Regulation of ADIPOR1 Function and Signaling by Adipokines in Breast Cancer MCF7 Cells

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By: **Christopher Theriau**

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Abstract

Breast cancer continues to be the number one cause of new cancer cases and second in mortality rate in Canadian women. For over 40 years researchers have shown a statistical link between obesity and breast cancer. Obesity has been linked to many non-communicable diseases but researchers are now showing several cancers are linked to obesity, including breast. Specifically, post-menopausal women are at increased risk to developing and dying from breast cancer with increased adipose "fat" tissue. Although adipose tissue in the past was considered an inert storage depot, we now know it produces and secretes the adipokines adiponectin and leptin. Lean individuals have been shown to have higher circulating levels of adiponectin and lower levels of circulating leptin, 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 which directly stabilizes the cell cycle inhibitor protein p27. The effects of both adiponectin and leptin on ADIPOR1 have yet to be shown. Therefore the purpose of this thesis was to look at ADIPOR1 function, stability and regulation in order to determine what affects both adiponectin and leptin have on ADIPOR1. The results show that adiponectin is able to increase ADIPOR1 protein content while increases the amount of pAMPK and decreasing the amount of pAkt. I also show that upregulating ADIPOR1 can overcome the deleterious effects of LEP treatment and a HFD/obesity on cell cycle regulating proteins.

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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
BAT	Brown Adipose Tissue
BMI	Body Mass Index (kg/m^2)
BSA	Bovine Serum Albumin
CC	Compound C
CD-CM	Chow Diet-Conditioned Media
Cdk	Cyclin-dependent Kinases
cDNA	Complementary Deoxyribonucleic Acid
cFBS	Charcol Stripped Fetal Bovine Serum
CKI	Cyclin-Dependent Kinase Inhibitors
CRM1	Exportin-1 (XPO1)
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E2	Estradiol
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
G_0	Gap Zero Phase
G1	Gap Phase 1
G2	Gap Phase 2

hKIS	Human Kinase Interacting with Stathmin
HFD-CM	High Fat Diet-Conditioned Media
IMEM	Improved Modification of Eagle's Minimum Essential Medium
INK	Inhibitor of Cdk-4/6
JAK2	Janus Tyrosine Kinase 2
KPC	KIP-1 ubiquitylation Promoting Complex
KIP	Kinase Inhibiting Protein
LEP	Leptin
LKB1	Liver Kinase B1
MAPK	Mitogen Activated Protein Kinase
NLS	Nuclear Localizing Signal
NF-κB	Nuclear Factor-κB
Ob-Rb	Long Form Leptin Receptor
PBS	Phosphate Buffered Saline
PI-3K	Phosphatidylinositol 3-OH-Kinase
PKB/Akt	Protein Kinase B
SRC	Non-Receptor Tyrosine Kinase of the Sarcoma Family
Rb	Retinoblastoma
SCF	Skp1, Cullin, F-box Containing Complex
SOCS3	Suppressor of Cytokine Signaling 3
STAT	Signal Transducers and Activators of Transcription
TGF-β	Transforming Growth Factor-β
TSP	Tumor Suppressor Protein
Ub	Ubiquitin
VEGF	Vascular Endothelial Growth Factor
WAT	White Adipose Tissue
WHR	Waist-to-Hip Ratio

1. INTRODUCTION

Cancer continues to be one of the leading causes of mortality in Canada, yet over the past 10 years these death rates have been steadily dropping. One explanation is better early detection as we begin to understand the mechanisms behind cancer development and the many factors which influence cancer progression. Breast cancer mortality specifically has been shown to have decreased by ≈ 2% since 1998 while the incidence rate continues to rise in part due to better detection [1]. This decrease in overall percent mortality should not be confused with an actual increase in the absolute number of deaths, further stressing the importance of breast cancer research.

Recent statistics from the Canadian Cancer Society predict in 2012 that an estimated 22,700 women will be diagnosed with breast cancer and 5,100 will die of it [1]. That equates to roughly 1 in every 9 women will develop breast cancer and 1 in every 40 dying from the disease. Breast cancer ranks 1st in incidence for Canadian women and only 2nd in mortality, closely behind lung cancer. As so many women become affected by this disease it has become important to look at the molecular events which cause breast cancer progression as well as epidemiological trends to better help prevent breast cancer development.

Cancer in its simplest form is a disease of the mammalian cell cycle. Under normal circumstances, the mammalian cell cycle is a tightly regulated series of events. The cell monitors extracellular signals and determines when appropriate to enter the

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. During the cell cycle there are check points where mistakes are noted and either fixed or the cell is terminated. A tumor cell on the other hand is no longer under proper function and control which can lead to the cell undergoing uncontrolled proliferation.

In order to properly understand how this transition from controlled growth to uncontrolled cell growth occurs, it is necessary to understand the events and mechanisms which govern a normal mammalian cell cycle as well as how these mechanisms are altered in cancer progression.

1. The Mammalian Cell Cycle

The mammalian cell cycle is an extremely important process which is tightly regulated and involves multiple stages for successful division. Originally the cell cycle was divided into to two primary phases known as mitosis (M phase) and interphase, the interlude between two M phases [2]. Cell division occurs within M phase where a cell undergoes a controlled division to produce two identical daughter cells. Although M phase is important for cell division, the majority of the time spent by our cells is within interphase. Interphase itself can be further divided into three stages known as gap one (G1), synthesis (S) and gap two (G2) phases. The replication of genetic material or DNA, occurs within a specific part of interphase known as S phase. S phase is preceded by G1 phase and followed by G2 phase, where a cell must prepare for DNA synthesis or

prepare for mitosis, respectively [3]. These gap phases allow for the cell to repair any DNA damage or replication errors. If the DNA damage cannot be repaired, under normal circumstances the cell will induce the process of cell death (apoptosis) [4]. It is also important to note that cells within G1 can enter a quiescent state known as G_0 but this is done before the cells commit to the replication of DNA [2]. The majority of cells in our body which are normally non-proliferating remain in this quiescent state of G_0 until they are stimulated by an external mitogenic signal to divide. Arguably one of the most important phases is the G1 phase as this is the point in the cell cycle where a cell commits to replicate the DNA and undergoes cell division [3]. G1 is the period where the cell evaluates its surroundings (ie. environment, stress) and determines whether it should continue to divide or regress back into G_0. Also within G1 phase, many signals intervene in order to influence cell division and the deployment of the cells developmental programme [3].

1.1 Regulation of the Cell Cycle

The transition between one phase to another is governed by different cyclin dependent kinases (cdks), which act as regulatory enzymes. These kinases are a family of serine/threonine kinases which are activated at specific points throughout the cell cycle in order for progression to occur [2]. Five cdks have been found to be active during certain phases of the cell cycle which include Cdk2/4/6 (G1), Cdk2 (S phase) and Cdk1 (G2), all of which when activated induce downstream processes by phosphorylating

certain proteins [5]. What's important to note is that cdks act in combination with unique regulatory cyclin proteins in order to form Cdk-activating complexes (Figure 1).

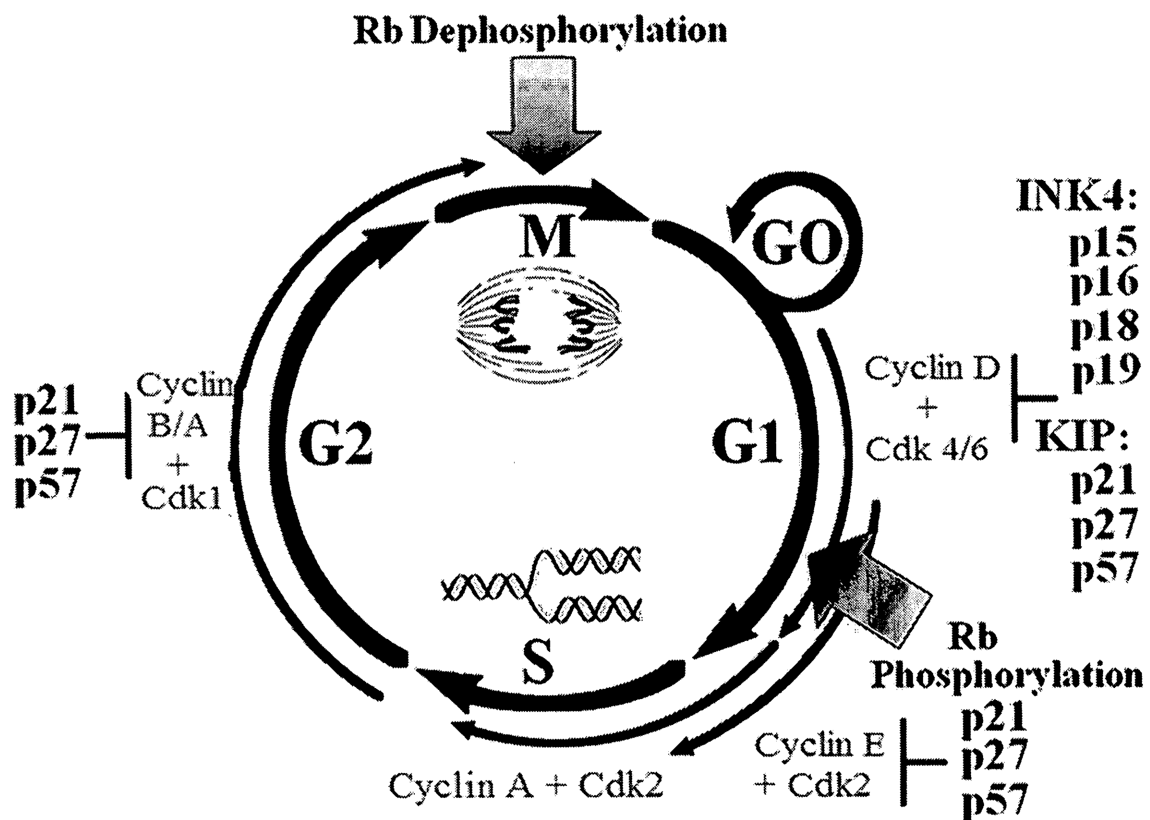


Figure 1: A simplified diagram of the mammalian cell cycle showing the cyclin/cdk complexes and the inhibitor proteins (INK4 and KIP) which affect them. Modified from Slingerland and Pagano, *J Cell Physiology, 2000*

The cdks require this binding to their cyclin subunit to allow it to become catalytically active [6]. Different members of the cdks, along with their regulatory cyclins, act as key switches throughout the cell cycle to allow for further progression. Cdk protein levels remain constant throughout the cell cycle which is in contrast to their

regulatory proteins the cyclins. As the name suggest, cyclin proteins are either accumulated or degraded during the cell cycle which allows them to periodically activate their cdks. The cyclins can be upregulated by cycles of transcription and translation to increase their protein levels or they can be degraded by ubiquitin mediated proteolysis to decrease protein levels [7]. An additional mechanism by which the cdks become active is through the phosphorylation of different residues within their activating segments which induces a conformational change to enhance its binding of the cyclins [4]. This includes the Wee1 kinases which phosphorylate cdks with an inactivating phosphorylation. The enzyme cdc25 is a phosphatase which dephosphorylates the same sites that are phosphorylated by Wee1 removing inhibition and allowing for cell cycle progression [8]. Once cdks become active, target proteins can then be phosphorylated on cdk consensus sites which leads to further cell cycle progression.

As shown in figure 1, the predominate G1 cyclin/cdk complexes are cyclin D/cdk4/6 and cyclin E/cdk2. Following the activation of these complexes there is the further activation of the cyclin A/cdk2 in S phase and cyclin A/cdk1 and cyclin B/cdk1 in G2 and M phases [4]. Although cyclin D is expressed periodically unlike the other cyclins it is synthesized as long as the growth factor stimulation continues [9].

Also shown in figure 1 are the two distinct families of cdk inhibitors (CKIs) that have been discovered, known as the inhibitors of cyclin dependent kinase 4 (INK4) and the kinases inhibitor protein (KIP) families. The INK4 family consists of $p15^{INK4}$, $p16^{INK4}$,

p18^{INK4} and p19^{INK4}, all of which specifically inactivate the G1 cdks which include cdk4 and cdk6. These CKIs form stable complexes with the cdk enzyme before the cyclin can bind, preventing its association with cyclin D [10]. The KIP family consists of p21^{CIP1}, p27^{KIP1} and p57^{KIP1} which binds and inhibits all cyclin/cdk complexes besides the cyclin D complexes [11]. CKIs are regulated both by internal and external signals, for example, the expression of p21 is under transcriptional control of the p53 tumor suppressor while the activation of p27 can occur due to transforming growth factor β (TGF-β), both contributing to the growth arrest of cells [12, 13].

In terms of cell cycle regulation, the sole target phosphorylated by cyclin D/cdk4/6 is the retinoblastoma tumor suppressor (Rb) (Figure 1). During early G1, Rb becomes phosphorylated 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 [14]. Once a cell reaches this state in late G1, the cell is at a "point of no return", called the restriction point. Following this, the cell is committed to enter the cell next phase of the cell cycle and can't return back to G1 until a full cell cycle is completed. Importantly, limiting the amount of cyclin E available keeps cells inactive until a mitogenic signal intervenes. Cyclin E expression is dependent on E2F transcription factors once they have been released from Rb. When a cell is in the G_0 quiescent state, Rb is in a hypophosphorylated state and bound to E2F.

Initially, these mitogenic signals as stated earlier, induce the expression of cyclin D and cdk 4/6 which then forms an active kinase state 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. This newly formed cyclin E/cdk2 then works through a positive feedback loop to cause Rb to become hyperphosphorylated thus causing E2F to fully activate its target genes and induce the transition into S phase and DNA replication.

1.2 Importance of $p27^{KIP1}$

As previously described, p27 is a key regulator of the G1-S phase transition. It serves to prevent premature activation of cyclin E/cdk2 in early G1 and also helps with the assembly and activation of cyclin D/cdk4/6 in late G1. Therefore p27 can be thought as a positive regulator of G1 progression by this assembly and nuclear import of cyclin D/cdk4/6 [11]. It is now believed that the balance between the levels of cyclin D1 and p27 are the main regulating mechanism for controlling proliferation of cells instead of their absolute levels within the cells [15]. Nakayama *et al.*(1996) discovered that p27 knockout mice develop multiorgan hyperplasia and parathyroid tumors which further supports the role of p27 in helping control cell proliferation and differentiation [16].

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 [17, 18, 19, 20]. 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 [21]. Mitogenic growth factor signals have been shown to cause a loss of p27 levels and activity. In contrast p27 has been shown to increase in response to differentiation signals [22, 23, 24], loss of adhesion to the extracellular matrix [9, 25, 26, 27], and signalling by growth inhibitory factors such as TGF-β [17, 19, 20].

As cells exit G_0 and enter G1, p27 that is bound to cyclin E initially must be degraded and newly synthesized p27 which is held within the cytoplasm helps facilitate the assembly and nuclear import of cyclin D/cdk. As described earlier, this cyclin D/cdk complex can then phosphorylate Rb in order to begin the transition into S phase. These effects in p27 are regulated by changes in its phosphorylation [28, 29]. It has been demonstrated that p27 mRNA levels do not change throughout the cell cycle but its protein levels are regulated by translational controls [22, 30] and also by ubiquitin mediated proteolysis [31]. p27 is also predominately located within the nucleus in quiescent cells where it can act to inhibit cell cycle progression through the inhibition of cyclin E/cdk2 [32]. Pagano *et al.* (1995) demonstrated that p27 levels as well as its 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 [31]. This decrease in p27 levels observed in the transition between G_0 and S phase is due mainly to the dramatic decrease in its half-life which is six to eight times longer in the quiescent cells compared to proliferating cells [31]. Another reason for the decrease in p27 can be attributed to the nuclear export of p27 following

Phosphorylation at S10 [33]. Therefore, changes in p27 levels are due to an increase in the degradation machinery rather than an actual decrease in p27 transcription.

1.3 Regulation of $p27^{KIP1}$

The mRNA expression of p27 as previously stated, does not change through the cell cycle but instead the protein content of p27 is regulated by stabilizing phosphorylation in G_0/ early G1 and inactivating phosphorylation in late G1/ early S phase (Figure 2) [22, 30].

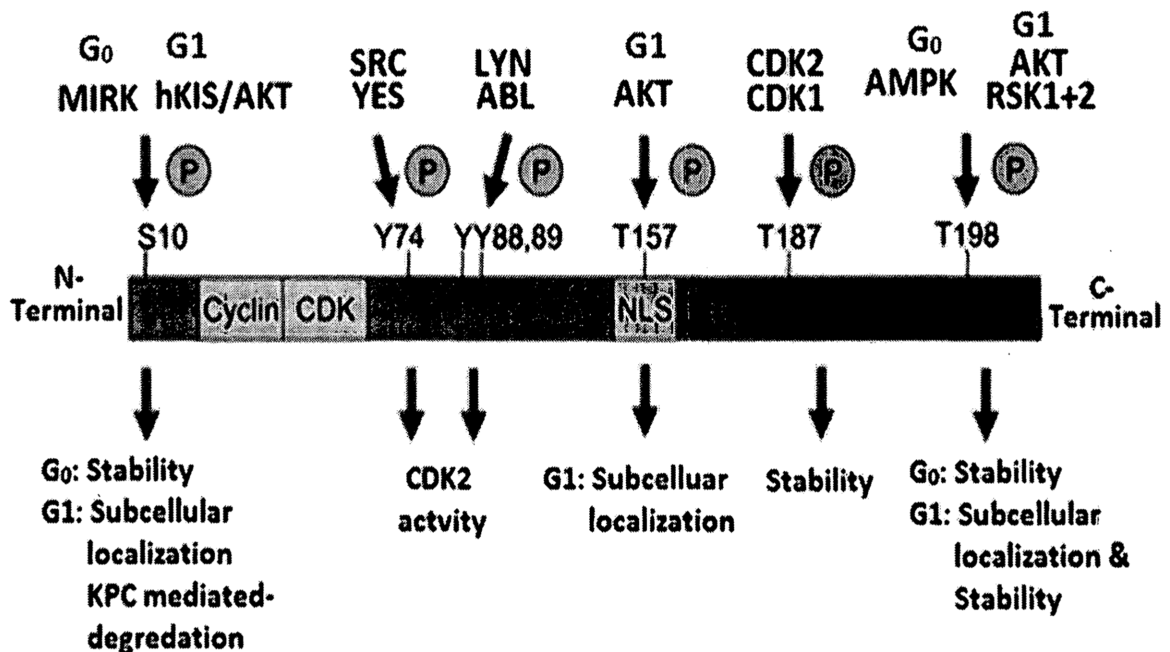


Figure 2: Summary of post translational phosphorylation sites observed in p27 with known kinases that modify the individual sites as well as functions associated with these post-translational modifications. Modified from Vervoorts et al., 2008

As described, p27 acts on the catalytic cleft of cyclin E inhibiting its interaction with Rb but recently, three tyrosine residues (Y74,Y88 & Y89) have been identified within the middle of the p27 protein which are vital for its binding with cyclin E/cdk2 via hydrophobic interactions [21]. Grimmler *et al.* (2007) discovered that these conserved tyrosine residues in the Cdk-binding domain of p27 can be phosphorylated by the SRC-family kinase and the oncogene product BCR-ABL in early G1 [21]. Once this occurs, the entire inhibitory helix of p27 is ejected from the cdk2 active site, thus partially restoring cyclin E/cdk2 activity. Once ejected, p27 undergoes other modifications which promote cytoplasmic localization, sequestration and/or proteolytic degradation [34].

One of the initial modifications of the p27 nuclear pool after ejection is in response to mitogenic dependent stimulation, such as the mitogen-activated protein kinase (MAPK), which causes p27 to undergo nuclear export dependent on the phosphorylation at serine 10 (S10) [33, 35, 36]. Human kinase interacting stathmin (hKis) and Akt/PKB have been shown to phosphorylate p27 at S10 causing its translocation out of the nucleus and into the cytoplasm [4, 37]. Due to this phosphorylation it has been discovered that p27 binds to the exportin CRM1 in early G1, allowing further G1 progression [33]. Connor *et al.* (2003) discovered that p27 contains a nuclear export signal (NES), and when mutated, causes a decrease in p27-CRM1 binding, nuclear export and p27 degradation [33]. Once in the cytoplasm in early G1, p27 is ubiquitinated and degraded by members of the KIP1-ubitquitylation promotion complex (KPC) 1 and 2 [21, 34].

This initial degradation of p27 is necessary in order to promote the loss of p27 needed to activate the cyclin E/cdk2 located in the nucleus and promote S phase entry [4, 37].

p27 proteolysis is regulated by a least two distinctly linked mechanisms with one mechanism already addressed; the KPC complex. In early G1, mitogens activate the export-linked degradation of p27 which is then followed in late G1/S phase by the cyclin E/cdk2 dependent degradation. Phosphorylation of p27 occurs on the threonine 187 (T187) by the newly assembled cyclin E/cdk2 complexes which targets the majority of p27 for ubiquitin mediated degradation [38]. Phosphorylation of T187 allows p27 to be recognized by its SCF-type E3 ligase complex which is composed of Skp1, Cul1, ROC1 and the F-box proteins Skp2 which mediates p27 ubiquitylation and degradation by the 26S proteasome [39, 40, 41]. Skp2 is the component of the F-box protein in the SCF-ubiquitin ligase that is responsible for p27 proteolysis in late G1.

Both of these independent mechanisms of p27 degradation are necessary in order to promote the loss of p27 needed in order to enter 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 3). 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 3) [32, 42].

Also important is another phosphorylation event that occurs on newly translated p27, occurring on threonine 157 (T157), phosphorylated by PKB/Akt [33]. By phosphorylating p27 at T157, this newly synthesized p27 is unable to be imported into the nucleus and is localized within the cytoplasm. As shown in figure 2, within the p27 nuclear localizing signal (NLS) there contains an Akt consensus site at T157 and once phosphorylated, it impairs p27 translocation to the nucleus. This is due to an interaction with the 14-3-3 proteins, a family of conserved regulatory molecules, that cause the T157 phosphorylated p27 to become sequestered in the cytoplasm [43]. Shin *et al.* (2005) discovered that S10 phosphorylation and CRM1 export can lead to Akt further phosphorylating p27 in the cytoplasm at T157 and the 14-3-3 protein family then impairs its interaction with importin-α [43] (Figure 3).

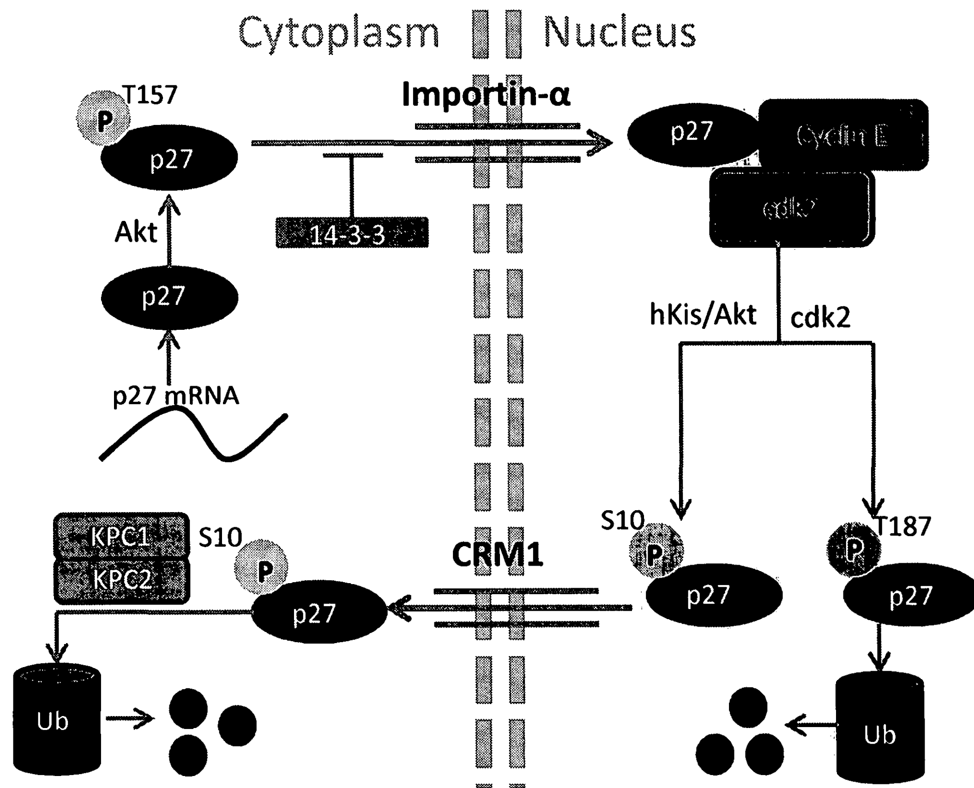


Figure 3: Summary of p27 degradation and translocation mechanisms. Phosphorylation sites on p27 with effects of hKis/Akt (S10), cdk2 (T187) and Akt (T157).

Not all phosphorylation sites on p27 cause ubiquitin-mediated degradation and/or nuclear export, as some facilitate p27 effects on cell cycle arrest and protect it from these events [44]. The most significant stabilizing phosphorylation to p27 is caused by AMP-activated protein kinase (AMPK) on threonine 198 (T198) [45, 46]. This stabilizing phosphorylation is important in quiescent and early G1 cells as this T198 phosphorylated p27 inhibits cyclin E/cdk2 leading to cell cycle arrest. Liang *et al.* (2007) discovered that in MCF7 cells, activated AMPK (pAMPK) directly phosphorylated p27 on

T198 (T198p27) [45]. This then causes cell cycle arrest by increasing the stability of the protein. Also interesting to note is that Rattan et al. (2005) demonstrated that AICAR; an AMP analog which can indirectly up-regulate AMPK through its upstream kinase, liver kinase B1 (LKB1), increases pAMPK, stabilizes p27 and p53 [47]. AICAR also inhibited the Akt pathway through its upstream activator phosphatidylinositol-3-OH kinase (PI3K), which when taken together, caused 70-80% of MCF7 breast cancer cells to undergo growth arrest in S-phase [47]. Phosphorylation on T198 increases total p27 protein half-life when compared to T198 mutated p27 cells when activated with AICAR [45]. The importance of this stabilizing phosphorylation on p27 is extremely important to cell cycle arrest and required for optimal cell cycle progression. As will be outlined in the next sections, alteration of one or more of these phosphorylation events on p27 can lead to the cell no longer being under proper cell cycle control, leading to uncontrolled proliferation and cancer.

1.4 Deregulation of the Cell Cycle in Cancer

The orchestration of the G1/S phase transition is elaborate and it is not surprising that several components of this transition can become involved in the progression of cancer. The G1/S 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 [4]. Many direct cell cycle oncogenes such as cyclin D

and cyclin E have been found to be no longer under proper function/control in many breast cancer cases as well as the tumor suppressor proteins (TSP) pRb, p53 and p27 [4].

Cyclin D1 has been discovered to be over expressed with or without gene amplification, in over 50% of breast cancers but is more prominent in estrogen receptor positive (ER+) breast cancers [48]. It has been found that cyclin D1 is required in order for mammary tumor formation by the Ras pathway, a small GTPase that acts as a potent mediator of cell growth and survival signals [49]. In early/late G1 as mitogenic signals cause an increase in Ras which in turn increases the production of cyclin D to further advance the cell cycle. These mitogenic signals, acting through different receptor tyrosine kinases or G-protein coupled receptors can not only activate Ras but also the PI3K pathway in order to stimulate cell proliferation and survival. PI3K promotes cell survival by means of Akt up regulation as it aids cdk activation by relieving two arresting constraints of the cell cycle. As previously described, Akt can phosphorylate p27 at both T157 and S10, which causes the nuclear export and ubiquitin mediated degradation of p27, respectfully. Secondly, Akt can also phosphorylate (S9) and inactivate glycogen synthase kinase 3β (GSK-3β) which acts to both directly and indirectly inhibit cyclin D [50]. GSK-3β achieves this inhibitory role on cyclin D by phosphorylating it at T286 causing its nuclear export and degradation [51]. Also, cyclin D can be inhibited indirectly by GSK-3β which has been shown to phosphorylate the transcription factor c-Myc at T58, causing it to become destabilized [52]. c-Myc induces the transcription of cyclin D as well as cyclin E and so GSK-3β can indirectly inhibit both cyclin proteins by inhibiting the upstream

transcription factor c-myc [53]. Also c-myc has also been found to be over expressed in over 50% of breast tumors and therefore losing control of c-myc leads to increased risk of breast cancer [54]. Stabilization of c-Myc is achieved by phosphorylation on S62 by MAPK which is a downstream effector of Ras [55]. All of these activations or inactivations of both Ras and c-Myc are shown in figure 4.

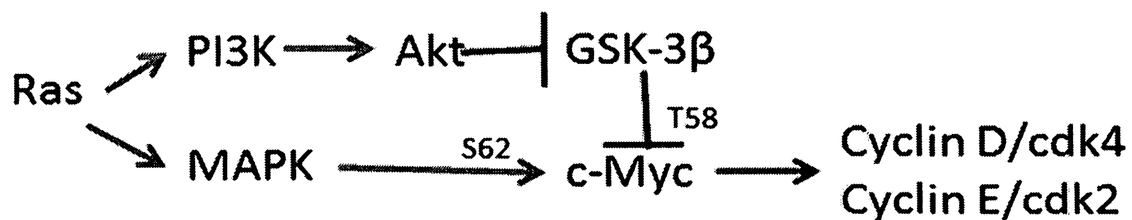


Figure 4: Summary of Ras and c-Myc activation and inactivation. Constituently active Ras or c-Myc can cause increased expression of cyclin D/E, leading to increased proliferation and tumor development.

The F-box protein Fbw7 is part of the SCF-ubiquitin ligase complex responsible for the ubiquitin mediated degradation of Ras, c-Myc abd cyclin E [56]. The loss of Fbw7 in some breast cancers leads to c-Myc activation and cyclin E stabilization which both promotes cell proliferation [56]. Therefore in many cancers, the over expression and/or amplification of either Ras or c-Myc can cause increased proliferation and cell cycle entry by increasing cyclin D and E. This increased activity of the G1/S phase cyclins promote this phase transition and can lead to tumor progression.

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

The TSP p27 is an important protein in cancer development and progression. Although p27 mutation is rare, loss of a single p27 allele occurs in some human malignancies, however silencing of the remaining allele is rare [57]. Most p27 dependent tumor issues occur thorugh accelerated proteolysis or cytoplasmic mislocalization. As stated in the previous section, increased Ras expression can cause an increase in Akt which in turn can increase both its proteolysis and cytoplasmic localization. This reduction in p27 protein levels has been shown in up to 60% of all cancers including breast [38].

p27 protein levels as well as 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 but a loss of p27 protein has frequently been demonstrated in breast cancer [58]. 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 [59]. This study was using older women with the mean age of 60 but 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 pateints under the age of 45, decreased p27 is a predictor of poor overall survival using multivariate analysis [60]. They 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

[60]. As one would expect, breast tumors with low p27 have been shown to also have high cyclin E/cdk2 activity [61]. A reduction in p27 has been found in premalignant and noninvasive ductal carcinoma in situ (DCIS), therefore appearing to precede the development of breast cancer invasion and progression [62, 63]. 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 [15]. Also it was found that at 5 years, 80% of the patients with high p27 expressing tumors were alive compared to only 50% of the patients with low p27 expressing tumors. Another study by Chappuis *et al.* (2000) showed that women with node negative breast cancer and reduced nuclear p27 was associated with a 10-fold increase in the risk of cancer relapse [64].

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. It has been reported that p27 has been found to be mislocalized within the cytoplasm in up to 40% of primary human breast cancers [65, 66, 67]. The best outcome for a breast cancer patient exist when there are high levels of exclusively nuclear p27, while the worst were those with reduced p27 levels as well as cytoplasmic localization [66]. In all cases with cytoplasmic p27 localization it has been significantly associated with Akt activation [65, 66]. The oncogenic activation of the PI3K/Akt pathway causes the Akt dependent

phosphorylation of p27 and presents an important mechanism underlying the cytoplasmic mislocalization in human cancers.

2. Obesity

The prevalence of obesity has been on the rise for the past 20 years and has more than doubled since 1980. More than 1.4 billion people worldwide are considered overweight and of that 500 million are considered obese [68]. Obesity can now be classified as an epidemic as these numbers are predicted to continue to rise.

In order to determine an individual's weight status, a worldwide index, known as the body mass index (BMI) is often used to classify adults as overweight and 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 it is just a rough guideline and does not equate the same level of "fatness" in all individuals. One fundamental flaw of BMI is it does not tell you where the fat is located. Increased obesity, particularly in the visceral (intra-abdominal) compartment, has been found to be associated with many negative health issues such as insulin resistance, hyperglycemia, dyslipidemia and hypertension [69].

Obesity in its simplest form can be thought as a prolonged state of positive energy balance. This may be due from excess dietary caloric consumption and or insufficient caloric expenditure though 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 on part an excess accumulation of white adipose tissue (WAT) which is primarily composed of adipocytes, fibroblast, macrophages, stromal cells and endothelial cells [70].

Adipose tissue is also a major site for the production of estrogen in obese postmenopausal women, most notably in tissues of the breast, abdomen, thighs and buttocks [71]. Estrogen biosynthesis is catalyzed by the enzyme aromatase (aromatase cytochrome P450) and obese postmenopausal women have been found to have increased adipose-dependent production of estrogens [72].

2.1 Link Between Obesity and Breast Cancer

From decades of research we now know that individuals who are considered obese (BMI>30) have been found to have the greatest health risks compared to their lean counterparts. An increased BMI is a risk factor for non-communicable diseases such as type 2 diabetes, cardiovascular diseases (heart disease, stroke), as well as some cancers including breast and prostate [73, 74].

For over 40 years researchers have found a statistical association that an increase in adiposity is correlated with an increased risk of cancer [75]. In 2007, reports from the American Institute for Cancer Research and the World Cancer Research Fund, predict that in 10 years obesity will overtake smoking as the leading cause of cancer [76]. Obesity causes an increased risk of breast cancer in postmenopausal women that is 30-50% greater than their lean counterparts [77]. Not only is the incidence higher in obese women but the death rate is also higher. A recent 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 [74]. Obese 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 [78]. In a meta-analysis by Harvie *et al.,* (2003), they calculated a 39% lower risk of breast cancer in postmenopausal women with the smallest waists compared with those who had the largest waists and 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 [79]. Premenopausal women also show an increase in breast cancer incidence with increases in visceral obesity while general obesity causes these increases when comparing postmenopausal women. 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 [80]. Independent of menopausal status, obese

women are more likely to suffer from metastatic breast cancer and have a poorer clinical outcome than non-obese women. These observations cannot be fully explained by alterations in sex hormone levels (estrogen) alone and therefore must be mediated by another mechanism.

There are theories as to the molecular mechanisms underlying this statistical link between obesity and breast cancer, with newer hypothesis placing adipocytes and their autocrine and endocrine functions at the forefront. Initially adipocytes were believed to be inert storage depots but we now know that they are highly active endocrine cells that secret many factors including growth factors, cytokines, extracellular matrix proteins and hormone like molecules known as adipokines [81, 82, 83]. 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 5, adipokines which are produced and secreted by the adipose tissue can enter the interstitial space and follow either two paths.

The adipokines can enter the blood stream, acting on distant targets of the body as an endocrine factor. They can also signal locally, binding to their respective membrane receptor of immediate surrounding tissue acting as a paracrine factor. This paracrine signalling is important to note as adipocytes make up the bulk of breast tissue and represent one of the most abundant cell types surrounding mammary epithelium. This could therefore play a large part in the stromal-ductal epithelial cell interactions within the mammary microenvironment [84]. This is important as many primary breast

tumors originate from ductal or intraductal epithelial cells. It is still not entirely clear how adipocytes influence breast tumor cell behaviour or whether any of the paracrine factors (adipokines) secreted by these adipocytes can change the phenotypic behaviour of malignant cells.

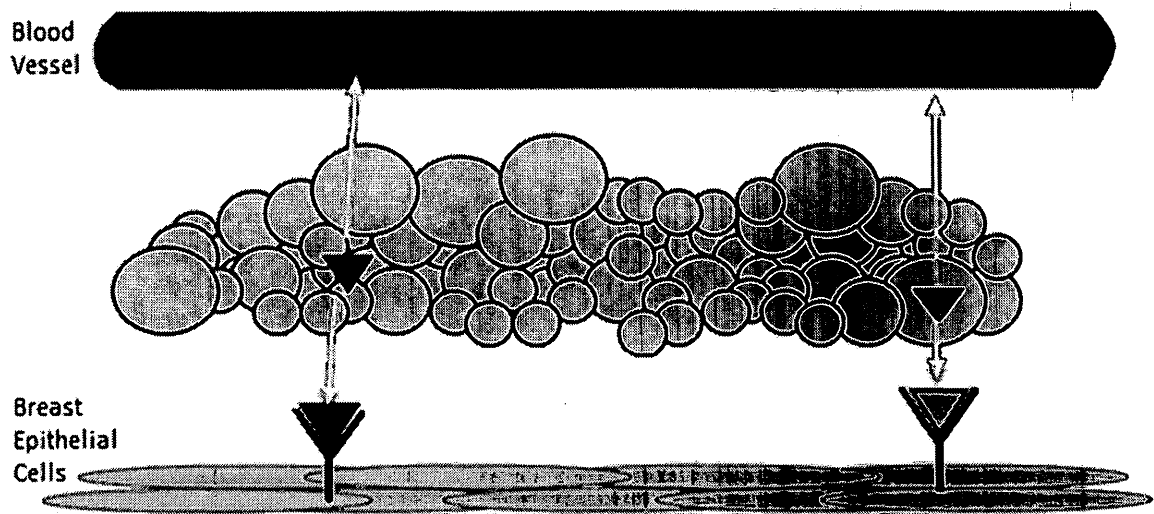


Figure 5: An example of how adipokines can be produced and secreted into the interstitial space where they can either act on a larger body wide scale through endocrine signalling or can act locally or surrounding microenvironment though paracrine signalling

2.2 Adipokines as Circulating Hormones in Breast Cancer

To date there have been over 50 different adipokines discovered that are produced and secreted by adipocytes. 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 [85, 86, 87]. With such a large

number of possible factors which possibly influence breast cancer development, two adipokines, namely adiponectin (ADIPO) and leptin (LEP) have come to the forefront of research.

One reason for this focus is that both ADIPO and LEP are 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* [84]. Interestingly, ADIPO and LEP effects on both metabolism and cell proliferation act as antagonists to one another [84, 88, 89]. LEP synthesis and plasma levels are increased with obesity and higher LEP levels have been significantly associated with increases in breast cancer incidence [90, 91]. Interestingly, LEP has also been shown to stimulate aromatase expression in MCF7 breast cancer cells which would help promote cell growth and cycling by converting androgens to estrogen [92]. ADIPO on the other hand, has the opposite effect, as levels in the serum decrease with increased obesity and an inverse relationship between serum ADIPO levels and breast cancer risk has been reported [93]. In a study observing Taiwanese breast cancer patients, Chen *et al.* (2006) discovered that the serum LEP:ADIPO ratio was significantly increased in breast cancer patients when compared to control patients without breast cancer [94]. Previous research has shown that the ratio between ADIPO:LEP is altered as a women becomes obese. The ratio calculated shows that lean women have an ADIPO:LEP ratio of 1588:1 while obese women show a significantly lower ratio of 163:1 [95].The status of the

estrogen receptor, HER2/neu or lymph node metastasis did not affect serum LEP or ADIPO levels in breast cancer patients and after adjusting for confounding factors, serum LEP and ADIPO levels were positively (r=0.323, P=0.001) and negatively (r=-0.333, P=0.001) correlated to BMI, respectively [94]. Figure 6 illustrates how the ratio between ADIPO and LEP shifts as an individual increases or decreases the size of adipose depots within the body.

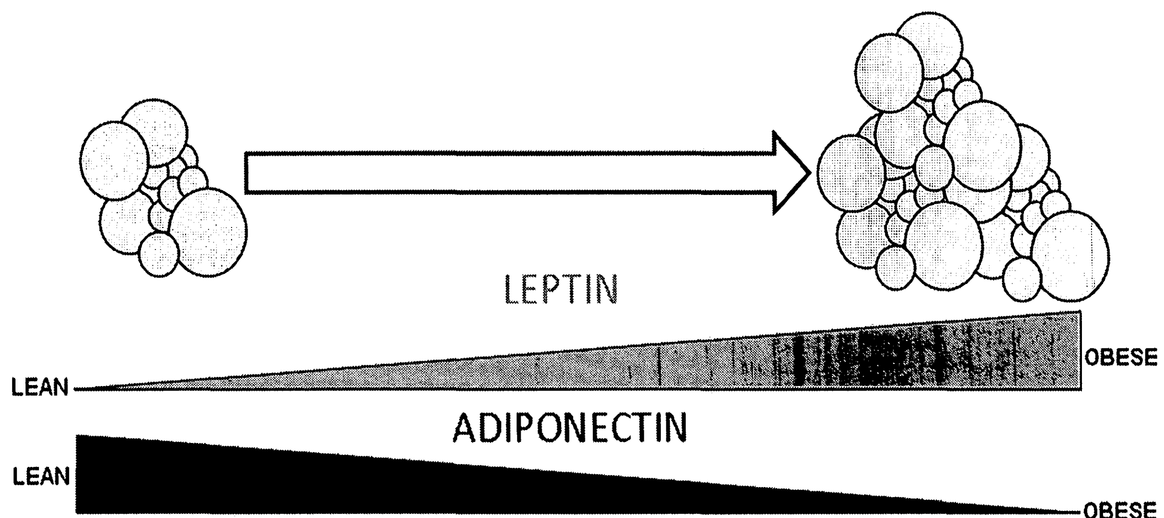


Figure 6: Relationship between ADIPO:LEP in lean and obese individuals. A lean individual has a higher ratio of ADIPO:LEP. As this individual becomes obese, the size and number of adipocytes increases while the ratio of ADIPO:LEP decreases. The ratio between ADIPO:LEP is key and not the absolute concentrations of adipokines.

2.3 Leptin (LEP)

LEP is a product of the obese (ob) gene and is a 16 kDa cytokine which was discovered in 1994 that acts through the hypothalamus as a regulator of body weight and energy balance [96]. This is achieved by inhibiting food intake and increasing energy

expenditure. In animals which have mutations in the gene encoding LEP, morbid obesity is observed. However, in humans these mutations very rarely occur and obesity is related to LEP resistance instead of LEP deficiency [97, 98, 99]. LEP mRNA has been primarily detected in both WAT and BAT, as well as a number of non-adipocyte tissues which synthesize and secret LEP, such as mammary epithelial cells [100]. The major factor which influences plasma LEP concentrations is the amount of adipose mass in humans. Circulating levels of LEP have shown a strong positive correlation with total body fat and a lesser degree with BMI [101], [90]. One important discovery was that serum LEP levels are significantly higher in women compared to men, even after adjusting for total body fat mass [102]. The negative effects of LEP can be lessened by caloric restriction and weight loss, since these cause circulating LEP levels to decline rapidly [103].

2.4 LEP Signaling and Cancer

LEP exerts its biological effects through binding to its membrane receptors. Several different receptors isoforms exist including the long form (Ob-Rb) as well as the short form (Ob-Ra) [104, 105]. Only the full length Ob-Rb has full signaling capabilities, whereas the short Ob-Ra lack major domains that are needed to recruit downstream effectors. Both the long and short isoforms of LEP have been discovered in the human breast cancer cell lines MCF7, T47D and MDA-MB-435 [106, 107].

Upon LEP stimulation, intracellular Janus tyrosine kinases (JAK2) are activated through transphosphorylation and phosphorylate tyrosine residues on the long isoform

LEP receptor and also on signal transducers and activators of transcription (STAT) proteins [108]. These phosphorylated STAT proteins can then dimerize and translocate to the nucleus where they activate numerous genes including those involved in cell proliferation. Also, LEP signaling through Ob-Rb has also been shown to activate the Ras/MAPK pathway as well as the PI3K/Akt pathway [109, 110]. From our cell cycle review we know that Ras/MAPK and Akt all increase cell cycle proliferation by affecting p27 localization or stability. Figure 7 shows the LEP signaling pathway and the events produced when bound to its transmembrane receptor Ob-Rb. LEP signaling has been shown to crosstalk with both polypeptide growth factor signaling and also with steroid receptor function. An example of this is how 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 the suppressor of cytokine signaling (SOCS3) [111, 112]. This occurs as LEP accumulates in the serum, binds to its receptor and activates the JAK2/STAT3 pathway. STAT3 can then dimerize and translocate to the nucleus where it upregulates the negative feedback gene SOCS3 which inhibits JAK2 [111, 112]. Although this LEP resistance can shut off the JAK2/STAT3 pathway, LEP signalling has been shown to continue affecting downstream proteins such as p27 even when the JAK2/STAT3 pathway is inhibited [113]. Therefore LEP appears to exhibit its cell cycle effects through another signaling pathway other than JAK2/STAT3, possibly the PI3K/Akt pathway. Also, LEP has been shown to enhance the stability of estrogen receptor α (ERα), which leads to maintenance of ER-dependent transcription in breast cancer cells, even in the

presence of antiestrogens [107]. All of these events are important in the development of breast cancer and to the understanding of why obese postmenopausal women are at such as an increased risk for breast cancer development.

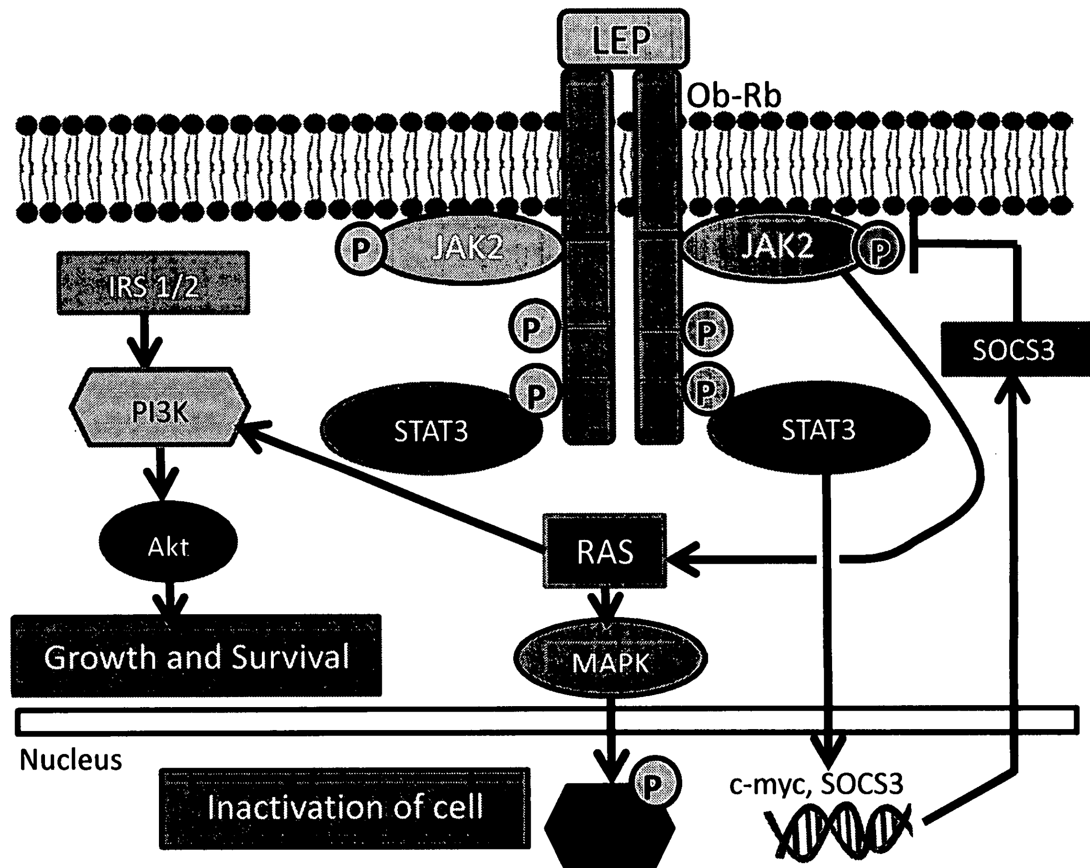


Figure 7: LEP receptor signaling. Showing how Ob-Rb stimulates a broad spectrum of intracellular signaling pathways. LEP stimulates both Akt and Ras/MAPK, both of which cause cell growth and proliferation by inhibiting cell cycle inhibitors $p27^{KIP1}$, Rb and p53. All of which once down regulated or inhibited cause cancer progression. STAT3 can also translocate into the nucleus and induce the transcription of genes including SOCS3 which acts as a negative feedback mechanism for JAK2.

LEP has been shown to act as a mitogen, transforming factor or also a migration factor for many different cell types including both normal and malignant mammary

epithelial cells [106]. Ishikawa *et al.* (2004) found that LEP was overexpressed in 92% of examined breast carcinomas but was absent when observing normal breast epithelium [114]. 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 [115, 116]. In a study by Yin *et al.* (2004), it was shown that LEP increases both cyclin D1/E by stimulating the expression of c-Myc [117]. Saxena *et al.* (2007) showed that treating MCF7 cells with LEP caused an increase in the number of cells which had entered S phase and a decrease in the number of cells which were still in G_0/G1 while also showing that LEP stimulated the growth of MCF7 cells in both a time and dose dependent manner [118]. From these studies it can be theorized that LEP causes an increasing in certain key components of the cell cycle machinery such as cyclin D/E possibly through an upregulation c-Myc. Through these affects as well as the decrease in certain key cell regulation proteins such as $p27^{KIP1}$, LEP is able to promote a positive growth environment of breast cancer cells possibly explaining why obese individuals have a higher incidence of breast cancer.

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 [119]. This suggest 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.

2.5 Adiponectin (ADIPO)

ADIPO is a 30 kDa adipocytokine which has been identified to be secreted primarily by white adipocytes and is abundantly present (2-20 μg/ml) in human plasma. Serum levels of ADIPO have been shown to be strongly inversely correlated with waist circumference and visceral fat; even more so than BMI [120]. ADIPO has also been found to have anti-inflammatory and anti-diabetic functions as well as suppression of the process of angiogenesis [121].

There are several different forms of ADIPO within the plasma that are capable of binding with varying affinity to ADIPOs receptors. Full length ADIPO exists as either a trimer known as low molecular weight (LMW), or as two larger multimers known as medium molecular weight (MMW) and high molecular weight (HMW) forms [122]. ADIPO can also be cleaved and exist in the serum as a smaller globular fragment known as globular ADIPO (gADIPO) [123]. 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.

2.6 ADIPO Signaling and Cancer

Two ADIPO receptor forms (ADIPOR1 and R2) have been cloned and shown to have unique distributions throughout the body and altered affinities for the different forms of circulating ADIPO [124]. Both receptors share a 67% sequence homology and represent the only two ADIPO receptors that have been identified to date. ADIPOR1 and R2 have been shown to exist as integral membrane proteins with an internal N-terminus and an external C-terminus [124]. This is of course opposite to the topology which is observed in G protein-coupled receptors.

gADIPO has been shown to have the highest affinity for ADIPOR1 [124], while ADIPOR2 has been shown to have intermediate affinity for both globular and full length ADIPO [125]. ADIPOR1 has been found to be most abundantly located in skeletal muscle [125], but has also been found present in healthy breast epithelial cells [126], in both invasive and pre-invasive breast cancer tissue [127] and in human adipocytes [128]. ADIPOR2 has been found located mainly in the liver while low amounts have been found in other tissue locations [125]. Important to note is that ADIPOR1 protein and mRNA have been detected in many primary breast cancer cell lines including MCF7, T47 and MDA while ADIPOR2 expression was found to be much lower [129, 130]. Furthermore, Rasmussen *et al.* (2006) have shown that ADIPOR1 mRNA expression is 10-15 times higher than ADIPOR2 in human isolated adipocytes [128]. Therefore, in my thesis ADIPOR1 functioning will be investigated exclusively due to its higher localization in both breast cancer cells and adipose tissue then ADIPOR2.

ADIPOR1 has been shown to mediate fatty-acid oxidation and glucose uptake once stimulated by ADIPO through the downstream phosphorylation-dependent activation of AMPK [131]. The activation of AMPK occurs when there is an increase in the AMP:ATP ratio. This increase in AMP then allows for AMP to bind to the γ Bateman domain causing a conformational change in AMPK exposing its catalytic domain on the α subunit [132]. With its catalytic domain exposed, the upstream AMPK kinase (AMPKK) can then phosphorylate AMPK at threonine 172 (T172) causing it to become active [132]. 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. AMPK has been shown to be phosphorylated (pAMPK, T172) with ADIPO stimulation in breast cancer cell lines, including MCF7, T47D and MDA-MB-231 [126, 129, 133, 134]. MCF7 cells treated with ADIPO were found to undergo both a time- [135, 134] and dose-dependent inhibition of proliferation [136]. ADIPO induces similar activation of pAMPK to that seen when MCF7 cells are incubated with AICAR, which causes an increase in the AMP analogue ZMP which then alters the ZMP:ATP ratio causing AMPK activation [129, 134]. From previous research in our lab in 2007, a study by Walker *et al.* found that increasing concentrations of ADIPO caused an increase in p27 while in contrast LEP showed a decrease in p27 protein [113]. This is important as the AMPK pathway assists in the stabilization of the cell cycle inhibitor p27 via a phosphorylation on threonine 198 (T198p27). AICAR has been shown to cause AMPK-dependent cell cycle arrest in a dose-dependent manner in many cancer cell lines including MCF7 cells. This in turn has been

shown to increase the amount of cells arrested in S-phase, inhibit the PI3K-Akt pathway and up-regulate the cell cycle regulator proteins p27 and p53 [47]. Besides 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 [129]. This is of course opposite to the affects observed in cell treated with LEP. The antagonism between the two adipokines and their effects on the cell cycle are determined by the amount of each within the serum, which favours LEP and cell proliferation in obese individuals. ADIPO may also inhibit the activation of nuclear factor-κB (NF-κB), a transcription factor which has been found to upregulate the pro-angiogenic factor vascular endothelial growth factor (VEGF) in breast cancer cells [137]. Figure 8 shows a simplified depiction of the ADIPO and AMPK activation in 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 [134]. The dephosphorylation of Akt by ADIPO was blocked once the AMPK inhibitor, Compound C was added to the ADIPO treatment while the use of a PP2A inhibitor, okadaic acid, caused all pAMPK effects on pAkt to be abolished. This indicates that AMPK working through PP2A could be causing the decrease in pAkt in MDA-MB-231 cells. Although this pathway has yet to be confirmed in MCF7 cells, it may be possible that ADIPO causes an increase in pAMPK, which in turn causes an increase in

T198p27 and a decrease in pAkt, both which would cause a decrease in cell proliferation.

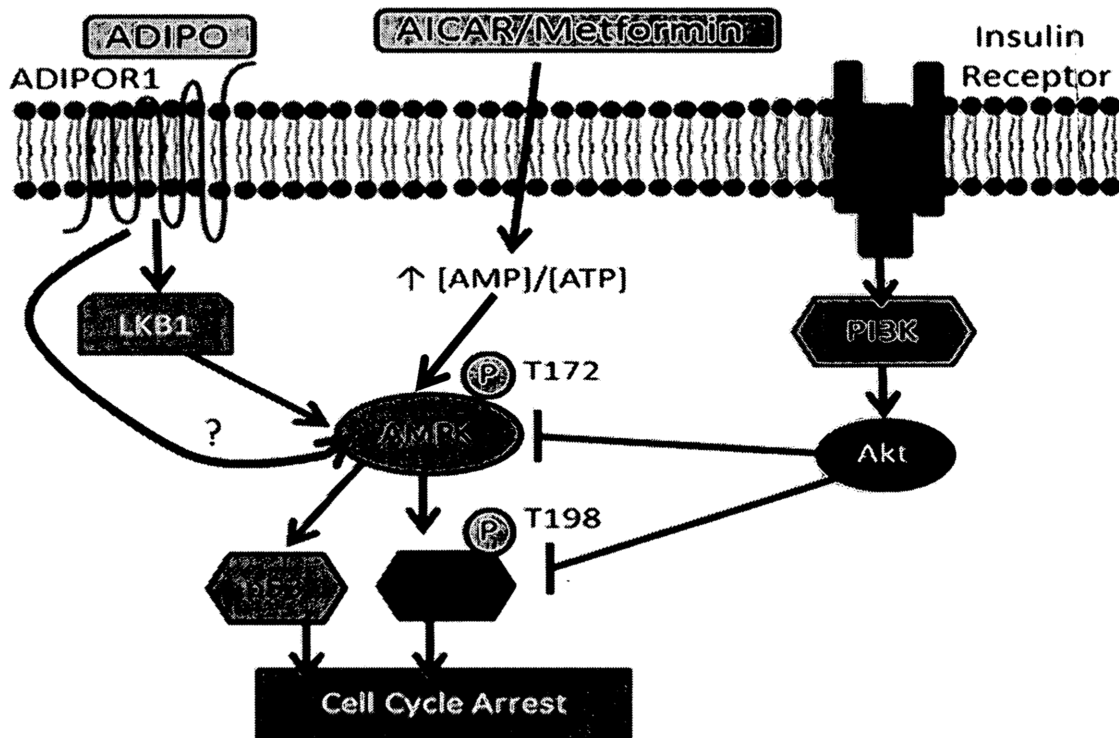


Figure 8: ADIPO and AMPK signaling. The energy sensing kinase AMPK is activated by ADIPO as well as by exercise and pharmacological agents. Once activated, AMPK phosphorylates and stabilizes p27 leading to cell cycle arrest in both normal and malignant cells.

2.7 Importance of ADIPO and ADIPOR1

As ADIPO has been shown to affect both pAMPK and the PI3K/Akt pathways, it is again not surprising that any alteration to ADIPO serum concentration or ADIPOR1

expression can lead to possible cell cycle problems. Mantroros et al. (2004) found an inverse relationship between circulating ADIPO levels and breast cancer in postmenopausal women which was independent of possible effects of insulin growth factor (IGF-1), LEP, BMI and other parameters [138].

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 [128]. In addition, the consumption of very low calorie diets (VLCD) 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 an increase expression of ADIPO in adipose tissue by 65% in humans [128]. Therefore, it can be theorized that a decrease in adiposity can increase the amount of ADIPOR1 leading to better utilization of existing ADIPO. This is important for obese women as there will be a decrease in ADIPO and ADIPOR1 while an increase in LEP, all of which have been shown to produce a positive growth environment.

At the present time, it is still unclear exactly how obesity plays a role in decreasing ADIPOR1 and how both the adipokines ADIPO and LEP play a role in this effect. The effects of ADIPO on cell cycle arrest have been shown but the effects that the adipokine has on ADIPOR1 are still to be uncovered. Examining the role of ADIPOR1 signalling and functioning in breast cancer cells I believe, may play an intricate role in the negative effects which are observed in obese breast cancer patients and the positive protective effects observed in lean individuals. Therefore, my thesis will look at the role

ADIPOR1 signaling plays in cell cycle regulation as well as how the receptor responds in an obese environment and how this plays a role in the regulation of proliferation in breast cancer cells.

3. Study Objectives

1. To determine the effects of the adipokines ADIPO and LEP on ADIPOR1 protein expression and downstream signaling molecules in MCF7 breast cancer cells.
2. To determine if an upregulation of ADIPOR1 in MCF7 cells can counteract the negative effects on cell cycle proteins observed with LEP and in co-culture with obese adipocytes.
3. To determine if any difference occurs between a chow diet (CD) and a high fat diet (HFD) in rats causes changes in adipocytes and ADIPOR1 protein content in both non-transfected MCF7 cells and ADIPOR1 transfected cells.

4. Hypothesis

1. LEP causes a decrease in ADIPOR1 through an upregulation of pAkt in MCF7 cells.
2. The ratio between ADIPO:LEP (rather than the absolute concentrations of each adipokine) is important to the stability of ADIPOR1
3. The 14-3-3 protein plays a protective role in ADIPOR1 protein stability protecting it from LEP induced destruction.
4. Upregulating ADIPOR1 protein expression in MCF7 cells will lessen the negative effects on cell cycle entry with LEP treatment, while increasing the cell cycle inhibitory effects of ADIPO treatment.
5. Adipocytes from HFD rats will deregulate the cell cycle in MCF7 cells and these effects will be tempered by overexpressing ADIPOR1.

5. Materials and Methods

Cell Line and Cell Culture. The human breast cancer cells, MCF7, were obtained from a pleural effusion and purchased from the American Tissue type Culture Collection (ATCC, Manassas, VA). These cells were maintained routinely in Alpha Modification of Eagle's Medium (AMEM, Wisent, St.Bruno, QC), 10% fetal bovine serum (FBS, Hyclone, Thermo Fisher Scientific, Whitby, ON), 2% anti-micotic/anti-biotic (Wisent), 1% 100mM sodium pyruvate (Sigma, Oakville, ON), 1% non-essential amino acids (Sigma), and 10 μg/ml insulin from human pancreas (Wisent) at 37°C and 5% CO_2. For arrested experiments, the cells were plated in AMEM for 48 hours, then media was aspirated, and the cells were washed twice with phosphate buffered saline (PBS, Wisent) and maintained in Improved Modification of Eagle's Medium without phenol red (IMEM, Wisent), 2% dextran-charcoal treated fetal bovine serum (c-FBS, Hyclone, Thermo Fisher Scientific, Whitby, ON), 2% anti-micotic/anti-biotic(Am/Ab) for 48 hours at 37°C and 5% CO_2.

Cell Growth. MCF7 cells were plated in 6 well plates in AMEM for 24 hrs. At approximately 70% confluence, cells were either maintained in AMEM (cycling experiments) or IMEM (arrested experiments) and treated with various concentrations (3-24 nM) of human globular ADIPO (Peprotech, Rocky Hill, NJ), or (25-200 nM) of human recombinant LEP (Peprotech) for 24 hrs. In co-culture experiments, cells were exposed to concentrations of either ADIPO (9 or 18 nM) or recombinant LEP (150 or 300

nM) for 24 hrs. The use of a pharmacological Akt inhibitor (1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-*sn* glycerocarbonate, EMD-Millipore, Billerica, MA) was used to look at Akt involvement in cell cycle signalling using various concentrations (1-10 nM) of the Akt inhibitor for 24 hrs. DMSO was added to cells not treated with Akt inhibitor as a vehicle control. In immunoprecipitation experiments, additional pharmacological treatments were done including estrogen (10 nM, Sigma), the AMPK agonist AICAR (5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, 1mM, Toronto Research Chemicals, Toronto, ON) and the AMPK inhibitor Compound C (AMPK inhibitor, 1mM, Millipore)

Cell Harvesting. The 6 well plates were aspirated to remove media and washed twice with cold PBS. Cells were collected using a 25 cm cell scraper and transferred into 1.7 mL Eppendorf tubes with 1 mL of cold PBS. Cells were centrifuged at 2300 rcf at 4°C for 10 minutes. The supernatant was discarded and the pellet was then re-suspended in TENT++ (0.2% Tent [TRIS, EDTA, NaCl, and 0.2% Triton x-100], Sigma protease inhibitor cocktail and Sigma phosphatase inhibitor cocktail 2). Samples were then sonicated for 5 seconds at 10% power and centrifuged for 10 minutes at 15700 rcf. The supernatant lysate was then extracted and stored at -84°C.

Western Blotting and Immunoprecipitation. The activation of ADIPO and LEP signalling pathways as well as expression of ADIPOR1 and $p27^{KIP1}$ were assessed by Western blotting or immunoprecipitation followed by Western blotting. The sample protein concentrations were determined by using a Bradford Assay (BioRad). 25 μg of

protein was loaded onto a 12% SDS polyacrylamide denaturing gels (PAGE) and proteins were separated and then transferred onto a PVDF membrane (Bio Rad, Mississauga, ON) at 4°C overnight. Blots were stained with amido black to determine protein transfer efficiency. Membranes were blocked for 2 hrs in 10% low fat milk. Blots were then washed with TBST (Tris-Buffered Saline with 0.5% Tween 20, TBST) for 4X10 min intervals and membranes were subsequently incubated with specific primary antibodies (Abs) at 4°C overnight. Following the primary incubation the membranes were washed with TBST for 4X10 min intervals. Next, membranes were incubated with specific secondary Abs (in 5% low fat milk) for 1 hr and then washed again in washed with TBST for 4X10 min intervals. Proteins of interest were then visualized by ECL Chemiluminescent HRP Substrate (Millipore) using the Kodak *In vivo* FX Pro Imager to detect the chemiluminescent signal which was quantified using Carestream imaging software.

For immunoprecipitations, 250 μg of protein lysates in TENT++ were incubated with primary antibodies for goat polyclonal anti-ADIPOR1 (1:140, Santa Cruz Biotech, Santa Cruz, CA) or rabbit polyclonal anti-$p27^{Kip1}$ (1:140, Santa Cruz Biotech) at 4°C for 1 hr. Then the antigen/Ab complexes were precipitated with IP/WB Optima B goat IP matrix (Santa Cruz Biotech) for AipoR1 or rabbit IP matrix (Santa Cruz Biotech) for $p27^{KIP1}$ at 4°C overnight. The immunoprecipitated proteins were washed three times with TENT buffer, separated on a 12% SDS PAGE gel, and processed by Western blotting.

Antibodies for Western Blotting and Immunoprecipitation. Proteins of interest were studied by Western blotting with primary Abs for mouse monoclonal anti-p27^{Kip1} (1:3000, BD Biosciences), rabbit polyclonal anti-T198p27^{KIP1} (1:2000, R&D Systems, Minneapolis, MN), rabbit polyclonal anti-p-Akt (1:500, Cell Signaling), rabbit polyclonal anti-Akt (1:3000, Cell Signaling), rabbit polyclonal anti-p-AMPK^{T172} (1:1000, Cell Signaling), rabbit polyclonal anti-AMPK (1:2000, Cell Signaling), goat polyclonal anti-ADIPOR1 (1:2000, Santa Cruz Biotech) and mouse monoclonal anti-β-actin (1:50000, Abcam, Cambridge, MA) for 24 hrs at 4°C. Secondary Abs (1:5000, rabbit, mouse or goat, Promega, Madison, WI) or β-actin (1:20000, mouse, Promega), were incubated with the membrane at room temperature in 5% low fat milk for 1 hr. All protein levels have been corrected with β-actin, to account for any errors due to uneven loading.

ADIPOR1 was immunoprecipitated to determine the amount of 14-3-3 bound. In this assay, 14-3-3 was detected using primary Ab monoclonal anti-pan 14-3-3 (1:1000, Santa Cruz Biotech) and ADIPOR1 immunoprecipitated using goat polyclonal anti-ADIPOR1 (1:2000, Santa Cruz Biotech). The amount of 14-3-3 bound p27^{KIP1} was also assessed using primary Ab mouse monoclonal anti-pan 14-3-3 (1:1000 Santa Cruz Biotech) and p27^{KIP1} levels were measured using mouse monoclonal anti-p27^{KIP1} (1:30000, Santa Cruz Biotech).

ADIPOR1 transfection was assessed by using a rabbit polyclonal myc tag (1:1000, Cell Signaling) and goat polyclonal anti-AdipoR1 (1:2000, Santa Cruz Biotech).

ADIPOR1 cDNA amplification. 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 myc-DDk flag tag. Transformed cells containing ADIPOR1 were streaked on LB bacterial plates supplemented with 25 µg/ml kanamycin (KAN), as per manufacturer recommendations. Streaked LB/KAN plates were stored at 37°C overnight. Colonies were selected and added to 500 mL LB/KAN liquid cultures and shaken at 37°C overnight. Liquid LB/KAN cultures were centrifuged for 15 min at 4°C and bacterial cells resuspended in 20 mL STE (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetrtraacetic acid (EDTA) pH 8.0). Plasma DNA was then purified by centrifugation through a cesium chloride gradient overnight at 100,000 rcf. DNA was quantified using UV spectrophotometry.

ADIPOR1 transfection and production of stable MCF7 cell lines. Following isolation and purification, ADIPOR1 cDNA (5 µg) was transiently transfected into MCF7 cells using ExGen 500 *in-vitro* transfection reagent (Fermentas, Burlington, ON). As per manufacturer's guidelines, 10.9 µl of ADIPOR1 cDNA was diluted in 589 µl 150 mM NaCl. The 16.45 µl of ExGen 500 was added to diluted cDNA and immediately vortexed for 10 sec. The mixture was then incubated at room temperature for 10 min and 600 µl was added to three 10 cm plates of proliferating MCF7 cells. Plates were then incubated at 37°C for 24 hrs upon which they were treated with the selection antibiotic G418 sulfate (400 µg/ml, Multicell), as transfected plasmids carried a G418 resistance gene. The concentration of G418 was determined by a killing curve with non-transfected MCF7

cells. A transfection using p27-YFP was also performed on separate MCF7 cells, in order to determine transfection efficiency. This was achieved by adding 6.6 µl of p27-yFP cDNA, diluted in 593 µl of 150 nM NaCl and 16.45 µl of ExGen 500 to MCF7 cells. Over the period of 4-10 days, MCF7 colonies remaining after G418 selection were picked using a microscope and a 200 µl pipette tip. Colonies were then immediately transferred to a 24 well plate in AMEM. After 24 hrs the 24 well plates were subjected to continuation of G418 selection at 200 µg/ml to allow growth but maintain selection pressure. As viable colonies grew, they were subsequently trypsanized and reseeded on a 6 well plate and later to 10 cm plates. Once confluence reached ≈70% on a 10 cm plate, transfected ADIPOR1 MCF7 cells were tested for myc tag expression and ADIPOR1 expression. From this, two viable stable ADIPOR1 transfected MCF7 cell lines were selected.

Lean and Obese Animal Models. All animal experiments were approved by the York University Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines. Five (age 10 weeks) male Sprague-Dawley rats and eight (age 3 weeks) male Sprague-Dawley rats were purchased from Charles River Laboratories (Montreal, QC, Canada). Only male rats were selected for this experiment because female rats have differences in reproductive hormones (estrogen) throughout their ovulatory cycle that can affect MCF7 cell growth (Lee et al., 2005). All animals had a 7 day habituation period to a 12 hr light-dark cycle (lights on at 08:00 hours and lights off at 20:00 hours) in a temperature (22-23°C) and humidity (50-60%) controlled room. The

5 rats received at 10 weeks age were given a normal chow diet (ad libitum) for the 7 day habituation period (housed in groups of 2) after which they were sacrificed (mean body weight 363.36 g ±4.357 SEM), while the 8 rats received at 3 weeks age were fed a high fat diet (60% HFD) for 7 weeks (housed in groups of 2), after which they were sacrificed (mean body weight 588.84 g ±12.36 SEM). The lean animal group is considered the chow-diet (CD) control animal group. Upon time of sacrifice, all animals were 11 weeks old. The 60% HFD was comprised of 60% fat, 21% carbohydrate, 18% protein and 5.1 calories/g ad libitum (Harlan Laboratories, Madison, WI). The composition of the fat in the HFD was as follows: saturated: 37%; monounsaturated: 47%; polyunsaturated: 16%. The normal chow diet was comprised of 14% fat, 54% carbohydrate, 32% protein and 3.02 calories/g, ad libitum. Food intake and body weight were measured every three days.

Adipose Tissue Collection and Conditioned Media. Visceral (epididymal) adipose tissue was harvested from both CD and HFD Sprague-Dawley rats at 11 weeks old. The epididymal fat was then minced with scissors and cultured in 10 cm plates with Medium 199 (Sigma-Aldrich, Oakville, Ontario) and supplemented with 2.5 nM dexamethasone (Sigma-Aldrich, Oakville, Ontario), 200 pM insulin (Humalog, Lilly, Toronto, Ontario) and 1% antibiotic/antimycotic (Wisent, ST-BRUNO, Quebec) for 24 hours in an incubator at 37°C with 5% CO_2. After 24 hrs, the media was switched to AMEM (Multicell) and again left for 24 hrs at 37°C with 5% CO_2, after which this new conditioned media was then strained of adipose tissue and stored at -84°C. All conditioned media was created by

Yaniv Shpilberg, but all subsequent MCF7 experiments using the conditioned media were done myself.

Normal MCF7 cells and the two ADIPOR1 transfected MCF7 cell lines were seeded in a 6 well plate with AMEM and left to seed for 24 hrs. The wells were then washed with warm PBS and conditioned media from either lean or obese animals was added for 24 hrs. Unconditioned media plates were also used as controls. Both conditioned and unconditioned media plates were untreated or incubated with 18 nM of ADIPO or 300 nM of LEP for a 24 hrs.

Statistical Analyses. All values are expressed as means ± SEM of four to eight separate experiments and statistical analysis was performed using a one-way ANOVA with Tukeys Multiple comparisons test used when significance found. A two-way ANOVA with Bonferroni post-test comparisons used when significance found was used for both CM and FBS co-culture experiments. Statistical analyses of treatments where $p \leq 0.05$ were considered to be significantly different.

6. Results

6.1 ADIPO affects pAMPK, $pAkt^{T308}$, p27 and ADIPOR1 in MCF7 breast cancer cells

ADIPO has been shown in previous studies to be decreased in obese breast cancer patients leading to a poorer prognosis[96] so we wanted to look at increasing concentrations of ADIPO to evaluate the effects on key cell signaling pathways (pAMPK and $pAkt^{T308}$), their downstream cell cycle regulator targets (p27) as well as ADIPOR1. AMPK was found to be activated via phosphorylation at residue 172 in a dose-dependent manner with ADIPO treatment. There was a significant increase seen with an ADIPO concentration of both 9 nM and 24 nM displaying a 2.5-fold increase in pAMPK (Figure 9 A,B). The opposite effects were observed for $pAkt^{T308,}$ with a negative dose-dependent effect evident with ADIPO treatment. ADIPO caused significant decreases in $pAkt^{T308}$ by 40% at 12 nM and 60% at 24 nM (Figure 9 A,C). AMPK and Akt total protein content does not change with increasing concentrations of ADIPO (Figure 9 A). ADIPO also caused a 1.6-fold increase in p27 (Figure 9 A,D), which was mirrored by a 1.5-fold increase in p27 phosphorylated at T198 by AMPK (Figure 9 A,E). Both total and T198 p27 attain significance with 12 nM ADIPO treatment, and this persists as ADIPO concentrations increase to 24 nM. ADIPO also caused a significant increase in ADIPOR1 (Figure 9 A,F). As little as 9 nM ADIPO elicited a 1.7-fold increase in adipoR1 protein and continued to increase up to 2.0-fold above untreated cells with 12 nM. These experiments were also used to determine the optimum concentration of ADIPO to be

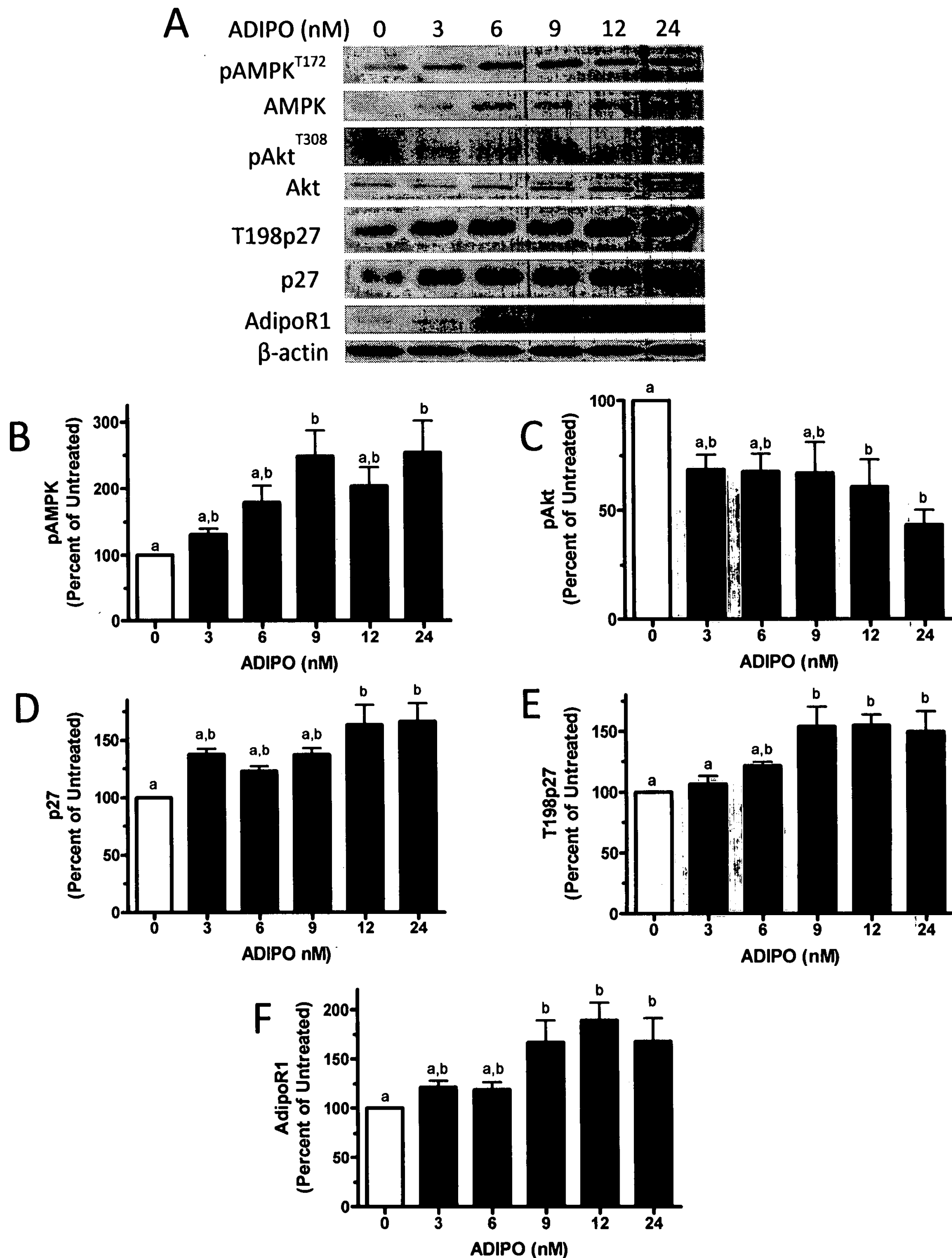


Figure 9: ADIPO 24 hr treatment effects on (B) pAMPK, (C) pAkt, (D) p27, (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Letters which differ represent a significant difference ($p<0.05$). n=6/group

used for future studies. A concentration of 9 nM and a mid-point between 12-24 nM (18 nM) was used for all other experiments.

***6.2** LEP affects pAMPK, pAkt^{T308}, p27 and ADIPOR1 in MCF7 cells*

LEP has been shown to be increased in obese breast cancer patients[96]. We determined the effects of increasing LEP concentrations on activating AMPK and Akt and determined the downstream ramifications on p27 and ADIPOR1. LEP caused a dose-dependent decrease in pAMPK becoming significant at 200 nM with a 50% reduction (Figure 10 A,B). In contrast LEP induced the opposite effects on pAkt^{T308} with increases of 2.2- and 2.6-fold at 150 nM and 200 nM, respectively (Figure 10 A,C). These results were not due to an increased total protein levels as neither changed with LEP treatment (Figure 10 A). p27 was found to be significantly lower at only 25 nM LEP by 30% and p27 levels remained low as LEP concentrations increased (Figure 10 A,D). The largest reduction in p27 was 35% at 200 nM LEP. Virtually identical results were observed for T198p27. As little as 25 nM LEP caused a significant 30% decrease in protein levels, which remained this same level with increasing concentrations of LEP (Figure 10 A,E). LEP appeared to decrease the protein level of ADIPOR1 (p=0.70) with increasing concentrations of LEP reaching an average of 73% of the level in untreated cells (Figure 10 A,F). From these experiments it was determined that the concentrations of 150 nM and 300 nM will be used in future experiments.

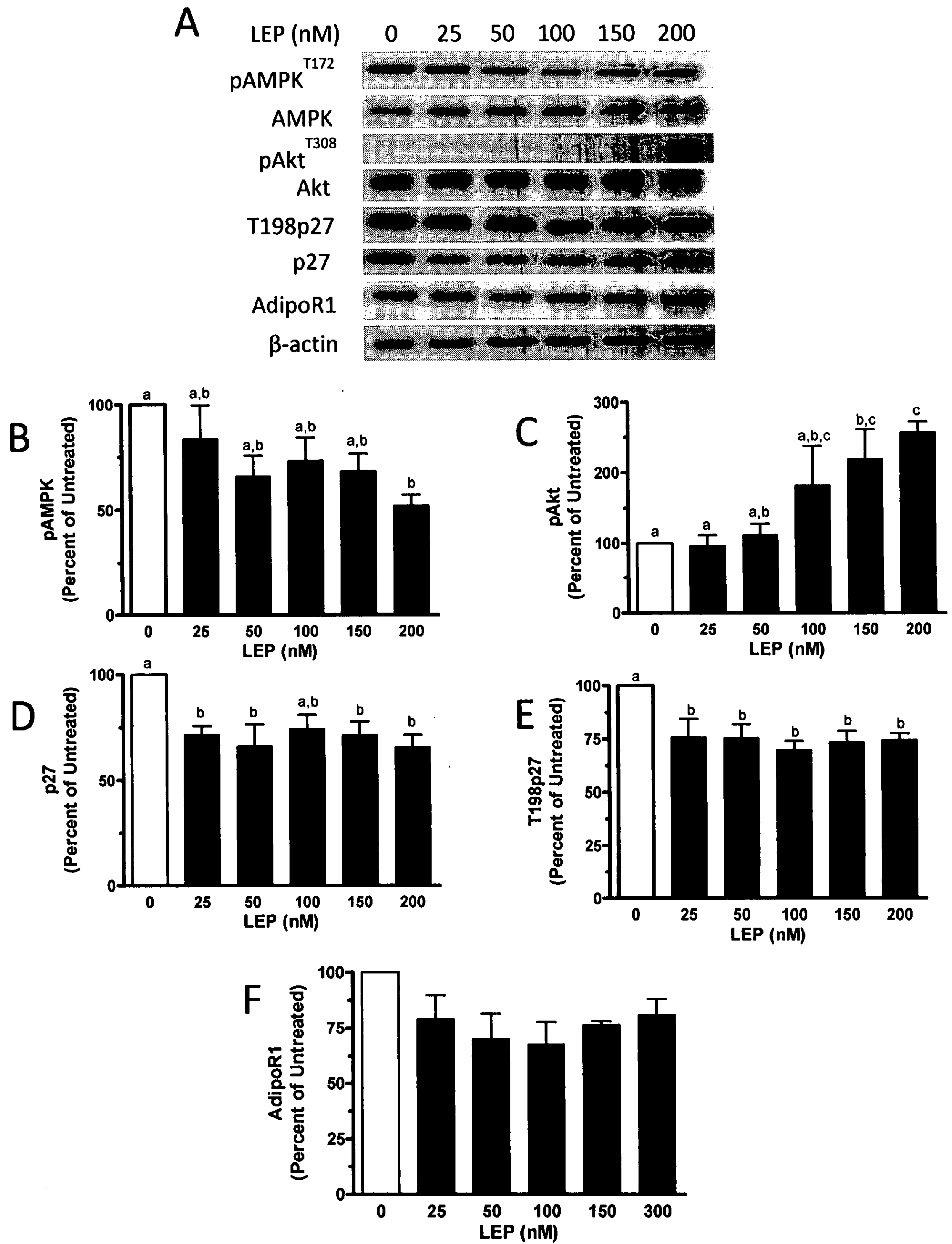


Figure 10: LEP 24 hr treatment effects on (B) pAMPK, (C) pAkt, (D) p27, (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Letters which differ represent a significant difference ($p<0.05$). n=6/group

***6.3** ADIPO antagonizes the effects of LEP on pAMPK, pAkt^{T308}, p27 and ADIPOR1 protein levels*

Next we wanted to co-incubate MCF7 cells with ADIPO and LEP to observe any antagonism between these two adipokines on AMPK and Akt signaling, in addition to any effects on p27, T198p27 and ADIPOR1 protein levels. As shown in figure 9, ADIPO significantly increased pAMPK, p27, T198p27 and adipoR1 protein while decreasing pAkt^{T308}. In contrast, LEP significantly increased pAkt^{T308}, while decreasing pAMPK, p27, T198p27.

First we looked at pAMPK and again found at a LEP concentration of 300 nM there was a significant 30% decrease in protein while at ADIPO concentrations of 9 and 18 nM there was a 1.4-fold increase in pAMPK. These alterations amount to a 1.7-fold higher pAMPK in ADIPO treated cells above those treated with LEP (Figure 11 A,B). With co-treatment ADIPO was able to counteract the inhibitory effects of LEP on pAMPK, even at concentrations as high as 300 nM LEP. Even in the presence of up to 300 nM LEP, pAMPK levels were not significantly different from cells treated with ADIPO alone, while they were significantly higher than cells treated with 300 nM alone. In response to 150 nM and 300 nM LEP pAkt^{T308} levels increased by 1.6 and 1.7-fold, respectively (Figure 11 A,C). ADIPO caused a significant 30% decrease in pAkt^{T308} at 18 nM as previously shown while with co-treatment, ADIPO cause pAkt^{T308} protein levels to not be significantly different then untreated. Thus, there was a 2.0-fold difference between pAkt^{T308} levels in ADIPO and LEP treated cells. LEP increased pAkt^{T308} in co-treatment

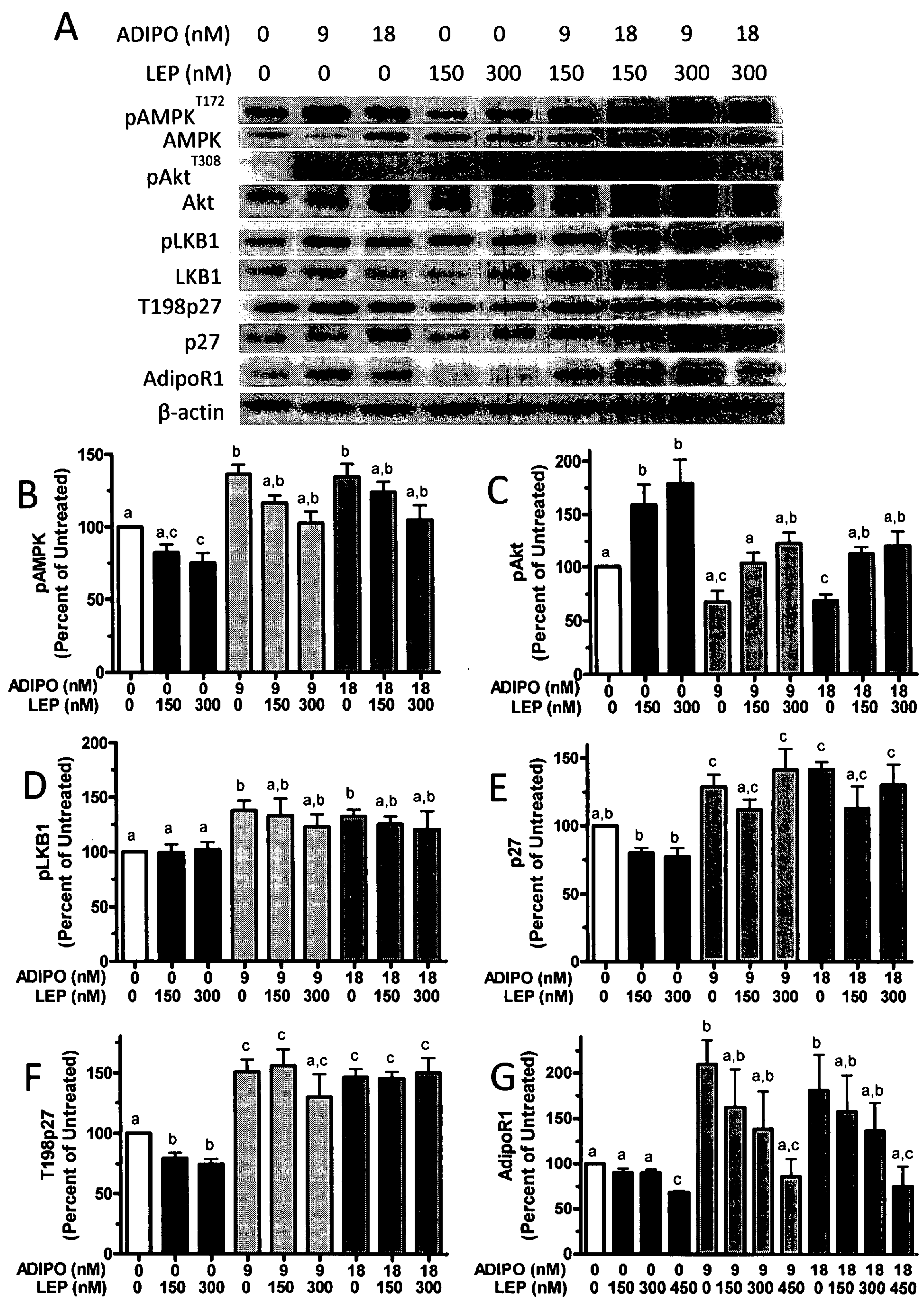

A
ADIPO (nM) 0 9 18 0 0 9 18 9 18
LEP (nM) 0 0 0 150 300 150 150 300 300
pAMPK T172
AMPK
pAkt T308
Akt
pLKB1
LKB1
T198p27
p27
AdipoR1
β-actin
B
pAMPK (Percent of Untreated)
C
pAkt (Percent of Untreated)
D
pLKB1 (Percent of Untreated)
E
p27 (Percent of Untreated)
F
T198p27 (Percent of Untreated)
G
AdipoR1 (Percent of Untreated)
ADIPO (nM)
LEP (nM)

Figure 11: ADIPO & LEP 24 hr co-treatment in untreated (open), LEP (black), ADIPO 9 nM co-treatments (stripped) and ADIPO 18 nM co-treatments (grey) with effects on (B) pAMPK, (C) pAkt, (D) pLKB1, (E) p27 and (F) T198p27 and (G) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Graphs have been rearranged to better show trends. Letters which differ represent a significant difference ($p<0.05$). n=6/group

experiments but was unable to restore $pAkt^{T308}$ to levels similar to cells treated with LEP alone, even with the addition of 300 nM LEP. pLKB1 was measured to see if the upstream activator of AMPK may be changing with ADIPO and LEP co-treatment. LEP had no effect on pLKB1 protein levels while ADIPO did cause a 1.4-fold increase (Figure 11 A,D). With co-treatment LEP attenuated the increase in pLKB1 proteins level such that they were not different from untreated and LEP treated cells. ADIPO also caused a 1.40-fold increase in p27 protein levels compared to untreated cells and was able to resist LEP-dependent reductions in p27, even at a maximum LEP concentration of 300 nM (Figure 11 A,E). These effects on total p27 were paralleled by changes in T198p27, as expected, with a 1.75-fold difference in T198p27 between ADIPO and LEP treatments. When co-treated with ADIPO and LEP, ADIPO was able to maintain elevated T198p27 protein levels in the presence of both 150 nM and 300 nM LEP. Lastly, we measured ADIPOR1 protein levels and demonstrated that ADIPO caused ADIPOR1 to increase to levels that were 2.35-fold higher than those in LEP treated cells (450 nM) (Figure 11 A,G). With co-treatment, LEP caused a dose-dependent decrease in ADIPOR1 in the presence of 9 or 18 nM ADIPO. Unlike the other proteins measured, LEP was able to completely antagonize ADIPO-dependent increases in ADIPOR1 protein levels.

Experiments using even higher LEP concentrations (450 nM) revealed that at this high concentration LEP was able to completely overcome ADIPO effects on ADIPOR1 protein levels at both 9 and 18 nM ADIPO concentrations.

***6.4** Akt Inhibition mimics the effects of ADIPO in MCF7 breast cancer cells*

MCF7 cells were treated with an Akt inhibitor to see whether Akt inhibition can mimic the effects of AMPK activation using ADIPO. Akt inhibition caused effects similar to ADIPO on pAMPK, $pAkt^{T308}$, p27 and ADIPOR1. pAMPK displayed dose-dependent increases reaching statistical significance (1.60-fold) at an Akt inhibitor concentration of 10 nM (Figure 12 A,B). As expected, the Akt inhibitor caused dose-dependent decreases in $pAkt^{T308}$ reaching levels that were 70% lower than those in untreated cells at 10 nM (Figure 12 A,C). Akt inhibition caused a 1.8-fold increase in p27 protein levels at 10 nM treatment (Figure 12 A,D), while T198p27 demonstrated similar effects with a 1.5-fold increase at 10 nM. ADIPOR1 also demonstrated increased protein levels following treatment with the Akt inhibitor. Treatment with only 4 nM induced a 2.10-fold increase in adipoR1 (Figure 12 A,F) and protein levels continued to rise reaching a maximum of 2.55-fold at 10 nM. These experiments illustrate the powerful effect that Akt inhibition has on ADIPOR1 protein levels in a manner similar to ADIPO treatment.

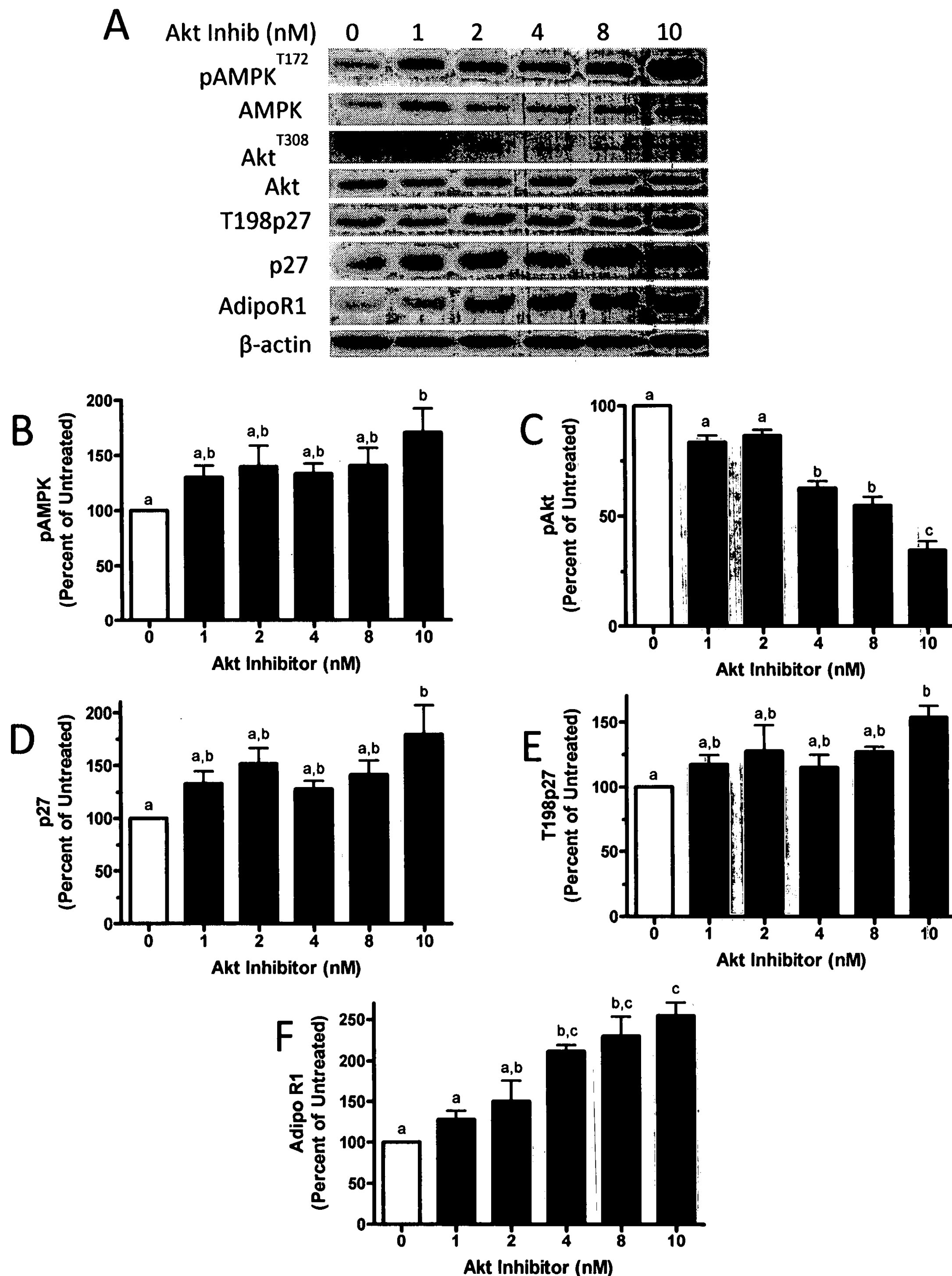


Figure 12: Akt inhibitor 24 hr treatment effects on (B) pAMPK, (C) pAkt, (D) p27, (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Letters which differ represent a significant difference ($p<0.05$). n=6/group

6.5 *Akt inhibition and ADIPO elicit additive effects on $pAkt^{T308}$ and p27 in MCF7 cells*

Since ADIPO and Akt inhibition lead to similar increases in pAMPK, p27 and T198p27, we wanted to observe whether co-treatment would lead to additive or synergistic effects on these protein levels. ADIPO (18 nM) increased pAMPK 1.75-fold while co-treatment of 18 nM ADIPO and 2 nM Akt inhibitor resulted in no further increases above ADIPO alone (Figure 13 A,B). Next we looked at the co-treatment on $pAkt^{T308}$. ADIPO decreased $pAkt^{T308}$ 30% and the addition of an Akt inhibitor resulted in a further decrease to 50% of the levels in untreated cells (Figure 13 A,C). As previously shown, ADIPO increased p27 1.5-fold, with an even greater increase evident with co-treatment, reaching levels that were 2.0-fold above those in untreated cells (Figure 13 A,D). ADIPO also caused a 1.4-fold increase in T198p27 but no further increase was observed with co-treatment (Figure 13 A,E). Lastly, ADIPO increased ADIPOR1 protein by 1.6-fold but the addition of an Akt inhibitor showed no further increases in ADIPOR1 protein levels (Figure 13 A,F).

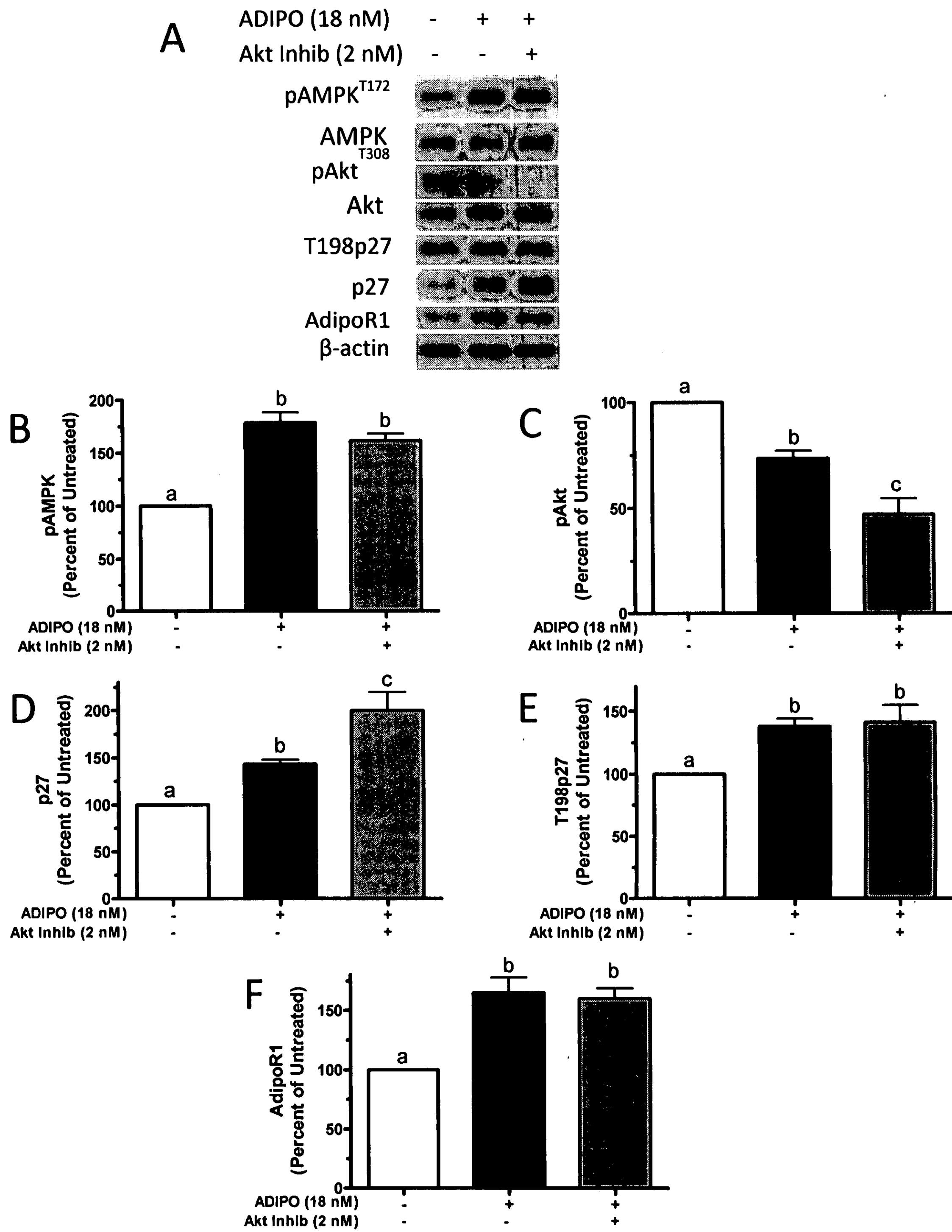


Figure 13: ADIPO & Akt inhibitor 24 hr treatment effects on (B) pAMPK, (C) pAkt, (D) p27, (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Letters which differ represent a significant difference ($p<0.05$). n=6/group

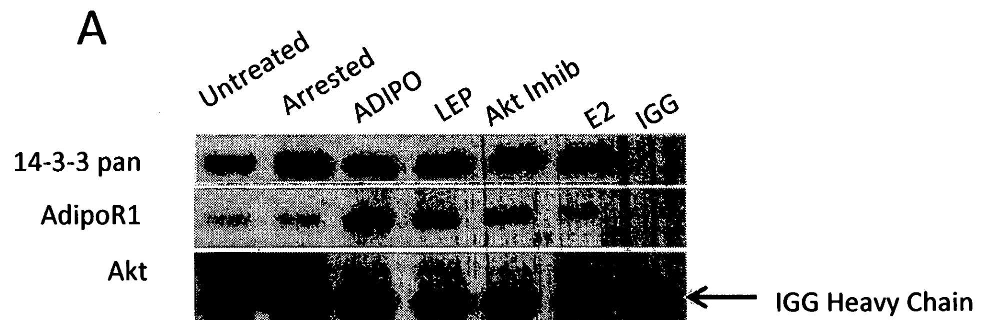
6.6 ADIPOR1 binds to 14-3-3 and Akt

Since ADIPOR1 contains a 14-3-3 and Akt consensus binding site at T68 I evaluated whether 14-3-3 and Akt did indeed interact with ADIPOR1 by immunoprecipitating ADIPOR1. The justification for this experiment is that LEP causes an increase in pAkt which may decrease the stability of ADIPOR1. Lysates from arrested MCF7 cells showed a 1.7-fold increased interaction between 14-3-3 and ADIPOR1 compared to untreated cells (Figure 14 A,B) This binding was higher than that observed for all other conditions except for the 18 nM ADIPO treatment. ADIPOR1/14-3-3 binding was evident in all other conditions but there was no significant difference in binding between conditions. In addition to a 14-3-3 binding site, there is also a potential Akt phosphorylation site (T68) on ADIPOR1. Akt was found bound to 14-3-3 in all conditions and may help confirm the possible interaction Akt has with ADIPOR1 (Figure 14 A).

6.7 14-3-3 binds to p27

Given that 14-3-3 interactions have been shown to affect protein stability, we evaluated the possible interaction of 14-3-3 with p27 during conditions where p27 proteins levels were changing. Only the 300 nM LEP and 1 mM Compound C treatments were found to be significantly different from untreated cells despite variability in the mean p27 values amongst groups. LEP was found to cause a 55% decrease while Compound C caused a 60% reduction in 14-3-3 binding to p27 (Figure 15 A,B). ADIPO treatment (18 nM), caused an approximate 2-fold higher amount of 14-3-3 bound to p27 than LEP and

Compound C and a 1.3-fold higher 14-3-3/p27 interaction observed with Akt inhibitor treatment (Figure 15 A,B). All other treatments were found to not display a significantly different amount of 14-3-3 binding to p27 compared to other treatments.



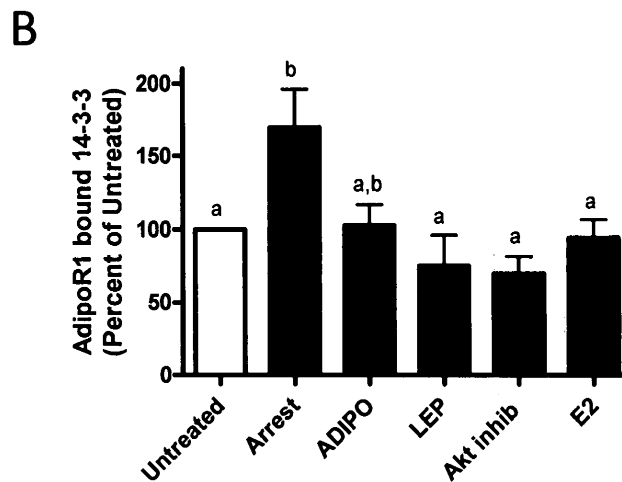
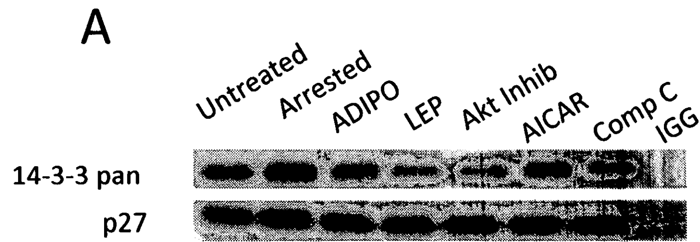


Figure 14: AdipoR1 immunoprecipitation for bound 14-3-3 protein levels. Effects of 14-3-3 bound AdipoR1 with treatments of Arrest, ADIPO 18 nM, LEP 300 nM, Akt Inhibitor 10 nM and Estrogen 10 nM. IGG is also shown. Blots standardized to AdipoR1 to correct for loading. Any letters which differ in treatment represent a significant difference ($p<0.05$). n=8/group

A



B

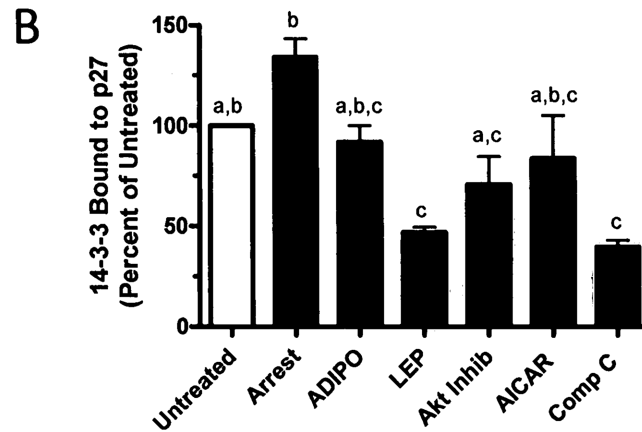


Figure 15: p27 immunoprecipitation for bound 14-3-3 protein levels. Effects of 14-3-3 bound p27 with treatments of Arrest, ADIPO 18 nM, LEP 300 nM, Akt Inhibitor 10 nM AICAR 1 mM and Compound C 1 mM. Blots standardized to p27 to correct for loading. Any letters which differ in treatment represent a significant difference ($p<0.05$). n=8/group

6.8 *Selection of a stable ADIPOR1 overexpressing MCF7 cell line*

To evaluate the role of ADIPOR1 in MCF7 cell cycle regulation, we created a cell line stably transfected with an adipoR1 expression plasmid. Seventeen individual stable transfected cell lines exhibiting G418 resistance were tested for Myc-tag protein levels/expression. Of the 3 cell lines which all had the highest expression of the Myc-tag, p31-3-2 was found to display the highest Myc-tag expression. Two other clones, p31-4-2-2 and p33-1-1 also showed high levels of Myc-tag expression (Figure 16 A,B). In addition, we wanted confirm increased plasmid expression by looking at the amount of ADIPOR1 protein. p31-3-2 was found to display a 1.3-fold higher ADIPOR1 compared to wild-type MCF7 cells, while p31-4-2-2 showed a 1.6-fold increase in ADIPOR1 protein levels (Figure 16 A,C). Although clone p33-1-9 showed the greatest increase in ADIPOR1 protein levels, it showed one of the lowest Myc-tag expressions and therefore we didn't use this clone for future experiments. p31-4-2-2 had a larger increase in adipoR1 protein levels than p31-1-1. Therefore, p31-4-2-2 and p31-3-2 were chosen as they showed the highest levels of both the Myc-tag and ADIPOR1 protein while providing a difference in ADIPOR1 protein levels to observe the result of different ADIPOR1 activation.

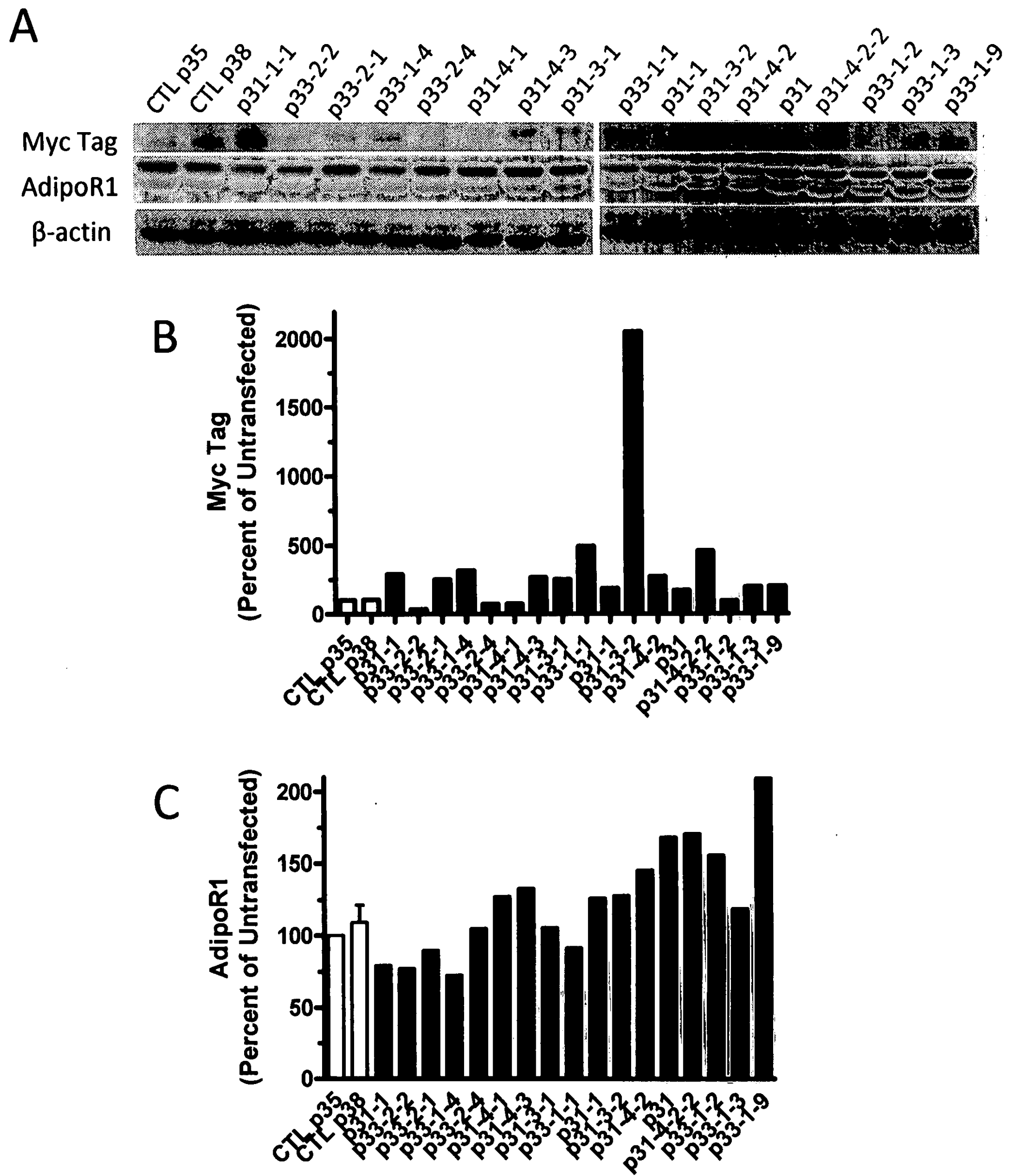
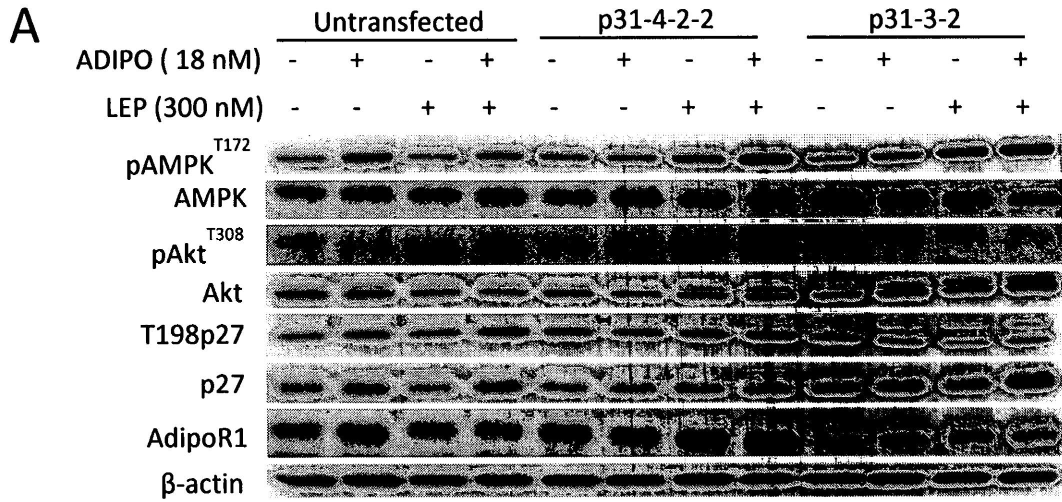
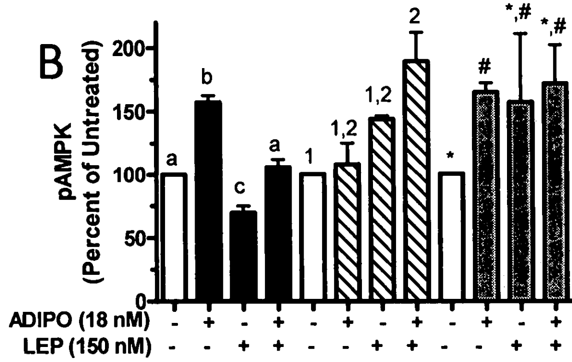


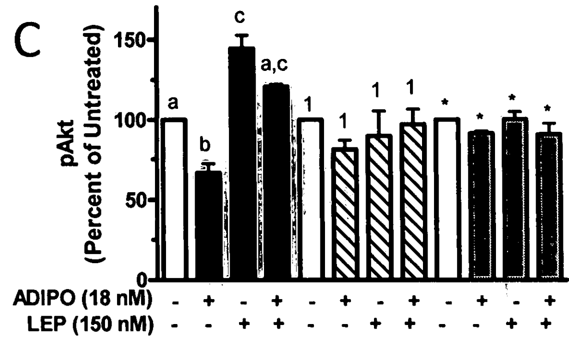
Figure 16: AdipoR1 transfection assessment. Successfully plated cell lines who survived G418 selection were tested for (B) Myc Tag and (C) AdipoR1. Two transfected cell lines (red) were then chosen for further experiments due to both increased Tag and adipoR1 protein levels. Blots standardized to β-actin to correct for loading.

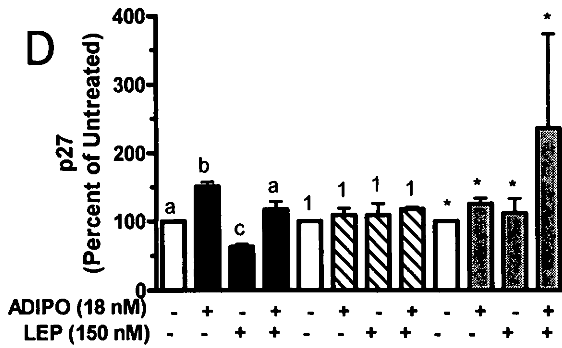
***6.9** AdipoR1 overexpression can abolish the LEP-dependent effects in breast cancer cells*

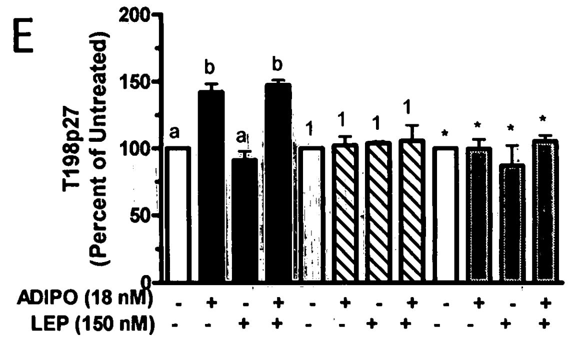
The stable cell lines created above were used in ADIPO/LEP co-treatment experiments. pAMPK levels were over 2-fold higher in ADIPO than in LEP treated cells (Figure 17 A,B). With co-treatment pAMPK was lower than with ADIPO treatment but higher than LEP treatment alone. In the p31-4-2-2 cell line, no difference between pAMPK levels in untreated, ADIPO and LEP were evident despite overall pAMPK levels being higher in this cell line, as expected (Figure 17 A,B). Only the co-treatment was found to be elevated, with a 1.8-fold increase compared to untreated, but no difference was observed when compared to ADIPO and LEP treatments alone. In the p31-3-2 transfected cell line, ADIPO caused a 1.65-fold increase in pAMPK. Both the LEP and co-treatment demonstrated pAMPK levels similar to ADIPO but they were not significantly different than those in untreated cells due to larger error (Figure 17 A,B). Next we evaluated pAkt^{T308}. pAkt^{T308} protein in ADIPO treated cells was 2.5-fold lower than in LEP treated cells (Figure 17 A,C). Co-treatment increased pAkt^{T308} protein levels higher than those in cells treated with ADIPO alone. Interestingly in both adipoR1 overexpressing cell lines there is no change in pAkt^{T308} levels between all treatment groups compared to the untreated condition (Figure 17 A,C). Changes in pAMPK and pAkt^{T308} protein levels were not due to increases in total protein levels (Figure 17 A). p27 levels were 2.5-fold higher in ADIPO treated cells compared to LEP treated cells (Figure 17 A,D). Co-treatment caused p27 to stay at the same level as untreated cells and a level that was significantly lower than ADIPO treatment alone. The p27 response in ADIPOR1


A
Untransfected
p31-4-2-2
p31-3-2
ADIPO (18 nM)
LEP (300 nM)
pAMPK T172
AMPK
pAkt T308
Akt
T198p27
p27
AdipoR1
β-actin


B
pAMPK
(Percent of Untreated)
ADIPO (18 nM)
LEP (150 nM)


C
pAkt
(Percent of Untreated)
ADIPO (18 nM)
LEP (150 nM)


D
p27
(Percent of Untreated)
ADIPO (18 nM)
LEP (150 nM)


E
T198p27
(Percent of Untreated)
ADIPO (18 nM)
LEP (150 nM)

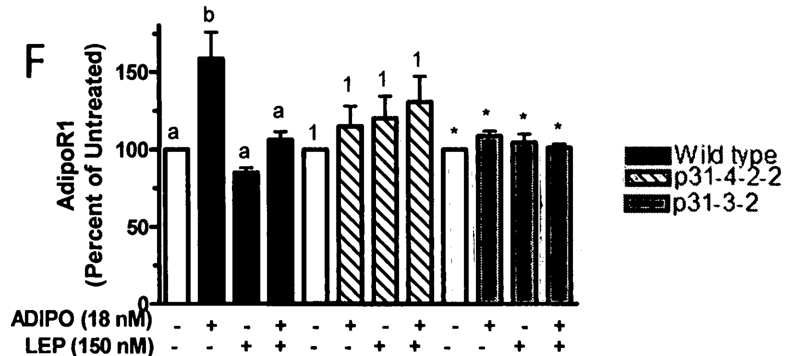

F
AdipoR1
(Percent of Untreated)
ADIPO (18 nM)
LEP (150 nM)
Wild type
p31-4-2-2
p31-3-2

Figure 17: ADIPO & LEP 24 hr co-treatment in wild type (black), p31-4-2-2 (stripped) and p31-3-2 (grey) with effects on (B) pAMPK, (C) pAkt, (D) p27 and (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. All 3 cell lines compared to same cell line untreated condition. Letters which are different represent a significant difference ($p<0.05$). n=3/group

overexpressing cells was similar to $pAkt^{T308}$. p27 levels in all conditions were not significantly different than in untreated conditions. When evaluating T198p27 protein levels, ADIPO caused an approximate 2.5-fold increase compared to LEP treated cells and co-treatment brought T198p27 levels up to levels similar to those seen in ADIPO treated cells (Figure 17 A,E). T198p27 protein levels followed the same pattern as $pAkt^{T308}$ and p27 with the two ADIPOR1 stable cell lines. All treatment conditions were not significantly different than untreated, including the LEP treatments. Lastly we again evaluated ADIPOR1 protein levels that ADIPO caused an almost 2-fold increase in ADIPOR1 expression compared to LEP treated cells (Figure 17 A,F). Co-treatment reduced ADIPOR1 levels to those seen with LEP treatment alone. There were no significant changes in either stably transfected cell lines with ADIPO, LEP or co-treatment. From these results we concluded that both transfected cell lines appear to counteract the effects of LEP and will both be used as we moved forward to the animal experiments.

6.10 *HFD fed animals had significantly more epididymal fat than the normal CD fed animals*

HFD feed animals showed rapid gains in mass for the entire protocol and were 1.7-fold heavier than their age matched CD fed animals at sacrifice (Figure 18 A). Consistent with this increased body weight was a significant 3.2-fold increase in epididymal fat pad mass (per 100 grams of body weight). These results clearly show that the HFD was effective in increasing body mass and visceral adiposity and represents an accurate obesity model.

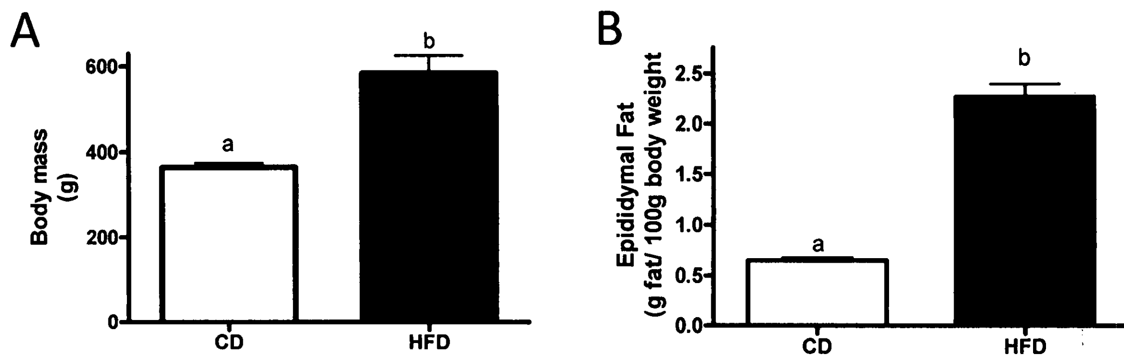
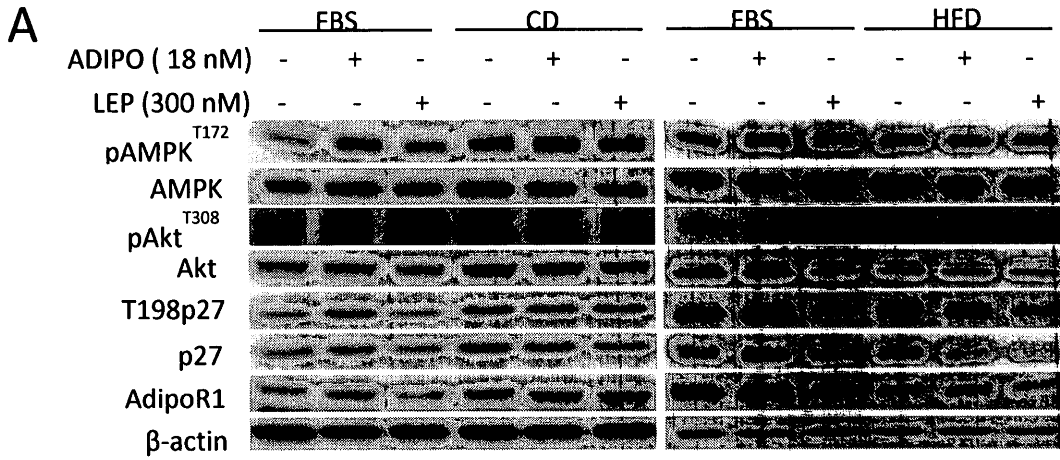
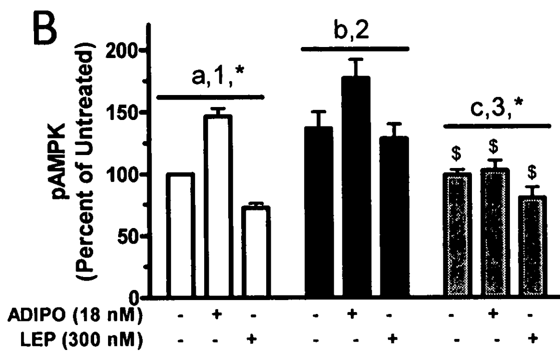


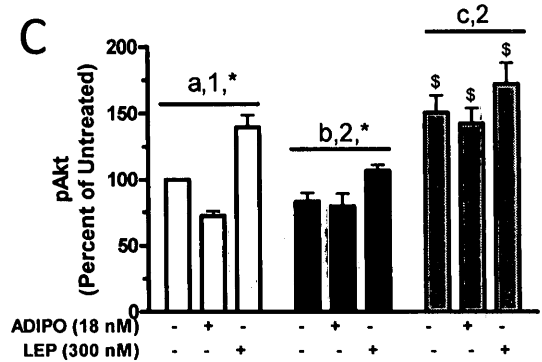
Figure 18: (A) Comparison of body mass between CD and HFD rats at time of sacrifice. (B) Comparison of the epididymal fat extracted per 100g body weight between CD and HFD rats Letters which are different represent a significant difference ($p<0.05$). CD n=5, HFD n=8

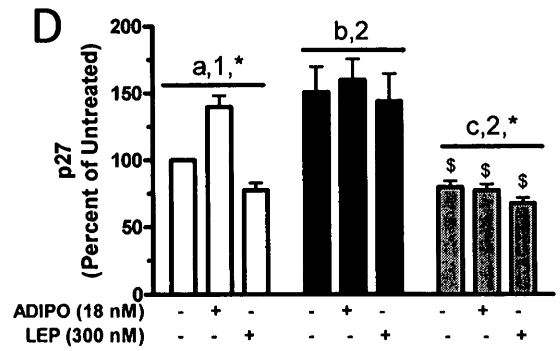
6.11 Conditioned media from adipocytes from HFD and CD fed animals induced effects on pAMPK, pAkt^{T308}, p27 and ADIPOR1 proteins in wild type MCF7 cells

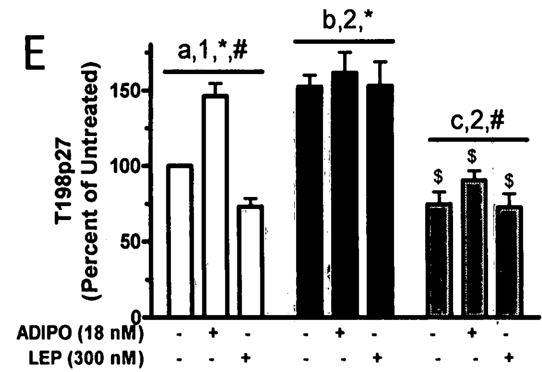
Our next experiments were designed to see if our ADIPO/LEP antagonism model persisted in an environment that more resembled an *in-vivo* condition where a wide variety of adipokines are present. To do this, we created conditioned media (CM) by culturing crude adipocyte preparations from CD and HFD fed rats and incubating proliferating MCF7 cells with either AMEM (containing 10% FBS alone) or CM for 24 hrs. pAMPK protein levels were higher in cells with CM from CD animals compared to cells in CM from HFD adipocytes (Figure 19 A,B). The effects of ADIPO and LEP in FBS and CD-CM treated cells were similar, however these treatment effects were abolished in HFD-CM treated cells. When comparing the effects of CM, HFD caused a reduction in pAMPK in UT, ADIPO and LEP treated cells compared to cells incubated with CD-CM. Most notable was a 75% decrease in pAMPK with ADIPO in HFD-CM compared to the CD-CM. Similarly, pAkt^{T308} levels were affected by CM (Figure 19 A,C). CD-CM and HFD-CM induced different effects on pAkt^{T308} then as seen in pAMPK protein levels. Cells incubated with AMEM FBS media showed similar results from previous studies with ADIPO and LEP treatments (Figure 9, 10). When comparing CD and HFD media conditions, we found that pAkt^{T308} levels in all three treatments were significantly increased compared to the same treatments with CD-CM. In response to ADIPO treatment we see that the HFD-CM increased pAkt^{T308} 1.60-fold above CD-CM treated cells. For both p27 and T198p27 we observed the same differences between both


A
FBS
CD
FBS
HFD
ADIPO (18 nM)
LEP (300 nM)
pAMPK T172
AMPK
pAkt T308
Akt
T198p27
p27
AdipoR1
β-actin


B
pAMPK
(Percent of Untreated)
a,1,*
b,2
c,3,*
ADIPO (18 nM)
LEP (300 nM)


C
pAkt
(Percent of Untreated)
a,1,*
b,2,*
c,2
ADIPO (18 nM)
LEP (300 nM)


D
p27
(Percent of Untreated)
a,1,*
b,2
c,2,*
ADIPO (18 nM)
LEP (300 nM)


E
T198p27
(Percent of Untreated)
a,1,*,#
b,2,*
c,2,#
ADIPO (18 nM)
LEP (300 nM)

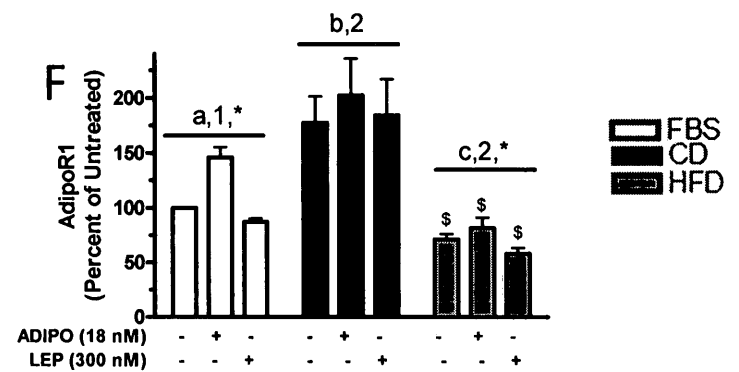

F
AdipoR1
(Percent of Untreated)
a,1,*
b,2
c,2,*
FBS
CD
HFD
ADIPO (18 nM)
LEP (300 nM)

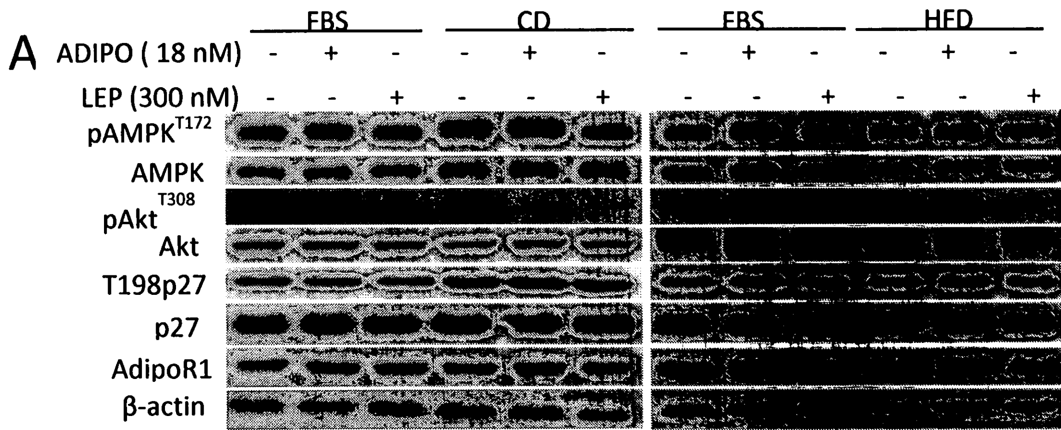
Figure 19: ADIPO and LEP 24 hr treatment using conditioned media from FBS (open), CD (black) or HFD (grey) in wild type cells with effects on (B) pAMPK, (C) pAkt, (D) p27, (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Letters which are different represent a significant difference in conditioned media, numbers significant difference in treatment and symbols represent an interaction ($p<0.05$). A $ indicates a significant difference in a treatment between CD-CM and HFD-CM ($p<0.05$). n=11 (FBS), n=5 (CD), n=6 (HFD)/group

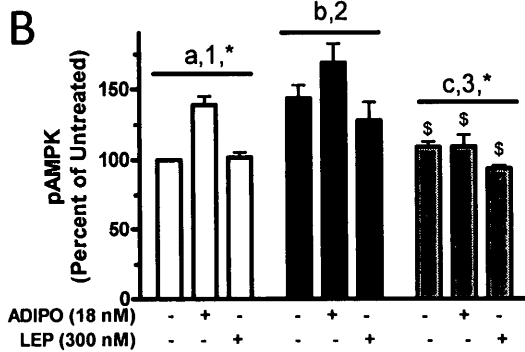
conditions and treatments, with CD-CM increasing p27 and T198p27 above HFD-CM. In CD-CM there was no effect of ADIPO or LEP on p27 or T198p27. HFD-CM also abolished the effects of ADIPO and LEP on these two proteins. HFD-CM altered the ADIPO effects on p27 and T198p27 protein levels with measured reductions of 85% and 75% in p27 and T198p27 protein levels, respectively. Lastly, CD-CM and HFD-CM showed the same effect on ADIPOR1 as p27 (Figure 19 A,F). ADIPOR1 protein levels were higher with CD-CM treatment compared to HFD-CM exposure. The effects of ADIPO and LEP on ADIPOR1 in FBS treated cells were abolished in both CD-CM and HFD-CM conditions. There is a significant 2.25-fold increase in adipoR1 protein with ADIPO treatment in CD-CM compared to HFD-CM experiments

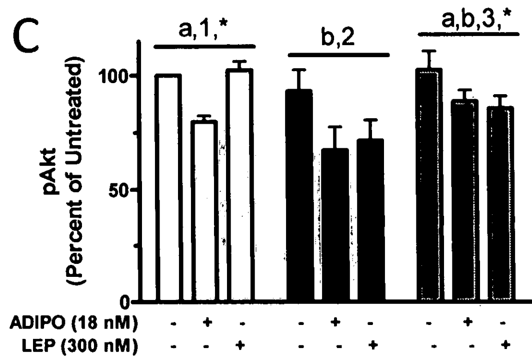
From these results, we see that the CD-CM increases pAMPK, p27, T198p27 and AdipoR1 while decreasing $pAkt^{T308}$. Each of these alterations likely lead to cell cycle exit and would be indicative of an inhibitory tumor growth microenvironment. The opposite is true of HFD-CM suggesting that obese adipocytes promote breast cancer proliferation.

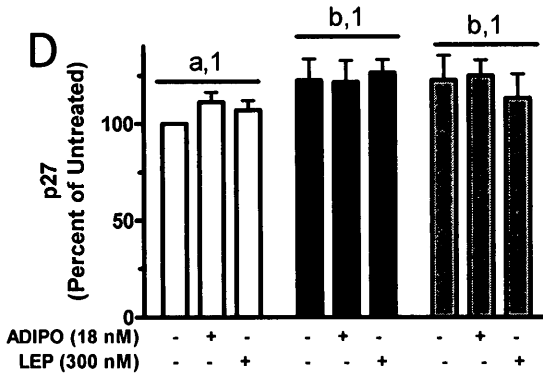
__6.12__ ADIPOR1 overexpression ameliorates the effects on protein levels caused by HFD

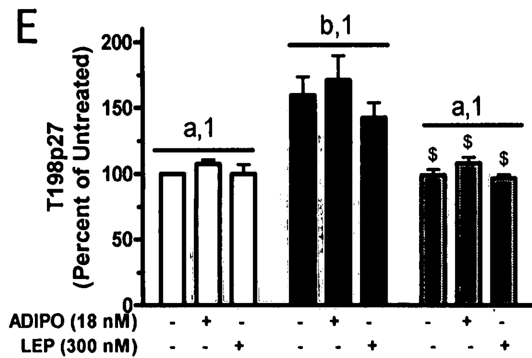
Following our previous results we wanted to determine what affect ADIPOR1 overexpression would have on HFD-CM effects. Our first experiments utilized the p31-3-2-2 cell line that had the highest ADIPOR1 expression. First we looked at pAMPK and found similar results to those seen in non-overexpressing cells (Figure 20 A,B vs. Figure 19, A,B). However, the pAMPK levels in all ADIPOR1 overexpressing cells were higher than in non-transfected cells. Most importantly, pAMPK in HFD-CM cells was higher than FBS treated non-transfected cells suggesting that increasing ADIPOR1 can lessen or even completely abolish HFD-dependent decreases in pAMPK. This effect of ADIPOR1 over-expression was evident for other proteins. In the p31-4-2-2 line there was no enhancement of $pAkt^{T308}$ with HFD-CM compared to FBS and CD-CM cells (Figure 20 A,C). Similarly, all of the decreases in p27, T1987p27 and ADIPOR1 induced by HFD-CM in wild-type MCF7 cells (Figure 19 A,D-F) were no longer evident when ADIPOR1 was over expressed (Figure 20 A, D-F). These results show that increasing ADIPOR1 expression can alter the tumor growth promoting environment created by HFD/obesity.


FBS
CD
FBS
HFD
A
ADIPO (18 nM)
LEP (300 nM)
pAMPK T172
AMPK
pAkt T308
Akt
T198p27
p27
AdipoR1
β-actin


B
pAMPK
(Percent of Untreated)
a,1,*
b,2
c,3,*
ADIPO (18 nM)
LEP (300 nM)


C
pAkt
(Percent of Untreated)
a,1,*
b,2
a,b,3,*
ADIPO (18 nM)
LEP (300 nM)


D
p27
(Percent of Untreated)
a,1
b,1
b,1
ADIPO (18 nM)
LEP (300 nM)


E
T198p27
(Percent of Untreated)
a,1
b,1
a,1
ADIPO (18 nM)
LEP (300 nM)

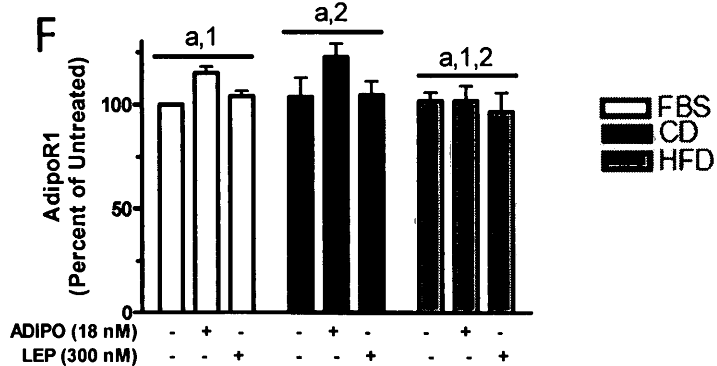
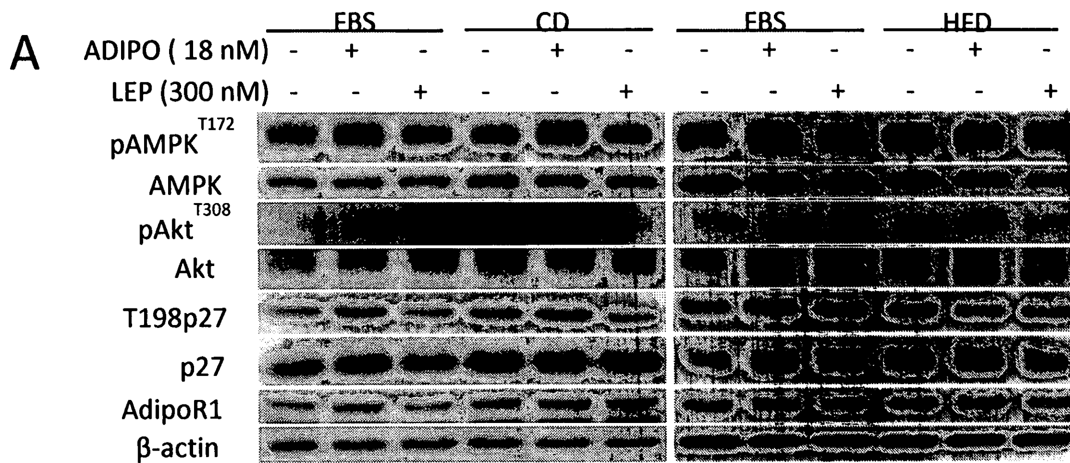
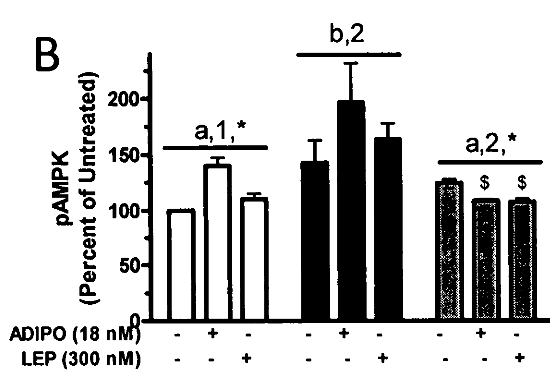
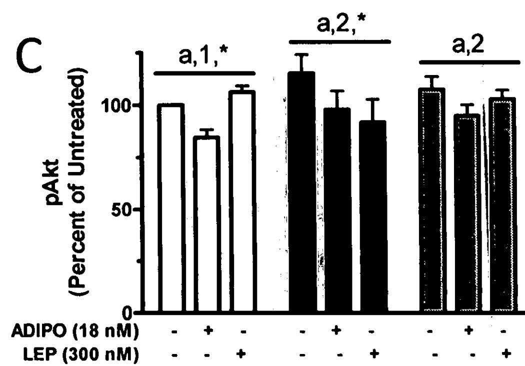

F
AdipoR1
(Percent of Untreated)
a,1
a,2
a,1,2
ADIPO (18 nM)
LEP (300 nM)
FBS
CD
HFD

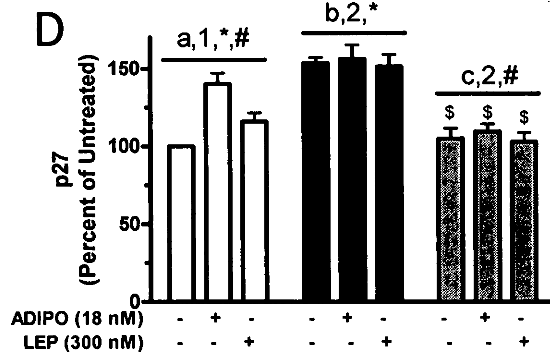
Figure 20: ADIPO and LEP 24 hr treatment using conditioned media from either FBS (open), CD (black) or HFD (grey) in p31-4-2-2 cells with effects on (B) pAMPK, (C) pAkt, (D) p27, (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Letters which are different represent a significant difference in conditioned media, numbers significant difference in treatment and symbols represent an interaction ($p<0.05$). A $ indicates a significant difference in a treatment between CD and HFD ($p<0.05$). n=11 (FBS), n=5 (CD), n=6 (HFD)/per group

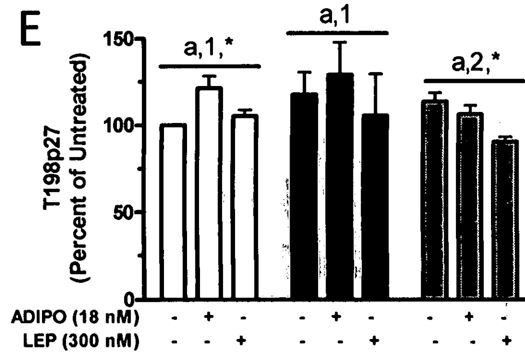
The final experiments utilized the stably transfected cell line p31-3-2 which had the highest myc-tag expression levels. Overall, we observed similar results to those seen using p31-4-2-2 cell line (Figure 20). For pAMPK, HFD-CM reduced protein levels compared to CD-CM (Figure 21 A,B), but even in the presence of HFD-CM pAMPK levels were higher in p31-3-2 cells than in FBS treated non-transfected MCF7 cells (Figure 19 A,B). HFD-CM did not increase $pAkt^{T308}$ above the levels seen in FBS and CD-CM experiments (Figure 21 A,C). HFD-CM decreased p27 levels compared to CD-CM treatment, but the absolute levels of p27 were higher in p31-3-2 cells treated with HFD-CM that in non-transfected MCF7 cells grown in FBS (Figure 19 A,D). T198p27 and ADIPOR1 protein levels were unaffected by HFD-CM (Figure 21 A,E,F). These results agree with those from our p31-3-2-2 line which had the highest ADIPOR1 protein levels (Figure 20). Taken together, these results suggest that activation of ADIPO signaling by increasing ADIPOR1 protein levels can counteract the growth promoting effects of adipocytes from obese animals.


A
FBS
CD
FBS
HFD
ADIPO (18 nM)
LEP (300 nM)
pAMPK T172
AMPK
pAkt T308
Akt
T198p27
p27
AdipoR1
β-actin


B
pAMPK
(Percent of Untreated)
a,1,*
b,2
a,2,*
ADIPO (18 nM)
LEP (300 nM)


C
pAkt
(Percent of Untreated)
a,1,*
a,2,*
a,2
ADIPO (18 nM)
LEP (300 nM)


D
p27
(Percent of Untreated)
a,1,*,#
b,2,*
c,2,#
ADIPO (18 nM)
LEP (300 nM)


E
T198p27
(Percent of Untreated)
a,1,*
a,1
a,2,*
ADIPO (18 nM)
LEP (300 nM)

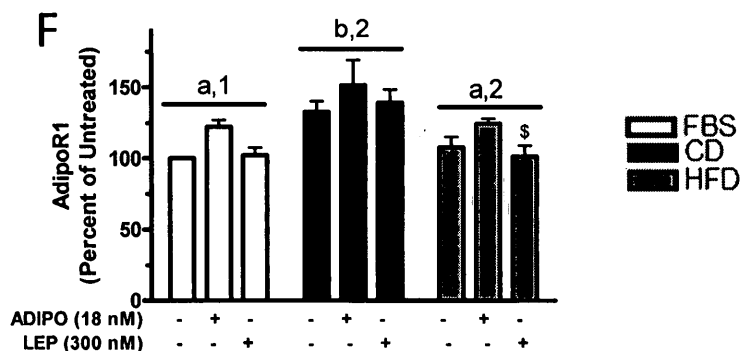

F
AdipoR1
(Percent of Untreated)
a,1
b,2
a,2
ADIPO (18 nM)
LEP (300 nM)
FBS
CD
HFD

Figure 21: ADIPO and LEP 24 hr treatment using conditioned media from either FBS (open), CD (black) or HFD (grey) in p31-3-2 cells with effects on (B) pAMPK, (C) pAkt, (D) p27, (E) T198p27 and (F) AdipoR1 protein levels. Blots standardized to β-actin to correct for loading. Letters which are different represent a significant difference in conditioned media, numbers significant difference in treatment and symbols represent an interaction ($p<0.05$). A $ indicates a significant difference in a treatment between CD and HFD ($p<0.05$). n=11 (FBS), n=5 (CD), n=6 (HFD)/group

7. Discussion

Breast cancer continues to be a growing concern in our society and due to the increase in the number of cases [1], partly due to better technology for early detection which also translates to an increased number of deaths. Despite the increase in the absolute number of deaths, early detection results in the discovery of lower grade tumors which respond better to therapies and decrease the overall percent mortality rate [1]. Breast cancer therapies employed in the clinic have remained relatively unchanged over the last 20 years. To address this, more emphasis has been placed on understanding the underlying molecular mechanisms which lead to cancer development and progression.

Obesity like breast cancer has been on the rise over the last 20 years. As previously stated, more than 1.4 billion people worldwide are considered obese and the numbers are continuing to increase [68]. This increase in obesity is becoming a growing concern as there has been a statistical association between increasing adiposity and an increased risk of cancer for over 40 years [75]. Thus, it is likely that the increase in breast cancer incidence may be due, in part to increases in obesity.

Adipose tissue has been traditionally considered to be an inert storage depot for excess energy but now has been shown to be a highly active endocrine tissue producing potential growth factors termed adipokines [82, 83]. The adipokines ADIPO and LEP have come to the forefront of research as they have been shown to be the most abundant

adipocytokines produced by adipoctyes and have been shown to affect the growth status of cells, including breast cancer cells [84]. Studies have shown that in breast cancer patients, the serum ADIPO:LEP ratio is significantly decreased which has been shown to occur in obese individuals [90, 93, 94]. Understanding the ramifications of this shift in the ratio between ADIPO and LEP is one of the key mechanisms studied in my thesis as well understanding how these two adipokines affect certain key cell cycle regulatory proteins.

Previous research by Dieudonne et al. (2006) showed how ADIPO causes a dose-dependent increase in pAMPK which has also been previously shown in our lab [129]. My findings again showed this with ADIPO causing a significant increase in pAMPK and a significant decrease in pAkt (Figure 9 A,B,C). LEP induced the opposite effect as shown in figure 9, producing a significant decrease in pAMPK [113] and a novel significant increase in pAkt (Figure 9 & 10 A,B,C). I believe that it is this interaction between ADIPO and LEP through both AMPK and Akt that may be causing the effects on cell proliferation status. With ADIPO and LEP co-treatment, I show that ADIPO is able to overcome both the inhibitory effects that LEP has on pAMPK and the increase observed in pAkt (Figure 11 A,B,C). It is this antagonism between ADIPO and LEP that I believe is key to all the downstream cell cycle effects, specifically with respect to the cell cycle regulator p27. It appears that it is the relative concentration, or ratio between the two adipokines, and not the absolute concentration that is of importance. If we can alter the ratio of ADIPO:LEP, which can be achieved naturally through weight loss by means of exercise or

energy restriction [139], it may create a negative growth environment for breast cancer cells. This would agree with a previous study that showed ADIPO activated AMPK can stimulate the dephosphorylation of Akt [134], which is consistent with my findings. It may be possible that once the ADIPO:LEP ratio reaches a high enough level, we see enough ADIPO to cause AMPK to lead to the dephosphorylation of Akt by PP2A. This mechanism only explains an increase in AMPK leads to a decrease in Akt but does not address the novel results I show of how increasing Akt plays a role in decreasing AMPK. It may be possible that AMPK and Akt also have direct antagonism of one another or that Akt may also activate a different phosphatase which de-activates AMPK.

In order to look at the downstream effects of this ADIPO:LEP antagonism, I observed the effects on both p27 as well as T198p27, which is directly phosphorylated by AMPK [45]. p27 is an important regulator of cell cycle progression and regulates the important G1-S phase transition [15, 19, 25, 32]. Research has shown that activation of AMPK leads to the phosphorylation of human p27 at T198, which increases the stability of p27 protein [45]. Previous and current research in our lab has shown that ADIPO causes a significant 1.6-fold increase in p27 while LEP produces the opposite effect (Figure 9 A,D & 10 A,D). Previous studies reveal that an increase in p27 protein concentration to the magnitude achieved (Figure 9 A,D) corresponds with full cell cycle arrest in MCF7 cells [113].

I show the same effects on T198p27 with ADIPO and LEP treatment (Figure 9 & 10 A,D,E). Interestingly with co-treatment, as opposed to pAMPK and pAkt, where LEP

did have slight effects on both proteins, p27 and T198p27 showed no change from ADIPO treatment and co-treatment of ADIPO and LEP. LEP was unable to antagonize the effects of ADIPO on p27 and T198p27.

In order to further understand how phosphorylation at T198 could lead to more overall p27 protein stability, we looked at the possibility of 14-3-3 interacting with and stabilizing p27. Previous work by Short *et al.* (2010), showed how activation of AMPK leads to the phosphorylation of p27 at the conserved sequence of the C-terminal threonine residue of the murine p27 (T197), similar to the human T198 binding site [140]. Mutation of T197 to D197 led to an increased interaction between p27 and 14-3-3 which resulted in increased p27 protein stability. They also discovered that this increased stability of p27 protein was dependent on T197 phosphorylation by introducing a T198A mutation [140]. From the use of an immunoprecipitation of p27, subsequently probed for 14-3-3, I found that both CC and LEP caused significant 60% decreases in the amount of 14-3-3 that was bound to p27 compared to arrested cells (Figure 15). My experiments have shown that LEP causes a significant decrease in active AMPK while previous research in our lab has shown the same inhibition of AMPK with CC treatment. If we compare the treatments back to the arrested cells we see that both LEP and CC treatments are significantly lower 14-3-3 binding to p27 and have a corresponding decrease in pAMPK and T198p27. We had expected ADIPO, AICAR and Akt inhibition to cause a significantly higher amount of 14-3-3 bound p27 than both LEP and CC as they have been shown to increase pAMPK as well as T198p27. When

comparing the relative differences in protein levels of both p27 and T198 (Figure 9 D,E & Figure 10 D,E) we see that they follow the same trend as they correlate with the amount of 14-3-3 bound to p27 (Figure 15). There is a doubling of pAMPK which causes double the amount of T198 with ADIPO treatment compared to LEP. This corresponds with the amount of 14-3-3 binding to p27, with slightly more than double the amount of 14-3-3 bound to p27 with ADIPO treatment compared to LEP treatment. This same trend was previously shown using ADIPO and CC in our lab when comparing pAMPK, T198 and p27 protein differences. ADIPO caused over double the amount of T198 phosphorylation, leading to over double the amount of p27/14-3-3 binding and a subsequent 2 fold increase in p27. These results agree with the molecular results by Short et al., (2010), who employed an *in vivo* model using transgenic mice expressing mutant forms of p27 [140]. Therefore, I believe that this pathway may be the means of which active AMPK causes the stabilization of p27 which agrees with the results from previous studies (Figure 22) [140]. This may also explain why decreasing active AMPK through CC or LEP decreases the amount of 14-3-3 binding and in turn decreases p27 stability. What is unclear with LEP treatment is whether it is the decrease in pAMPK or the increase in pAkt that is causing this decrease in 14-3-3 binding and p27 protein levels. Although mutations give insight into the potential role of phosphorylation into protein functions, they are not truly representative of physiological cellular function. By treating cells with ADIPO and LEP and showing similar results, I have given physiological relevance to this

AMPK dependent phosphorylation on p27/14-3-3 interaction and defined a potential role in human breast cancer.

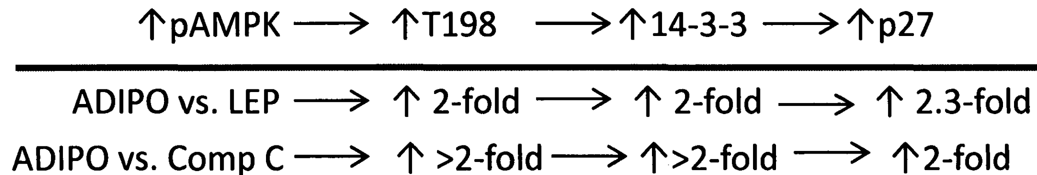


Figure 22: Possible relationship between increases in active AMPK, leading to increased phosphorylation of T198, increased binding of 14-3-3 to p27 causing increased stability.

Also novel to this study was the discovery of the effects of ADIPO and LEP on ADIPOR1 protein. I show that ADIPO causes a significant increase in ADIPOR1 in a positive feedback manner while LEP appears to decrease ADIPOR1 trend (Figure 9 & 10 A,F). The co-treatment displayed an important result as LEP caused a significant decrease in ADIPOR1, even in the presence of ADIPO (Figure 11 A,F). Therefore I believe that it is this LEP-dependent decrease in ADIPOR1 that may be driving the proliferative effects induced by LEP in MCF7 cells. This is supported, in part through a study by Pfeiler *et al.* (2010) which showed that women with the most aggressive breast tumors had decreased ADIPOR1 protein expression [127]. Furthermore, ADIPOR1 expression is 62% lower in obese women compared to lean women in visceral adipose tissue [128]. I believe that this decrease in ADIPOR1 either through a decrease in pAMPK or an increase in pAkt could be regulating ADIPOR1 signaling. Thus, in an obese individual, where the

ratio of ADIPO:LEP is decreased, this compounding effect on decreasing ADIPOR1 would only worsen the effectiveness of ADIPO which already exists at lower levels in obese individuals.

In order to determine if Akt was directly responsible for these effects on ADIPOR1, pAMPK, p27 and T198p27, a pharmacological Akt inhibitor was utilized. I was able to show for the first time that Akt appears to play an important role in ADIPOR1 protein stability. As shown in figure 12, the use of an Akt inhibitor caused the same effects on ADIPOR1 as ADIPO treatment, mirroring the exact same fold protein changes. These results also resemble previous research done in our lab which looked at AMPK activation through AICAR, an AMP mimetic. Therefore, the same overall effects on pAMPK, pAkt, p27 and T198p27 are achieved by either activating AMPK or inactivating Akt. Interestingly, I showed that Akt may play a pivotal role in ADIPOR1 stability, as there is a significant dose-dependent increase in ADIPOR1 with Akt inhibition similar to that seen with ADIPO treatment.

I wanted to determine whether given these changes in ADIPOR1 in response to AMPK activation and Akt inhibition, increasing the amount of ADIPOR1 would have any effect on p27 in MCF7 cells. In order to do so, I created numerous stably ADIPOR1 transfected cell line which showed a significant increase of ADIPOR1 protein compared to non-transfected MCF7 cells (Figure 16 A,B,C). With two of these transfected cell lines, I show that an increase in ADIPOR1 protein and resultant ADIPOR1 downstream signaling abolished all of the LEP-dependent effects on p27 (Figure 17). By increasing the

amount of ADIPOR1 present in the cells they are resistant to LEP, possibly by utilizing more of the existing ADIPO present in the FBS, even in the presence of high LEP concentrations. Also important to note is the overall protein levels in the ADIPOR1 overexpressing cells compared to the non-transfected MCF7 cells. If we look at figure 17 A, the transfected cell lines have more pAMPK, p27, T198p27 and of course more adipoR1 while displaying less overall pAkt then the non-transfected cells. Given that increasing pAMPK, p27 and T198p27 are associated with cell cycle inhibition/withdrawal, increasing ADIPOR1 protein may inhibit proliferation of breast cancer cells, even in an obese patient with a low ADIPO:LEP ratio.

Akt inhibition caused a large dose-dependent increase in ADIPOR1 protein and I wanted to determine if this possible increase was caused by Akt signaling affecting the interaction with potential stabilizing proteins. To accomplish this, a stringent Scansite blast search (scansite.mit.edu) was used to identify potential protein-protein interaction sites with ADIPOR1. It was discovered that there may be a potential Akt consensus site (RXRXXS/T) at T68 which shares homology with the consensus 14-3-3 binding motif (RSXS/TXP). It was then hypothesised that Akt may phosphorylate ADIPOR1 at T68 which then affects 14-3-3 binding to the same consensus site, thereby affecting ADIPOR1 protein stability. Through the use of an immunoprecipitation of ADIPOR1, I probed for 14-3-3 and found that in arrested MCF7 cells there is a significant 1.70-fold increase in 14-3-3 bound to ADIPOR1 then in untreated proliferating cells (Figure 14). Interestingly, differences in 14-3-3 bound to ADIPOR1 with LEP treatment were similar

to decreases in ADIPOR1 protein with LEP treatment. If Akt were directly responsible for 14-3-3/ADIPOR1 interaction, Akt inhibition should cause an increase in the amount of 14-3-3 bound to ADIPOR1. This was not evident and therefore I believe that 14-3-3 binding to ADIPOR1 affects ADIPOR1 stability but is probably not mediated through Akt. Although I didn't see the results hypothesised with the use of an Akt inhibitor, I did show that Akt does bind to ADIPOR1 in all experimental conditions. One other possibility may be that there is also a consensus site for pyruvate dehydrogenase kinase 1 (PDK1) on ADIPOR1 (Scansite). Previous research has shown that PDK1 acts as the upstream activator of Akt [141] and it may be that PDK1 mediating 14-3-3 binding to ADIPOR1. Therefore, Akt may still phosphorylate ADIPOR1 at T68 but more work needs to be done in order to precisely determine direct Akt effects on ADIPOR1.

Excess obesity, especially visceral adipose tissue, has been found to be associated with many negative health factors, including some forms of cancer [69, 73, 74]. The decrease in ADIPO:LEP of an obese woman is one possibility that likely drives the proliferation of breast cancer in obese patients [84, 88, 89]. Previous research has shown that serum LEP and ADIPO levels are positively and negatively correlated to BMI, respectively [94]. I wanted to determine if the proliferating effects of LEP achieved by increasing ADIPOR1 in MCF7 cells could either be increased or decreased depending on the adipocyte profile it was exposed to. To evaluate this, we cultured primary crude adipose tissue from obese HFD fed rats and generated conditioned MCF7 growth media (HFD-CM). We also generated CM from lean age matched normal chow diet fed animals

(CD-CM). This allowed us to compare the effects of increasing ADIPOR1 protein expression in the presence of a complete adipokine profile that a breast cancer would be exposed to. The CD-CM activated AMPK and increased p27 and T198p27 while inhibiting the activation of Akt, which was also the case in cells treated with ADIPO alone. The HFD-CM produced exactly the opposite effects. This HFD work agrees with previous research conducted in our lab which showed that adipocytes from ZDF rats induced cell cycle entry in MCF7 cells [113]. Non-transfected MCF7 cells in CD-CM were capable of blunting LEP effects on p27 while ADIPO was able to counteract the reduction in p27 caused by the HFD-CM (Figure 19). The CD-CM caused an increase in pAMPK and a subsequent decrease in pAkt, which agrees with the idea that a lean diet creates a growth inhibitory tumor microenvironment possibly by increasing the ADIPO:LEP ratio [94]. The opposite effect is observed with HFD-CM, creating a positive tumor growth environment promoting proliferation. Importantly, even in the presence of all other adipokines and factors within CD-CM and HFD-CM, the results follow the ADIPO:LEP pattern I have previously shown (Figure 19). Therefore I believe that it is a distinct possibility that ADIPO and LEP are the two main adipokines which are driving the results observed throughout this study, possibly through the changes in the ratio of pAMPK/pAkt. This is beneficial for breast cancer patients because if these are the two main adipokines driving the growth environment of their tumors, previous work has shown that weight loss can increase the ADIPO:LEP ratio [139] and increase ADIPOR1 expression in visceral and subcutaneous adipose tissue [128]. Each of these factors increase

p27 by phosphorylation via AMPK which will ultimately lead to cell cycle arrest. In addition, even in an obese environment, increasing the amount of ADIPOR1 can minimize the reduction in p27 caused by LEP. Thus maintenance of ADIPOR1 signaling either pharmacologically or naturally via diet and exercise, can potentially inhibit breast cancer proliferation and improve patient prognosis.

The changes produced in this study are stable changes which are not specific for a certain tumor type. Although I used MCF7 breast cancer cells, many other cancers including colon cancer [142], endometrial cancer [143], prostate cancer [144] and acute myelogenous leukaemia [145] to name a few, have been shown to be increased with a decrease in the ADIPO:LEP ratio. The method of controlling the ADIPO:LEP ratio and subsequent positive cell cycle effects would act as a blanket inhibition of tumor growth, possibly irrelevant of the genetic situation of the specific tumor.

The data obtained from these experiments have provided sufficient information to develop a working model of the possible signalling cascade of events which occur from both obese and lean adipocytes in relation to cell cycle status (Figure 23). From previous work in our lab which supports my data, I believe that active AMPK helps stabilize ADIPOR1. We see that ADIPOR1 protein is increased with ADIPO and AICAR treatment while CC causes a significant reduction in ADIPOR1, displaying the importance of AMPK in regulating ADIPOR1 protein levels. By increasing ADIPOR1 expression, a positive feedback loop is created, producing more active AMPK which in turn help stabilize p27 through 14-3-3 binding/interaction, leading to cell cycle arrest. This

increased AMPK also may control active pAkt levels which acts in an opposing manner to promote cell cycle progression subsequently via decreasing p27 and its phosphorylation. Akt may also play a role in further inhibiting AMPK and ADIPOR1 protein levels and stability. Therefore, an individual who is obese has a decrease in their ADIPO:LEP ratio, causing increases in active Akt, decreases in active AMPK and a subsequent decreases in p27 leading to cell cycle entry. They also now seem to have less ADIPOR1, which would decrease the positive feedback loop produced by ADIPO to increase/stabilize ADIPOR1 protein levels. If we can activate AMPK to increase ADIPOR1 via AICAR, the use of ADIPO treatment or diet and exercise, we may be able to create a more negative growth microenvironment for the tumor. Fully understanding the relationship between ADIPO and LEP may link a physiological/molecular relationship between obesity, breast cancer and cell cycle regulation.

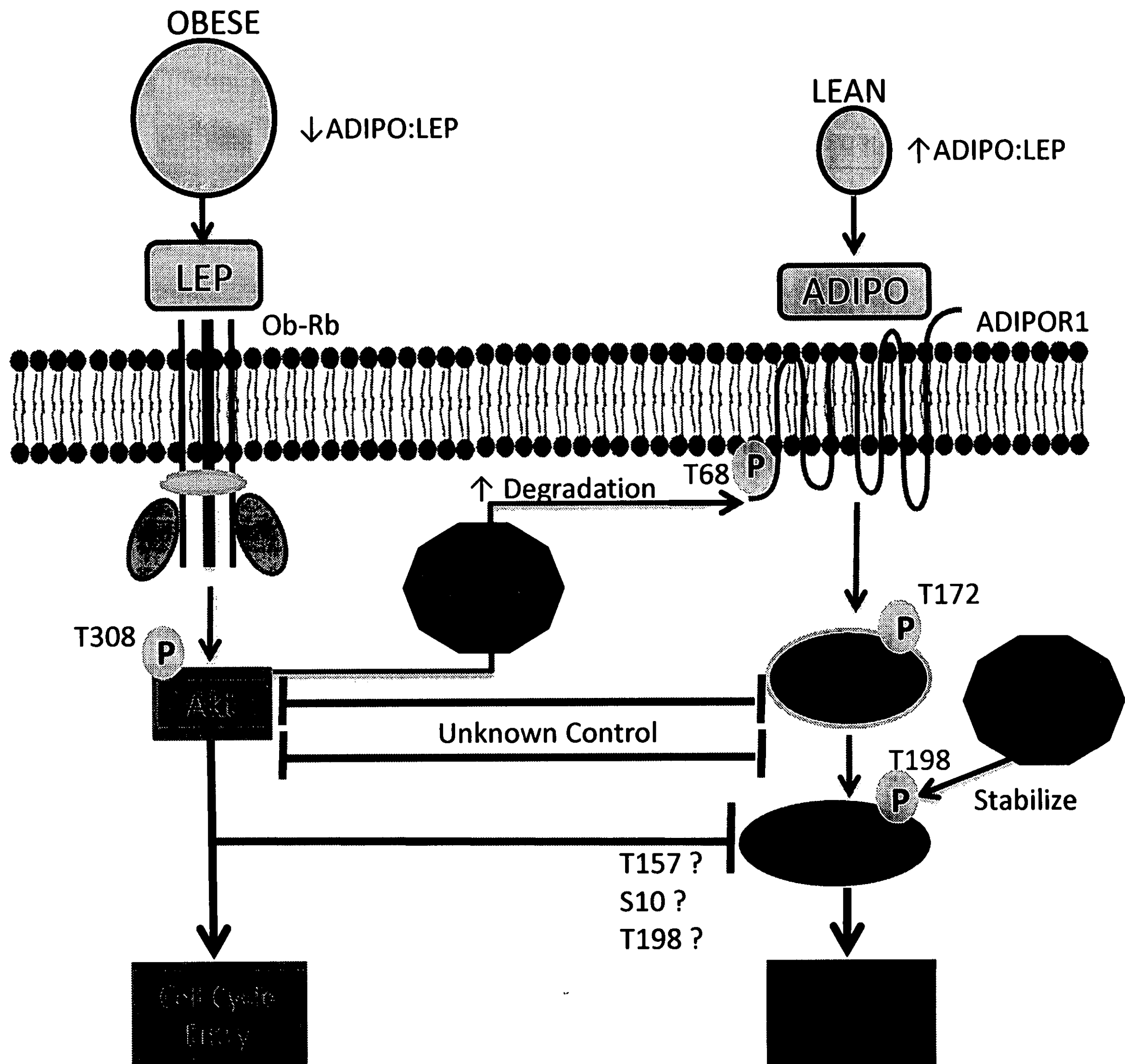


Figure 23: Current working model based on data collected. The ratio between ADIPO:LEP based on weight and adipokine profile affect downstream pathways AMPK and Akt. Unclear is whether AMPK is controlling Akt or vice versa. 14-3-3 seems to play a stabilizing role with both p27 and adipoR1 while Akt seems to play a role in destabilizing ADIPOR1

8. Future Directions and Limitations

This study tries to further the research into the effects of the two adipokines ADIPO and LEP on breast cancer development and progression. There are other possible means by which I can further support my data that has been collected. First, I will be utilizing the MCF7 cells that I have collected during the conditioned media study which were placed in ethanol in order to run a FACS analysis. The cells were collected at the same time as the treatments in both non-transfected and transfected MCF7 cells. This will confirm my hypothesis that the HFD media is causing more cells to enter the cell cycle while the CD-CM caused more cells to arrest in G1. Also, I would want to determine if the transfected cells caused more cells to enter G1 with the CD-CM than the non-transfected MCF7 cells. Also interesting would be whether these ADIPOR1 transfected cells increased the number of cells in G1 even with the HFD-CM. This would only further stress the importance of ADIPOR1 and the environment created by the adipocyte.

In the future I would like to run a study looking at cells treated with LEP and the Akt inhibitor. I showed that the Akt inhibitor only caused even greater increases in p27 protein with co-treatment with ADIPO. If I find that all of LEP negative effects on the cell cycle proteins, specifically on ADIPOR1, are abolished I can then say more concretely that the effects discovered with LEP are due to increases in Akt.

Also in the future I plan on looking at serum samples from both obese and lean breast cancer patients in order to determine if the same results discovered with the adipocyte conditioned media persist with the *in-vivo* samples. I will also look to add on to my current HFD and CD rat study by adding an exercise group in order to determine if I can cause an increase in ADIPOR1 in non-transfected MCF7 cells. Also interestingly will be whether this exercise group can further increase the positive effects ADIPOR1 transfection had on cell cycle proteins and whether it can further overcome the negative effects caused by a HFD.

With all studies there are limitations to my study and it is important to understand them in order to make future studies even stronger. Although I utilized an adipocyte conditioned media experiment, it doesn't classify as a direct *in-vivo* experiment. Although all the adipokines are present in the adipose tissue are in the media, the system itself is not present. I think it would be beneficial to utilize a direct *in-vivo* model, by injecting both non-transfected and ADIPOR1 transfected MCF7 cells subcutaneously into immunocompromised rats. We could then determine whether the increase in ADIPOR1 causes the tumor cells to grow slower by utilizing more ADIPO from within the circulation *in-vivo*.

Another limitation could be the concentrations of ADIPO that were used in the study. The concentrations used are considered subphysiological and could be viewed as a limitation to the study. The decision to utilize 9 nM (150 ng/mL) and 18 nM (300 ng/mL) concentrations of globular ADIPO came from the ADIPO optimization

experiment done with pAMPK in my thesis as well as previous work done within our lab on MCF7 cells [113]. The LEP concentrations of 150 nM (2500 ng/mL) and 300 nM (5000 ng/mL) used was also determined from the LEP optimization experiment as well as previous research in our lab that look at LEP effects on pAMPK and p27 protein levels [113]. Although the levels of ADIPO are below that which is considered physiological, I show that the ratio between ADIPO and LEP is of importance and not the absolute concentrations and that the same results persist even at physiologically relevant conditions in the CM experiments.

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. Further investigation, I believe will elicit the exact mechanisms by which ADIPO and LEP elicit their response on the cell cycle regulatory proteins.

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