (46) and LEP has been shown to down regulate AdipoR1 mRNA in breast

cancer cells (26). We found that overexpressing AdipoR1 was able to abolish the effects of the HFD-CM on all cell cycle proteins probed (Fig. 8.3 vs. 8.4) as well as overall cell cycle status (Fig. 8.5C vs. D). This strengthens the notion that regardless of whether estrogen is present, increasing AdipoR1 protein levels increases anti-proliferative effects of ADIPO, thereby supressing tumor growth. This highlights AdipoR1 stabilization as a target for novel breast cancer pharmacological therapeutics regardless of menopausal status.

Taken together, these results highlight the importance of adipose tissue in controlling the tumor growth microenvironment surrounding breast cancer cells. We show that the adipose tissue appears to have a greater effect on controlling the tumor growth microenvironment when estrogen levels are low (57), compared when circulating estrogen is elevated and appears to alter the secretome and subsequent growth environment of breast cancer cells. These results agree with a large number of studies which suggest that obesity may not have as strong of an impact on tumor growth in premenopausal women as compared to postmenopausal women (11, 27, 31) and we do not observe any protective effects due to obesity, as suggested by some studies (6, 39, 62). Regardless of estrogen status, PA can counteract the effects of obesity on promoting breast cancer cell proliferation. AdipoR1 plays an important role in the regulation of MCF7 cell growth and stabilizing the receptor, especially in an obese phenotype, and presents a possible method of slowing obesity-dependent breast cancer cell growth regardless of menopausal status. Although our work used ADIPO and LEP as markers of adipokine secretion profile, we are in no way suggesting that these are the only adipokines of the more than 400 produced by adipocytes that underlie adipose-dependent effects. In fact, for premenopausal women there appears to be a more evident contribution of these other adipokines. Estrogen appears to alter the adipose tissue, which affects the growth microenvironment that a breast cancer is exposed too. However, it is clear that

ADIPO and LEP still represent prime candidates for accurately predicting the growth microenvironment that a breast cancer in an obese patient is exposed to in both post and premenopausal women. Therefore, therapies that alter the levels/ratio of these adipokines may represent interventions that can alter tumor growth microenvironment, increasing the chance of success in obese breast cancer patients regardless of menopausal status.

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Table 8.1. *ADIPO:LEP ratio for adipose-derived conditioned media*

Group	ADIPO (ng/ml)	LEP (ng/ml)	ADIPO:LEP	Estradiol (pg/ml)
HFD	438.7 ± 71.5	1.85 ± 0.21	128.8 ± 28.2	46.8 ± 4.0
CD	1166.0 ± 169.0*,†	0.31 ± 0.09*,†	2051.0 ± 336.3*,†	42.2 ± 3.0
HFD+LPA	569.4 ± 111.2	0.96 ± 0.44*	341.2 ± 91.1*	49.8 ± 7.2
HFD+HPA	889.6 ± 82.4*	0.43 ± 0.15*,†	1250.0 ± 467.5*,†	48.9 ± 4.7
CD+HPA	1692.0 ± 584.7*,†	0.39 ± 0.18*,†	2398.0 ± 459.0*,†	51.4 ± 12.2

HFD, high fat diet; CD, chow diet; HFD+LPA, high fat diet + low physical activity; HFD+HPA, high fat diet + high physical activity; CD+LPA, chow diet + low physical activity; * indicates significantly different from HFD, † indicates significantly different from HFD+LPA ($p<0.05$, n=6/group).

FIGURE LEGENDS

Fig 8.1. HFD increases total visceral fat and is ameliorated with PA. Body mass changes over the 6 week protocol *(A)*. Body weight adjusted total visceral fat mass in CD (open bar), HFD (black bar), HFD+LPA (light grey bar), HFD+HPA (dark grey bar) and CD+LPA (hatched bar) animals *(B)*. Body weight adjusted muscle mass of the gastrocnemius *(C)*, soleus *(D)* and tibialis anterior *(E)* muscles. Physical activity alters cytochrome C oxidase enzyme activity in the gastrocnemius muscles of CD, HFD, HFD+LPA, HFD+HPA and CD+LPA animals *(F)*. * in *Fig. A* indicates different from HFD and HFD+LPA animals, ** indicates different from all other groups ($p<0.05$). Different letters *(Figs. B-F)* indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 8.2. ADIPO is decreased, LEP and E2 increased in serum of HFD animals and reversed with HPA. Circulating serum ADIPO (open bar; µg/ml), LEP (closed bar; ng/ml) and E2 (grey bar; ng/ml) concentrations at time of sacrifice *(A)*. Circulating serum ADIPO:LEP ratio at time of sacrifice in CD (open bar), HFD (black bar), HFD+LPA (light grey bar), HFD+HPA (dark grey bar) and CD+LPA (hatched bar) animals at time of sacrifice *(B)*. Plotting of serum E2 (pg/ml) vs. serum ADIPO (µg/ml) *(C)*. Plotting of serum E2 (pg/ml) vs. serum LEP (ng/ml) *(D)*. Plotting of ADIPO:LEP ratio in CM vs. daily km run *(E)*. Dotted line indicates divider between HPA and LPA groups. Western blots showing levels of Akt and β-actin in adipose tissues from the indicated groups *(F)*. * indicates different from HFD ($p<0.05$, n=6/group).

Fig. 8.3. Physical activity abolishes the effect of a HFD on adipose-dependent tumor growth microenvironment. Representative western blots for selected proteins showing the effects of

treatment with CM prepared from CD (open bar), HFD (black bar), HFD+LPA (light grey bar), HFD+HPA (dark grey bar) and CD+LPA (hatched bar) animals on MockT MCF7 cells *(A)*. Graphical representations of multiple experiments showing the effects of CM on $pAMPK^{T172}$ *(B)*, $pAKT^{T308}$ *(C)*, p27 *(D)*, $p27^{T198}$ *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 8.4. Overexpression of AdipoR1 can counteract the effects of HFD and accentuates the effects of PA. Representative western blots for selected proteins showing the effects of treatment with CM prepared from CD (open bar), HFD (black bar), HFD+LPA (light grey bar), HFD+HPA (dark grey bar) and CD+LPA (hatched bar) animals on AdipoR1 transfected (p31-4-2-2) MCF7 cells *(A)*. Graphical representations of multiple experiments showing the effects of CM on $pAMPK^{T172}$ *(B)*, $pAKT^{T308}$ *(C)*, p27 *(D)*, $p27^{T198}$ *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=6/group).

Fig. 8.5. Adipose-dependent growth environment causes cell cycle changes in CM experiments. Typical cell cycle profiles in MockT MCF7 cells *(A)* and stably transfected AdipoR1 overexpressing MCF7 cells treated with HFD-CM *(B)*. Graphical representation of multiple cell cycle profile experiments observing effects of diet on CM effects in CD (open bar), HFD (black bar) animals in MockT MCF7 cells *(C)* and in MCF7 cells stably overexpressing AdipoR1 *(D)*. Graphical representation of multiple cell cycle profiles showing the effects of exercise and diet CD+LPA (hatched bar), HFD+HPA (dark grey bar) and HFD+LPA (light grey bar) on MockT

MCF7 cells *(E)* and MCF7 cells stably overexpressing AdipoR1 *(F)*. * indicate groups that are significantly different from HFD treated cells ($p<0.05$, n=6/group).

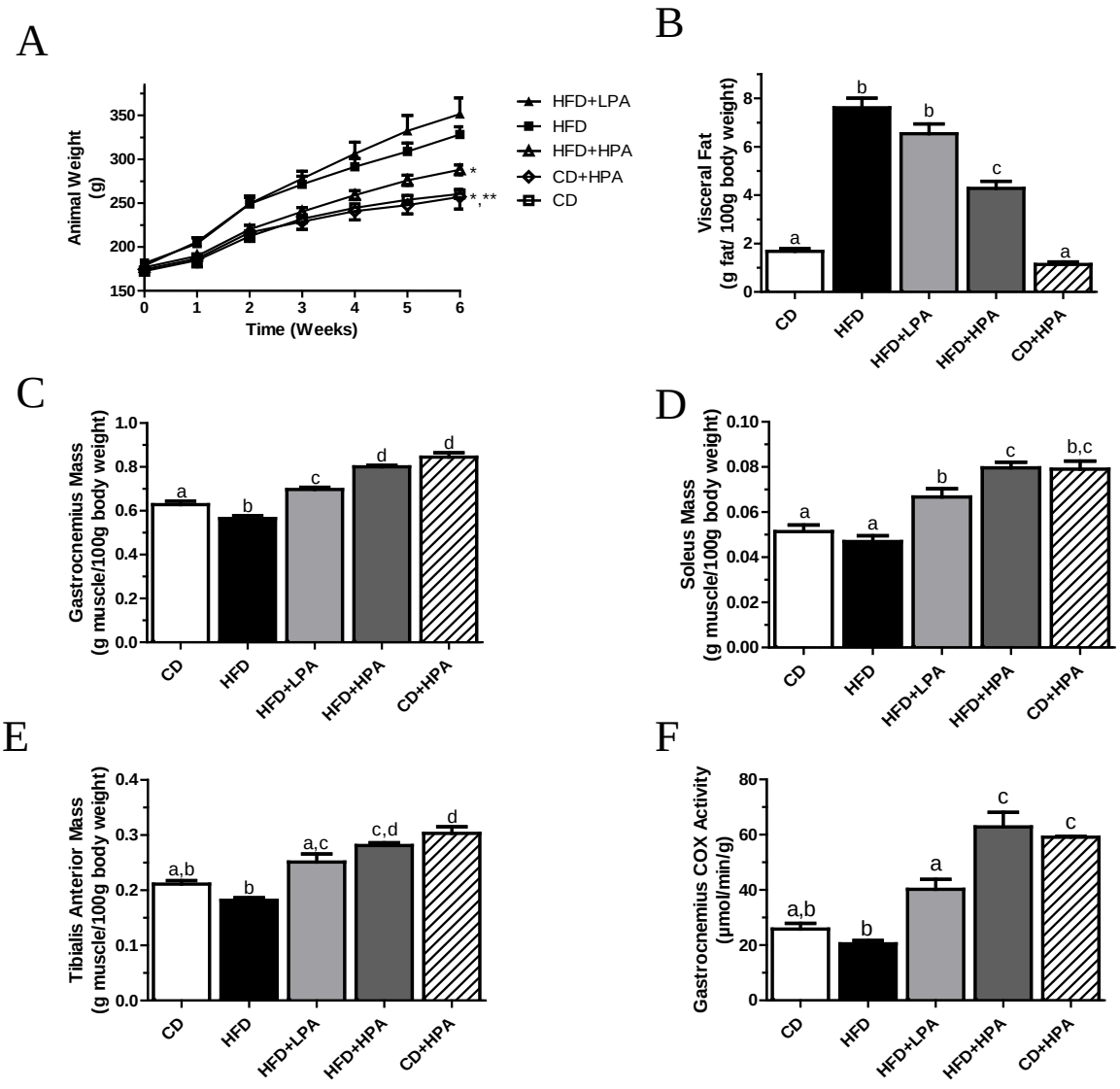
A
Animal Weight (g)
Time (Weeks)
HFD+LPA
HFD
HFD+HPA
CD+HPA
CD
*
*,**
B
Visceral Fat (g fat/ 100g body weight)
C
Gastrocnemius Mass (g muscle/100g body weight)
D
Soleus Mass (g muscle/100g body weight)
E
Tibialis Anterior Mass (g muscle/100g body weight)
F
Gastrocnemius COX Activity (µmol/min/g)
CD
HFD
HFD+LPA
HFD+HPA
CD+HPA

Figure 8.1

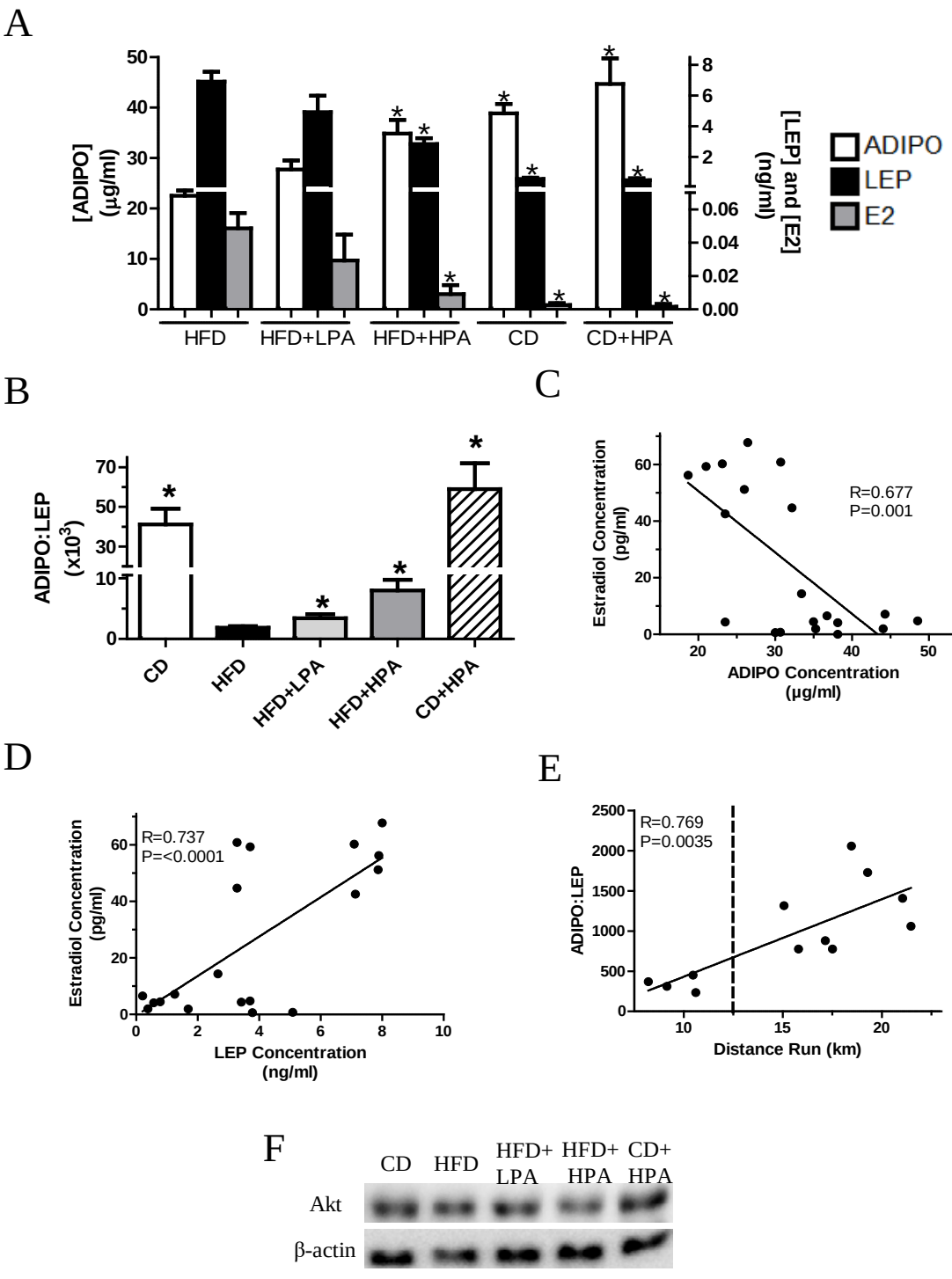
A
[ADIPO] (µg/ml)
[LEP] and [E2] (ng/ml)
ADIPO
LEP
E2
HFD
HFD+LPA
HFD+HPA
CD
CD+HPA
B
ADIPO:LEP (x10³)
C
Estradiol Concentration (pg/ml)
R=0.677
P=0.001
ADIPO Concentration (µg/ml)
D
R=0.737
P=<0.0001
LEP Concentration (ng/ml)
E
R=0.769
P=0.0035
ADIPO:LEP
Distance Run (km)
F
CD
HFD
HFD+ LPA
HFD+ HPA
CD+ HPA
Akt
β-actin

Figure 8.2

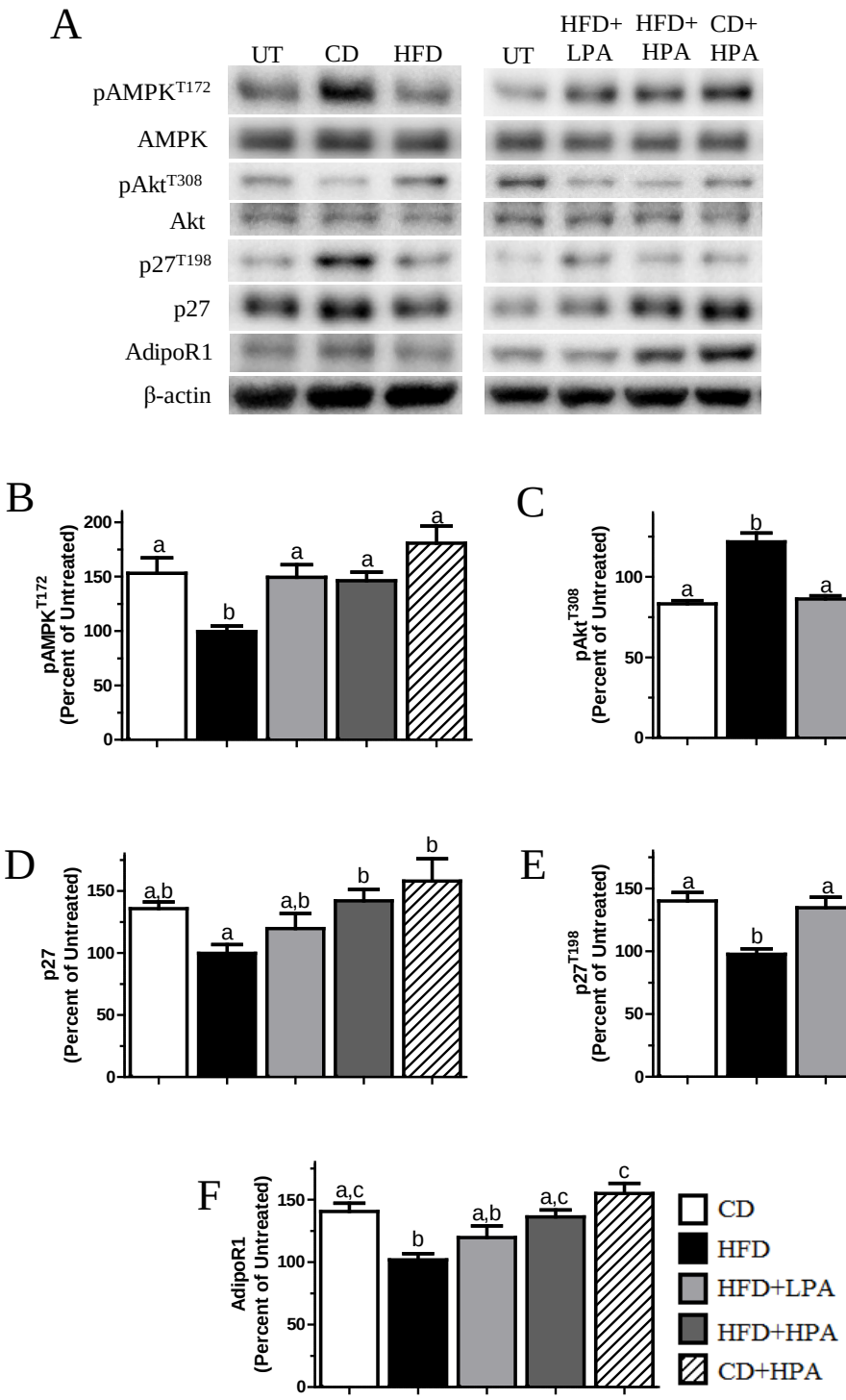
A
UT
CD
HFD
UT
HFD+ LPA
HFD+ HPA
CD+ HPA
pAMPK^{T172}
AMPK
pAkt^{T308}
Akt
p27^{T198}
p27
AdipoR1
β-actin
B
pAMPK^{T172} (Percent of Untreated)
C
pAkt^{T308} (Percent of Untreated)
D
p27 (Percent of Untreated)
E
p27^{T198} (Percent of Untreated)
F
AdipoR1 (Percent of Untreated)
CD
HFD
HFD+LPA
HFD+HPA
CD+HPA

Figure 8.3

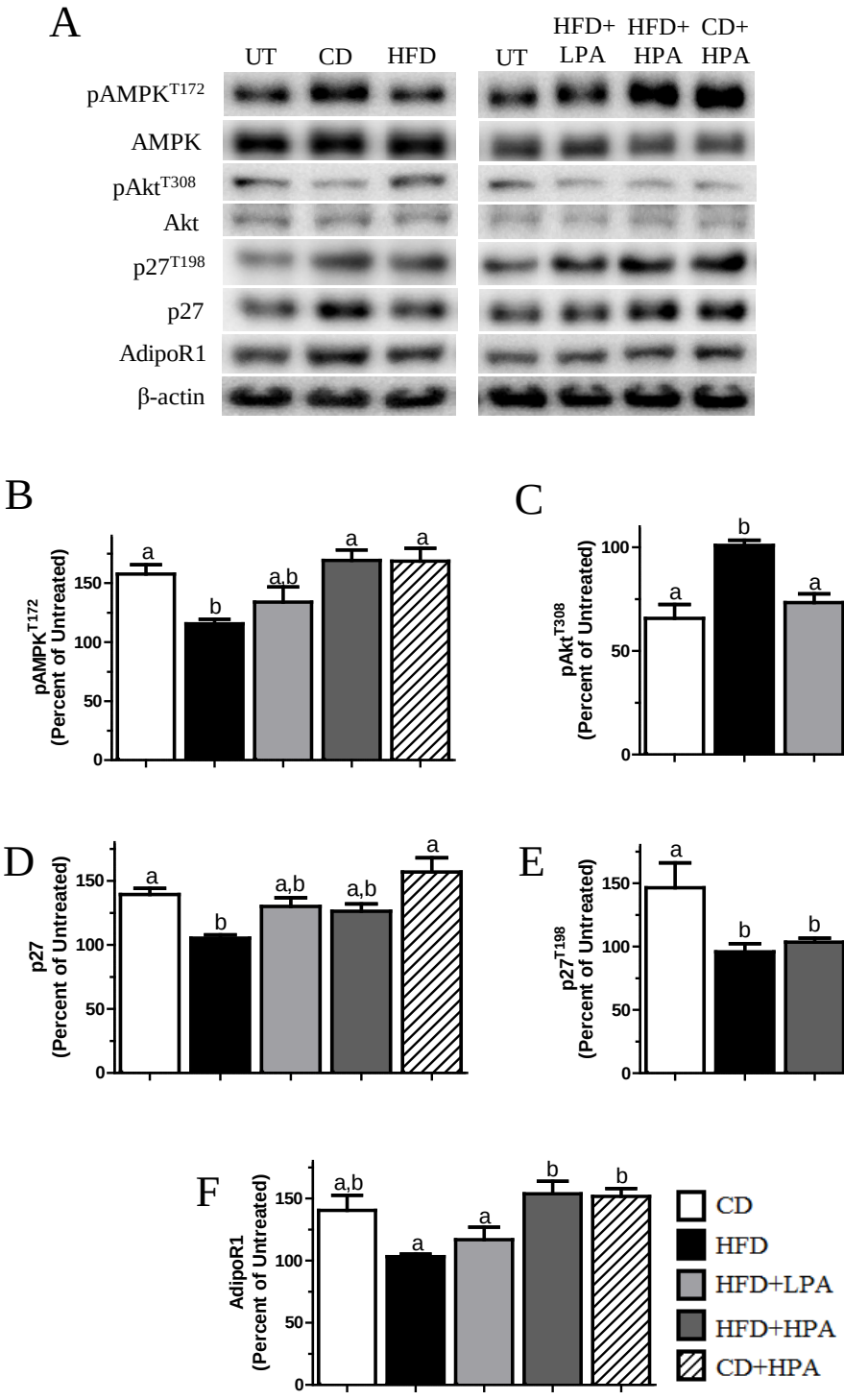
A
UT
CD
HFD
UT
HFD+ LPA
HFD+ HPA
CD+ HPA
pAMPK^{T172}
AMPK
pAkt^{T308}
Akt
p27^{T198}
p27
AdipoR1
β-actin
B
pAMPK^{T172} (Percent of Untreated)
C
pAkt^{T308} (Percent of Untreated)
D
p27 (Percent of Untreated)
E
p27^{T198} (Percent of Untreated)
F
AdipoR1 (Percent of Untreated)
CD
HFD
HFD+LPA
HFD+HPA
CD+HPA

Figure 8.4

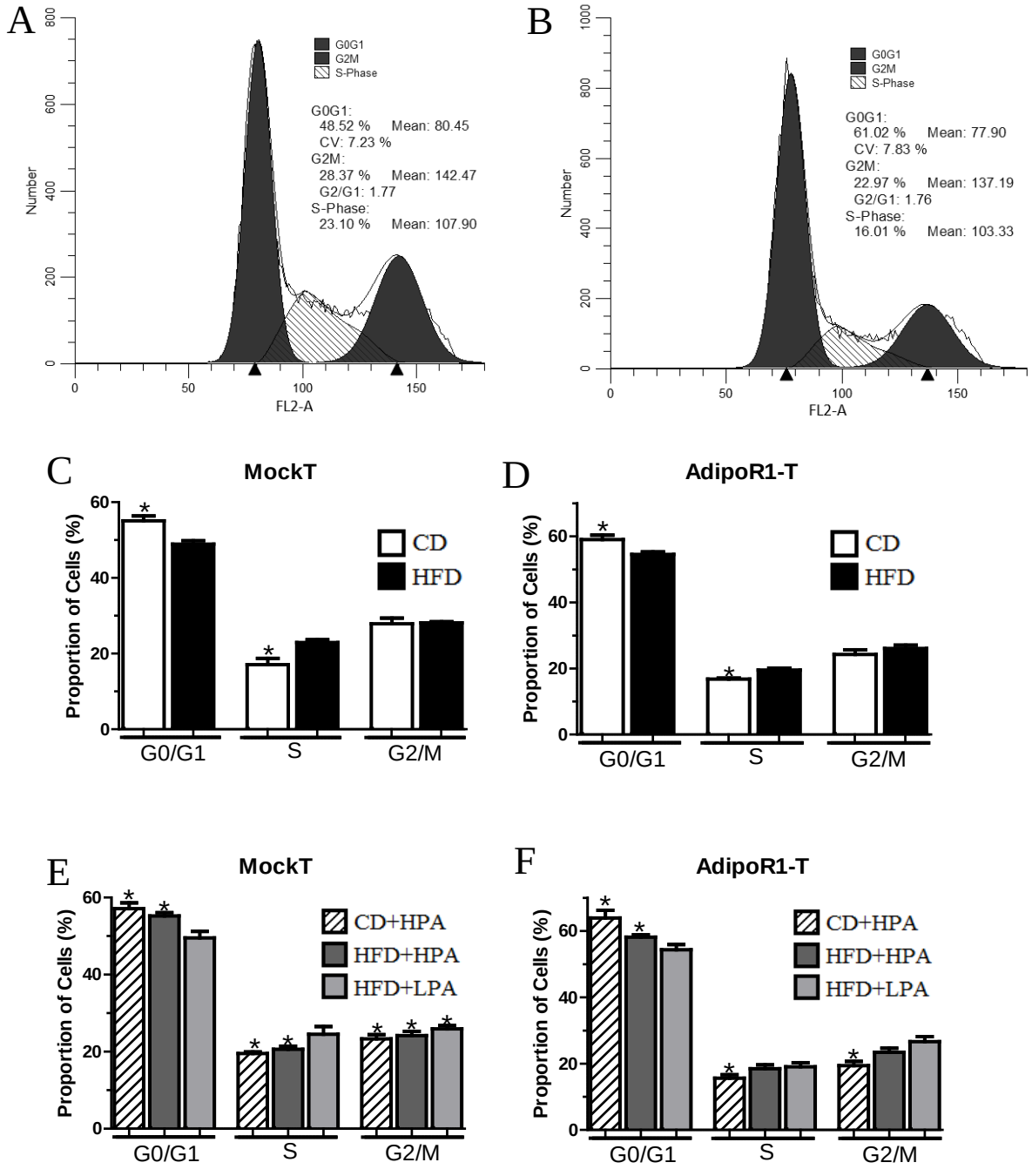
A
G0G1
G2M
S-Phase
G0G1:
48.52 % Mean: 80.45
CV: 7.23 %
G2M:
28.37 % Mean: 142.47
G2/G1: 1.77
S-Phase:
23.10 % Mean: 107.90
Number
FL2-A
B
G0G1
G2M
S-Phase
G0G1:
61.02 % Mean: 77.90
CV: 7.83 %
G2M:
22.97 % Mean: 137.19
G2/G1: 1.76
S-Phase:
16.01 % Mean: 103.33
Number
FL2-A
C
MockT
Proportion of Cells (%)
CD
HFD
G0/G1
S
G2/M
D
AdipoR1-T
Proportion of Cells (%)
CD
HFD
G0/G1
S
G2/M
E
MockT
Proportion of Cells (%)
CD+HPA
HFD+HPA
HFD+LPA
G0/G1
S
G2/M
F
AdipoR1-T
Proportion of Cells (%)
CD+HPA
HFD+HPA
HFD+LPA
G0/G1
S
G2/M

Figure 8.5

9. Academic Research Paper #3

Paracrine and Endocrine Proliferative Effects of Subcutaneous Adipose Tissue from Obese Animals on MCF7 cells are Ameliorated by Resveratrol Supplementation[1]

Breast cancer continues to be the number one cause of new cancer cases and second in mortality rate among North American women. For almost 50 years researchers have shown a statistical link that with increased adiposity there is an increased risk of breast cancer. Specifically, post-menopausal women are at an increased risk of developing breast cancer with increased adipose tissue mass. This link between obesity and breast cancer may be in part due to the adipocyte derived proteins adiponectin (ADIPO) and leptin (LEP). The purpose of this paper was to determine if obesity can alter the adipoctyes adipokine secretion profile, thereby altering the paracrine growth microenvironment that a breast cancer cell (MCF7) is exposed to. Furthermore, we examined whether resveratrol (RSV) supplementation of obese (ZDF) rats can counteract the effects of obesity on adipose function and maintain an inhibitory MCF7 growth microenvironment. Currently we show that subcutaneous adipocytes from ZDF rats promoted cell cycle entry in MCF7 cells and that this effect was counteracted by RSV supplementation. Conditioned media

[1] **Christopher F. Theriau, O'Llenecia S. Sauvé, Marie-Soleil Beaudoin, David C. Wright and Michael K. Connor**
STATEMENT OF LABOUR:
Christopher Theriau:
Wrote thesis manuscript and created all figures/table, Performed co-culture experiments using scAT and MCF7 cells Performed all condition media and MCF7 incubation experiments, Performed and analysed all western blot (MCF7), FACS (MCF7) and ELISA (conditioned media; ADIPO/LEP) experiments
O'Llenecia Sauvé:
Performed co-culture experiments using scAT and MCF7 cells, Performed ELISA (ADIPO/LEP) on co-cultured MCF7 cell media, Performed cyclin E probed western blots (MCF7)
Marie-Soleil Beaudoin:
Collected all anthropometric data on animals throughout experiments and at time of sacrifice Administered resveratrol supplementation to animals, Performed all animal tissue extraction and serum collection, Created all conditioned media using scAT

(CM) prepared from the adipose of ZDF rats supplemented with RSV had a higher ratio of ADIPO:LEP compared to ZDF-CM. This RSV–induced increase in the ADIPO:LEP ratio led to increased levels of pAMPK^{T172}, p27, p27^{T198} and AdipoR1 while decreasing pAkt^{T308} in MCF7 cells grown in RSV-CM compared to those grown in ZDF-CM. These effects on cell cycle proteins resulted in an increased number of cells in G0/G1 and fewer cells in S-phase compared to ZDF-CM treated cells. Overall we show that the use of RSV, a nutritional supplement which increases the ratio of ADIPO:LEP produced/secreted by adipocytes, may possibly lead to an altered adipose-dependent growth environment, which can ultimately have beneficial effects by slowing tumor proliferation and augmenting the benefits of exercise for obese breast cancer patients.

Keywords: Adiponectin, leptin, resveratrol, AMPK, Akt

INTRODUTION

Breast cancer is a dynamic, multi-factorial and inherently complex disease. Each patient likely possesses a unique and specific carcinoma making developing a single global cancer therapeutic strategy difficult, as tumors with different underlying etiologies may be resistant to similar therapies. Unlike the heterogeneity of breast cancers, the growth environment that exists within each patient is more stable and uniform since the majority of factors that contribute to this environment are produced by stable and predictable components of patient physiology, which has distinct advantages over targeting the genetically unstable and ever changing tumor itself. Thus, targeting this growth microenvironment therapeutically may result in more predictive and reliable treatment outcomes. Given that the vast majority of all tumors are surrounded by adipocytes and adipocytes serve as an active endocrine tissue, there may be important endocrine and/or paracrine effects of adipose derived factors (adipokines) on tumor proliferation and progression, potentially representing avenues for the development of a viable target tissue for novel cancer therapies.

Obesity has been statistically linked to breast cancer incidence for almost 50 years with increased adiposity being associated with an increased risk of breast cancer development (35). Despite this long association, the exact mechanisms underlying this relationship have not been fully elucidated. Previous research has shown a 50% increased risk of breast cancer development in obese postmenopausal women compared to their lean counterparts (6, 41). Regardless of menopausal status, obese women are more likely to suffer from metastatic breast cancer and have a poorer clinical outcome than non-obese women (6). These effects therefore cannot be fully explained by the effects of estrogen alone, implying a contribution of additional mechanisms in disease progression. Traditionally adipocytes were thought of as an inert storage depot for excess energy, but it is now clear that adipose tissue produces and secretes over 400 different adipokines

into the extracellular space which ultimately make their way into the systemic circulation (43). Two adipokines, adiponectin (ADIPO) and leptin (LEP), have both been shown to elicit growth effects on tumor cells and also have demonstrated altered production/secretion with changing adiposity (11, 12, 19). ADIPO is a 30 kDa protein whose production is inversely proportional to adiposity and has been shown to induce cell cycle exit in MCF7 cells by activating AMPK by directly phosphorylating p27 at T198, increasing p27 stability and inducing G1 arrest (11, 15, 22). There exists an antagonistic relationship between the cell cycle inhibitor $p27^{KIP1}$ and the cell cycle promoting protein cyclin E that governs cell transition from G1 to S-phase, and regaining control over this antagonism is critical in inhibiting tumor proliferation. LEP (16 kDa) is an adipokine that elicits the opposite cell cycle effects to those of ADIPO. LEP production/secretion is directly proportional to adiposity and induces cell cycle entry by activating Akt, which phosphorylates p27 at T157, denying p27 entry into the nucleus, thereby preventing it from inhibiting cyclin E/cdk2 and inducing cell cycle entry (12, 14, 23). Obese individuals produce low levels of ADIPO and higher levels of LEP which is correlated with a greater incidence of tumor formation (8, 26). Independent of adiposity, serum ADIPO has also been found to be reduced while LEP is increased in women with breast cancer compared to women without the disease (20, 27, 32). In addition, microdialysis of breast cancer tissue demonstrated that tumor tissues had lower ADIPO and higher LEP levels compared to normal adjacent breast tissue in the same patient (28), a situation that is exactly the same when comparing obese women to lean women. The association of each adipokine alone with breast cancer development/progression is not always evident, making the use of either of these adipokines as a sole predictor mediating the obesity/breast cancer association somewhat unreliable. Given that ADIPO and LEP activate antagonistic intracellular signaling pathways (AMKP vs. Akt) (37), the ratio of ADIPO:LEP might be a more reliable predictor of cancer

incidence and outcome in breast cancer patients (8). Previous work in our lab has shown that visceral adipose tissue of "obese" high fat diet fed (HFD) animals promoted breast cancer cell cycle entry by decreasing pAMPK^{T172}, p27, p27^{T198} and AdipoR1 protein levels while increasing pAkt^{T308} (37). These effects of HFD were exactly opposite to those elicited by adipose tissue from "lean" animals fed a normal chow diet. The proliferative differences elicited by these different adipose depots were correlated with a higher ADIPO:LEP ratio secreted by lean adipose than secreted by obese adipose tissue. These results point to the tumor growth microenvironment produced by the adipocytes of obese patients playing an important role in regulating the proliferation of breast cancer cells and identifies adipose as a target tissue for breast cancer therapy.

The search for novel and effective cancer chemo-preventative substances has expanded to include the study of various non-pharmacologic naturally occurring compounds. Resveratrol (RSV; *trans*-3-4',5-trihydroxystilbene), is a phytoalexin which is produced by plants and is contained in high proportions within the skin of red grapes. The effects of RSV with respect to metabolism have begun to be unraveled, but are far from completely characterized. Concomitant feeding of rodents with a high fat diet and RSV resulted in decreases in overall body weight gain, fat mass and alterations in the adipokine profile (2, 25). In addition, RSV increases the circulating levels of ADIPO (3, 31), while concomitantly lowering the levels of LEP in rodents (3, 36). The metabolic effects of RSV appear to be mediated in part by AMPK within the adipose tissue, possibly by an AMPK-dependent increased production/secretion of ADIPO by the adipocytes (31, 39).

The purpose of the current study was to examine the effects of dietary RSV supplementation treatment on adipokine secretion in white adipose tissue from Zucker Diabetic Fatty (ZDF) rats, a genetic model of LEP insensitivity and obesity. We hypothesized that adipose

from genetically induced obese animals (ZDF) will create a growth promoting tumor growth microenvironment for MCF7 cells and that the adipocytes would be the primary component of the adipose tissue contributing to this microenvironment. Furthermore, we feel that RSV supplementation will counteract these growth effects on MCF7 cells by altering the adipokine secretion profile of the obese adipose tissue. These effects would highlight a potential for RSV to be used as an adjuvant therapy for breast cancer patients.

METHODS

Animals. All protocols followed Canadian Council on Animal Care guidelines and were approved by the Animal Care Committees at the University of Guelph and York University. For the initial adipocyte/MCF7 co-culture experiments, five 10 week old ZDF and age matched lean Zucker rats (Charles River, St. Constant, QC, Canada) were singly housed in standard clear, plastic cages. Male rats were used in order to create an estrogen-free environment and allow for the delineation of the effects of obesity alone on the microenvironment created by the adipose tissue adipokine secretion profile (37). All animals had a 7 day habituation period to a 12 hour light-dark cycle (lights on at 0600) in a temperature (22°C) and humidity (50-60%) controlled room. The animals were given a standard show diet (Purina 5012 Chows, Ralston Purina, St. Louis, MO) and water *ad libitum*.

For the RSV supplementation experiments, four-week old male ZDF rats weighing ~100g were individually housed in wire-bottom cages and provided with food and water *ad libitum* as previously described (4). After a 10-day acclimatization period, ZDF rats were randomly selected into two groups and fed either a standard powdered chow diet (Purina 5008 diet; Purina; ZDF+chow) or the same chow diet supplemented with ~200mg/kg body weight RSV (Cayman

Chemical, Ann Arbor, MI, USA; ZDF+RSV) for 6 weeks. RSV was mixed directly into the powered diet on a weekly basis, based on predicted body weight and food intake for that week. We acknowledge that dose of RSV used in the present study is likely not attainable through diet alone. However, while the RSV dose is large, it is similar to many other rodent-based reports in the literature (21, 29, 39). Zucker rats were used as lean controls and fed a standard show diet *ad libitum*. Food intake was recorded 3 times weekly while body weight was assessed weekly.

Adipocyte isolation, co-culture and conditioned media. Initial experiments were designed to evaluate the specific role of purified adipocytes on MCF7 cell cycle regulation. Following the 7 day habituation period, age matched ZDF and lean Zucker animals were weighed and adipose tissue (AT) from the inguinal subcutaneous (scAT) depot was harvested. We isolated functional purified adipocytes using a technique based on previously published protocols (7, 13). Briefly, adipocytes from scAT were isolated under sterile conditions and adipose tissue was subsequently minced and treated with type II collagenase (0.4 mg/ml) for 10 minutes at 37°C in a shaking water bath. Following collagenase treatment, adipocytes were passed through a coarse metal sieve to remove large, undigested and unseparated fat pieces. The filtered adipocytes were washed with complete Alpha Modified Eagle's Medium (AMEM; Wisent, Montreal, PQ, CAN) supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific, Whitby, ON), 2% anti-micotic/anti-biotic (Wisent), 1% 100 mM sodium pyruvate (Sigma, Oakville, ON), 1% non-essential amino acids (Sigma), and 10 µg/ml insulin from human pancreas (Wisent) and centrifuged for 20 seconds at 1000 x g. The wash media was then aspirated to remove non-adipocyte particulate matter from the preparation. Adipocytes were washed 3 times in AMEM and allowed to equilibrate in culture media for 45 mins. Following isolation, adipocytes were counted according to published protocols (7, 13). Average adipocyte cell volume was calculated

and a measure of the concentration of adipocytes (number of cells/ml) in each preparation was determined. Pre-determined adipocyte volumes (cell volume x cell number) were added to 6-well plates containing MCF7 cells for 24 hours. Upon harvesting, the cells were washed three times with warm PBS to remove the adipocytes by preventing the adipocytes from congealing and adhering to the plate, ensuring MCF7 protein extracts were free of any adipocyte proteins and that any measurements of cell cycle characteristics were representative of the intracellular millieu within the MCF7 cells alone.

Subsequent to the isolated adipocyte experiments we conducted experiments using conditioned media (CM) prepared from crude whole adipose preparations. All CM was prepared from inguinal adipose depots as previously described (37). Briefly, whole scAT depots containing adipocytes, fibroblast, macrophages, stromal cells and endothelial cells were excised from ZDF, ZDF+RSV and lean Zucker rats. Adipocytes were weighed, minced into ~5-10mg pieces and immediately placed in 50 ml vented conical tubes containing Media 199 (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 2.5% FFA-free BSA, and incubated at 37°C with 5% CO_2 for 24hrs. After this time the CM was separated from the fat and stored at -84°C for future experiments. The use of CM to affect MCF7 cell growth has been previously used to examine the effects of adipose on breast cancer growth. This type of preparation yields similar CM preparation conditions among groups, as measured comparing specific protein contents among the adipose used in each CM preparation (37).

Conditioned media Adipokine Measurements. The levels of ADIPO and LEP produced and secreted into the CM created from scAT in second experiments was determined using a rat adiponectin sandwich ELISA kit (BioVision, Milpitas, CA) and a mouse/rat leptin quantikine sandwich ELISA kit (R&D Systems, Minneapolis, MN), respectively, as per manufacturer

instructions. Aliquots of CM were analysed against standard curves and the levels of each adipokine were calculated in ng/ml and nM values.

Cell culture. MCF7 cells were purchased from the American Tissue type Culture Collection (ATCC, Manassas, VA) and were maintained in complete AMEM at 37°C and 5% CO_2. For co-culture experiments MCF7 cells were cultured in 6-well plates in complete AMEM for 48 hours prior to the addition of increasing scAT adipocyte volumes. Furthermore, based on pilot experiments establishing a dose-response curve for ADIPO, 9 nM human globular adiponectin (gADIPO; Peprotech, Rocky Hill, NJ) was added to wells containing the highest adipocyte content both lean Zucker and ZDF co-cultures. For CM experiments, MCF7 cells were plated in 6 well plates in Media 199 (Sigma-Aldrich, Oakville, ON, Canada) for 24 hrs after which they were treated with either ZDF-CM or ZDF+RSV-CM for a further 24hrs prior to cell harvesting. MCF7 cells grown in AMEM supplemented with 10% FBS served as untreated controls (UT).

Immunoblotting. The effects of both co-cultured adipocytes and scAT created CM (ZDF, ZDF+RSV) on specific proteins was measured using standard SDS-PAGE protocols using 12% polyacrylamide gels. Following overnight transfer to polyvinylidene fluoride (PVDF) membranes (Bio-rad, Mississauga, ON, CAN) membranes were blocked (10% skim milk powder in tris-buffered saline/Tween 20) and subsequently incubated overnight with primary antibodies: $p27^{Kip1}$ (BD Biosciences); $p27^{T198}$ (R&D Systems, Minneapolis, MN); cyclin E, phospho-Akt^{T308}, Akt, phospho-$AMPK^{T172}$ and AMPK (Cell Signaling, Pickering, ON,CAN); AdipoR1 (Santa Cruz Biotech, Santa Cruz, CA) and β-actin (Abcam, Cambridge, MA). Anti-mouse and anti-rabbit (Promega, Madison, WI) and anti-goat (Santa Cruz) horseradish peroxidase conjugated secondary antibodies were used and protein levels were determined using

Immobilon enhanced chemiluminescence substrate (Millipore, Whitby, ON, CAN), detected on a Kodak *In Vivo* Pro imaging system (Marketlink Scientific, Burlington, ON, CAN) and quantified using Carestream Software (Carestream Health, Rochester, NY, USA).

Cell cycle analyses. MCF7 cells isolated from 6-well plates following trypsinization were fixed by drop wise addition of ice-cold 70% ethanol. Cells were washed in PBS and re-suspended in a propidium iodide/RNAse solution and subjected to FACS analyses (FACSCalibur, BD Biosciences, Mississauga, Canada). Cell cycle profiles were determined using Mod-fit software (Verity Software House, Topsham, ME), by fitting curves to profiles and measuring the areas under the curve to determine relative numbers of cells in G1, S and G2/M phases.

Statistical Analyses. All values are expressed as means ± SEM of three to six separate experiments (as indicated) and statistical analyses were performed using a one-way ANOVA with Tukey's multiple comparisons test used when significance found. Values of $p \leq 0.05$ were considered to be significantly different. For the isolated adipocyte experiments, 3-4 animals were used/group while 5-6 animals/group were used for CM experiments.

RESULTS

Adipocytes from lean animals induce cell cycle arrest while adipocytes from obese ZDF adipocytes promote cell cycle entry in MCF7 cells. Media from adipocyte/MCF7 cell co-culture experiments contained altered ADIPO and LEP levels depending on the origin of the adipocytes used. "Lean" animals weighed 342±5.2 g (n=3) while "obese" ZDF animals were significantly heavier, weighing 542±22.6 g (n=3). Media from "lean" adipocyte co-cultures contained 258.2±2.5 and 0.51±0.06 ng/ml of ADIPO and LEP, respectively (Fig. 9.1A). This resulted in a stoichiometric ADIPO:LEP ratio of 280.7±35.8 in the growth microenvironment (media) created by the "lean" adipocytes. Conversely, the media from co-culture experiments using "obese" ZDF

adipocytes contained 125.7±4.3 and 1.18±0.13 ng/ml of ADIPO and LEP, respectively (Fig. 9.1A). This resulted in an approximate 80% reduction in the ADIPO:LEP ratio to 58.2±7.1 in the growth microenvironment created by the "obese" adipocytes compared to that seen in "lean" co-culture media.

In order to determine whether this altered ADIPO and LEP secretion from "obese" ZDF adipocytes elicited any growth effects on the MCF7 cells in co-culture experiments, we measured cell cycle profiles using flow cytometric analyses (Figs. 9.1B-C). When incubated with adipocytes from scAT of ZDF rats, 56.7±2.3% of the MCF7 cells were in G1, which represents a 22% reduction compared to cells that were co-cultured with adipocytes from "lean" animals (Fig. 9.1D). Concomitantly, 24.6±4.3% of MCF7 cells were in S-phase when co-cultured with "obese" ZDF adipocytes, which is a 1.7-fold increase compared to MCF7 cells cultured with "lean" adipocytes. Taken together, this suggests that "obese" adipocytes create a growth environment that induces MCF7 cell cycle entry while "lean" adipocytes promote cell cycle exit in these same cells. To examine the effect of altering the ADIPO:LEP ratio alone on tumor growth environment, we increased this ratio by adding 9 nM gADIPO to the "obese" ZDF co-cultures. The addition of ADIPO caused a reduction in the number of cells in S-phase and an increase in the number of cells in G1, almost completely removing the proliferative effects of the "obese" adipocytes (Fig. 9.1D).

Co-culture with purified "lean" and "obese" scAT adipocytes differentially affects intracellular signaling and cell cycle proteins in MCF7 cells. Following 24 hr co-culture with "lean" or "obese" purified scAT adipocytes MCF7 cells were harvested and the levels of signaling and cell cycle proteins were measured. Purified "lean" scAT adipocytes increased pAMPK^{T172}, p27, p27^{T198} and AdipoR1 protein levels in the MCF7 cells while decreasing pAkt^{T308} protein levels in a dose dependent manner (Fig. 9.2A-F). The level of the cell cycle protein cyclin E was

undetectable in all MCF7 "'lean" scAT co-cultures. Addition of gADIPO to the co-cultures with the highest adipocyte numbers (4.4×10^6) caused no further increases in pAMPK^{T172} (Fig. 9.2A,B), p27 (Fig. 9.2A,D), p27^{T198} (Fig. 9.2A,E), AdipoR1 (Fig. 9.2A,F) or decrease to pAkt^{T308} (Fig. 9.2A,C). No changes in total AMPK and Akt were evident. These results partially identify some of the mechanisms responsible for the cell cycle withdrawal effects elicited by "lean" scAT on MCF7 cells (Fig. 9.1D).

In contrast to the observations in "lean" adipocyte/MCF7 co-culture experiments, purified scAT adipocytes from "obese" ZDF animals activated intracellular signaling pathways and responses that suggest the induction of cell cycle entry. Co-culture with "obese" scAT adipocytes elicited dose–dependent decreases in pAMPK^{T172}, p27, p27^{T198} and AdipoR1 protein levels, while increasing pAkt^{T308} and cyclin E protein levels in MCF7 cells (Fig. 9.3A-F). Addition of gADIPO to the co-cultures with the highest adipocyte volume ($1.6\text{x}10^5$) pAMPK^{T172}, pAkt^{T308}, p27^{T198}, cyclin E and AdipoR1 protein levels were "rescued" reaching levels that were no different than those in untreated MCF7 cells in the absence of adipocytes, completely abolishing the effect of the "obese" adipocytes (Fig. 9.3A-F last lane vs. first lane). Interestingly, the level of p27 climbed to levels that were significantly higher than those in UT cells (Fig. 9.3A&E). No changes in total AMPK and Akt were evident in any of the treatment conditions.

RSV supplementation of ZDF animals alters the adipokine secretion profile of scAT which affects MCF7 cell cycle profiles. After determining that adipocytes alone can alter the tumor growth environment by altering their adipokine secretion profile with adiposity, we set out to determine whether these effects were similar when all of the components of adipose tissue are present and whether RSV supplementation can alter the effects of obesity on MCF7 cell growth. To do this we used a model where crude adipose preparations were used to create a conditioned

media (CM) and MCF7 cells were subsequently incubated with the CM. This model is representative of the endocrine functions of adipose tissue, as opposed to the paracrine functions that are represented in the adipocyte co-culture model (Figs. 9.1-9.3). We employed 3 groups for these experiments (lean, ZDF and ZDF+RSV). ZDF animals were heavier than the lean animals but RSV supplementation did not affect body mass at all (4). CM prepared from scAT of ZDF animals (ZDF-CM) contained LEP levels (3.7±0.8 ng/ml) that were 4.5-fold higher than those in CM prepared from scAT of lean Zucker rats (lean-CM; 0.8±0.1 ng/ml; Fig. 9.4A). In addition, lean-CM contained ADIPO levels (580.7±181.7 ng/ml) that were 2.6-fold higher than those in ZDF-CM (225.1±32.5 ng/ml; Fig. 9.4A). RSV supplementation in ZDF animals appeared to elicit alterations in the adipokine profile secreted by scAT into the conditioned media (ZDF+RSV-CM). RSV supplementation caused a 2.3-fold increase in the amount of ADIPO in the ZDF+RSV-CM (527.3±130.7 ng/ml) compared to CM prepared from the scAT from ZDF animals, attaining levels that were not different than those in lean-CM (Fig. 9.4A). Furthermore, ZDF+RSV CM demonstrated an approximate 32% lower level of LEP (2.6±0.8 ng/ml) compared to ZDF-CM (Fig. 9.4A). These observed changes resulted in vast differences in the overall stoichiometric ADIPO:LEP ratios among groups. ZDF-CM contained the lowest ADIPO:LEP ratio (34.5±12.8) while lean-CM had the highest ADIPO:LEP ratio (388.0±164.3). RSV supplementation managed to alter the adipokine secretion profile such that the ADIPO:LEP ratio was more than 3-fold higher (115.3±51.8) than that seen in ZDF-CM.

Given that our overall intent was to determine whether RSV supplementation altered the tumor growth environment created by adipose tissue we measured the cell cycle profiles of MCF7 cells grown in the CM from the various treatment conditions using propidium iodide staining and flow cytometric analyses (Figs. 9.4B,C). ZDF+RSV CM induced an increase in the proportion of

MCF7 cells in G0/G1 to 62.5±2.3%, a level that was 24% higher than cells grown in ZDF-CM (50.5±2.8%; Fig. 9.4D). In addition, ZDF+RSV-CM reduced the number of cells in S-phase compared to ZDF-CM treated MCF7 cells by 43% to 9.7±2.8% from 17.3±2.1%, respectively (Fig. 9.4D). These observations highlight the important effects that altering the ADIPO:LEP ration in the tumor growth microenvironment can impart on breast cancer cells.

RSV-dependent alterations in scAT adipokine secretion profile counteracts obesity related proliferation enhancement. Given the effects RSV supplementation elicited on adipose regulation of MCF7 cell cycle growth, we evaluated intracellular signaling and cell cycle proteins to unravel the molecular mechanisms underlying these changes. As found using our co-culture model, ZDF-CM was found to decrease pAMPK^{T172}, p27, p27^{T198} and AdipoR1 in MCF7 cells compared to those cells grown in cells growing lean-CM by 47%, 51%, 31% and 37%, respectively. In addition, ZDF-CM increased MCF7 cell pAkt^{T308} by 39% compared to cells grown in lean-CM (Figs. 9.5A-F). As was evident in cell cycle profiles, ZDF+RSV-CM altered protein expression in MCF7 cells. Compared to ZDF-CM, cells grown in ZDF+RSV-CM showed increased levels of pAMPK^{T172} (43%), p27 (85%), p27^{T198} (30%) and AdipoR1 (22%), while exhibiting decreased levels of pAkt^{T308} (34%) proteins (Fig. 9.5A-F). Thus, RSV supplementation of obese animals altered the growth microenvironment created by scAT to a point that it elicited almost the same anti-proliferative effects on MCF7 cell growth as did scAT from lean animals.

DISSCUSION

A growing literature is pointing to adipose tissue, and its associated adipocytokines, as being potential influences on the progression of numerous cancers. ADIPO and LEP are the two most abundant adipokines produced within the human body and have been shown to affect the

growth status of breast cancer cells (18), making these proteins prime candidates for mediating the molecular link between obesity and breast cancer. In obese breast cancer patients, the serum ADIPO:LEP ratio is decreased compared with lean patients and is associated with more aggressive tumors and a poorer patient prognosis (9, 40). It has been proposed that the ratio of ADIPO:LEP may be a more reliable predictor of obesity-dependent breast cancer than either adipokine alone (1, 8, 37). It is known that ADIPO and LEP activate AMPK (11, 42) and Akt (12) signaling pathways, respectively, and these pathways antagonize each other in breast cancer cells. Our data supports this, as scAT from "obese" ZDF rats elicited decreases in $pAMPK^{T172}$ and increases in $pAkt^{T308}$ in MCF7 cells compared to scAT from lean animals in both co-culture (Fig. 9.3) and CM experiments (Fig. 9.5). The downstream cell cycle effects of this AMPK/AKT antagonism was manifested by decreases in the levels of p27, $p27^{T198}$ and AdipoR1 and increases in cyclin E which are all indicative of increased cell proliferation, something that was confirmed using cell cycle profile analyses. Thus, it appears in an obese vs lean tumor growth microenvironment that the decision whether to enter or exit the cell cycle lies in the relative effects that the adipose tissue exerts on these two pathways via receptors on the cell surface. Although we identify ADIPO and LEP as major potential regulators of this external milieu as they activate the AMPK and Akt pathways, respectively, the possibility exists that a multitude of the more than 400 adipokines secreted by adipose tissue also play a role in mediating the obesity-breast cancer link. However, it appears that ADIPO and LEP can serve as reliable predictors of the overall tumor growth microenvironment in any individual, regardless of their overall adiposity (37). Interestingly, RSV imparted all of these beneficial effects on adipose-dependent tumor growth microenvironment in the absence of any changes in body weight (4), suggesting that the functionality of the adipose rather than the absolute amount of adipose tissue is the driving factor in the adipose-dependent

regulation of the growth environment, noting that the functionality is often correlated with the amount of fat.

Adipose tissue is comprised of many cell types including adipocytes, fibroblasts, macrophages, stromal cells and endothelial cells. Since each of these cells are capable of producing and secreting growth factors (16), determining the specific component of the adipose tissue that is the major regulator of the adipose-dependent tumor growth microenvironment in lean and obese breast cancer patients is difficult. Much research suggests that it is the associated inflammatory response that is associated with obesity that is responsible for the endocrine/paracrine cancer effects. However, the current results point to the adipocytes themselves as being the major contributor to the adipose-dependent effects on cancer cell proliferation. This is inferred by the fact that the resultant effects from isolated adipocytes on MCF7 cell proliferation are the same as those using whole adipose preparations (Figs. 9.1-9.3 vs Figs. 9.4-9.5). Furthermore, the effects of RSV on adipokine secretion profile and cell cycle regulation occurred in the absence of any weight loss or changes in inflammatory markers including TNFα and IL-6 (4). Taken together, these observations suggest that the intracellular signaling and cell cycle effects of the adipose tissue on tumor growth microenvironment come from primarily the adipocytes and not the adipose tissue as a whole. When examining paracrine vs. endocrine effects questions often arise around the adipose depot being studied. These are valid concerns as different cancers will share proximity to different adipose tissue depots. For example, breast cancers will be exposed to scAT while prostate cancers will be exposed to visceral adipose tissue. This is important because evidence suggests that visceral and subcutaneous adipose depots can respond differently to the same stimulus. Currently, we demonstrate the responses/effects of scAT, which has a relevance to breast cancer location. However, recent work has shown that

induction of adiposity via high-fat diet elicits the exact same responses that we report here using scAT, suggesting a homogeneity of response between the two adipose depots with respect to their relative contributions to overall tumor growth microenvironment (37).

RSV directly inhibits breast cancer cell growth in cell culture, but these effects have been shown to have controversial results in an *in-vivo* model (17, 24, 33). In addition, naturally occurring analogs of RSV have been shown to down-regulate the PI3K/Akt signaling cascades in MCF7 cells (38) leading to decreases in cell proliferation (30). However, the direct effects of RSV on breast cancer cells may only be a small portion of the overall protective effects that RSV can elicit on cancer growth, since we show that RSV effects on MCF7 cell proliferation are brought about indirectly by altering the adipokine secretion profile of adipose tissue *in-vivo* (Fig. 9.5; 4). RSV supplementation altered adipocyte function such that the CM produced using adipose from ZDF-RSV animals abolished the effects elicited on intracellular proteins by ZDF-CM on MCF7 cells, resulting in a decrease in cell proliferation as evidenced by an increase in the number of cells in G0/G1 and a decrease in the number of cells in S-phase. RSV has been previously shown to increase the amount of circulating ADIPO and increased the production and secretion of ADIPO from scAT (4) and our results confirm this. Given the antagonism between ADIPO and LEP signaling pathways we chose to evaluate the ADIPO:LEP ratio as a measure of the tumor growth microenvironment. RSV managed to ameliorate the effects of obesity in a similar manner as low volume voluntary wheel running (37). However, RSV elicited greater changes in ADIPO than LEP secretion by adipose tissue, while exercise caused greater changes in LEP than ADIPO secretion. Yet, the overall effect of these two obesity-targeted interventions on MCF7 cell proliferation was similar, despite the mechanisms behind each effect being somewhat different in nature. If we just examined one of these adipokines, the underlying pathways responsible may

appear to be different for RSV and exercise. But given the antagonistic nature of the intracellular effects of ADIPO and LEP in combination with the fact that a cancer cell will be exposed to both adipokines *in vivo*, the case for using the ADIPO:LEP ratio as a predictor of tumor growth microenvironment appears to be more reliable than using either on their own.

Targeting the stable components of a cancer patient's physiology as part of a therapeutic strategy has lost some of its lustre, despite the angiogenesis work pioneered by Folkman's group beginning in the early 1970s. The idea behind this strategy is that these stable components will respond reliably and predictably both over time and among patients. This is in contrast with therapies directed at the tumor itself, which due to the inherent genetically unstable nature of cancers makes them unique among patients and malleable over time. The long standing relationship between obesity and cancer has led us to evaluate the role of adipose tissue, and its associated adipokines, in contributing to the growth environment that any tumor is exposed to. This is because adipokine secretion profile is altered with obesity and numerous interventions including diet, exercise and nutritional supplements can alter adipose function. RSV alters adipokine secretion (increased ADIPO:LEP ratio) without an effect on body weight and this results in alterations in tumor growth microenvironment such that it supports cell cycle exit of the cancer cells. This is a similar end result to when exercise is used as an obesity intervention, although the specifics of the response are slightly different with high levels of activity also causing weight loss (37). Since obese breast cancer patients have been shown to have a lower circulating ratio of ADIPO:LEP (8, 34) and a higher incidence of and associated mortality from breast cancer compared to their lean counterparts (5, 6, 10, 41), targeting adipose function as an adjuvant therapy with exercise, diet and/or supplementation seems a viable avenue moving forward. The current work identifies RSV as a potential supplement to augment cancer treatment, but in no way is it

suggested that it replace exercise as an adjuvant co-therapy. Exercise carries numerous benefits to a patient (i.e. decreased stress, pain management, increasing skeletal muscle mass) that are not brought by diet or nutritional supplementation alone. However, cancer patients represent a group that may be advanced in age and burdened with other difficulties that make higher intensity exercise unattainable. Addition of dietary and nutritional supplementation may allow lower levels of exercise to be prescribed while maintaining a highly effective, multi-faceted approach to targeting the adipose-dependent tumor growth microenvironment.

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FIGURE LEGENDS

Fig 9.1. Co-cultured subcutaneous adipocytes elicit cell cycle changes in MCF7 cells. Graphical representation of ADIPO and LEP concentration (ng/ml) in media co-cultured with isolated subcutaneous adipocytes and MCF7 cells *(A)*. Typical cell cycle profiles in MCF7 cells treated with lean Zucker adipocytes (4.4×10^6) *(B)* ZDF adipocytes (1.6×10^5) *(C)*. Graphical representation of multiple cell cycle profile experiments observing effects of adipocyte co-culture with lean Zucker (closed bar), ZDF (grey bar) and ZDF+ 9 nM ADIPO (grey bar) in MCF7 cells *(D)* * indicate groups that are significantly different from ZDF co-cultured cells ($p<0.05$, n=5/group).

Fig. 9.2. Lean Zucker scAT co-culture positively affects MCF7 cell cycle proteins in does-dependant manner. Representative western blots for selected proteins showing the effects of treatment with UT (open bar) or lean Zucker scAT increasing adipocyte number (closed bars) or the highest adipocyte number (4.4×10^6) plus ADIPO (18 nM; grey bar) co-culture treatment for 24 hours in MCF7 cells *(A)*. Graphical representations of multiple experiments showing the effects of lean Zucker scAT increasing adipocyte number and the addition of ADIPO (9 nM) on $pAMPK^{T172}$ *(B)*, $pAKT^{T308}$ *(C)*, p27 *(D)*, $p27^{T198}$ *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=5/group).

Fig 9.3. ZDF scAT co-culture negatively affects MCF7 cell cycle proteins in does-dependant manner. Representative western blots for selected proteins showing the effects of treatment with UT (open bar) or ZDF scAT increasing adipocyte number (closed bars) or the highest adipocyte number (1.6×10^5) plus ADIPO (18 nM; grey bar) co-culture treatment for 24 hours in MCF7 cells

(A). Graphical representations of multiple experiments showing the effects of ZDF scAT increasing adipocyte number and the addition of ADIPO (18 nM) on pAMPK^{T172} *(B)*, pAKT^{T308} *(C)*, p27 *(D)*, p27^{T198} *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=5/group).

Fig 9.4. RSV supplementation causes cell cycle exit in MCF7 cells compared to ZDF CM. Cell cycle profiles determined in MCF7 cells. Typical cell cycle profiles in MCF7 cells grown in conditioned media (CM) prepared from scAT of ZDF *(A)* or ZDF+RSV supplementation *(B)* for 6 weeks. Graphical representation of multiple cell cycle profile experiments observing effects of RSV supplemented animals CM (grey bar) and ZDF animals CM (black bar) in MCF7 cells *(C)* Different letters indicate groups that are significantly different from each other ($p<0.05$, n=5-10/group).

Fig 9.5. ZDF rats supplemented with RSV prevents the deleterious effects of ZDF CM on cell cycle proteins. A: Representative western blots for selected proteins showing the effects of treatment with CM prepared from UT (open bar), ZDF (black bar), ZDF+RSV supplemented (grey bar) and lean Zucker (hatched bar) animals on MCF7 cells *(A)*. Graphical representations of multiple experiments showing the effects of CM on pAMPK^{T172} *(B)*, pAKT^{T308} *(C)*, p27 *(D)*, p27^{T198} *(E)* and AdipoR1 *(F)* protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other ($p<0.05$, n=5-10/group).

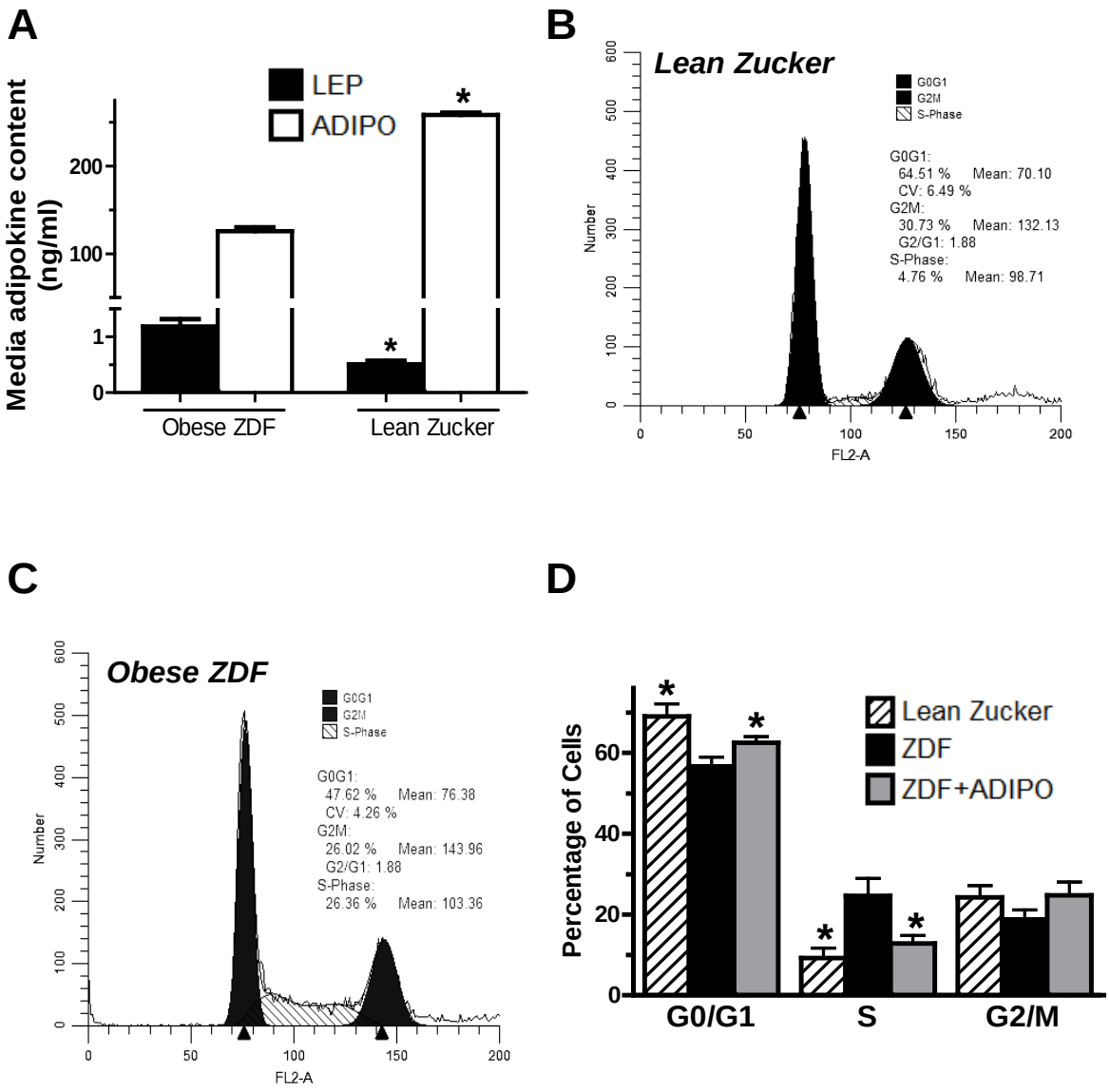
A
Media adipokine content (ng/ml)
LEP
ADIPO
200
100
1
0
*
*
Obese ZDF
Lean Zucker
B
Lean Zucker
G0G1
G2M
S-Phase
G0G1:
64.51 %
Mean: 70.10
CV: 6.49 %
G2M:
30.73 %
Mean: 132.13
G2/G1: 1.88
S-Phase:
4.76 %
Mean: 98.71
Number
FL2-A
C
Obese ZDF
G0G1
G2M
S-Phase
G0G1:
47.62 %
Mean: 76.38
CV: 4.26 %
G2M:
26.02 %
Mean: 143.96
G2/G1: 1.88
S-Phase:
26.36 %
Mean: 103.36
Number
FL2-A
D
Percentage of Cells
Lean Zucker
ZDF
ZDF+ADIPO
G0/G1
S
G2/M

Figure 9.1

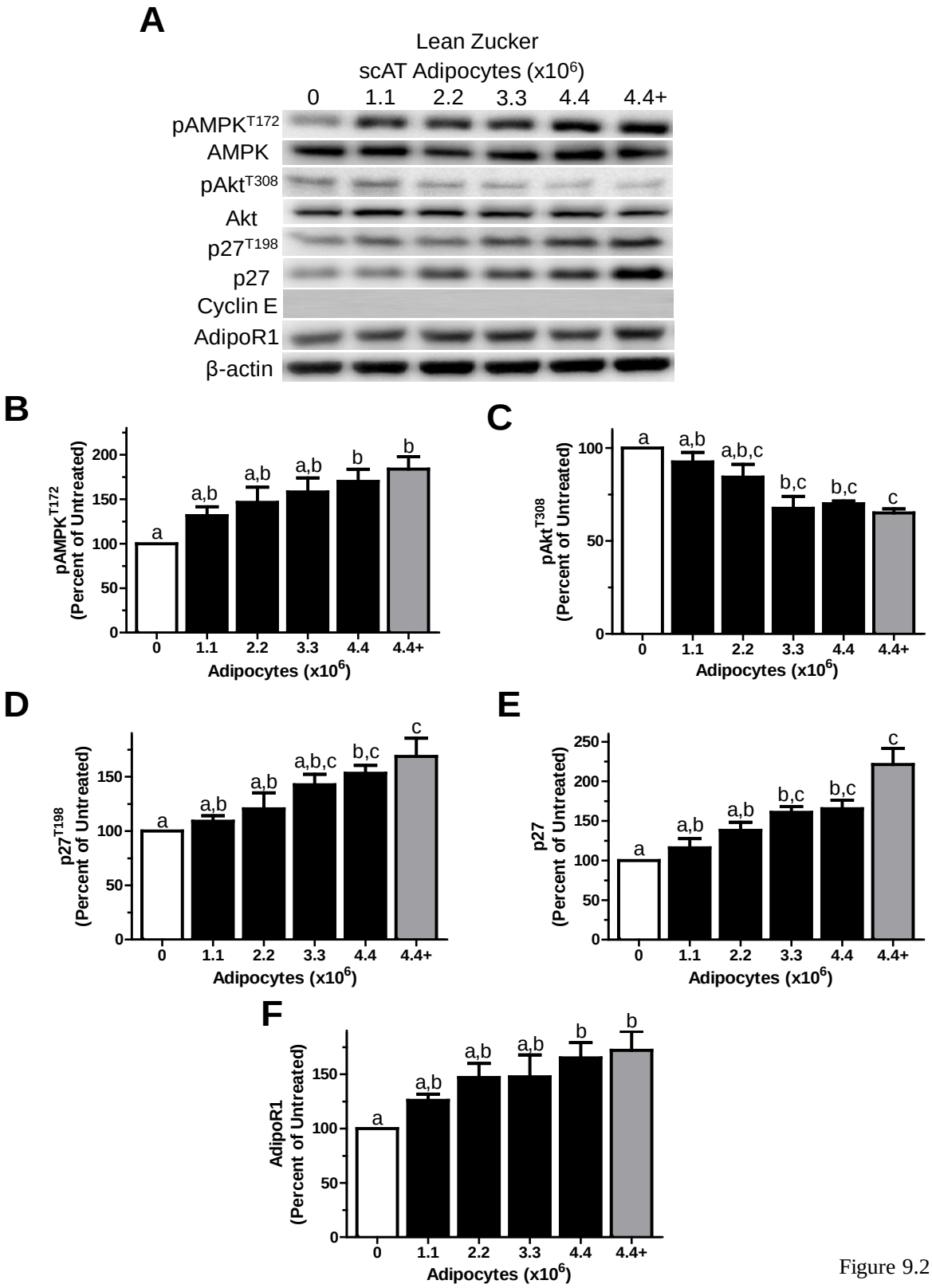
A
Lean Zucker
scAT Adipocytes (x10^6)
0
1.1
2.2
3.3
4.4
4.4+
pAMPK T172
AMPK
pAkt T308
Akt
p27 T198
p27
Cyclin E
AdipoR1
β-actin
B
pAMPK T172 (Percent of Untreated)
C
pAkt T308 (Percent of Untreated)
D
p27 T198 (Percent of Untreated)
E
p27 (Percent of Untreated)
F
AdipoR1 (Percent of Untreated)
Adipocytes (x10^6)

Figure 9.2

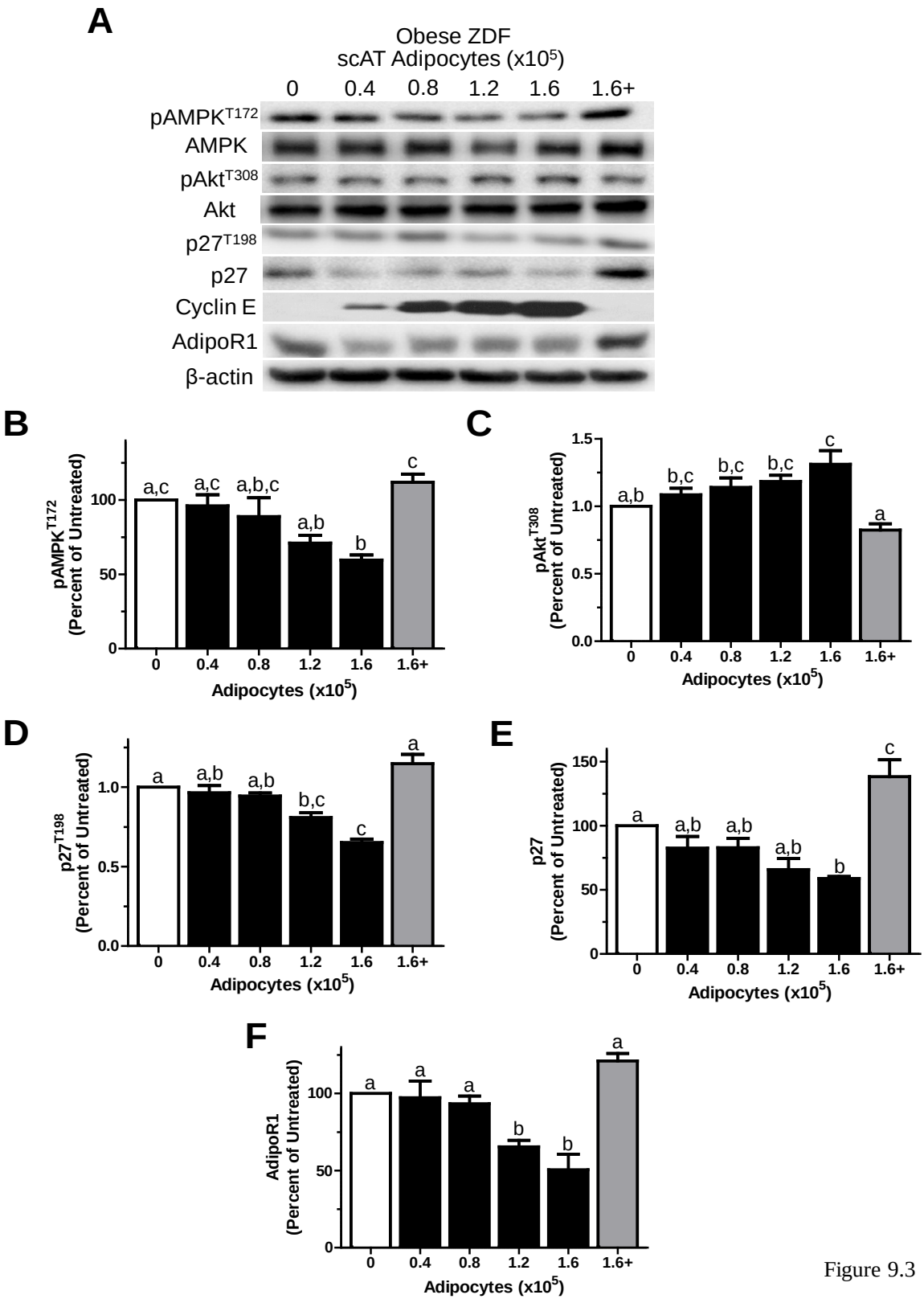
A
Obese ZDF
scAT Adipocytes (x10^5)
0 0.4 0.8 1.2 1.6 1.6+
pAMPK T172
AMPK
pAkt T308
Akt
p27 T198
p27
Cyclin E
AdipoR1
β-actin
B
pAMPK T172 (Percent of Untreated)
a,c a,c a,b,c a,b b c
Adipocytes (x10^5)
C
pAkt T308 (Percent of Untreated)
a,b b,c b,c b,c c a
Adipocytes (x10^5)
D
p27 T198 (Percent of Untreated)
a a,b a,b b,c c a
Adipocytes (x10^5)
E
p27 (Percent of Untreated)
a a,b a,b a,b b c
Adipocytes (x10^5)
F
AdipoR1 (Percent of Untreated)
a a a b b a
Adipocytes (x10^5)

Figure 9.3

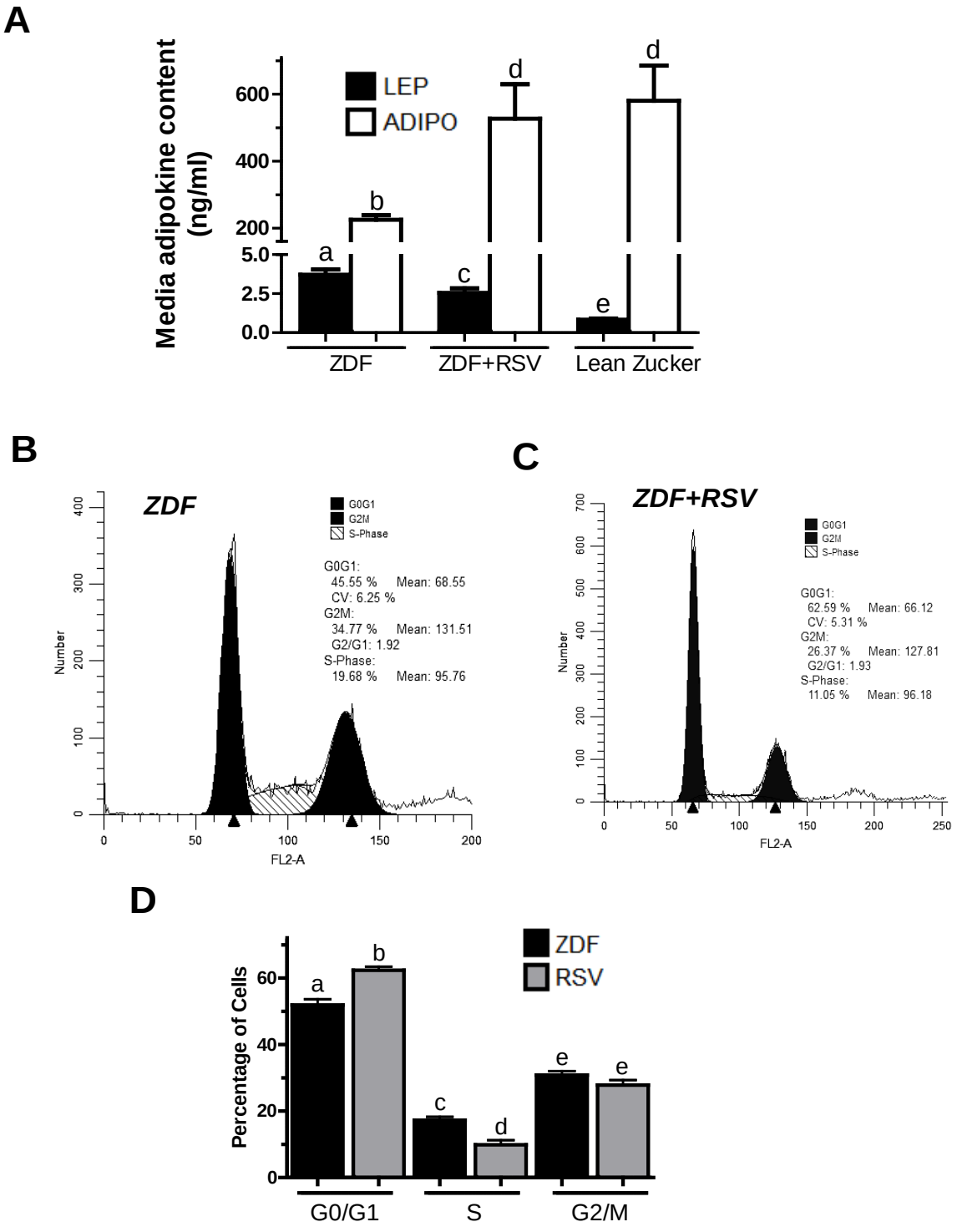
A
Media adipokine content (ng/ml)
LEP
ADIPO
600
400
200
5.0
2.5
0.0
a
b
c
d
e
d
ZDF
ZDF+RSV
Lean Zucker
B
ZDF
G0G1
G2M
S-Phase
G0G1:
45.55 % Mean: 68.55
CV: 6.25 %
G2M:
34.77 % Mean: 131.51
G2/G1: 1.92
S-Phase:
19.68 % Mean: 95.76
Number
FL2-A
C
ZDF+RSV
G0G1
G2M
S-Phase
G0G1:
62.59 % Mean: 66.12
CV: 5.31 %
G2M:
26.37 % Mean: 127.81
G2/G1: 1.93
S-Phase:
11.05 % Mean: 96.18
Number
FL2-A
D
ZDF
RSV
Percentage of Cells
a
b
c
d
e
e
G0/G1
S
G2/M

Figure 9.4

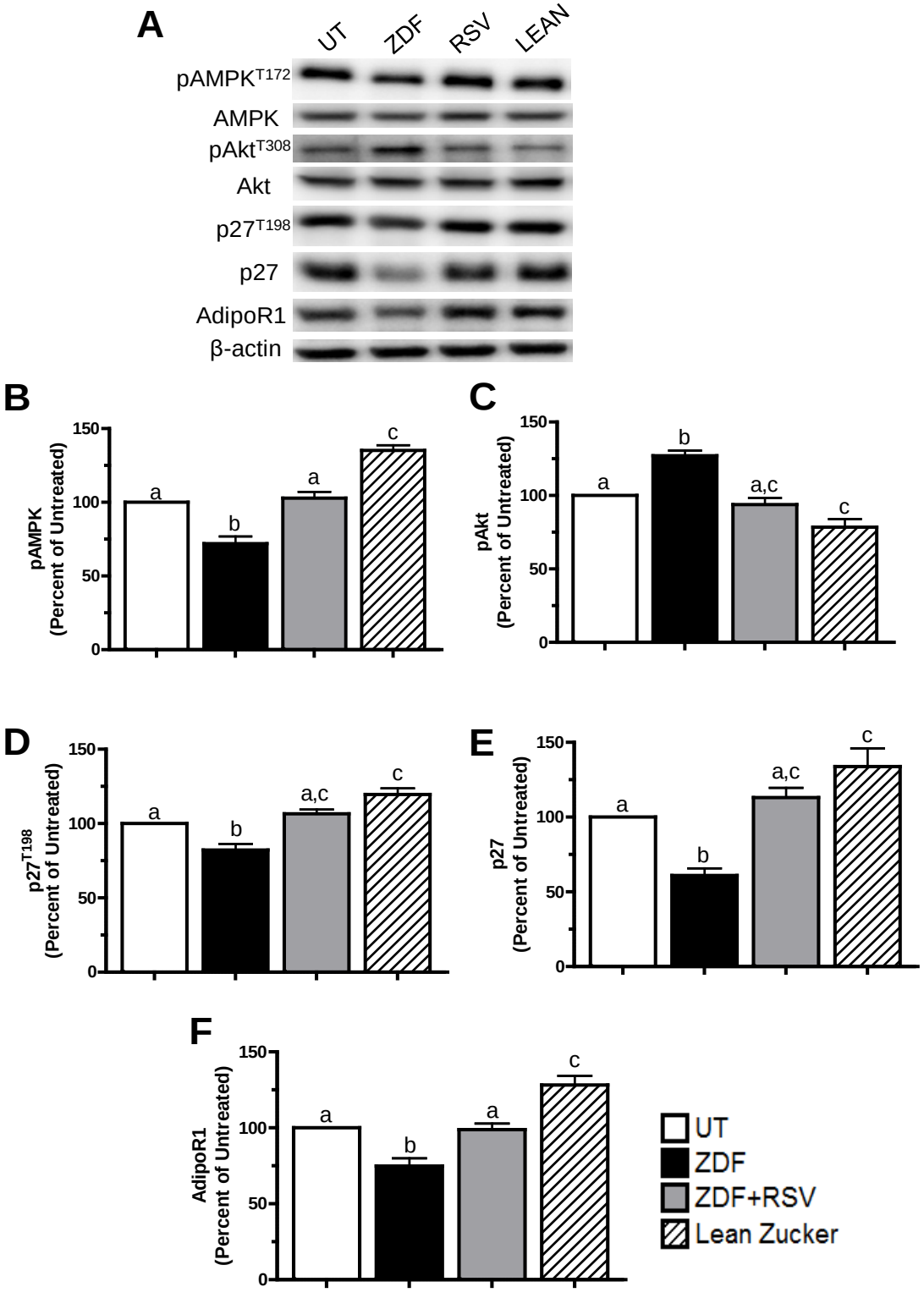
A
UT
ZDF
RSV
LEAN
pAMPK^{T172}
AMPK
pAkt^{T308}
Akt
p27^{T198}
p27
AdipoR1
β-actin
B
pAMPK (Percent of Untreated)
C
pAkt (Percent of Untreated)
D
p27^{T198} (Percent of Untreated)
E
p27 (Percent of Untreated)
F
AdipoR1 (Percent of Untreated)
UT
ZDF
ZDF+RSV
Lean Zucker

Figure 9.5

10. Thesis Discussion

Breast cancer is one of the most intensively investigated malignancy with respect to how body weight (obesity) acts as a risk factor, especially in postmenopausal women. For almost 50 years now researchers have shown a statistical link between increased adiposity and risk of breast cancer (27). Still it remains unclear as to the exact mechanisms behind this link as well. In addition, confounding the variables include growing discrepancy between menopausal status, obesity and breast cancer. Local circulating estrogen produced by the peripheral fat depots via aromatization has been suggested as a possible mechanism for the obesity-breast cancer link (6, 8). However the effects of estrogen alone are not directly correlated to breast cancer growth rate. Recently, adipocytes and their autocrine, endocrine and paracrine functions have moved to the forefront of breast tumorigenesis (16). Other growth factors such as adipokines may play a larger role in obesity-related cancers, as the majority of these tumors are surrounded by adipose tissue. Paracrine signaling from adipose tissue may play an important role in the breast, as adipocytes make up the bulk of breast tissue and represent one of the most abundant cell types surrounding mammary epithelium (16). This thesis focuses on the effect of the tumor growth microenvironment produced by the adipose tissue as well as its endocrine hormones and how this microenvironment can be altered in order to produce a less proliferative secretome. Adipose tissue plays an important role on breast cancer cell proliferation as shown in this thesis in agreement with others (9, 10, 12). As shown in all three studies in this thesis (chapters 7, 8 & 9), adipose tissue clearly has an effect on controlling breast cancer cell proliferation. Increasing visceral (chapters 7 & 9) or subcutaneous (chapter 8) adiposity, whether through high fat diet feeding or genetic obesity (ZDF animals) increases breast cancer cell proliferation. In all three studies, I found a clear link that increasing adiposity led to overall increases in breast cancer cell proliferation as confirmed via FACS

analysis. Most importantly, I show that altering adiposity through simple interventions such as diet and physical activity (PA; chapters 6 & 8) can have profound results on the proliferative effects of the secretome created by the adipose tissue. By decreasing adiposity these proliferative effects on breast cancer cells were blunted. This secretome appears to have a direct paracrine effect on surrounding breast tissue and can lead to tumor progression via a constant growth promoting microenvironment surrounding transformed ductal epithelial cells and support the growth of pre-existing malignant cells. Interestingly, I also found that it appears the adipocytes may represent the most important factor in controlling the tumor growth microenvironment rather than other components of the adipose tissue as a whole. While adipose tissue is comprised of several structures, our lab has shown that results using co-cultured isolated adipocytes (chapter 7) produce similar effects on breast cancer cell growth to those using whole adipose tissue (chapters 6 & 8). This further indicates the importance of adipocytes on affecting tumor growth microenvironment and how altering these adipocytes can lead to a more beneficial tumor anti-growth microenvironment. This has several implications, given that each cancer patient likely possess a unique and specific carcinoma making the development of individualized cancer cell directed therapies extremely difficult and potentially costly. However, the adipose tissue in obese cancer patients represents a stable and predictable component of a patient's physiology. Alterations in the secretome or adipokine secretion profile in obese individuals may represent a molecular link between obesity and cancer. Figure 10.1 shows the effect of obesity on altering the adipokine profile and the subsequent endocrine signaling effects on breast cancer cells. This effect can be blunted or even eliminated by decreasing adiposity.

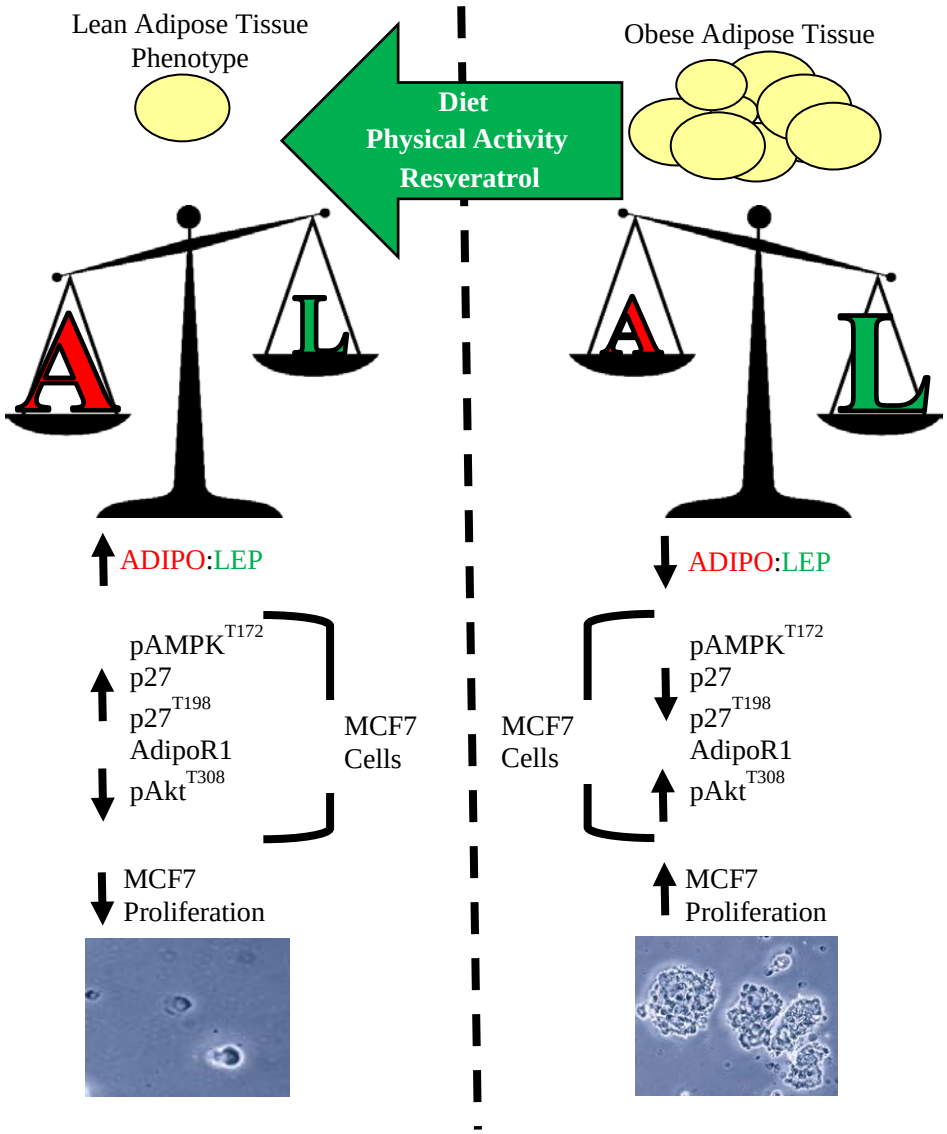

Figure 10.1: The effect of obesity on altering the ADIPO:LEP ratio and subsequent effect on MCF7 cell cycle proteins and proliferation. Also shown are methods of altering the adipokine growth microenvironment profile from an obese individual to a lean adipose tissue phenotype.

Altering the adipose tissue leads to an altered growth pattern of breast cancer cells, but the next question is why? Adipose tissue serves as an active organ producing adipokines which can act in an autocrine, endocrine and/or paracrine fashion, providing multiple potential mechanisms underlying the obesity-cancer link. Over 400 adipokines have been discovered and several have

been shown to become dysregulated in obese individuals (35). Of the over 400 different adipokines I chose to focus on ADIPO and LEP as both are among the most abundantly produced/secreted, are altered by obesity and have been shown to have cell cycle regulatory effects on breast cancer cells (9, 10). Although ADIPO and LEP on their own have been shown to be associated with cancer, emerging evidence, including (chapters 7, 8 & 9), now suggests that the ADIPO:LEP ratio may be a more reliable predictor of tumor growth microenvironment (1, 7, 28). Clinical studies in postmenopausal women show that decreased ADIPO:LEP ratios rather than the levels of each individual adipokine, are stronger predictors of breast cancer risk (21). Decreased ADIPO and increased LEP in circulation of obese women creates a microenvironment that promotes tumor growth by accelerating cell cycle entry (7, 17). A recent study by *Morad et al.* (2014) found that local extracellular LEP was higher, while ADIPO and ADIPO:LEP ratio was lower in breast tumors compared to normal adjacent breast tissue (20). Work from my thesis, as well as that of others, has shown that ADIPO and LEP antagonize one another through differing metabolic pathways (LEP via Akt, ADIPO via AMPK) resulting in breast cancer cell proliferation or arrest, respectively. Modulation of the adipokine profile by altering the secretion profile of obese adipose tissue may provide a novel method of protecting obese women who are at high risk of developing breast cancer. Several methods have been shown in this thesis including diet, PA and nutraceutical agents (RSV) which previously have been shown to increase the ratio of ADIPO:LEP in both rodent and human studies. In agreement with other studies, I show throughout my thesis (chapters 7, 8 & 9) that all three intervention methods increased the ratio of ADIPO:LEP within the animals. This increased ratio of ADIPO:LEP was found to cause breast cancer cell cycle arrest compared to obese animals. The magnitude of this anti-proliferative effect on breast cancer cells was in proportion to the magnitude of the increase in the ADIPO:LEP ratio compared to obese animals.

An alternate method of observing the ADIPO:LEP ratio is using a crude measure of obesity such as BMI. I found that ethnicity, at least between CA and SEA women, had no effect on the proliferation of breast cancer cells and rather a linear relationship between BMI or other measures of adiposity (waist circumference, percent body fat) and the subsequent proliferative influence of the patient's serum was found (supplemental data). This is in agreement with all animal data from this thesis in that any alteration in the level of obesity and subsequent ADIPO:LEP ratio appears to drive the adipose-dependent effects on breast cancer cell proliferation whether in animals or in humans. Data from all three animal studies are in agreement with other obesity preclinical animal models for other cancers addressing the interaction of ADIPO and LEP on tumorigenesis. One such study found obese animals had a 5-fold lower ADIPO:LEP ratio compared to lean counterparts which lead to a higher rate of spontaneous metastasis of Lewis lung carcinoma (34). Although I am unable to say that only ADIPO and LEP matter in predicting the proliferative tumor growth microenvironment, other adipose-derived factors that have been shown to be altered with obesity and affect breast cancer cell growth have been shown in this thesis to be unaffected. In chapter 8, the circulating levels of both IL-6 and TNF-α were determined and found to be unaffected by RSV supplementation in obese animals, yet overall breast cancer effects were found. The ADIPO:LEP ratio was found to be increased further pointing to these as two adipokines that have predictive power as important adipose tissue derived factors in breast tumor progression. One interesting result to come out of my studies was the possible individual importance of ADIPO and LEP depending on circulating estrogen. In an estrogen free environment (chapter 7), I found that ADIPO appeared to drive the changes in the ADIPO:LEP ratio compared to LEP, evident by greater changes in ADIPO compared to LEP. In contrast, when the adipose tissue was subjected to circulating estrogen (chapter 9), LEP appeared to drive the changes in the ADIPO:LEP ratio

compared to ADIPO. Despite this, the important end result is that the ADIPO:LEP ratio appears to be the best predictor regardless of estrogen status. Further study is warranted to determine whether the effects on tumor microenvironment in the presence of estrogen is reliant on one of the adipokines in particular, and vice-versa.

PA represents a simple intervention strategy for obesity that has been shown to be protective against breast cancer regardless of menopausal status (3, 5). Moderate PA (>0.64 MET-hours/day) reduces the incidence of breast cancer, with women who are physically active exhibiting a 20-30% reduction in the relative risk of developing breast cancer compared to their sedentary counterparts (22, 25, 26, 29). The effect of PA is also important in improving patient survival in breast cancer as seen by an up to 40% reduction in cancer-related death and cancer recurrence in physically active women (13). In addition, there are dose-dependent (intensity and duration) relationships among physical activity, cancer risk and overall survival in breast cancer patients (33). Breast cancer patients participating in physical activity consisting of walking as little as one hour/week was associated with improved survival compared to sedentary women (13). These effects were more pronounced in women who engaged in moderately intense exercise between 3-5 hours per week. When exercise intensity was increased further (running >1.8 MET-hours/day) breast cancer patients had an almost 90% lower risk of cancer mortality compared to women who walked (<1.07 MET-hours/day) (33). Thus, previous data suggest that PA can not only prevent breast cancer but also affects overall survival once a woman has been diagnosed with the disease and is dependent on the volume of PA they engage in. Data from my thesis agrees with this, as the effects of voluntary PA on both the ADIPO:LEP ratio and subsequent breast cancer cell proliferation were dependent on the volume of PA (chapters 7 & 9). We found a positive linear relationship between the amount of PA an animal performed and its subsequent ADIPO:LEP ratio

within the secretome, regardless of the presence of estrogen. I show that even at a low volume of PA, an overall increase in ADIPO:LEP ratio is found in the secretome (chapters 7 & 9) as well as the serum (chapter 9). Despite any anthropometric differences between the two studies, what remains clear was that PA is an extremely effective intervention/prevention strategy for obesity-linked cancers, regardless of whether estrogen is present or not.

The effect of estrogen directly on breast cancer cells has been extensively studied yet the indirect effect of how estrogen alters the adipose and subsequent secretome has received far less attention. Estrogen plays a confounding role in the link between breast cancer and obesity as there have been conflicting results in premenopausal women. I set out to determine what effect circulating estrogen would have on the adipose tissue secretome and how this secretome differed compared to when estrogen was absent (chapter 7 vs. chapter 9). As previously stated, estrogen has been shown to have antagonistic effects on the adipose production of ADIPO (decreases) and LEP (increases). Higher plasma estradiol has been correlated in premenopausal women to lower local tumor extracellular ADIPO and ADIPO:LEP ratio and positively correlated with local tumor extracellular LEP (20). Interestingly, we found that the presence of circulating estrogen caused a blunted effect of obesity on breast cancer cell growth compared to when estrogen was not present. Although this agrees with a large body of epidemiological research that report premenopausal women do not show as strong a correlation between obesity and breast cancer cell severity, we did not see any protective effect of obesity (chapter 9), as previously shown (4, 19, 30). This blunted effect of obesity on breast cancer cell growth could be due to several other adipokines and factors which estrogen has been shown to alter. Several other adipokines and inflammatory cytokines have been hypothesized to affect the tumor growth microenvironment. Only a few have been found to have an altered production within the adipose tissue due to the presence or absence of estrogen,

possibly explaining the proliferative difference. Resistin may be the best prime candidate in explaining why this altered effect is observed in the presence of estrogen. Ovariecotomized rats injected with subcutaneous estradiol found that resistin mRNA and protein content from isolated visceral adipocyte depots was reduced compared to estrogen free animals (14). Resistin has been shown to cause increased proliferation in breast cancer cells through the PI3K/Akt pathway similar to as we show with LEP (28). Resistin levels have also been shown to be higher in postmenopausal breast cancer patients compared to aged matched controls, while this increased level of resistin is not found in premenopausal breast cancer patients compared to women without cancer (2). Therefore, in the presence of higher circulating levels of E2, if the levels of resistin are lower compared to when estrogen is low there would be a decreased proliferative effect of obesity on breast cancer cells. Regardless, the ADIPO:LEP ratio was still found to be a reliable and consistent predictor of the proliferative tumor growth microenvironment created by the adipose tissue although a greater change occurred in the ratio of the two adipokines in the presence of circulating estrogen. Other factors besides ADIPO and LEP may also need to be monitored in obese premenopausal women with increased risk of breast cancer in order to better determine the proliferative potential of their respective adipose derived microenvironment.

Obesity and the resultant adipose tissue phenotype present with two distinct problems in regards to its effects on the proliferative nature of breast cancer cells. As shown in chapters 7, 8 and 9 as well as by others, obesity causes a decrease in circulating ADIPO (18, 32) and also causes a decrease in AdipoR1 protein in both adipocytes (24) and in breast cancer cells (23, 28). Although ADIPO is the most abundantly secreted protein from adipose tissue, obesity not only decreases the adipokine but also the potential binding sites on target tissues, blunting the anti-proliferative effects of ADIPO even further in any cancer cell. I show that regardless of the composition of the

tumor growth microenvironment, stabilizing AdipoR1 within breast cancer cells can completely abolish all proliferative effects of obesity (chapters 7 & 9). This leads into one potential avenue of cancer therapy which is now being explored as different means of activating the energy sensing AMPK pathway. As previously stated, the AMPK pathway becomes activated in times of decreased available energy substrates or periods of cellular stress. One primary problem with cancer cells is their shift in metabolism. Through the Warberg effect, cancer cells will opt to undergo aerobic glycolysis in order to facilitate the uptake and incorporation of nutrients into biomass rather than by generating energy through oxidative phosphorylation (31). If we can "trick" a cancer cell into thinking that its surroundings are not conducive to proliferation by activating the AMPK pathway and mitochondrial biogenesis via ADIPO signaling, we may be able to slow and even stop cancer cell growth. In this thesis I show that ADIPO can activate AMPK through AdipoR1 in breast cancer cells leading to cell cycle arrest (chapters 7, 8 & 9). This is important because if we can find ways to stabilize AdipoR1 (pharmacologically) or naturally as I show (chapters 7 & 9) in agreement with another study through diet or PA (24), we may be able to utilize more of the remaining ADIPO within the system without needing to alter the level of the adipokine. In addition, natural methods (diet and exercise) of increasing AdipoR1 have also been shown to increase circulating ADIPO resulting in an added benefit of slowing the progression of a proliferating neoplasm and producing a microenvironment which is not conducive to prompting the initiation of neoplastic growth.

The data obtained from this thesis has provided sufficient information in order to generate a working model between the interplay of ADIPO and LEP signaling in response to changes in tumor growth microenvironment brought about by changes in adiposity. This model consists of the possible signaling cascade of events due to an alteration, within the adipocytes, between an

obese and lean individual in relation to the effects on breast cancer cell cycle status (Figure 10.2). For this thesis, the focus was on utilizing methods of altering the tumor growth microenvironment created by obese adipose tissue and how this altered secretome would affect breast cancer cell cycle regulation. I found that both diet and voluntary PA have the ability to alter the proliferative nature of obese adipose tissue on breast cancer cells. Specifically, I believe that an alteration in the ratio of ADIPO:LEP plays an important role in the overall proliferative capacity of not only breast cancer cells but other obesity related cancers. I show that low volume daily PA without any weight loss is beneficial in creating an adipose-dependent less proliferative environment for breast cancer cell growth than is created by the adipose of obese sedentary animals. A low fat diet, both low and high relative daily PA and RSV supplementation altered the secretion profile of adipose tissue compared to obese animals. Specifically, I found that in all three cases where the secretome caused an altered growth pattern compared to the animals obese counterpart, the ADIPO:LEP ratio was increased. Although this does not definitively prove that the ratio of the two adipokines provides the most precise measure of the proliferative capacity of surrounding breast cancer cells, it does give an accurate indication of what growth pressures a breast cancer is exposed too. It also supports the notion that the ratio of ADIPO:LEP may need to become an additional method of monitoring women who are at high risk of developing breast cancer and are also obese. Studying the dynamic nature of adipose tissue and its local role on tumor formation and growth provides a therapeutic avenue for future cancer research. Fully understanding the relationship between ADIPO and LEP may link a physiological/molecular relationship between obesity, breast cancer and cell cycle regulation.

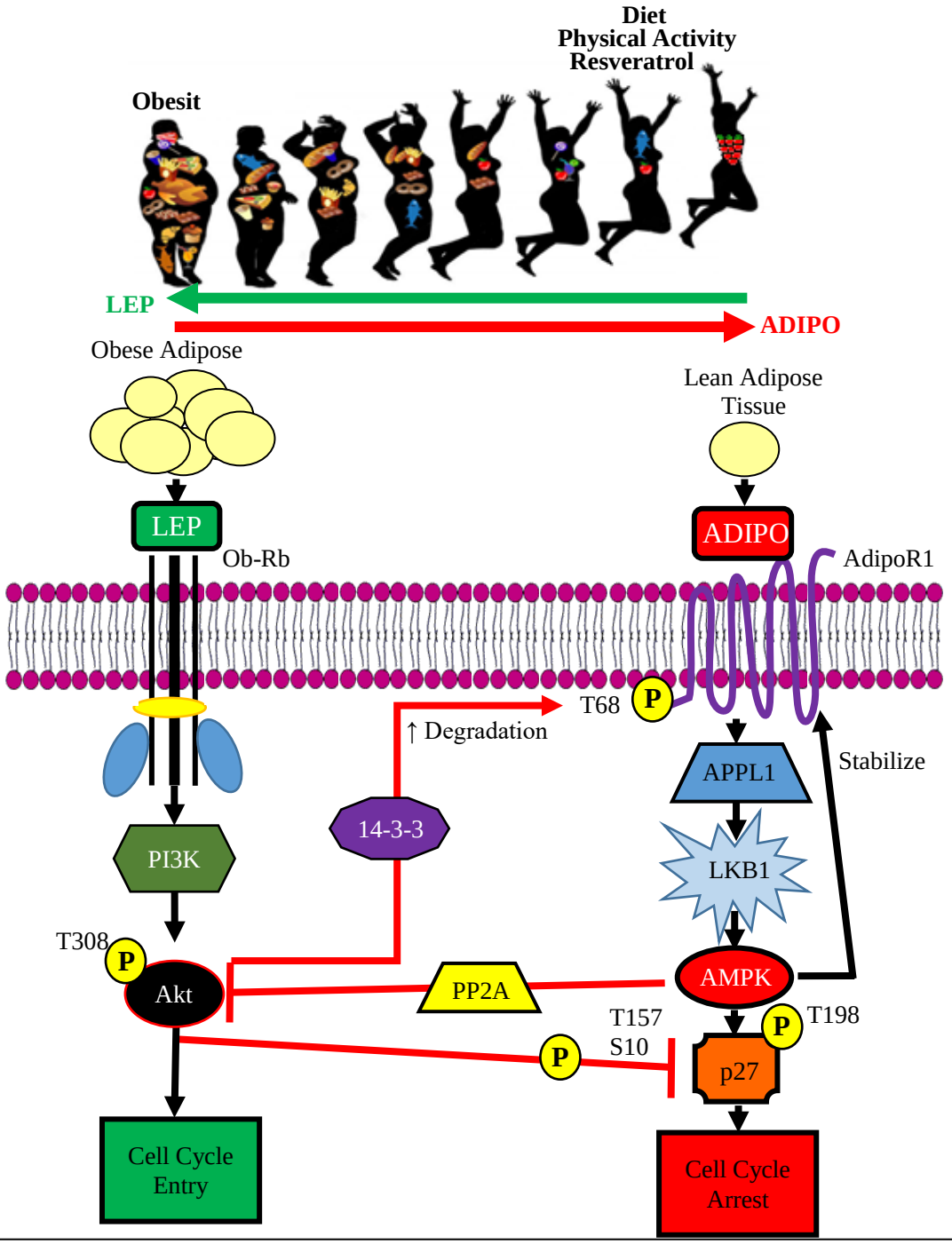

Figure 10.2: Final working model combining ADIPO and LEP cell signaling cascades leading to breast cancer cell entry or arrest. Effect of upstream intervention mechanisms which can alter the ADIPO:LEP ratio and subsequent breast tumor growth microenvironment. Top image modified from *Dreamstime.com*

11. Limitations and Future Direction

11.1 Limitations

The purpose of this study was to try to further research into the effects of the two adipokines ADIPO and LEP on breast cancer development and progression, how certain interventions could be used to alter the physiological concentrations of the two adipokines and how this altered secretome profile would affect breast cancer growth. As with any study there are limitations to my research and it is important to recognize and understand them in order to strengthen future experiments. Although we utilized an adipocyte CM protocol for the majority of the experiments it does not directly classify as a direct *in-vivo* experiment. While using a CM or the co-culture protocol to study the effects on breast cancer cell growth does have all adipokines which would be present in an obese breast cancer patient, the system itself is not represented. Without having the direct animal model with circulating and local adipose derived adipokines, such as in an xenograft model, we cannot make a direct comparison to the effects of diet, PA, RSV and the resulting ADIPO:LEP profile on slowing the growth of a tumor within a living organism. I think it would be beneficial to utilize a direct *in-vivo* model, by injecting both MOCKT and AdipoR1-T MCF7 cells subcutaneously into immunocompromised rats. I believe that the xenograft model would produce a more physiological, yet not perfect, model then other studies referenced in this paper which use a chemical means to produce breast cancer (MNU). We could then determine whether stabilizing AdipoR1 in conjunction with our three interventions (diet, voluntary wheel running and RSV) causes the tumor cells to grow slower by utilizing more ADIPO from within the system both circulating (endocrine) and surrounding the tumor (paracrine) *in-vivo*.

We also utilized only one type of breast cancer cell line. Because we only used one type of breast cancer cell line, the results cannot be fully concluded for all breast cancer cell types.

Although previously unpublished work in our lab using BT20 breast cancer cells which are triple negative (ER-, PR-, p53-) has shown that the antagonism between ADIPO and LEP occurs, much higher levels of ADIPO were needed in order to slow the growth of this very aggressive breast cancer cell line. This may also hold true for our model as low PA may not have produced the necessary alterations in the ADIPO:LEP ratio needed to slow the growth of more aggressive breast cancer cell lines.

Another limitation to the studies was the use of an animal model in predicting similar effects in humans. Although we use human breast cancer cells which have been shown and we again show are reactive to rat ADIPO and LEP, the model of obesity, PA and other interventions are based on an animal model that cannot be directly linked to what may happen in humans. Also with the animal model we used young rats both male and female and we know that the effects of breast cancer occur at a much later age even when correcting the animals age to human years. We used younger animals because older rats are much less likely to undergo voluntary PA. We wanted to utilize a voluntary PA model as forcing the animals to exercise added an additional variable and element that we wanted to keep from our study. Because of this, the changes in the adipokine profile due to obesity and subsequent intervention methods cannot be stated for older animals.

Lastly, because we used voluntary PA as a method of exercise intervention, we are unable to precisely prescribe a dose and intensity of exercise that can be prescribed to a patient. Although in our postmenopausal animal model paper (chapter 7) we did use previously published data with comparable CYTOX data in order to estimate the prescribed dose and intensity our animals engaged in, we cannot say with certainly the necessary VO_2 needed in order to reproduce our results in humans. The purpose of the PA papers was more about determining if low volume/daily

distance PA could counteract the effects of obesity on the adipokine profile of the animals rather than determining a precise dose of exercise necessary to observe changes.

11.2 Future Directions

From both my Masters and PhD research, we now have a clearer picture of how obese adipose tissue alters the growth of breast cancer cells and how can we physiologically alter this growth microenvironment and the ADIPO:LEP ratio. There are now several avenues of future direction that can be taken in order to further elude the diagnostic significance of the ADIPO:LEP ratio regardless of menopausal status. Now that we have shown the effects of diet and voluntary PA in animals with circulating estrogen and those without, the next possible step would be moving now towards using human samples. First if we could look to obtain healthy breast adipose tissue from both lean and obese women. Healthy obese breast adipose tissue would be relatively easy to obtain from breast reduction clinics but healthy lean breast adipose tissue may be more difficult to obtain. The key would be obtaining tissue from women in order to try and age match each sample in order to reduce possible variation. Working with key cancer clinics and hospitals, we could also obtain breast adipose tissue from women diagnosed with breast cancer both lean and obese from mastectomy surgeries. In both studies, the adipose tissue would be used to create CM as previously utilized in this study and co-treated with breast cancer cells. We would determine the ADIPO:LEP ratio and if again any alterations in this ratio between lean and obese as well as within patients leads to an altered growth pattern in breast cancer cells.

In order to fully conclude that alterations in the ADIPO:LEP ratio brought about by diet, PA or other means can be used to predict the tumor growth microenvironment of all breast cancer cells, we must utilize the same CM protocol in other cell lines. By utilizing cells such as BT20

(ER-, PR- and p53-) and MDA-MB-453 (ER-, PR-, p53+) in order to determine the broader effects of utilizing the ADIPO:LEP ratio for predicting the proliferative nature of the tumor growth microenvironment for all or only certain types of breast cancer. This can simply be done by utilizing frozen CM samples and treating the respective breast cancer cell lines in order to determine if similar effects persist or whether an altered growth pattern is present.

Lastly, if we wanted to continue using an animal model, it may be beneficial in duplicating the study but utilizing older animals in order to determine what effects age has on the alterations of the adipokine profile as well as what effect age will have on altering caloric output for the voluntary PA groups.

Overall, I believe this thesis gives insight into the mechanisms underlying the adipokine status of breast cancer patients. This study further stresses the importance of the ratio between ADIPO:LEP and how obesity can play a pivotal role in creating a positive growth environment for breast cancer cells. Also this thesis shows that alterations to the secretome of adipose tissue can be used in order to slow. Further investigation in humans I believe may produce a novel mechanism to attacking tumor growth in obese breast cancer patients and also present a target adipose microenvironment for women at risk of developing the disease.

12. References (Discussion)

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13. Supplemental Data

Effect of Race and Anthropometric Measures on Proliferative Capacity from Human Serum on MCF7 Breast Cancer Cells

Previous research has suggested that a racial difference in terms of BC incidence exists between Caucasian (CA) women and Southeast Asian (SEA) women (4, 5). Along with this racial difference, it has also been suggested that adipose tissue differs between the two races with SEA women showing an adipokine profile at a lean BMI similar to that of an overweight CA woman (Low ADIPO:LEP ratio) (2, 3). The purpose of this study was to determine if a difference does exist between the proliferative capacity of serum from both CA and SEA women on MCF7 cells. Also to be determined was whether measures of obesity or cardiovascular fitness (BMI, percent body fat, waist circumference, VO_2) are a better predictor of the proliferative nature of serum on MCF7 BC cells. We found no difference in the proliferative nature of serum from both SEA and CA women. BMI, %BF and WC were found to have similar predictive value in determining the effect of their respective serum on MCF7 growth.

Methods

All methods for both cell cycle proteins analyzed and FACS analysis were completed identically to those as previously published (6). With the exception that instead of MCF7 cells treated with CM, the cells were treated with serum (5% serum concentration + 5% FBS) from each patient. The study was ran blind with only each patient being given a number. At the completion of the study, protein and FACS analysis were then subdivided into SEA and CA women and matched to the patient's respective anthropometric data BMI, WC, %BF and VO_2).

Results

Table S1: Anthropometric Data for Caucasian and Southeast Asian Women

Ethnicity	Average Age	BMI	%BF	WC	Peak VO2
CA	61.8 ± 1.2	27.6 ± 1.2	37.0 ± 1.5	90.2 ± 2.9	28.1 ± 1.5
SEA	58.7 ± 0.9	32.2 ± 2.2	40.3 ± 2.5	104.6 ± 4.3*	22.6 ± 2.3

CA, Caucasian; SEA, Southeast Asian; BMI, body mass index; %BF, percent body fat; WC, waist circumference; * indicates significantly different from CA ($p<0.05$, n= 11 CA, 10 SEA/ group). **Data collected by Jamnik Lab for each patient, table created by Theriau CF.**

Anthropometric data for the 21 patients who were initially run through the cell cycle status (FACS) and western blot (protein) data with only a patient number ID and no indication of ethnicity or obesity status. We found that the only difference between the two groups was that the SEA women had a higher WC than CA women. All other anthropometric data were found to be similar between the two groups.

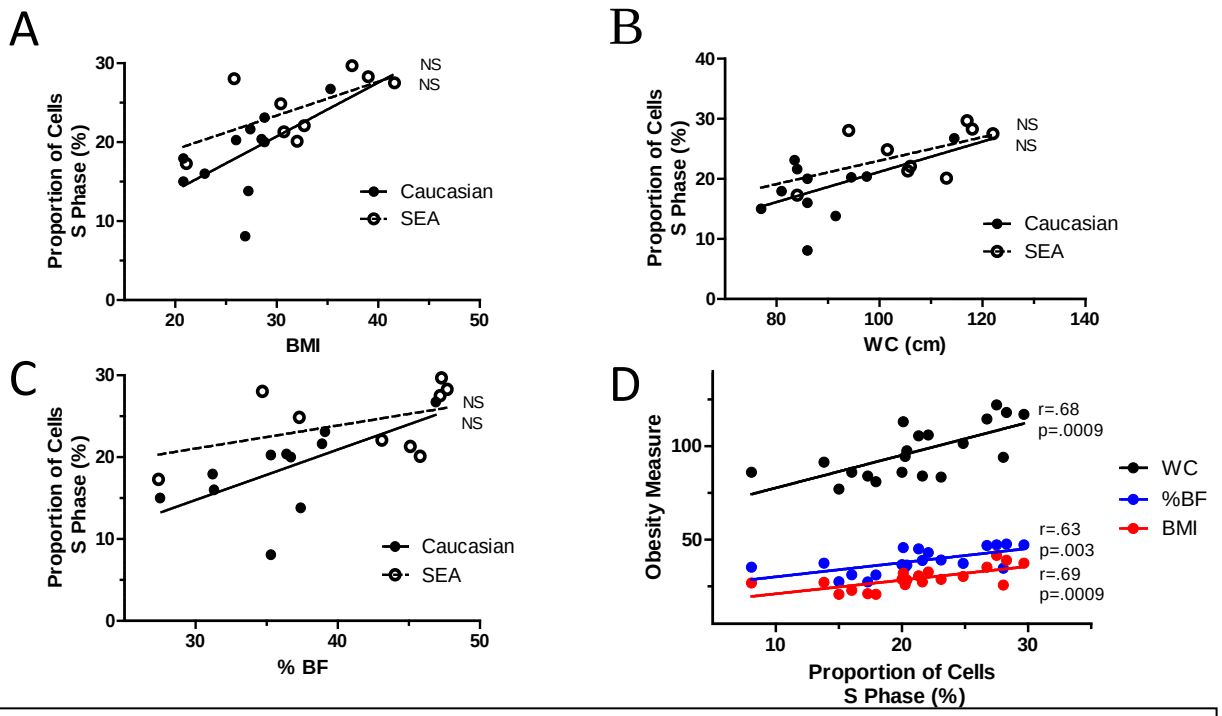

Figure 13.1: No difference between SEA and CA women serum proliferative capacity on MCF7 breast cancer cells. Scatter plot and linear regression of proportion of MCF7 cells in S-phase depending on BMI between CA (closed circle) and SEA (open circle) *(A)*. Scatter plot and linear regression of proportion of MCF7 cells in S-phase depending on WC between CA (closed circle) and SEA (open circle) *(B)*. Scatter plot and linear regression of proportion of MCF7 cells in S-phase depending on %BF between CA (closed circle) and SEA (open circle) *(C)*. Scatter plot and linear regression of proportion of MCF7 cells in S-phase depending on obesity measure of BMI (closed), %BF (blue) and BMI (red). ($p<0.05$, n=11 CA, 10 SEA/group).

We first wanted to determine if the serum from CA and SEA from both lean and obese women caused a different proliferative profile on MCF7 cells as previously thought. We found no difference in proliferation between MCF7 cells treated with serum from CA and SEA women (Fig. 13.1 A, B & C). Number of cells in S-phase increased with measures of adiposity (BMI, WC, %BF) reaching the highest values at the greatest obesity levels, regardless of ethnicity. Due to this lack of difference between racial groups, we pooled the data due to low patient numbers to determine which measure of obesity (%BF, BMI & WC) better predicted the proliferative capacity of the patient's serum on MCF7 breast cancer cells. First we found that VO_2 showed no correlation

in predicting the proliferative capacity of serum on breast cancer cell growth (not shown). Between the remaining three obesity measures, all showed a significant linear relationship effect of serum proliferation of MCF7 cells compared to obesity measures (Fig. 13.1D). Due to this, we decided to move forward showing the effect of the serum of cell cycle proteins previously studied in this thesis using BMI as our measure of obesity as the predictor. This is because BMI is such an extensively used measure of obesity for breast cancer patients even though it has been shown to be a flawed measure.

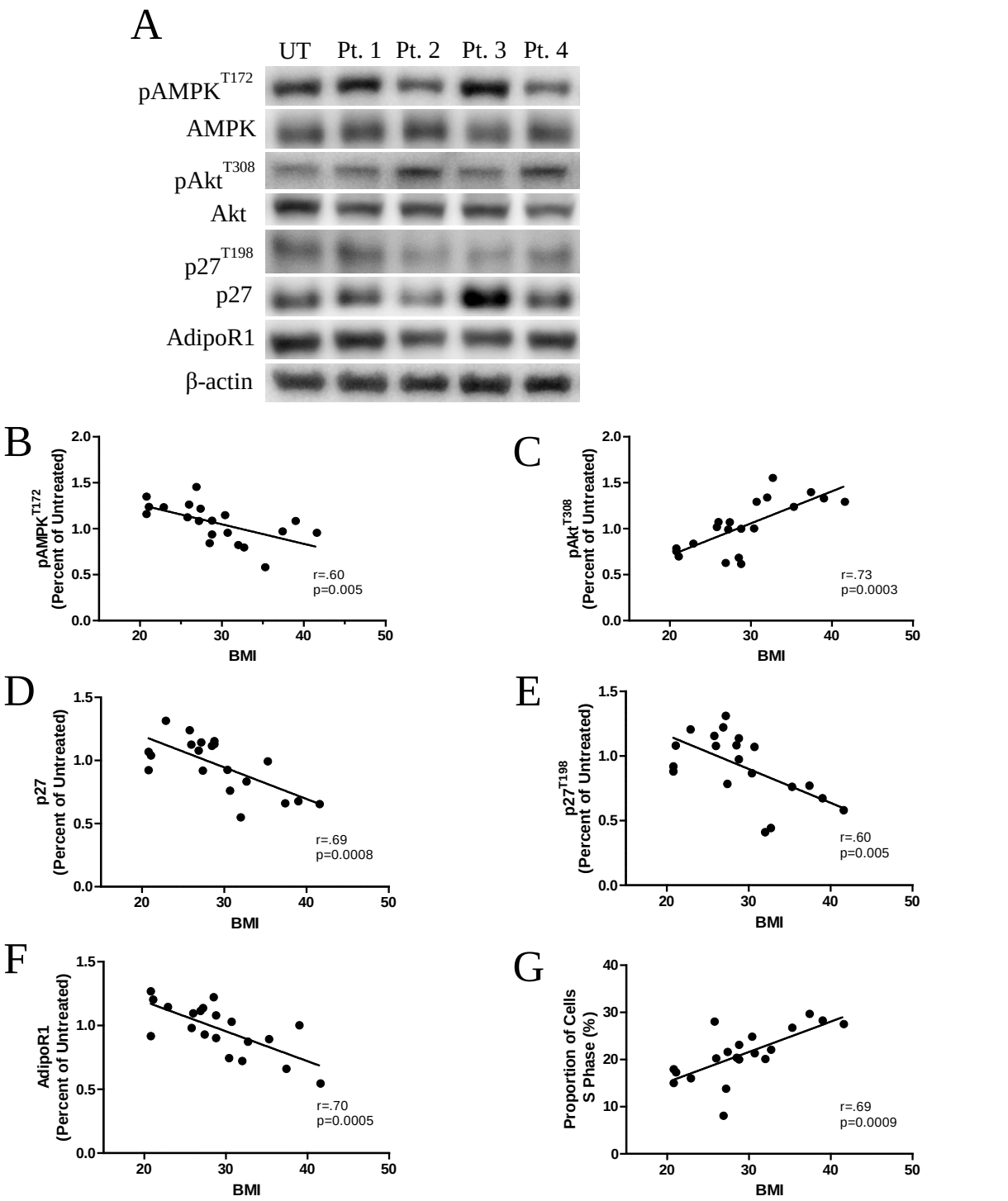

Figure 13.2: BMI is a strong predictor of combined CA and SEA serum treatment on MCF7 cell cycle proteins. Representative western blots for the first four numbered patients for selected proteins showing the effects of treatment with serum taken from both CA and SEA women on MCF7 cells *(A)*. Scatter plot and linear regression representation of multiple experiments showing the effects of serum on pAMPK^{T172} *(B)*, pAkt^{T308} *(C)*, p27 *(D)*, p27^{T198} *(E)* and AdipoR1 *(F)* protein levels using BMI as an obesity measure predictor. β-actin was used as a loading control. ($p<0.05$, n=11 CA, 10 SEA/group).

Using BMI as a predictor of the proliferative potential of serum from lean and obese women on MCF7 cells, we found an inverse correlation between BMI and all proteins measured (Fig. 13.2 A, B, D, E) except for $pAkt^{T308}$ (Fig. 13.2C) which changed proportionately with BMI. BMI appears to be able to reliably predict how the serum from both lean and obese will affect the proliferation of MCF7 cells. We see that serum from women of a lean BMI (<25) were found to increase $pAMPK^{T172}$, p27, $p27^{T198}$ and AdipoR1 in MCF7 breast cancer cells compared to women of an overweight/obese BMI (>25). The opposite effect of BMI was found for $pAkt^{T308}$. Probed proteins in MCF7 cells treated with lean and obese serum were also shown in overall cell cycle effects (Fig. 13.2G). BMI was also predictive for proliferation, as MCF7 cells grown with serum from lean women (BMI<25) had a lower proportion of cells in S-phase than those cells grown in serum from obese women (BMI>30).

Conclusions

Overall, this study found that contrary to some published data, we show no difference in the proliferative potential of serum from lean and obese women of CA and SEA nationalities. These results complement previously published data which demonstrated serum ADIPO has a negative (r=-0.33, p=0.001) and LEP a positive (r=0.323, p=0.001) correlation to BMI in SEA women (1). Although we did not determine the ADIPO:LEP ratio within the serum of patients, we do show an effect as serum from lean women BMI (<25) which caused a lower proliferative capacity (lower number of cells in S-phase) compared to women who had an obese BMI (>30). This is novel as although BMI is known as a flawed measure of obesity and cancer progression,

BMI does appear to play a predictive role in regards to how serum from patients may affect the proliferation on BC cells, regardless of race.

14. References (Supplemental Data)

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