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Obesity and Postmenopausal Breast Cancer:

Determining the Role of Local Aromatase Expression

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Obesity and Postmenopausal Breast Cancer:

Determining the Role of Local Aromatase Expression

by

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Obesity and Postmenopausal Breast Cancer: Determining the Role of Local Aromatase Expression

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Obesity is associated with a worse breast cancer prognosis, particularly in estrogen receptor alpha (ERα) positive, postmenopausal patients. It has also been correlated with elevated levels of inflammation, which can stimulate adipose tissue aromatase expression and subsequent estrogen production. Given that obese patients have a lower response rate to aromatase inhibitor treatment and greater mammary tissue aromatase levels, it was hypothesized that obesity promotes ERα positive postmenopausal breast cancer progression in part via an inflammation-induced increase in mammary tissue aromatase expression and ERα activity. These studies utilized an *in vitro* model of obesity in which cultured cells were exposed to postmenopausal breast cancer patients' serum samples, pooled by body mass index category (Obese (OB): ≥30.0 kg/m^2; Normal weight (N): 18.5-24.9 kg/m^2). OB versus N patient sera induced greater breast cancer cell (BCC)

aromatase expression, as well as higher ERα activity and cell viability in the presence of testosterone, the substrate for aromatase. Pre-adipocytes exposed to OB versus N patient sera also indirectly stimulated greater BCC aromatase expression, ERα activity, and viability. A retrospective review of breast cancer patient data demonstrated that daily use of non-steroidal anti-inflammatory drugs, which inhibit cyclooxygenase-2 (COX-2) activity, is associated with reduced ERα positive breast cancer recurrence in obese and overweight women. The mechanisms mediating this effect were examined using the same *in vitro* model. Exposure to OB versus N patient sera stimulated greater macrophage and BCC COX-2 expression and prostaglandin E2 production, leading to enhanced pre-adipocyte aromatase expression. These OB patient sera-induced effects were further linked with greater BCC ERα activity, proliferation, and migration in the presence of testosterone, and these differences were eliminated or reduced with aromatase inhibition. Based on these findings, prospective studies designed to examine the clinical benefit of NSAID use in obese ERα positive postmenopausal breast cancer patients are warranted. Additional obesity-associated mechanisms that may also promote breast cancer progression were also explored, including enhanced cross-talk between non-genomic ERα and growth factor signaling pathways, reduced estrogen receptor beta expression, and resistance to chemotherapy. Finally, the impact of obesity on tumor incidence and

characteristics in the MMTV-Wnt1 mouse model of mammary carcinogenesis was explored.

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Chapter 1: Introduction

1.1 Breast Cancer Prevalence and Mortality

Breast cancer is the most common form of cancer in females, accounting for almost one out of every three cancers diagnosed in United States women. Based on current incidence rates, the National Cancer Institute estimates that 12.4% of women born in the United States today will develop breast cancer at some point in their lives (1). Rates of both in situ and invasive breast cancer have surged over the last 30 years (1), largely due to improved detection with the growth of wide-spread mammography screening (2). However, increases in the prevalence of some breast cancer risk factors, including obesity, may have also contributed to this trend (3-6). In contrast with this rise in incidence, breast cancer mortality has been decreasing, with a 2.2% decline per year between 1990 and 2007 (1). This improvement has also been attributed to increased early detection with mammography (7-9), though a recent study found that such screening does not promote breast cancer survival (10). Advancements in the treatment of breast cancer are a likely additional driver of the decline in mortality.

While numerous factors contribute to an individual woman's breast cancer risk, the strongest risk factor is age. Consequently, breast cancer is more prevalent among postmenopausal women, with the highest incidence rates found among women 75-79 years of age (1). However, younger women tend to have more aggressive tumors and lower survival rates (11). Postmenopausal women are most

likely to develop the luminal A subtype of breast cancer (12), which expresses both estrogen receptor alpha (ERα) and the progesterone receptor (PR). Luminal A cancers, the least aggressive of the four major breast cancer subtypes, are associated with a favorable prognosis, largely because they tend to respond well to endocrine therapies targeting estrogen production or ERα itself (13). Patients with tumors that do not express ERα/PR have a 1.5 to 2-fold higher risk of mortality (14). Listed in order of increasing aggressiveness, the other subtypes are luminal B (ERα positive, PR negative), HER2 receptor over-expressing (ERα/PR positive or negative), and triple negative (ERα, PR, and HER2 negative) (13). While HER2 over-expressing tumors are still considered to be more aggressive than Luminal A or B, the prognosis for women with this breast cancer subtype has greatly improved in recent years with the introduction of drugs that specifically target the HER2 receptor (15). In contrast, triple negative breast cancers continue to be associated with a negative prognosis due to the lack of druggable targets in these tumors. While patients with luminal A cancers do tend to have the best prognoses, there are additional factors that can contribute to a poor breast cancer outcome, including obesity.

1.2 The Obesity Epidemic

Multiple measures of adiposity can be employed in order to define obesity, including waist to hip circumference ratio and body fat percentage. The latter may be assessed by skin caliper measurements, dual-energy x-ray absorptiometry, bioelectrical impendence, hydrostatic weighing, or air-displacement

plethysmography. However, body mass index (BMI) is typically used by organizations that track obesity trends, like the United States' Centers for Disease Control and Prevention and the World Health Organization, because of its relative ease of collection. BMI is defined as the ratio of an individual's mass in kilograms divided by her height in meters squared (kg/m^2). The resulting values are used to characterize individuals as underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2), or obese (≥ 30 kg/m^2). Certain individuals, particularly those with a large amount of lean muscle mass, may be misclassified by these BMI categories. However, for the majority of people, BMI is strongly correlated with body fat percentage as well as chronic disease risk and mortality (16).

Regardless of the measure used, there is little doubt that obesity has become a significant global health problem over the past 30 years. In the United States, the adult obesity rate has risen over that time period to a current rate of 35.7% (17). Similar trends are evident around the world and are no longer unique to wealthy, industrialized countries (18). The rise in obesity rates is alarming given the association between excess adiposity and an increased incidence of and mortality from numerous chronic diseases, including certain types of cancer.

1.3 Obesity and Breast Cancer

In postmenopausal women, obesity increases breast cancer risk by approximately 40% (3-5). While most research has indicated that obesity actually protects premenopausal women from breast cancer, two recent studies suggest

that the interaction may be more complex, differing with presence of other risk factors (6). A large body of evidence has established that obesity is also associated with a worse breast cancer prognosis for both pre- and postmenopausal women. One prospective study found that the breast cancer mortality rate escalates with each successive increase in BMI category, (19). Another study showed a significantly greater risk for disease recurrence within 10 years of diagnosis in breast cancer patients who were obese at the time of treatment in comparison to non-obese patients (20). These effects could be due to later diagnosis in the obese population, resulting in more advanced disease at the time of diagnosis. This hypothesis was initially supported by data from a large cohort of patients followed for a 20 year period; Majed et al. (21) reported that the obese patients presented with more advanced tumors, suggesting that diagnosis had been delayed. However, the authors ultimately found that multivariate statistical analysis demonstrated an independent effect of obesity on breast cancer prognosis, regardless of tumor stage at time of diagnosis. Survival analysis revealed increased metastatic recurrence as well as decreased disease-free interval and overall survival in the obese patient population. Additional studies have produced similar findings supporting the link between obesity and a worse prognosis (22, 23).

While obesity has been shown to negatively impact breast cancer outcome for both pre- and postmenopausal patients, the most prominent effects are seen in ERα positive postmenopausal patients, a finding confirmed by a recent

retrospective analysis of the German BRENDA-cohort (24). Obesity has been hypothesized to negatively impact outcomes in this patient population by promoting adipose tissue expression of aromatase, the rate-limiting enzyme in the production of estradiol from testosterone. Due to an abundance of this aromatase-expressing tissue, obese postmenopausal women typically have higher levels of circulating estradiol (25-27), and researchers have posited that this may contribute to the observed increase in breast cancer risk, recurrence, and mortality in this population.

1.4 Aromatase, Obesity, and Breast Cancer

1.4.1 Aromatase Expression and Regulation

Aromatase is a member of the cytochrome P450 superfamily of enzymes that is localized in the endoplasmic reticulum of estrogen-producing cells. It is an enzyme complex composed of two polypeptides: aromatase cytochrome P450, the product of the *CYP19A1* gene, and NADPH-cytochrome P450 reductase, a flavoprotein that is ubiquitously distributed in most cells. In premenopausal women, ovarian granulosa cells are the primary site of aromatase expression, while the adipose (specifically immature adipose fibroblasts, also called pre-adipocytes) is the principal aromatase-expressing tissue following menopause. Aromatase is also expressed at low levels in the placenta, bone, skin, brain, blood vessels, testicular Leydig cells, and the breast (28).

Aromatase expression is regulated by multiple partially-tissue specific promoters (29). Ovarian aromatase expression is induced by follicle stimulating hormone through the potent PII promoter (28), while aromatase levels in disease-free adipose tissue are governed by the relatively weak I.4 promoter, which requires combined stimulation by a glucocorticoid plus a class I cytokine like interleukin 6 (IL-6) or tumor necrosis factor alpha (TNFα). However, in the presence of the pro-inflammatory eicosanoid prostaglandin E2 (PGE2), pre-adipocytes will utilize the stronger I.3 and PII aromatase promoters. Breast cancer cells can also express aromatase via these promoters, but the mechanisms by which they are regulated differ from pre-adipocytes and remain poorly understood (28, 29).

It is known that paracrine interactions between these two cell types contribute to a substantial increase in local breast tissue aromatase expression and estrogen production with the development of breast cancer. Malignant breast epithelial cells secrete a large quantity of cytokines, including TNFα and interleukin-11, which inhibit the differentiation of pre-adipocytes into mature adipocytes. This promotes the desmoplastic reaction, an expansion in the population of adipose fibroblasts that creates a dense population of these aromatase expressing, estrogen-producing cells around the tumor (28, 29). In addition, the pre-adipocytes are stimulated to express higher amounts of aromatase via the I.3 and PII promoters by breast cancer cell-derived PGE2. Pre-adipocyte aromatase expression is also induced by additional unknown factors that the breast cancer cells produce (30).

While PGE2 is known to be independently capable of stimulating aromatase expression in pre-adipocytes, the other breast cancer cell secretory products make significant contributions to this paracrine effect. In fact, stimulation of pre-adipocyte aromatase expression via MCF-7 breast cancer cell conditioned media exposure persists with inhibition of the cancer cells' COX-2 activity, clearly showing that these other factors can independently promote pre-adipocyte aromatase expression (30, 31). The pro-angiogenic tumor environment also encourages the proliferation of endothelial cells that express aromatase via the I.7 promoter and contribute to estrogen production within the tumor microenvironment. Finally, the cancer cells themselves express additional aromatase via the I.3 and PII promoters (28, 29). The result of these paracrine interactions is a 5-fold elevation in aromatase expression in the tumor and associated adipose tissue in comparison to disease-free tissue. There is a corresponding increase in breast tumor estradiol levels, which can be up to 10-fold higher than systemic serum concentrations, suggesting that this estrogen is being produced locally and not taken up from circulation (32). In support of the hypothesis that paracrine interactions between the tumor and adipose tissue contribute to this local production, O'Neill et al. [33] demonstrated in their analysis of 12 breast samples that the highest aromatase activity is consistently found in the tumor-containing breast quadrant in comparison to the adjacent and distal quadrants.

1.4.2 Aromatase and Obesity

These findings suggest that an obesity-associated elevation in systemic estrogen levels will probably not have a significant impact on breast tumor ERα activity, given that a much larger local source of estrogen is already available in the tumor microenvironment and adjacent adipose tissue. However, aromatase and estrogen may still be key factors in the link between obesity and poor prognosis in ERα positive, postmenopausal breast cancer patients. Obesity is associated with increased circulating levels of several growth factors, cytokines, and adipokines that may enhance the paracrine interactions described above, resulting in a further elevation in local aromatase levels and estrogen production. For example, serum concentrations of IL-6 and TNFα are generally increased with obesity (34), and these cytokines have been shown to promote PGE2 production in multiple cell types via their effects on cyclooxygenase-2 (COX-2) expression (35-37). In addition, obesity is accompanied by elevated levels of free insulin-like growth factor (IGF-1) (38, 39), which can directly regulate the expression of aromatase in breast cancer epithelial cells. IGF-1 treatment *in vitro* increases aromatase expression in both MCF-7 and MDA-MB-231 breast cancer cells (40).

Recent studies by Dannenberg et al. confirmed the presence of elevated aromatase expression in the breast tissue of overweight and obese women with and without breast cancer. Crucially, they demonstrated high congruence between aromatase levels, obesity, and local breast tissue inflammation, as measured by the number of crown-like structures (CLS-B). CLS-B are inflammatory foci

composed of a necrotic adipocyte surrounded by macrophages, which produce excess amounts of several pro-inflammatory mediators, including PGE2 (41-43). In addition to promoting an increased number of these CLS-B, obesity is associated with another factor that may further enhance PGE2 production in adipose-infiltrating macrophages. Saturated fatty acids can promote COX-2 expression and PGE2 secretion by cultured macrophages (43, 44), and the increased lipolysis that accompanies obesity results in a higher concentration of circulating free fatty acids (45-47). Consequently, the obesity-associated elevation in breast tissue aromatase levels may also be due to free fatty acid-induced macrophage PGE2 production. Overall, this obesity-induced local breast aromatase expression and estrogen production may be a significant contributor to the worse outcome observed in obese postmenopausal patients with ERα positive breast cancer.

1.4.3 Obesity and Response to Aromatase Inhibitors

This hypothesis is supported by an analysis of data from the ATAC trial by Sestak et al. (48), which found that obese breast cancer patients receiving the aromatase inhibitor anastrozole had a significantly greater risk of recurrence. In agreement with these findings, Schmid et al. (49) demonstrated that obese patients have a reduced response rate to another aromatase inhibitor, letrozole, in comparison to lean (11 vs 35%). The ATAC trial also showed that while anatrozole treatment resulted in significantly greater recurrence-free survival in comparison

to the selective erstrogen receptor modulator tamoxifen, this advantage was lost in the obese cohort (48). In addition, while three extra years of anastrozole treatment has been shown to decrease normal weight patients' risk of disease recurrence and death by half, overweight and obese patients did not benefit from this treatment (50). Furthermore, plasma estradiol and estrone sulfate levels in obese patients remain significantly elevated in comparison to non-obese patients following letrozole treatment (51), suggesting that this reduced response rate is related to suboptimal inhibition of obesity-associated aromatase activity. It is possible that an adjustment in the aromatase inhibitor dosage, which is prescribed at a fixed amount, may improve obese patient prognosis. However, that inference is confounded by two phase III clinical trials of anastrozole that found no overall benefit from a 10 mg dose (versus 1 mg), indicating that an increased dosage may not be effective in overcoming obesity-induced resistance to aromatase inhibitors (52, 53).

1.5 Obesity and Endocrine Resistance

As discussed above, obese breast cancer patients do not respond as well as lean patients to aromatase inhibitor treatment. This resistance to aromatase inhibitor therapy may be mediated by increased local aromatase expression alone, but it is likely due to the activation of multiple signaling pathways. In general, endocrine therapy resistance can develop through a number of different mechanisms. Frequently, aberrant signaling from growth factor receptors, particularly the insulin-like growth factor 1 receptor (IGF-1R) and the HER family

of receptors, is responsible. These receptors can engage in bidirectional crosstalk with ERα, leading to increased nongenomic ERα activity, ligand-independent activation of ERα, and dysregulated cell cycle and apoptotic signaling. Nongenomic ERα activity results in the activation of the MAPK and PI3K/Akt signaling pathways, and these can in turn activate ERα via phosphorylation, leading to enhanced genomic ERα activity (54, 55).

Over the past decade, a number of researchers have successfully characterized several of the nongenomic interactions that occur between ERα and other signaling molecules in the cytoplasm. For example, Song et al. (56, 57) discovered that, in the presence of estradiol, ERα undergoes translocation to the plasma membrane and complexes with IGF-1R and the adaptor protein Shc, resulting in MAPK pathway activation. Down-regulation of IGF-1R prevents ERα translocation to the membrane, suggesting that IGF-1R signaling is necessary for nongenomic ERα activity. Ligand-bound ERα can also directly bind Src as well as p85, the regulatory subunit of PI3K, resulting in Akt activation downstream (58, 59). In addition, p85 can bind IGF-1R, leading to speculation that ERα may complex with both of these molecules upon activation by estradiol (60, 61). The receptor for leptin, an obesity-associated adipokine, has also been shown to crosstalk with IGF-1R, resulting in greater IGF-1R activation and an upregulation of Akt and ERK1/2 phosphorylation (62). This interaction could potentially enhance IGF-1R/ERα crosstalk. Activated Akt and ERK1/2 can in turn activate

ERα via phosphorylation at serine 167 and 118, respectively, within the receptor's AF-1 domain, leading to enhanced genomic ERα activity (63, 64).

Obesity is typically accompanied by elevated circulating levels of insulin, bioavailable IGF-1 and leptin, as well as a series of pro-inflammatory cytokines (34, 38, 39, 65). All of these obesity-associated circulating factors are able to activate the PI3K/Akt and/or MAPK pathways, potentially increasing the ERα crosstalk pathways described above and leading to endocrine resistance and breast cancer progression (66-70). In fact, the metabolic alterations associated with obesity, including changes in insulin and insulin-like growth factor binding protein 1 (IGFBP-1) serum levels (which result in increased circulating free IGF-1 levels), are significantly correlated with breast cancer recurrence and mortality (71). High serum concentrations of pro-inflammatory cytokines and leptin have been similarly linked to a worse breast cancer outcome (72-74). In addition, because obesity enhances local breast tissue aromatase expression and aromatase inhibitors fail to effectively suppress estrogen production in many obese patients, more estradiol is also available in the tumor microenvironment to activate these nongenomic ERα signaling pathways. Overall, obesity creates a complex metabolic imbalance accompanied by chronic inflammation, enriching the blood with a number of signaling molecules that may promote breast cancer progression and adversely affect outcome.

1.6 Dissertation Objectives

Given that obesity rates are climbing globally and have exceeded a third of the adult population in the United States, it is expected that the incidence and severity of many obesity-associated diseases will also continue to rise, including breast cancer. Obesity negatively impacts breast cancer prognosis across all ages and breast cancer subtypes. Consequently, while breast cancer mortality rates have improved in recent years, the obesity epidemic may be mitigating the positive impact of treatment advancements and other factors on survival rates. In addition, obesity rates in the United States are highest among older women, and it is in this postmenopausal population that the strongest link between obesity and a poor breast cancer prognosis is seen. Thus, this project was designed to expand our understanding of the mechanisms by which obesity promotes postmenopausal ERα positive breast cancer, with a focus on examining the role of obesity-induced local aromatase expression and estrogen production.

We hypothesized that obesity promotes postmenopausal breast cancer progression by enhancing local estrogen production via an inflammatory cytokine-induced elevation in aromatase expression and activity in the mammary stroma and epithelium. An *in vitro* model of obesity (Illustration 1.1) was designed that utilized serum samples from postmenopausal breast cancer patients pooled by BMI category (normal weight: 18.5-24.9 kg/m2; obese: ≥30 kg/m2). Various cultured cells, including breast cancer cells, pre-adipocytes, and macrophages, were exposed to the pooled sera alone and in co-culture in order to mimic the

impact of obesity on the tumor microenvironment. We examined the direct impact of obese patient sera on breast cancer cell and pre-adipocyte aromatase expression as well as the ability of the obese patient sera to promote pre-adipocyte, breast cancer cell, and macrophage production of aromatase-promoting factors like IL-6 and PGE2. Through elucidation of the signaling pathways responsible for the obesity-associated elevation in local aromatase expression, our ultimate goal was to identify a more effective chemotherapeutic regimen for postmenopausal, ERα positive breast cancer patients.

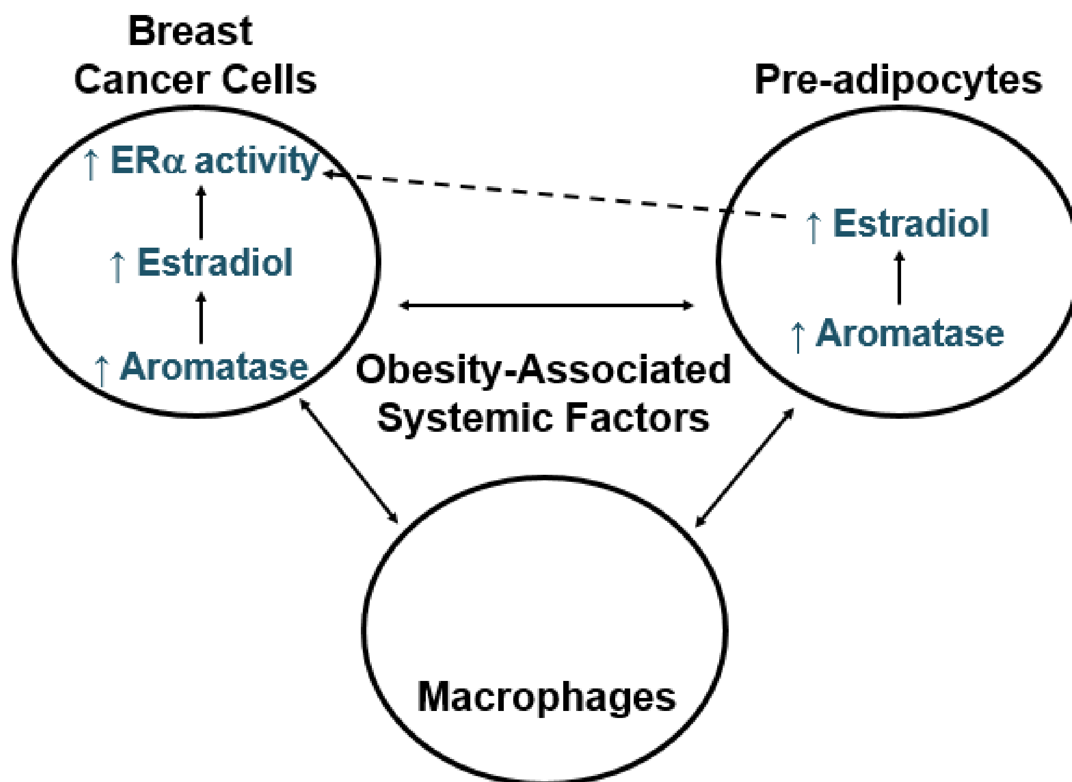

Illustration 1.1 An *in vitro* model of obesity and the tumor microenvironment

Studies examining the role of docetaxel resistance and a HER2-mediated reduction in ERβ expression in the obesity-breast cancer progression link were also initiated, along with an investigation of the obesity's impact on tumor incidence and characteristics in the MMTV-Wnt1 mouse model of mammary carcinogenesis. Preliminary results from these studies are presented here.

Chapter 2: Breast cancer cell aromatase expression is stimulated by obesity-associated PI3K/Akt, MAPK, and interleukin-6 signaling

2.1 Introduction

According to the United States Centers for Disease Control and Prevention, over a third of U.S. women are currently obese, defined as a body mass index (BMI) ≥30 kg/m^2 (17). This indicates that there is a substantial need for better understanding of the mechanisms linking obesity with various chronic diseases, including breast cancer. In postmenopausal women, epidemiological studies have shown that obesity increases the risk of developing breast cancer, with one study demonstrating a 41% elevation in risk between the lowest and highest BMI quintiles (3, 75). It has also been established that obesity is associated with a worse breast cancer prognosis for both pre- and postmenopausal women. One prospective study found that the mortality rate due to breast cancer was amplified with each successive increase in BMI category (19). Other research has shown a significantly greater risk for disease recurrence within 10 years of diagnosis in breast cancer patients who were obese at the time of treatment in comparison to non-obese patients (20). One mechanism for this negative effect may be reduced response to endocrine therapy (22), as researchers have found that obese postmenopausal patients' response rate to the aromatase inhibitor letrozole is significantly lower in comparison to lean patients' (11% vs 35%) (49).

The key enzyme responsible for the conversion of androgens to estrogens is aromatase, encoded by the *CYP19A1* gene (28). Following menopause, the primary site of aromatase activity shifts from the ovarian granulosa cells to the adipose tissue (76). The majority of breast tumors in obese postmenopausal women are estrogen receptor alpha (ERα) positive, suggesting that elevated estrogen synthesis by the adipose tissue may be the primary mediator of breast tumorigenesis in this population (77). This is supported by a recent study demonstrating that free plasma estradiol levels rise by approximately 20% for each 5 kg/m^2 increase in BMI among postmenopausal women (78). However, aromatization of androgens into biologically active estradiol also occurs in the breast tissue, indicating that elevated local estrogen production may also be a factor in the increased breast cancer risk and worse prognosis seen in the obese postmenopausal population. In fact, examination of breast carcinoma tissue samples has revealed estradiol concentrations ten-fold greater than circulating plasma levels and two times the concentration found in normal breast tissue. This is correlated with a four to five-fold increase in aromatase expression within the breast tumor (32). Taken together, this data has led many researchers to hypothesize that factors associated with obesity may be driving local aromatase expression and activity even higher, thereby promoting greater local estrogen production and activity. However, the specific mechanisms by which this occurs are not clearly understood.

The current study examined the impact of obesity-associated circulating factors on the expression of aromatase in breast cancer cells, utilizing an *in vitro* model of obesity. Systemic obesity-associated factors were shown to both directly and indirectly enhance breast cancer cell aromatase expression, resulting in subsequent increases in ERα activity and cell viability. Through better understanding of the mechanisms mediating these effects, an improved chemotherapeutic regimen for the obese postmenopausal population may be developed.

2.2 Materials and Methods

2.2.1 Serum Samples

Serum was collected from 25 postmenopausal breast cancer patients under an IRB approved biorepository collection protocol at the Cancer Therapy and Research Center of University of Texas Health Science Center at San Antonio. The collection and use of these biological samples was conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained prior to participation, and all samples and data were de-identified prior to release to maintain patient confidentiality. BMI was calculated, and serum was pooled according to the BMI category of the patient (normal weight (N): 18.5-24.9 kg/m^2; obese (OB): ≥30.0 kg/m^2). The free IGF-1 concentration of each patient's serum sample was measured using the MILLIPLEX MAP Human IGF-1 Single Plex Metabolism Assay, while the MILLIPLEX MAP Human Serum Adipokine

Panel A and B kits were used to assess patient serum concentrations of insulin, interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), leptin, and adiponectin (EMD Millipore, Billerica, MA).

2.2.2 Cell Lines and Reagents

MCF-7 breast cancer cells (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS). Pre-adipocytes isolated from women undergoing elective surgical procedures were a generous gift from Dr. Rong Li, UTHSCSA, and have been described previously (79). They were maintained in DMEM/F12 1:1 media (GIBCO Life Technologies) plus 10% FBS. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) was purchased from Sigma-Aldrich (St. Louis, MO). Testosterone, anastrozole, LY 294,002, and PD 98,059 were also obtained from Sigma-Aldrich. The Akt II inhibitor and IL-6 depleting antibody were produced by EMD Millipore (Billerica, MA). The primary antibodies for pAkt (s473), tAkt, pERK1/2, and tERK1/2 were purchased from Cell Signaling (Beverly, MA).

2.2.3 Pre-adipocyte Conditioned Media

Pre-adipocyte conditioned media (CM) was generated by seeding $2x10^5$ pre-adipocytes per well in 6-well plates, allowing them to grow to confluence, then serum-starving the cells for 18 hours. The pre-adipocytes were then exposed to a 2% concentration of the pooled N or OB sera in serum-free media (SFM) for three hours, the sera removed and cells washed once with phosphate buffered saline

(PBS) followed by incubation in SFM for 24 hours. Over the course of this 24 hours, the cells release various factors into the SFM, enriching it into CM, which was then collected and stored at -20°C for subsequent *in vitro* assays.

2.2.4 Quantitative RT-PCR

MCF-7 cell *CYP19A1* (aromatase) mRNA levels were assessed following a 24-hour incubation in 2% N or OB patient sera (in SFM) or pre-adipocyte CM. The MCF-7 cells were serum-starved for 18 hours prior to sera or CM exposure. To assess the role of the PI3K, Akt and MAPK signaling in the sera's effects on aromatase expression, the MCF-7 cells were pre-treated with LY 294,002, Akt II inhibitor, and PD 98,059 for one hour prior to as well as during sera exposure. The pre-adipocyte CM was incubated with an IL-6 depleting antibody for one hour prior to its addition to the MCF-7 cells in order to examine the impact of pre-adipocyte-secreted IL-6 on MCF-7 cell aromatase expression. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer sequences are as follows: *CYP19A1*: forward, 5'-GCCGAATCGAGAGCTGTAAT-3'; reverse, 5'-GAGAATTCATGCGA GTCTGGA-3'.

2.2.5 ERE Luciferase Assay

A luciferase reporter gene driven by a 3X ERE-tk promoter was utilized to measure ERα transcriptional activity. Transient transfections were performed in triplicate wells three times. MCF-7 cells were seeded at a density of 1.5 x 10^4 in 24 well plates and concurrently transfected with the ERE luciferase and renilla

plasmids after 24 hours of growth using Fugene 6 from Promega (Madison, WI) at a 1:3 ratio. The cells were serum-starved for six hours the following day, then exposed for 48 hours to 2% patient sera in SFM or pre-adipocyte CM +/- testosterone (100 nM) +/- anastrozole (1 uM). Luciferase activity was then measured using Promega's Dual Luciferase Reporter Assay System, with the fluorescence read on a FLUOstar Omega Spectrometer (BMG Labtech, Offenberg, Germany). Relative ERα activity was calculated by dividing each fluorescence value (standardized to renilla) by the value for cells grown in N patient sera or CM with testosterone.

2.2.6 MTT Assay

MCF-7 cells were seeded at a density of $8x10^3$ in 96 well plates. After 24 hours of growth in the 10% FBS media, the cells were exposed to 2% patient sera in SFM or pre-adipocyte CM +/- testosterone (100 nM) +/- anastrozole (1 uM) for 48 hours. MTT reagent in PBS (5 mg/ml) was then added to each well to a final concentration of 0.5 mg/ml. After two hours of incubation at 37°C, the media was removed and 50 ul DMSO added to each well to lyse the cells. Absorbance was read at 570 nm on a FLUOstar Omega Spectrometer (BMG Labtech, Offenberg, Germany). Relative cell viability was calculated by dividing each absorbance value by the absorbance for cells grown in N patient sera or CM with testosterone.

2.2.7 Western Blot Analysis

The MCF-7 cells were grown to 80% confluence, then serum-starved for 18 hours and exposed to 2% patient sera in SFM for 15 minutes or one hour. Kinase lysis buffer was used for protein extraction. Protein content of the lysates was measured using the BCA Protein Assay kit from Thermo Scientific Pierce (Rockford, IL). Images were acquired using a Syngene G:BOX Chemi (Frederick, MD). Relative protein levels were calculated by first standardizing phosphorylated protein to total protein levels for each experimental condition, then dividing the standardized protein level for each condition by that of cells grown in N patient sera. Data from at least three independent experiments was compiled for each protein to calculate the average protein level, standard error of the mean and statistical significance, with one representative image for each protein shown.

2.2.8 Statistical analysis

Differences between cells exposed to two different experimental conditions were measured using Student's *t* test. Two-way ANOVA was used for experiments with two independent variables. A p value of <0.05 was considered significant.

2.3 Results

2.3.1 Patient Characteristics

Table 2.1 describes the postmenopausal breast cancer patients that provided the sera utilized in this study, which was pooled into two groups by BMI category, obese (OB) and normal weight (N). There was no significant difference in the average patient age between groups. The average patient BMI in the OB group

was significantly higher than the N group ($p<0.01$), and this was accompanied by significantly higher levels of IL-6 ($p<0.05$), TNFα ($p<0.05$), and leptin ($p<0.01$), as well as lower levels of adiponectin ($p<0.05$). In addition, the OB group's serum insulin levels were almost five-fold greater, a difference that approached significance ($p=0.10$), but there was no difference in free IGF-1 concentration. Sixty percent of the patients in the OB group were Hispanic, while the N group was predominantly white. Diabetes and hypercholesterolemia were found in at least 25% of the patients in the OB group, but were not present in the N group. Those diagnosed with these conditions had all been prescribed metformin and statins, respectively, two drugs with possible anti-cancer effects (80-85). The majority of patients in both groups were receiving either aromatase inhibitor or tamoxifen treatment.

Table 2.1 Patient Characteristics

	Obese	Normal weight
Number of patients	20	5
Average Age (years)	59.7 (6.28)	59.5 (4.76)
Average BMI (kg/m^2)	36.6 (5.04)**	21.1 (2.17)
Average Serum Concentrations		
Free IGF-1 (ng/ml)	4.92 (0.925)	5.43 (1.80)
Insulin (pg/ml)	742.9 (156.8)	157.4 (44.1)
Interleukin 6 (pg/ml)	4.6 (0.58)*	1.6 (0.29)
TNF-α (pg/ml)	7.1 (0.51)*	4.6 (0.19)
Leptin (ng/ml)	36.6 (4.67)**	6.40 (3.86)
Adiponectin (ug/ml)	54.5 (6.55)*	97.5 (32.0)
Ethnicity/Race		
Hispanic	12	0
White	6	3
African-American	2	0
Asian	0	1
Not Available	0	1
Confounding Conditions		
Diabetes	5	0
Hypercholesterolemia	8	0
Hypertension	14	1
Medications		
Aromatase Inhibitor	9	1
Tamoxifen	5	2
Chemotherapy	6	2
Metformin	5	0
Statin	8	0

(Standard error of the mean); *, $p<0.05$; **, $p<0.01$ in comparison to normal weight

2.3.2 Obesity-associated circulating factors stimulate breast cancer cell aromatase expression

In order to examine the direct impact of obesity-associated circulating factors on breast cancer cell aromatase expression, MCF-7 cells were cultured in pooled serum from obese (OB) or normal weight (N) postmenopausal breast cancer patients. MCF-7 cell aromatase expression was 43% higher ($p<0.05$) after a 24 hour incubation in OB versus N patient sera (Figure 2.1a). When the cells were simultaneously exposed to sera and testosterone, the substrate for aromatase, ERα activity was 2.5 fold greater ($p<0.05$) under OB versus N conditions. In contrast, there was no difference in ERα activity when the cells were exposed to OB versus N patient sera alone. This indicates that any variance between the OB and N sera in estradiol concentration was not sufficient to induce a difference in breast cancer cell ERα activity. It also suggests that MCF-7 cell-produced estradiol is responsible for the difference in ERα activity seen with the addition of testosterone. In support of this conclusion, aromatase inhibition with anastrozole during sera and testosterone exposure eliminated the difference between the OB and N patient sera conditions (Figure 2.1b). To examine whether this OB patient sera-induced aromatase expression and ERα activity could promote breast cancer progression, MCF-7 cell viability was measured. There was a non-significant difference in viability when the cells were exposed to OB versus N patient sera alone, but the addition of testosterone resulted in OB patient sera-induced viability

levels that were significantly elevated in comparison to N. Aromatase inhibition again eliminated the difference between the OB and N plus testosterone conditions (Figure 2.1c).

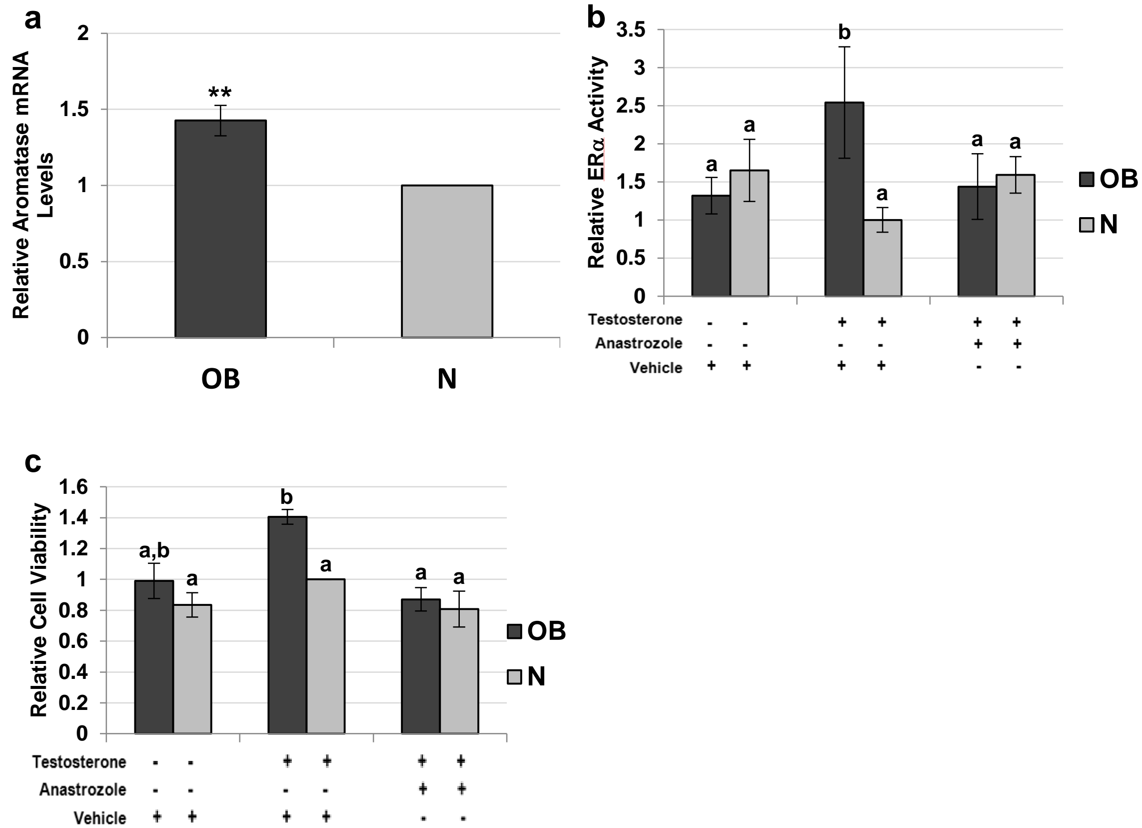

Figure 2.1 Obesity-associated circulating factors promote breast cancer cell aromatase expression, ERα activity, and viability. (a) Aromatase expression in MCF-7 cells exposed to 2% obese (OB) or normal weight (N) patient sera for 24 hours. (b) ERα activity, measured by ERE luciferase, in MCF-7 cells cultured in 2% OB or N patient sera +/- vehicle, testosterone, and/or anastrozole for 48 hours. (c) MCF-7 cell viability, assessed by MTT assay, following culture in the same conditions as (b) for 48 hours. **, $p<0.01$; different letters indicate significant differences, $p<0.05$.

2.3.3 Akt and MAPK pathway signaling mediates the effects of obese patient sera on breast cancer cell aromatase expression

Obesity is associated with elevated levels of numerous growth factors, adipokines, and inflammatory cytokines that can activate the Akt and MAPK signaling pathways, which could mediate the effects of the OB patient sera on breast cancer cell aromatase expression (34, 38-39, 65-70). To investigate this hypothesis, activation of these two pathways was first examined by western blot analysis. MCF-7 cells exposed to OB patient sera for 15 minutes and 1 hour had 2-fold ($p<0.01$) and 55% ($p<0.05$) higher levels of pAkt (ser473), respectively, in comparison to cells incubated in N patient sera. pERK1/2 levels were 79% and 33% greater ($p<0.05$) after Ob versus N patient sera exposure for the same time periods (Figure 2.2a). The role of these pathways in OB sera-induced aromatase expression was examined using three inhibitors: LY 294,002 (LY, a PI3K inhibitor), Akt II inhibitor (AktII), and PD 98,059 (PD, a MEK inhibitor). MCF-7 cell treatment with AktII or PD significantly inhibited OB sera-induced aromatase expression, bringing it down to the level of the N sera condition. Intriguingly, all three inhibitors significantly increased MCF-7 cell aromatase expression when combined with N patient sera exposure (Figure 2.2b).

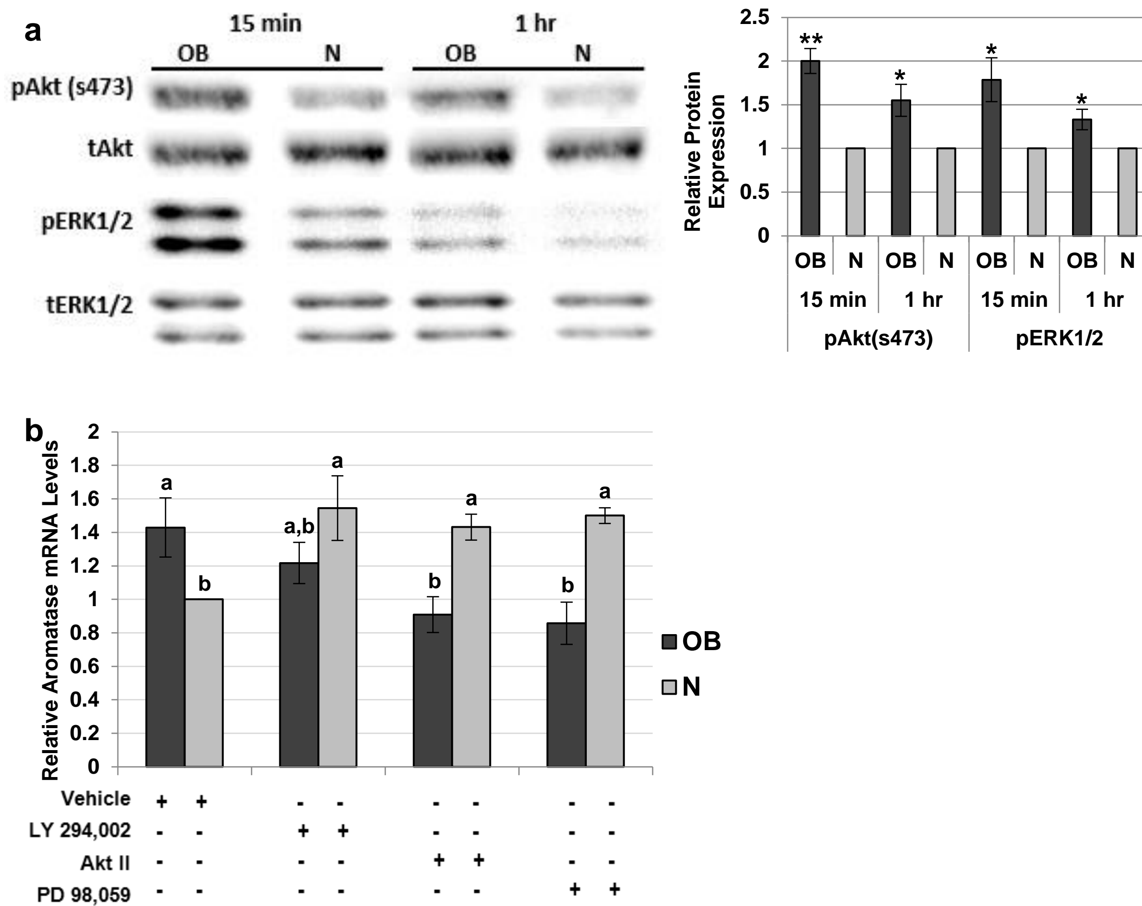

Figure 2.2 Obesity-associated systemic factors stimulate breast cancer cell aromatase expression via Akt and MAPK signaling pathways. (a) MCF-7 cells were exposed to 2% obese (OB) or normal weight (N) patient sera for 15 min or 1 hr; pAkt (ser473) and pERK1/2 protein levels were then measured by western blot and standardized to tAkt and tERK1/2 protein levels, respectively. Densitometry data from at least three independent experiments were compiled for each protein and cell line to calculate the average protein level, standard error of the mean and statistical significance, with one representative image for each protein shown. (b) Aromatase expression in MCF-7 cells exposed to 2% OB or N patient sera +/- vehicle, LY 294,002, Akt II inhibitor (AktII), or PD 98,059 for 24 hr. *, $p<0.05$; **, $p<0.01$; different letters indicate significant differences, $p<0.05$.

2.3.4 Obesity-associated systemic factors indirectly promote breast cancer cell aromatase expression via stimulation of pre-adipocyte interleukin-6 production

Researchers have previously established that aromatase expression is upregulated in breast cancer cells that have been co-cultured with patient-derived intra-tumoral stromal cells (86). Adipose fibroblasts, also known as pre-adipocytes, are one type of stromal cell found within breast tumors and the adjacent mammary tissue. These cells are known to produce inflammatory factors, including interleukin-6 (IL-6), that promote aromatase expression (28). Consequently, we next examined the impact of OB patient sera exposure on pre-adipocyte production of aromatase-promoting factors. MCF-7 cells cultured in conditioned media (CM) from pre-adipocytes exposed to OB versus N patient sera expressed 74% more ($p<0.01$) aromatase (Figure 2.3a). While there was no difference in MCF-7 cell ERα activity when the cells were exposed to OB versus N CM alone, the addition of testosterone to the CM resulted in a significant elevation in OB CM-induced ERα activity. This suggests that the OB sera-induced elevation in MCF-7 cell aromatase expression can lead to greater estradiol production and ERα activity. The significant suppression of OB CM-induced MCF-7 cell ERα activity with anastrozole treatment supports this conclusion (Figure 2.3b). MCF-7 cell viability under the same conditions mirrored ERα activity, with OB versus N CM producing similar levels of viability. The addition of testosterone led to elevated OB CM-induced viability in comparison to N CM ($p<0.05$), and

aromatase inhibition again significantly reduced viability in the OB CM condition, neutralizing the difference between OB and N CM (Figure 2.3c). To investigate whether this obesity-associated aromatase-promoting paracrine interaction is mediated by an increase in pre-adipocyte IL-6 production, the CM was depleted of IL-6 prior to use. IL-6 neutralization decreased OB CM-induced aromatase expression by 63% ($p<0.05$), eliminating the difference between OB and N CM (Figure 2.3d).

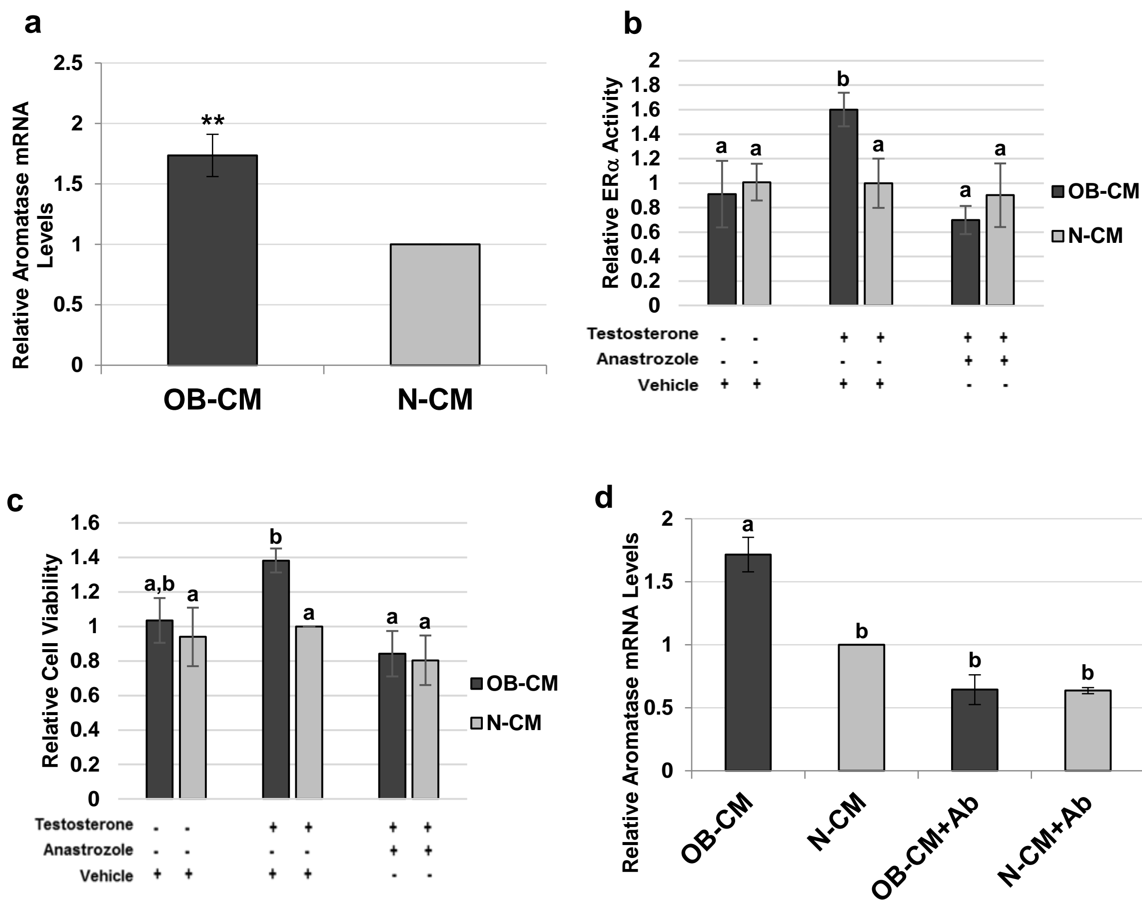

Figure 2.3 Breast cancer cell aromatase expression is enhanced by obesity-induced pre-adipocyte interleukin-6 production. (a) Aromatase expression in MCF-7 cells cultured for 24 hours in pre-adipocyte conditioned media (CM) generated by exposure to obese (OB) or normal weight (N) patient sera (OB-CM, N-CM). (b) ERα activity, assessed by ERE luciferase assay, in MCF-7 cells after a 48 hour incubation in pre-adipocyte OB-CM or N-CM +/- vehicle, testosterone, and anastrozole. (c) MCF-7 cell viability, measured by MTT assay, following a 48 hour exposure to the same conditions as (b). (d) Aromatase expression in MCF-7 cells cultured for 24 hours in OB-CM, N-CM or CM generated with sera depleted of IL-6 with a neutralizing antibody (OB+Ab-CM, N+Ab-CM). **, $p<0.01$; different letters indicate significant differences, $p<0.05$

2.4 Discussion

The role of aromatase expression and estradiol production in the obesity-breast cancer link has been the subject of numerous studies in recent years. Many have attributed the association between obesity and increased postmenopausal breast cancer risk and progression to an excess of aromatase-expressing adipose tissue. However, this theory fails to consider how obesity impacts estradiol production in the local breast tissue once a tumor has already formed, where both the pre-adipocytes and malignant breast epithelial cells are significant sources of aromatase.

In the current study, we demonstrated that obesity-associated circulating factors directly and indirectly promote breast cancer cell aromatase expression. Sera from obese versus normal weight postmenopausal breast cancer patients stimulated greater MCF-7 breast cancer cell aromatase expression via Akt and MAPK-mediated signaling pathways. Obesity is associated with elevated levels of numerous circulating growth factors, adipokines, and inflammatory cytokines that can activate the Akt and MAPK signaling pathways, including insulin, IGF-1, leptin, and IL-6, and *in vitro* and animal studies have established that these obesity-associated factors play a role in breast tumorigenesis (34, 38-39, 65-70, 87). IGF-1 has specifically been shown to regulate the expression and activity of aromatase in breast cancer epithelial cells. Aromatase expression in both ERα positive MCF-7 and negative MDA-MB-231 breast cancer cells is increased by IGF-1 treatment (40). In addition, IGF-1 enhances aromatase activity in the aromatase-

overexpressing MCF-7aro and T47Daro cell lines via post-transcriptional mechanisms (88). While there was no difference in serum free IGF-1 levels between the obese and normal weight patients in our study population, insulin can also activate the IGF-1R, and there was a difference in serum insulin levels that approached significance. Leptin has also been shown to directly upregulate MCF-7 cell aromatase expression via MAPK pathway activation of AP-1 (89). Given that serum leptin concentrations were significantly higher in our obese versus normal weight patients, it is possible that the effects of the obese patient sera on MCF-7 cell aromatase expression are at least partly mediated by leptin. IL-6 signaling may also be playing a role, though, as the obese patients had significantly higher serum concentrations of this inflammatory cytokine, and it is known to stimulate breast cancer cell aromatase expression (28). In sum, the direct aromatase-inducing effects of the obese patient sera could be mediated by a number of obesity-associated circulating factors, and it is likely that these effects are due to a combination of the various factors discussed above.

Intriguingly, inhibition of the PI3K, Akt, and MAPK pathways during normal weight sera exposure actually enhanced MCF-7 cell aromatase expression. This may be related to the inhibition of negative feedback loops that normally act to attenuate signaling via these pathways. For example, IGF-1R/insulin receptor-mediated PI3K/Akt signaling is typically suppressed by a negative feedback loop in which S6K, following activation by mTOR, inhibits the insulin receptor substrate 1/2 (IRS1/2) proteins located upstream of PI3K/Akt (90). Inhibition of PI3K or Akt

prevents activation of this loop, possibly resulting in continued stimulation of the aromatase-promoting pathway. Similarly, inhibition of MAPK signaling has also been shown to upregulate PI3K/Akt signaling in several breast cancer cell lines (91). However, it remains unclear why these effects are only seen in the cells exposed to normal weight versus obese patient sera.

We have also demonstrated that the obese patient sera indirectly promotes breast cancer cell aromatase expression by stimulating pre-adipocyte IL-6 production. IL-6 is known to induce breast cancer cell aromatase expression (28). Pre-adipocytes, found in abundance within the tumor microenvironment and adjacent breast tissue, produce a number of inflammatory cytokines, including IL-6 (92). Expression of the gene for IL-6 is regulated by tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), and IL-6 itself, among other factors. We did not measure serum IL-1 levels in our patient population, but serum TNFα and IL-6 concentrations were both significantly greater in the obese patients. Consequently, one or both of these two cytokines may be responsible for the obese patient sera's promotion of this aromatase-inducing paracrine interaction.

Obesity has been significantly and independently associated with postmenopausal breast cancer progression and a worse outcome, but the specific molecular mechanisms responsible for this link remain unclear. As obesity rates among women continue to rise, improvements in breast cancer mortality rates could be compromised, suggesting that further research regarding the link between obesity and breast cancer is critical. This study demonstrated that

obesity-associated circulating factors promote breast cancer cell aromatase expression via multiple pathways. Further elucidation of the mechanisms mediating these effects may inform the development of a more effective chemotherapeutic regimen for this high risk population.

Chapter 3: NSAID use reduces breast cancer recurrence in overweight and obese women: Role of prostaglandin-aromatase interactions

3.1 Introduction

Obesity has become a significant global public health problem in the past 30 years (18). More than 35% of Americans have a body mass index (BMI) of ≥30 kg/m^2 (17), an alarming percentage given obesity's association with an increased incidence of and mortality from numerous chronic diseases, including breast cancer. Several studies have demonstrated a link between obesity and a worse breast cancer prognosis in both pre- and postmenopausal women, including a prospective study of almost 500,000 women that found a progressive escalation in the risk of breast cancer mortality with each successive increase in BMI category (19). Other studies have established positive correlations between obesity and breast cancer recurrence (20), a shorter disease-free survival and lower rates of overall survival, independent of tumor stage at diagnosis (21-23).

Obesity is hypothesized to negatively impact outcomes in postmenopausal, hormone-responsive breast cancer patients by promoting adipose tissue expression of aromatase, the rate-limiting enzyme in estradiol production. This theory has been supported by the results of clinical studies with anastrazole and letrozole showing a reduction in the efficacy of these aromatase inhibitors with increasing BMI in postmenopausal breast cancer patients (48-49). Plasma estradiol and estrone sulfate levels in obese patients remain significantly elevated

in comparison to non-obese patients following letrozole treatment (51), suggesting that this reduced response rate is related to suboptimal inhibition of obesity-associated aromatase activity. Recent findings by Dannenberg et al. confirm the presence of elevated aromatase expression in the breast tissue of overweight and obese women (52-53). Crucially, they have demonstrated high congruence between aromatase levels and local breast tissue inflammation, as measured by the number of crown-like structures (CLS-B). CLS-B is an inflammatory focus composed of a necrotic adipocyte surrounded by macrophages, which produce excess amounts of several pro-inflammatory mediators. These include COX-2 derived prostaglandin E2 (PGE2), a potent stimulant of aromatase expression in pre-adipocytes, the predominant site of aromatase expression within adipose tissue (41-43). This obesity-inflammation-aromatase axis may be a significant contributor to the reduced aromatase inhibitor response and increased mortality rate observed in obese postmenopausal patients. Collectively, these studies suggest that obese postmenopausal women with estrogen receptor alpha (ERα) positive breast cancer may benefit from combining hormone therapy with a drug targeting the COX-2 pathway.

In this study, we retrospectively examined the association between non-steroidal anti-inflammatory drug (NSAID) use and recurrence rate in a hormone-responsive breast cancer patient population in order to determine whether this COX-2 pathway-targeting drug group improves prognosis. NSAID use reduced the recurrence rate by approximately 50% and extended the disease-free survival by

more than two years in our largely overweight/obese, postmenopausal population of women. Furthermore, to assess whether inflammation-associated pre-adipocyte aromatase expression may be responsible for this effect, we utilized an *in vitro* model of the obese patient's tumor microenvironment. Here we demonstrated that obesity-associated circulating factors enhance macrophage-derived PGE2 induction of pre-adipocyte aromatase expression and estradiol production, resulting in greater breast cancer cell ERα activity, proliferation and migration.

3.2 Materials and Methods

3.2.1 Subjects

Review of medical records dated between 01/01/1987 and 1/12/2011 from the Cancer Therapy and Research Center (CTRC) at the University of Texas Health Science Center at San Antonio (UTHSCSA) and the START Center for Cancer Care clinic in San Antonio, Texas, yielded a patient population of 440 women diagnosed with invasive, ERα positive breast cancer. Exclusion criteria included diagnosis of carcinoma *in situ*, hormone therapy refusal or non-compliance, unavailable treatment information, discontinuation of adjuvant therapy due to insurance issues or health problems, diagnosis of triple negative breast cancer, breast cancer metastasis at the time of diagnosis, unavailable diagnosis dates, and underweight status. The collection of patient data from these medical records was approved by the Institutional Review Boards (IRB) of UTHSCSA and

START and conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained from all patients prior to participation.

3.2.2 Study Design and Data Collection

This exploratory study utilized retrospective data collected from patient charts. Information regarding demographics, tumor stage, ERα and progesterone receptor status, medications, baseline weight (or the earliest recorded weight), height, and menopausal status was extracted. Body mass index (BMI) was calculated and patients classified as normal weight (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2), or obese (≥30.0 kg/m^2). Perimenopausal women were classified as premenopausal because they receive tamoxifen as their first-line hormonal therapy (93-94). Patients were designated as NSAID users if progress notes described daily use of aspirin, ibuprofen, celecoxib, naproxen, meloxican or another COX-2 inhibitor in the list of medications. Use of analgesics that do not target COX-2, such as acetaminophen and hydrocodone, as well as prn COX-2 inhibitor use did not qualify for inclusion in the NSAID user group. Recurrence was defined as any local, contralateral, distant tumor or metastasis of the primary breast cancer. Second primaries were not classified as recurrent breast cancer.

3.2.3 Serum Samples

Serum was collected from 25 postmenopausal breast cancer patients under an IRB approved biorepository collection protocol at the CTRC of UTHSCSA. The

collection and use of these biological samples was conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained prior to participation, and all samples and data were deidentified prior to release to maintain patient confidentiality. Serum was pooled according to the BMI category of the patient (normal weight or obese). Serum from five normal weight and five obese postmenopausal women, who were ineligible control subjects in the Polish Women's Health Study, was also utilized. The design of this study has been described elsewhere (95). The serum from these patients was not pooled.

3.2.4 Cell Lines and Reagents

The ERα positive MCF-7 and T47D and ERα negative MDA-MB-231 breast cancer cell lines (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS). Pre-adipocytes isolated from women undergoing elective surgical procedures were a generous gift from Dr. Rong Li, UTHSCSA, and have been described previously (79). They were maintained in DMEM/F12 1:1 media (GIBCO Life Technologies) plus 10% FBS. U937 monocytes (ATCC) were cultured in RPMI supplemented with 10% FBS and matured to macrophages at the time of seeding for experimentation by incubating the cells in 10.0 ng/ml phorbol 12-myristate 13-acetate (TPA) for 48 hours. Testosterone, anastrozole, celecoxib, and TPA were purchased from Sigma-Aldrich.

3.2.5 Conditioned Media

Macrophage conditioned media (CM) was generated by seeding $4x10^5$ U937 monocytes per well in six-well plates, stimulating them to mature into macrophages with TPA, then serum-starving the cells for six hours. The macrophages were then exposed to a 2% concentration of the pooled or individual sera samples for one hour, the sera removed and cells washed once with phosphate buffered saline (PBS) followed by incubation in serum-free media (SFM) for 24 hours. Over the course of this 24 hours, the cells release various factors into the SFM, enriching it into CM, which was then collected and stored at -20°C for subsequent *in vitro* assays. To assess the impact of macrophage COX-2 inhibition, the macrophages were pre-treated with 30 uM celecoxib for one hour prior to as well as during sera exposure. Macrophage/pre-adipocyte co-culture CM was generated by first stimulating $2x10^5$ U937 monocytes per well to mature into macrophages on top of confluent pre-adipocytes seeded in six-well plates. The co-culture was serum-starved for six hours, exposed to a 2% concentration of the pooled sera for one hour, then washed once with PBS and incubated for 24 hours in SFM with or without the addition of testosterone (100 nM) and/or anastrozole (1 uM). The enriched SFM, now considered CM, was then collected and stored at -20° C for use in subsequent *in vitro* assays.

3.2.6 Quantitative RT-PCR

Macrophage *PTGS2* (COX-2) mRNA levels were measured following a six-hour serum-starvation period and a one-hour exposure to a 2% concentration of obese or normal weight pooled patient sera. To determine whether macrophage

COX-2 expression continued to change during the 24-hour SFM incubation period of CM generation, mRNA levels were also assessed after the six-hour serum-starvation period to establish a baseline level (prior to sera exposure) and after 12 and 24 hours in SFM (following the removal of the sera). Pre-adipocyte *CYP19A1* (aromatase) mRNA levels were assessed following a 24-hour incubation in macrophage CM. MCF-7 and T47D *TFF1* (pS2) and *CCND1* (cyclin D1) mRNA levels were measured after a 24-hour incubation in macrophage/pre-adipocyte co-culture CM supplemented with 2% charcoal-stripped (CS)-FBS. All cells were serum-starved for six hours prior to exposure to sera or CM. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer sequences are as follows: *PTGS2*: forward, 5'-CCCTTGGGTGTCAAAGGTAA-3'; reverse, 5'-GCCCTCGCTTATGATCT GTC-3'; *CYP19A1*: forward, 5'-GCCGAATCGAGAGCTGTAAT-3'; reverse, 5'-GAGAATTCATGCGAGTCTGGA-3'; *TFF1*: forward, 5'-GGTCGCCTTGGAGCAGA-3'; reverse, 5'-GGGCGAAGATCACCTTGTT-3'; *CCND1*: forward, 5'-TGGAGGTCTGCGA GGAACAGAA-3'; reverse, 5'-TGCAGGCGGCTCTTTTTCA-3'. The manufacturer's recommended cycling conditions for the QuantiFast SYBR Green PCR kit (Qiagen) were used. Data shown represents the average of at least three independent experiments, with the exception of pre-adipocyte aromatase expression measured following culture in macrophage CM generated with the individual serum samples from the Polish Women's Health Study.

3.2.7 Prostaglandin E2 and Estradiol Concentrations

The concentration of prostaglandin E2 in macrophage CM was measured using the Prostaglandin E2 Parameter Assay Kit from R&D Systems (Minneapolis, MN). The estradiol concentration in macrophage/pre-adipocyte co-culture CM was analyzed with the Estradiol EIA Kit from Cayman Chemical (Ann Arbor, MI).

3.2.8 ERE Luciferase Assay

A luciferase reporter gene driven by a 3x ERE-tk promoter was utilized to measure ERα transcriptional activity. Transient transfections were performed in triplicate wells three times. MCF-7 and T47D cell lines were seeded at a density of 1.5 x 10^4 in 24 well plates and concurrently transfected with the ERE luciferase and renilla plasmids after 24 hours of growth using Fugene 6 from Promega (Madison, WI) at a 1:3 ratio. The cells were serum-starved for six hours the following day, then exposed for 48 hours to the macrophage/pre-adipocyte co-culture CM supplemented with 2% CS-FBS. Luciferase activity was then measured using Promega's Dual Luciferase Reporter Assay System, with the fluorescence read at 570 nm on a FLUOstar Omega Spectrometer (BMG Labtech, Offenberg, Germany). Relative ERα activity was calculated by dividing the fluorescence value (standardized to renilla) from cells grown in each experimental condition by that from cells grown in CM generated with normal weight sera exposure followed by incubation in SFM plus testosterone. Data shown represents the average of at least three independent experiments.

3.2.9 Cell Proliferation and Migration

MCF-7, T47D, and MDA-MB-231 cell proliferation was measured using cell counting by hemocytometer following a 48-hour incubation in macrophage/pre-adipocyte co-culture CM supplemented with 2% CS-FBS. The cells were seeded in 24-well plates at a density of $1x10^4$ cells/well for MCF-7 and MDA-MB-231 cells and $1.5x10^4$ cells/well for T47D cells, then serum-starved for six hours the following day. For each cell line, the relative number of cells after 48 hours was calculated by dividing the cell number for each experimental condition by that for cells grown in CM generated with normal weight sera exposure followed by incubation in SFM plus testosterone, then multiplying that number by $1x10^5$. Migration of these same cell lines was assessed using the wound healing assay after a 24-hour incubation in macrophage/pre-adipocyte co-culture CM supplemented with 2% CS-FBS. The cells were seeded in six-well plates and grown to 100% confluence, then serum-starved for six hours. After adding the CM to the cells, a scratch was made down the center of each well and the baseline width of the scratch in three locations was measured using Spot Imaging software (Diagnostic Instruments, Sterling Heights, MI, USA). Images were captured with a Motic AE31 inverted microscope and Spot Idea 3cMP color digital camera (GMI, Minneapolis, MN, USA). At 24 hours, follow-up measurements at the same locations were taken, and the percent wound closure was calculated by dividing the difference of the two measurements by the baseline and multiplying by 100. Data shown represents the average of at least three independent experiments.

3.2.10 Statistical analyses

Patient data: The data was examined for normality. Duplicates were also analyzed to guarantee that one patient was not recorded twice in the event of referrals or transfer of medical care to the other clinic. Pearson's chi-squared tests were used to analyze categorical variables of NSAID users and nonusers. Student's t-test was used to examine mean differences in numerical variables. Time to recurrence in the NSAID users and nonusers that developed disease recurrence was assessed by Wilcox non-parametric test. Age at diagnosis and tumor stage were included as *a priori* confounding factors. Logistic regression was used to predict recurrence. A p value of <0.05 was considered significant. Statistical analyses were performed in R Foundation for Statistical Computing (version 2.10.1, Vienna, Austria, 2009).

***In vitro* data:**

Differences between cells exposed to two different experimental conditions were measured using Student's *t* test. One-way ANOVA was used to analyze differences between more than two experimental conditions. A p value of <0.05 was considered significant.

3.3 Results

3.3.1 Patient Characteristics

The patient population consisted of 440 women with invasive, ERα positive breast cancer. NSAID users did not significantly differ from nonusers by BMI

category, tumor stage, hormone receptor status, tumor type, race or type of surgery (lumpectomy versus mastectomy). Not surprisingly, NSAID users were more likely to be older, postmenopausal, and diabetic. The majority of patients in our population were overweight/obese (BMI≥25 kg/m^2), such that the average BMI for both users and nonusers was >30 kg/m^2 (Table 1).

Table 3.1 Descriptive characteristics of breast cancer patients (n=440)

Characteristics	NSAID nonusers (n=281)	NSAID users (n=159)	p-value
Age at diagnosis, years (mean±SD)	55.5 ± 10.3	60.7 ± 10.7	< 0.001
BMI, kg/m^2 (mean ± SD)	30.7 ± 6.21	31.9 ± 6.70	0.072
Time to recurrence, months (median)[a]	50.6	78.5	0.464
	[----------------------------N(%---)	--------------------]	**χ^2, p-value for trend**
Recurrence			
Yes	34(12.1)	10(6.3)	0.050
No	247(87.9)	149(93.7)	
BMI category			
Normal	50(17.8)	25(15.7)	0.132
Overweight	94(33.4)	41((25.8)	
Obese	137(48.8)	93(58.5)	
Menopausal status			
Premenopausal	110(39.2)	32(20.1)	<0.001
Postmenopausal	171(60.8)	127(79.9)	
Race			
Hispanic	134(47.7)	74(46.5)	0.135
White	111(39.5)	57(35.8)	
African-American	5(1.8)	10(6.3)	
Other	4(1.4)	1(0.7)	
Missing/Unavailable	27(9.6)	17(10.7)	
Tumor stage			
I	101(35.9)	60(37.8)	0.389
II	107(38.1)	64(40.2)	
III	56(19.9)	23(14.5)	
Missing/Unavailable	17(6.1)	12(7.5)	
Hormone receptor status			
ER+/PR+	246(87.6)	133(83.6)	0.255
ER+/PR-	35(12.4)	26(16.4)	
Histological type			
Ductal	218(77.6)	121(76.1)	0.156
Lobular	33(11.7)	21(13.2)	
Mucinous	1(0.4)	2(1.2)	
Missing/Unavailable	29(10.3)	15(9.5)	
Type of surgery			
Lumpectomy	128(45.5)	72(45.3)	0.405
Mastectomy	122(43.4)	69(43.4)	
Bilateral mastectomy	26(9.3)	12(7.5)	
Other excision[b]	5(1.8)	6(3.8)	
Adjuvant hormonal therapy			
Aromatase inhibitor	171(60.9)	125(78.6)	<0.001
Tamoxifen	110(39.1)	34(21.4)	
Type of NSAID[c]			
Aspirin	-	129(81.1)	
Other	-	30(18.9)	
Diabetes status			
Diabetic	56(19.9)	57(35.9)	<0.001
Not diabetic	225(80.1)	102(64.1)	
Diabetes drug			
Metformin	37(66.1)	33(57.9)	0.036
Other	19(33.9)	24(42.1)	
Omega-3 fatty acid use			
Yes	32(11.4)	40(25.1)	<0.001
No	249(88.6)	119(74.9)	
Statin use			
Yes	65(23.1)	67(42.1)	<0.001
No	216(76.9)	92(57.9)	

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; SD, standard deviation; BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor. [a] Wilcox non-parametric test used to analyze time to recurrence analysis in 44 patients who had a recurrence. [b] Other excision includes segmentectomy and quadrantectomy. [c] Calculations pertinent to the 159 patients classified as NSAID users.

3.3.2 NSAID use is associated with a reduced risk of recurrence and longer disease-free survival

The recurrence rate in NSAID users was 52% lower than non-NSAID users (OR, 0.48; 95% CI, 0.22-0.98) (Table 2). NSAID users remained disease-free for an average of 78.5 months, while non-users averaged 50.6 months, a difference of more than two years (Table 1). Overweight/obese patients had a 1.86-fold higher risk of recurrence versus normal weight when controlling for NSAID use and type of hormone therapy (OR, 1.86; 95% CI, 0.76-5.62), a trend that approached significance (Table 2). Unfortunately, the small number of normal-weight patients, combined with their low rate of recurrence, precluded our ability to examine whether NSAID use was more effective in preventing recurrence in patients with an elevated BMI. However, despite the small sample size, we found that NSAID was associated with a substantial reduction in recurrence rate in this predominantly overweight/obese postmenopausal patient population. Larger studies have observed more modest effects (96-98).

Table 3.2 Logistic regression model to predict breast cancer recurrence

Predictors	OR	95%CI	p-value
NSAID use			
Users	0.48	0.22-0.98	0.05
Nonusers	reference		
BMI category			
Normal	reference		
Overweight + Obese	1.86	0.76-5.62	0.11

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; OR, odds ratio; CI, confidence interval; BMI, body mass index

3.3.3 Obesity stimulates pre-adipocyte aromatase expression and estradiol production via elevated macrophage PGE2 production

We next sought to determine whether the NSAID-associated reduction in recurrence rate observed in the overweight/obese patient population may be due to the effect of these drugs on local aromatase expression and estrogen production. To this end, we utilized an *in vitro* model in which cultured cells were exposed to pooled sera samples from obese (OB) or normal weight (N) postmenopausal breast cancer patients in order to mimic the tumor microenvironment of obese versus normal weight women. The characteristics of the serum donors, including serum concentrations of insulin-like growth factor 1 as well as various cytokines and adipokines, have been previously described (99). Following a one hour exposure to OB sera, COX-2 expression in cultured macrophages was a modest 24% higher than cells exposed to N sera (Figure 1a). However, exposure to OB sera for one hour increased macrophage PGE2 production five-fold versus N sera in the 24 hours following sera removal (Figure 1b). Consequently, we assessed whether COX-2 expression may continue to increase in macrophages exposed to OB sera during that 24-hour period following sera removal. While COX-2 expression rises in both OB and N sera-exposed macrophages during this period, after sera removal mRNA levels were 68% and 92% greater in OB versus N sera-exposed cells at 12 and 24 hours, respectively. (Figure 1c). Pre-adipocytes cultured in conditioned media (CM) from macrophages exposed to OB versus N sera had 52% greater aromatase expression (OB-CM, N-

CM) (Figure 1c). Treatment of the macrophages with celecoxib during sera exposure (OB+C-CM, N+C-CM) neutralized the difference in pre-adipocyte aromatase expression, indicating that OB sera-induced macrophage PGE2 production is responsible for this effect. Pre-adipocyte aromatase expression was also measured following growth in CM from macrophages exposed to serum from ten individual OB or N postmenopausal women, allowing us to examine whether similar results are obtained using non-pooled serum with the data averaged by BMI category. Serum from ineligible control subjects in the Polish Women's Health Study, a breast cancer case-control study that has been described previously (95), was used, enabling us to additionally determine whether our results are unique to sera obtained from a specific patient population. The average pre-adipocyte aromatase expression was over two-fold greater following culture in macrophage CM generated with OB versus N subjects' sera (OB-CM, N-CM) (Figure 1d), supporting the findings obtained using pooled sera from the CTRC patients. Finally, the OB sera-induced increase in aromatase expression was correlated with a 16-fold amplification in pre-adipocyte estradiol production in the presence of exogenous testosterone, the substrate for aromatase (OB+T-CM, N+T-CM) (Figure 1e). The addition of the aromatase inhibitor anastrozole to the macrophage/pre-adipocyte co-culture following sera exposure (OB+T+AI-CM, N+T+AI-CM) completely nullified the difference between OB and N, demonstrating that the increase in estradiol production was solely due to the OB sera's effect on aromatase expression.

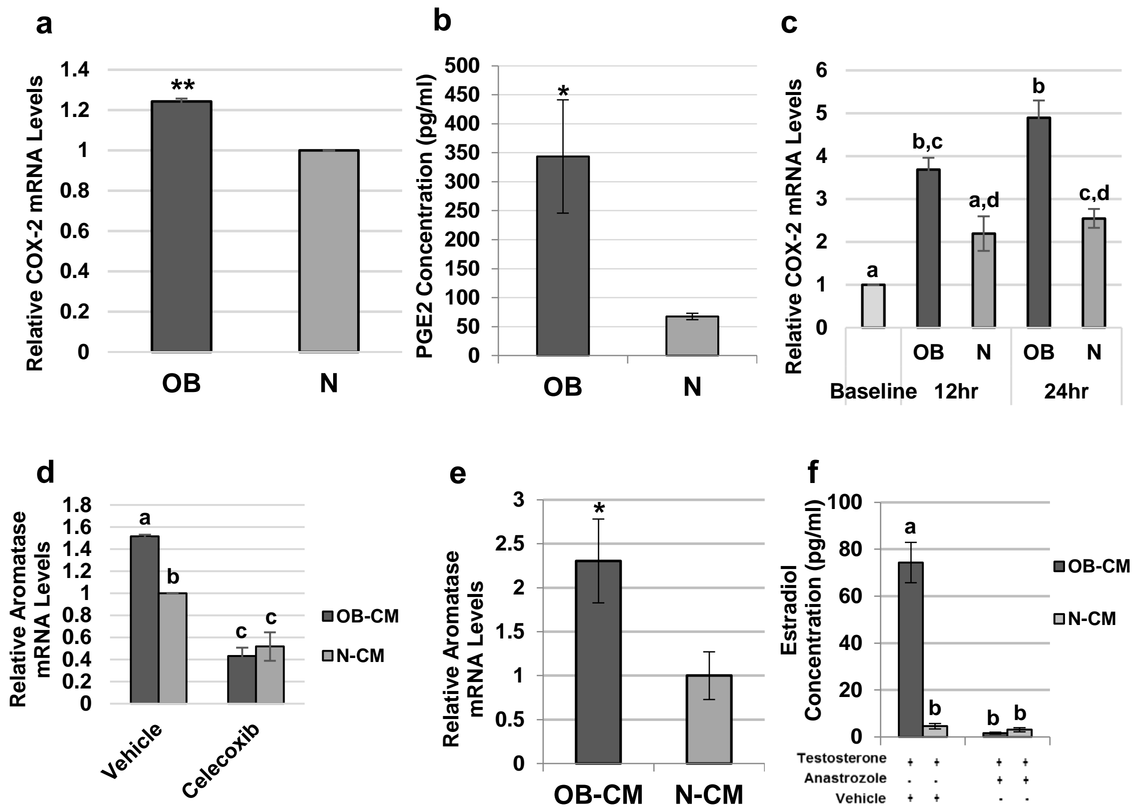

Figure 3.1 Obesity stimulates macrophage PGE2 production, resulting in elevated pre-adipocyte aromatase expression and estradiol production. (a) COX-2 expression in U937 cells matured to macrophages following one hour exposure to obese (OB) versus normal weight (N) patient sera. (b) PGE2 concentration in conditioned media (CM) from macrophages following exposure to OB or N patient sera. (c) COX-2 expression in U937 cells (matured to macrophages) after a six-hour serum-starvation period (Baseline), 12 hours after removal of the sera (OB and N, 12hr), and 24 hours after removal of the sera (OB and N, 24hr). (d) Aromatase expression in pre-adipocytes incubated in macrophage CM generated following patient sera exposure (OB-CM, N-CM) with vehicle or celecoxib treatment. (e) The impact of macrophage CM on pre-adipocyte aromatase expression when individual patient serum samples were utilized for CM generation, with the results averaged according to patient BMI category. (f) Estradiol concentration in CM from macrophage/pre-adipocyte co-cultures exposed to patient sera, then incubated in serum-free media with vehicle, testosterone, and/or anastrozole. Data shown represents the average of at least three independent experiments, with the exception of (d). *, $p<0.05$; **, $p<0.01$ in comparison to N or N-CM; different letters indicate significant differences, $p<0.05$.

3.3.4 Obesity-associated pre-adipocyte aromatase expression promotes greater breast cancer cell ERα activity

After establishing that obesity-associated circulating factors stimulate pre-adipocyte aromatase expression and estradiol production via their induction of macrophage PGE2 generation, we sought to determine whether this elevation in estradiol results in enhanced breast cancer cell ERα activity. ERα positive breast cancer cells cultured in CM from OB versus N sera-exposed macrophage/pre-adipocyte co-cultures (OB+T-CM, N+T-CM) exhibited greater ERα activity, as measured by estrogen response element (ERE) luciferase assay, with 29% and 42% differences in MCF-7 and T47D cells, respectively (Figures 2a and b). Co-culture treatment with anastrozole after sera exposure (OB+T+AI-CM, N+T+AI-CM) significantly decreased the OB-induced ERα activity in both cell lines, eliminating the difference between OB+T-CM versus N+T-CM-induced breast cancer cell ERα activity and mirroring the drug's effect on sera-induced pre-adipocyte estradiol production. OB and N co-culture CM produced without testosterone (OB-CM, N-CM) stimulated MCF-7 and T47D cell ERα activity that was statistically equivalent to that induced by CM from co-cultures treated with testosterone and anastrozole. Similar results were obtained when the CM was generated with anastrozole treatment but no testosterone (OB+AI-CM, N+AI-CM). This shows that the presence of testosterone during co-culture CM generation is required for the OB-induced elevation in breast cancer cell ERα activity, further demonstrating that this effect is due to pre-adipocyte aromatase activity. We

utilized pS2 and cyclin D1 expression as additional measures of ERα activity, obtaining analogous results. Following growth in OB+T-CM versus N+T-CM, pS2 expression was 49% higher in MCF-7 cells and 63% greater in T47D cells (Figures 2c and d) while cyclin D1 expression was elevated by 70% in MCF-7 cells and 78% in T47D cells (Figures 2e and f). Co-culture treatment with anastrozole neutralized these differences in pS2 and cyclin D1 expression in both cell lines and, as seen with the ERE luciferase assays, CM generated without testosterone stimulated equivalent expression. Together, these results demonstrate that the obesity-associated, PGE2-induced increase in pre-adipocyte aromatase expression and estradiol production can enhance breast cancer cell ERα activity.

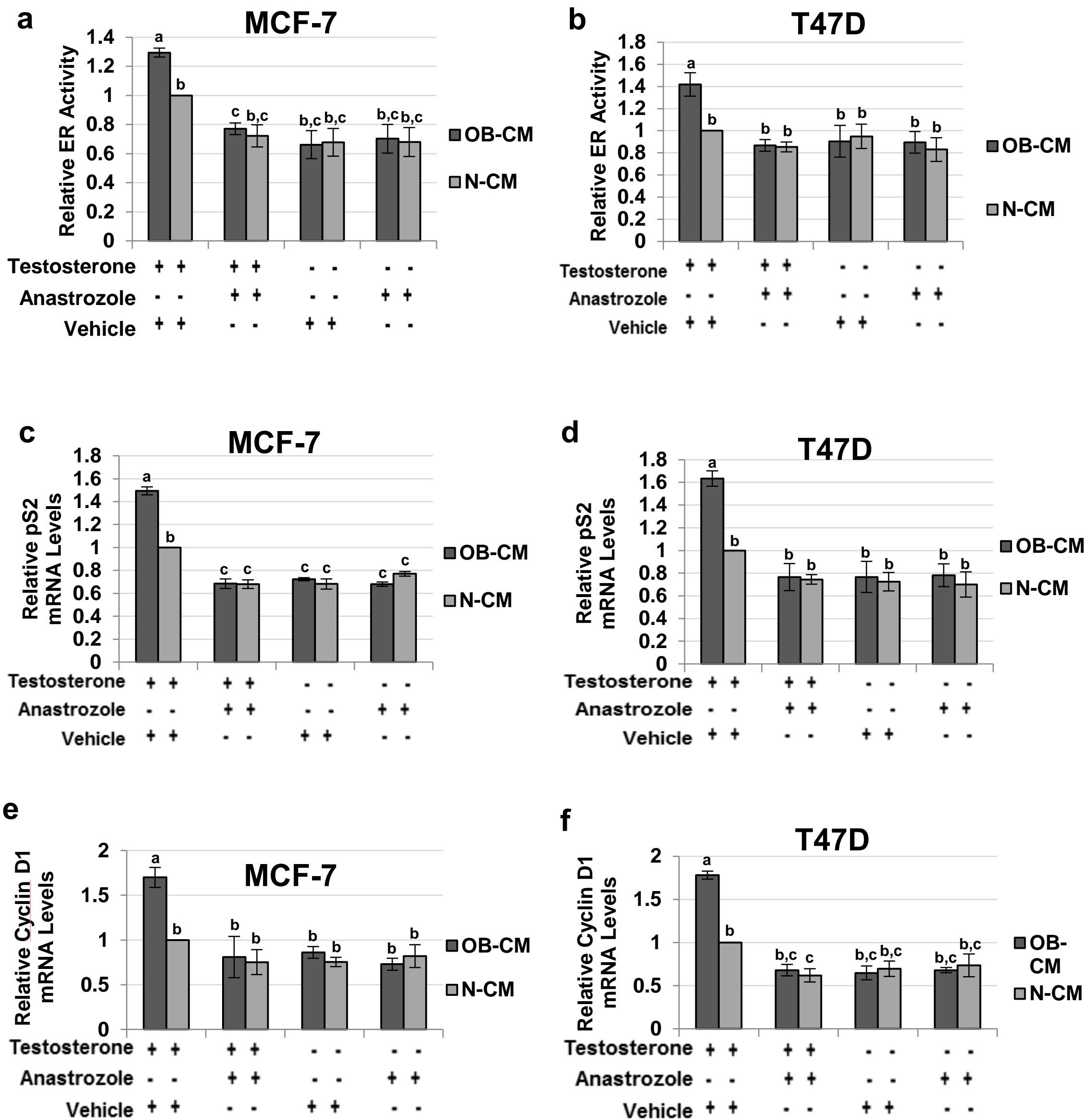

Figure 3.2 Obesity-associated pre-adipocyte aromatase expression promotes greater breast cancer cell ERα activity. MCF-7 and T47D breast cancer cell ERα activity after culture in macrophage/pre-adipocyte co-culture CM, as measured by ERE luciferase reporter (a, b) and qPCR analysis of pS2 (c, d) and cyclin D1 (e, f) expression. Experimental conditions include CM from co-cultures exposed to obese (OB) or normal weight (N) patient sera (OB-CM, N-CM) followed by incubation in serum-free media with vehicle, testosterone, testosterone and/or anastrozole. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences, $p<0.05$.

3.3.5 Breast cancer cell proliferation and migration are induced by obesity-associated pre-adipocyte aromatase expression

In order to determine whether the obesity-associated PGE2-induced elevation in pre-adipocyte aromatase expression and breast cancer cell ERα activity could result in enhanced disease progression, we assessed the impact of the macrophage/pre-adipocyte co-culture CM on breast cancer cell proliferation and migration. Culturing MCF-7 and T47D cells in OB+T-CM versus N+T-CM increased proliferation by 59% and 55%, respectively (Figures 3a and b). Treatment of the co-culture with anastrozole during CM generation (OB+T+AI-CM, N+T+AI-CM) eliminated the difference between OB and N, demonstrating that the OB+T-CM's elevated estradiol concentration is responsible for its effect on cell proliferation. This conclusion is further supported by the lack of any difference in proliferation levels in MDA-MB-231 cells, an ERα negative breast cancer cell line, following culture in the four CM conditions (Figure 3c). A similar trend was seen when we examined the impact of the co-culture CM on ERα positive breast cancer cell migration. OB+T-CM stimulated 57% more MCF-7 and 46% greater T47D cell migration in comparison to N+T-CM (Figure 4a and b). This effect was neutralized in the T47D cells by treatment of the co-culture with anastrozole during CM production (OB+T+AI-CM, N+T+AI-CM). However, while aromatase inhibition significantly decreased OB-induced MCF-7 cell migration, these cells still migrated farther than those cultured in N+T+AI-CM. Intriguingly, the migration of MDA-MB-231 cells was also significantly enhanced by culture in OB+T-CM versus N+T-CM

(Figure 4c). Consistent with this cell line's ERα negative status, anastrozole treatment during CM generation did not affect the CM's impact on migration. Overall, these findings strongly suggest that obesity-associated circulating factors may promote ERα positive breast cancer progression via stimulation of macrophage PGE2 production and the subsequent increase in pre-adipocyte aromatase expression.

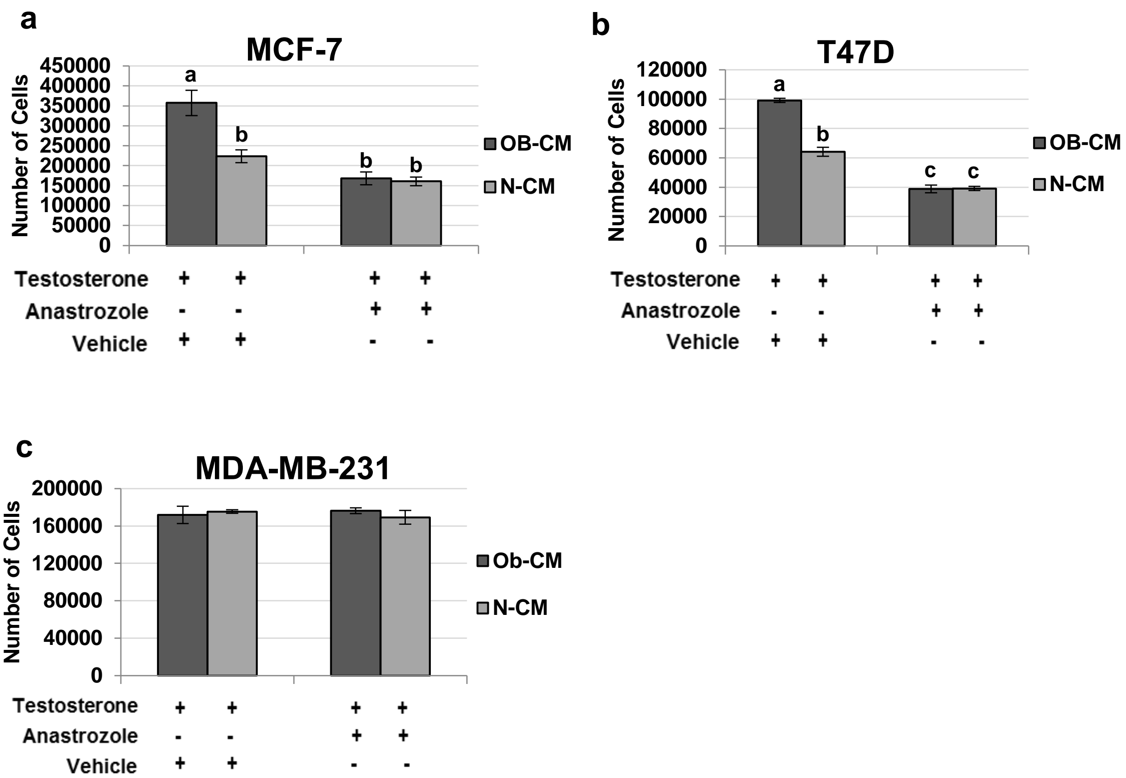

Figure 3.3 ERα positive breast cancer cell proliferation is induced by obesity-associated pre-adipocyte aromatase expression. MCF-7 (a), T47D (b), and MDA-MB-231 (c) breast cancer cell proliferation in response to culture in macrophage/pre-adipocyte co-culture CM. Experimental conditions include CM from co-cultures exposed to obese (OB) or normal weight (N) patient sera (OB-CM, N-CM) followed by incubation in serum-free media with vehicle, testosterone, and/or anastrozole. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences, $p<0.05$.

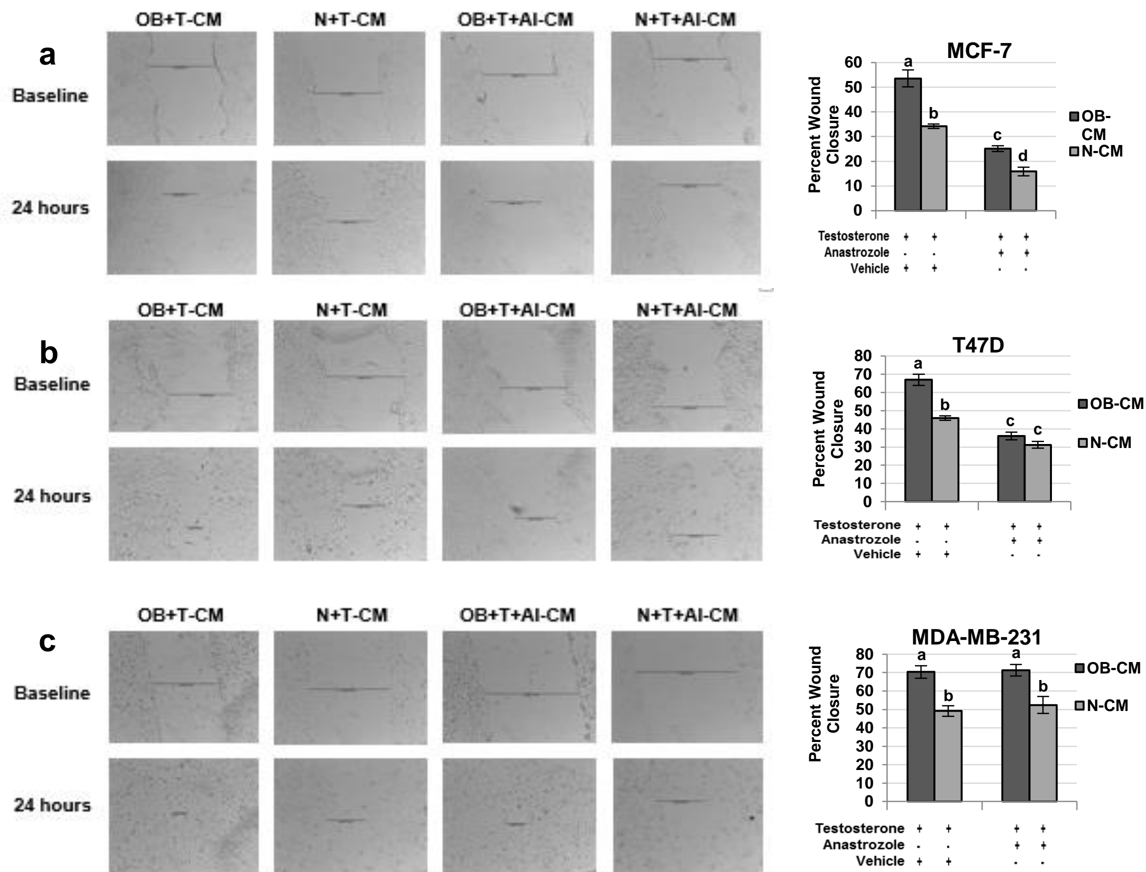

Figure 3.4. Obesity-associated pre-adipocyte aromatase expression promotes breast cancer cell migration. MCF-7 (a), T47D (b), and MDA-MB-231 (c) breast cancer cell migration during incubation in macrophage/pre-adipocyte co-culture CM. Experimental conditions include CM from co-cultures exposed to obese (OB) or normal weight (N) patient sera (OB-CM, N-CM) followed by culture in serum-free media with vehicle, testosterone and/or anastrozole. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences, $p<0.05$.

3.4 Discussion

Suboptimal pharmacologic aromatase inhibition in postmenopausal patients with ERα positive breast cancer has detrimental consequences to clinical outcome. COX-2 is a critical node for the convergence of various upstream pathways of inflammation, including IL-1, IL-6, and TNFα signaling (100-101), and it appears to be a key mediator of biologic processes affecting treatment failure, such as PGE2 synthesis and the resulting aromatase expression and estrogen production. Our study is the first to specifically examine the molecular mechanisms that may mediate the impact of daily NSAID use on recurrence rate and time to disease progression in patients with invasive breast cancer receiving adjuvant endocrine therapy. We have demonstrated that the NSAID users in this patient population had a 48% lower recurrence rate. A similar trend was seen after controlling for patient age and tumor stage at diagnosis, though the strength of the association was reduced (data not shown). This is not surprising given that late-stage tumors, as well as the aggressive tumors that disproportionately develop in younger women, seem less likely to significantly benefit from the modest effects of this drug group. NSAID users also remained disease-free for more than two years longer than non-users, a difference that was not statistically significant but may be a clinically relevant variance in outcome.

Similar results have been obtained in some larger prospective studies examining NSAID use after breast cancer diagnosis. Holmes et al. (96) showed that aspirin use 6-7 days/week was associated with a significant reduction in the

risk of recurrence (RR, 0.57; 95% CI, 0.39-0.82). Utilizing the Nurses' Health Study's substantial pool of subjects, the authors found no change in these results after stratifying by BMI, menopausal status, and ER status. In an analysis of postmenopausal breast cancer patient outcomes, Blair and colleagues (98) observed that any amount of regular NSAID use was correlated with a lower risk of breast cancer death (HR, 0.64; 95% CI, 0.39-1.05). Adjustment for ERα status, but not BMI category, increased the HR and reduced the statistical significance of this association. In contrast with these studies, the recurrence rate in a population of pre and postmenopausal patients was not decreased by aspirin use ≥3 days/week (RR, 1.09; 95% CI, 0.74-1.61), but was affected by use of ibuprofen (RR, 0.56; 95% CI, 0.32-0.98) (97). Controlling for BMI, menopausal status, and ERα did not alter these results.

The variability in design and patient population among these studies makes any comparison with our results difficult. Cumulatively, they appear to indicate that NSAID use may be an effective addition to adjuvant breast cancer treatment, regardless of BMI, ERα, or menopausal status. However, the association between NSAID use and a 50% lower disease recurrence in our study, despite a relatively small patient population, led us to hypothesize that the overwhelming prevalence of overweight/obesity among this population may have increased the NSAID benefit. Our patient population was also largely postmenopausal and included only hormone responsive patients. Because obesity and overweight status are associated with higher PGE2-induced aromatase expression in female breast

tissue (41-42), it seems likely that the efficacy of COX-2 inhibitors in a postmenopausal, ERα positive patient population would increase with greater adiposity. Perhaps the lack of variation in effect among BMI categories in previous studies is due to their failure to stratify the data by BMI, ERα, and menopausal status simultaneously. This question could potentially be addressed by previous trials assessing the clinical benefit of a celecoxib/aromatase inhibitor combination treatment for postmenopausal, hormone-responsive breast cancer. Most of these studies showed a modest benefit with at least three months of combination treatment, including trends towards more clinical complete response, longer duration of clinical benefit, and greater progression-free survival (102-104). Unfortunately, none of these trials analyzed the treatment benefit by BMI category, so it is impossible to determine from this data whether overweight/obese women in this patient population are more likely to benefit from COX-2 inhibition.

Given that obesity has been associated with a worse breast cancer prognosis (19-23) as well as a reduced response to aromatase inhibitor therapy among postmenopausal, ERα positive patients (48-49), determination of whether this specific population will benefit from COX-2 inhibition is an important question. Dannenberg et al. have demonstrated an obesity-associated, PGE2-induced elevation in pre-adipocyte aromatase expression using both pre-clinical models and patient breast tissue (41-43). Our aim was to confirm this phenomenon, using an *in vitro* model of the obese patient's breast tumor microenvironment, as well as determine whether this increased pre-adipocyte aromatase expression can

promote greater epithelial cell ERα activity and subsequent proliferation and migration, two *in vitro* measures of cancer progression. We show that exposure to sera from obese postmenopausal women stimulates significantly greater macrophage COX-2 expression and a five-fold increase in PGE2 production. Saturated fatty acids can promote COX-2 expression and PGE2 production in cultured macrophages (43, 105), and the increased lipolysis that accompanies obesity results in a higher concentration of circulating free fatty acids (106-108). Consequently, this difference in macrophage COX-2 expression and PGE2 production may be due to elevated levels of free fatty acids in the obese patient sera. However, there is also some evidence that the inflammatory cytokines IL-6 and TNFα, found in higher concentrations in our obese patient serum samples (99), can induce COX-2 expression (36-37, 101). This suggests that more than one mechanism may be responsible for the obesity-associated upregulation of macrophage COX-2 expression and PGE2 production seen in this study.

Taken together, our *in vitro* data provides compelling evidence that macrophage-derived PGE2 stimulates greater pre-adipocyte aromatase expression and estradiol production, resulting in an elevation in breast cancer cell ERα activity, proliferation, and migration. Neutralization of the obesity-induced elevation in ERα activity and cell proliferation by the addition of aromatase inhibitor treatment validates our hypothesis that these effects are due to pre-adipocyte aromatase expression and estradiol production. However, while incubation of the ERα negative MDA-MB-231 cells in OB versus N macrophage/pre-adipocyte co-

culture CM did not differentially affect cell proliferation, it did stimulate significantly greater cell migration. In addition, OB CM continued to promote more extensive MCF-7 cell migration in comparison to N CM with aromatase inhibition during CM generation, though anastrozole did significantly decrease the OB CM-induced migration. These results indicate that, while locally produced estradiol clearly plays a role in mediating obesity-associated breast cancer cell migration, one or more additional signaling molecules produced by macrophages and/or pre-adipocytes is involved in this effect. Macrophage-derived PGE2 is one possibility, as it is known to promote breast cancer cell migration (109-110). Several studies have concluded that it also stimulates breast cancer cell proliferation based on the ability of COX-2 inhibitors to hinder proliferation (111-112). However, we saw no differential proliferation in the MDA-MB-231 cells following incubation in OB versus N CM, and Robertson et al (113) demonstrated that treatment of MDA-MB-231 cells with exogenous PGE2 does not increase proliferation. The role of additional locally produced signaling molecules in the link between obesity-associated inflammation and breast cancer progression is an area that deserves further exploration in future studies.

Our current investigation strongly suggests that PGE2-induced local estradiol production may be a key mediator in the link between obesity and postmenopausal, hormone-responsive breast cancer progression. This conclusion is supported by the clinical observation of a 50% lower recurrence rate and 28-month extension in time to recurrence with NSAID use in a largely

overweight/obese and postmenopausal patient population with ERα positive disease. Collectively, our results provide a strong rationale for further studies regarding the clinical benefit of aromatase inhibitor/COX-2 inhibitor combination treatment for obese, postmenopausal breast cancer patients.

Chapter 4: Obesity promotes pre-adipocyte aromatase expression via breast cancer cell prostaglandin E2 production in an *in vitro* model of the breast tumor microenvironment

4.1 Introduction

The majority of breast cancer cases occur in postmenopausal women (1), though tumors in this population are typically less aggressive than those occurring in younger women (11). However, the obesity rate in women ≥60 years of age in the United States is currently 42.3%, the highest of any gender/age category (17), and this condition has been associated with a worse breast cancer prognosis. A 2010 meta-analysis of 43 studies found that obesity at diagnosis is associated with poorer breast cancer specific and overall survival (23), and others have shown that the strength of the latter association increases with each successive increase in body mass index (BMI) category (19). Links between obesity and an increased risk of breast cancer recurrence and shorter disease-free survival (20, 22), independent of tumor stage at diagnosis (21), have also been established.

Postmenopausal obesity is generally accompanied by an elevation in circulating estrogen levels because adipose tissue is the primary site of aromatase expression following menopause (25-27). Consequently, some researchers have hypothesized that this excess estrogen may link obesity with more aggressive estrogen receptor alpha (ERα) positive breast tumors in postmenopausal women,

a theory supported by studies demonstrating a reduced response to aromatase inhibitor treatment in obese patients (48-49, 51). However, this explanation has been confounded by studies demonstrating that estrogen levels in breast tumor tissue can be up to 10-fold higher than serum concentrations (32). A corresponding 5-fold elevation in aromatase expression in the tumor and associated adipose tissue in comparison to disease-free tissue (32) suggests that this estrogen is being produced locally, likely via a paracrine interaction between the adipose and tumor tissues. In support of this possibility, O'Neill et al. (33) demonstrated in their analysis of 12 breast samples that the highest aromatase activity is consistently found in the tumor-containing breast quadrant. Zhou et al. (30) later established that breast cancer cell-secreted prostaglandin E2 (PGE2), as well as other factors, stimulates aromatase expression in pre-adipocytes, the primary aromatase-expressing fraction of the adipose tissue. These findings indicate that an elevation in systemic estrogen levels will probably not have a significant impact on breast tumor ERα activity, given that a much larger local source of estrogen is available in the tumor microenvironment and adjacent adipose tissue.

However, aromatase and estrogen may still be key factors in the link between obesity and poor prognosis in ERα positive, postmenopausal breast cancer patients. Obesity is associated with increased circulating levels of several growth factors, cytokines, and adipokines that may enhance the paracrine interaction described above, resulting in a further elevation in local aromatase levels and estrogen production. For example, serum concentrations of interleukin-6 (IL-6),

an inflammatory cytokine secreted by both immune cells and adipocytes, are generally increased with obesity (34), and this cytokine has been shown to promote PGE2 production in multiple cell types via its effects on cyclooxygenase-2 (COX-2) (35-37). In the current study, we utilized an *in vitro* model of obesity to investigate the impact of obesity-associated systemic factors on the aromatase-promoting paracrine interaction between ERα positive breast cancer cells and pre-adipocytes. After establishing that obesity does enhance this interaction, we demonstrated that it results in greater breast cancer cell ERα activity and proliferation, suggesting that it may be one mechanism by which obesity promotes a worse breast cancer prognosis.

4.2 Materials and Methods

4.2.1 Serum samples

Serum was collected from 25 postmenopausal breast cancer patients under an IRB approved biorepository collection protocol at the CTRC of UTHSCSA. The collection and use of these biological samples was conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained prior to participation, and all samples and data were de-identified prior to release to maintain patient confidentiality. BMI was calculated, and serum was pooled according to the BMI category of the patient (normal weight (18.5-24.9 kg/m^2) or obese ($\geq$30.0 kg/m^2).

4.2.2 Cell lines and reagents

The ERα positive MCF-7 and T47D breast cancer cell lines (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS). Pre-adipocytes isolated from women undergoing elective surgical procedures were a generous gift from Dr. Rong Li, UTHSCSA, and have been described previously (79). They were maintained in DMEM/F12 1:1 media (GIBCO Life Technologies) plus 10% FBS. Celecoxib, human recombinant insulin, testosterone, and anastrozole were purchased from Sigma-Aldrich (St. Louis, MO) and human recombinant IL-6, tumor necrosis factor alpha (TNF-α), leptin, and insulin-like growth factor 1 (IGF-1) from R&D Systems (Minneapolis, MN). The IL-6 depleting antibody was produced by EMD Millipore (Billerica, MA).

4.2.3 MCF-7 cell conditioned media

MCF-7 cell conditioned media (CM) was generated by seeding $2x10^5$ MCF-7 cells per well in 6-well plates, allowing them to grow for 24 hours, then serum-starving the cells for 18 hours. The MCF-7 cells were then exposed to a 2% concentration of the pooled sera samples in serum-free media (SFM) for one hour, the sera removed and cells washed once with phosphate buffered saline (PBS) followed by incubation in SFM for 24 hours. Over the course of this 24 hours, the cells release various factors into the SFM, enriching it into CM, which was then collected and stored at -20°C for subsequent *in vitro* assays.

4.2.4 MCF-7/pre-adipocyte conditioned media

MCF-7/pre-adipocyte co-culture CM was generated by first seeding $1x10^5$ MCF-7 cells on top of confluent pre-adipocytes seeded in six-well plates. After a 24 hour incubation to allow the MCF-7 cells to adhere, the co-culture was serum-starved for 18 hours, then exposed to a 2% concentration of the pooled sera in SFM for one hour. Following this, the cells were washed once with PBS and incubated for 24 hours in SFM + testosterone (100 nM) + anastrozole (1 uM) or vehicle. The enriched SFM, now considered CM, was then collected and stored at -20° C for use in subsequent *in vitro* assays.

4.2.5 Quantitative RT-PCR

MCF-7 cell *PTGS2* (COX-2) mRNA levels were measured following a 18 hour serum-starvation period (baseline level) and a one-hour exposure to a 2% concentration of pooled patient sera in SFM. To determine whether MCF-7 cell COX-2 expression continued to change during the 24-hour SFM incubation period of CM generation, mRNA levels were also assessed after 12 and 24 hours in SFM (following the removal of the sera). Pre-adipocyte *CYP19A1* (aromatase) mRNA levels were assessed following a 24-hour incubation in MCF-7 CM. To assess the impact of MCF-7 cell COX-2 inhibition on pre-adipocyte aromatase expression, the MCF-7 cells were pre-treated with 30 uM celecoxib for one hour prior to as well as during sera exposure when generating the MCF-7 CM. We previously measured the concentrations of several obesity-associated circulating factors, including IL-6, TNF-α, leptin, insulin, and free IGF-1, in the obese versus normal weight patient sera samples (99). To examine whether these factors contribute to the aromatase-

promoting paracrine interaction between breast cancer cells and pre-adipocytes, each factor was added back to the pooled normal weight patient sera. With the exception of IGF-1, the amount of each factor added was determined by the difference in average concentration between the obese and normal weight patients' serum samples. There was no significant difference in free IGF-1 found in our samples, so it was added in an amount typically used in cell culture experiments (20 ng/ml) in order to examine whether IGF-1 could have an effect. Aromatase expression was then measured in pre-adipocytes exposed to MCF-7 cell CM generated with this enhanced normal weight sera as well as MCF-7 CM generated with non-enhanced obese and normal weight patient sera. To further examine the role of serum IL-6, the pooled sera samples were incubated with or without an IL-6 depleting antibody (10 ug/ml) for one hour at room temperature prior to use of the sera for MCF-7 cell CM generation and subsequent aromatase expression measurement in pre-adipocytes exposed to this MCF-7 CM. MCF-7 and T47D *TFF1* (pS2) and *CCND1* (cyclin D1) mRNA levels were measured after a 24-hour incubation in MCF-7/pre-adipocyte co-culture CM supplemented with 2% charcoal-stripped (CS)-FBS. All cells were serum-starved for 18 hours prior to exposure to sera or CM. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer sequences are as follows: *PTGS2*: forward, 5'-CCCTTGGGTGTCAAAGGTAA-3'; reverse, 5'-GCCCTCGCTTATGATCT GTC-3'; *CYP19A1*: forward, 5'-GCCGAATCGAGAGCTGTAAT-3'; reverse, 5'-

GAGAATTCATGCGAGTCTGGA-3'; *TFF1*: forward, 5'-GGTCGCCTTGGAGC AGA-3'; reverse, 5'-GGGCGAAGATCACCTTGTT-3'; *CCND1*: forward, 5'-TGG AGGTCTGCGA GGAACAGAA-3'; reverse, 5'-TGCAGGCGGCTCTTTTTCA-3'. The manufacturer's recommended cycling conditions for the QuantiFast SYBR Green PCR kit (Qiagen) were used. Data shown represents the average of at least three independent experiments.

4.2.6 Prostaglandin E2 and estradiol concentrations

The concentration of prostaglandin E2 in MCF-7 CM was measured using the Prostaglandin E2 Parameter Assay Kit from R&D Systems (Minneapolis, MN). The estradiol concentration in macrophage/pre-adipocyte co-culture CM was analyzed with the Estradiol EIA Kit from Cayman Chemical (Ann Arbor, MI).

4.2.7 Cell proliferation

MCF-7 and T47D cell proliferation was measured using cell counting by hemocytometer following a 48-hour incubation in MCF-7/pre-adipocyte co-culture CM supplemented with 2% CS-FBS. The cells were seeded in 24-well plates at a density of $1x10^4$ cells/well for MCF-7 cells and $1.5x10^4$ cells/well for T47D cells, then serum-starved for six hours the following day. For each cell line, the relative number of cells after 48 hours was calculated by dividing the cell number for each experimental condition by that for cells grown in CM generated with normal weight sera exposure followed by incubation in SFM + testosterone, then multiplying that number by $1x10^5$.

4.2.8 Statistical analysis

Differences between cells exposed to two different experimental conditions were measured using Student's *t* test. Two-way ANOVA was used to analyze experiments with two independent variables. A p value of <0.05 was considered significant.

4.3 Results

4.3.1 Pre-adipocyte aromatase expression is induced by obesity-associated circulating factors via stimulation of breast cancer cell PGE2 production

Given that breast cancer cells are known to produce factors, including PGE2, that promote pre-adipocyte aromatase expression, we began by examining whether obesity-associating circulating factors could enhance this paracrine interaction. To mimic the tumor microenvironment of an obese versus normal weight patient, we utilized an *in vitro* model in which cultured cells were exposed to postmenopausal breast cancer patient sera samples pooled according to BMI category (normal weight (N): 18.5-24.9 kg/m^2; obese (OB): ≥30 kg/m^2). The characteristics of the serum donors have been previously described (23). Conditioned media (CM) from MCF-7 breast cancer cells exposed to OB or N patient sera (OB-CM or N-CM, respectively) for one hour was generated. Pre-adipocytes cultured in OB-CM had 89% higher aromatase expression in comparison to N-CM ($p<0.05$), demonstrating that OB patient sera promotes greater breast cancer cell production of factors that stimulate pre-adipocyte

aromatase expression (Figure 4.1a). One factor known to induce aromatase expression is PGE2, which is produced from arachidonic acid via a reaction catalyzed by COX-2. Consequently, we next measured the impact of patient sera exposure on MCF-7 cell COX-2 expression and PGE2 production. Following a one hour exposure to OB versus N patient sera, there was no significant difference in MCF-7 cell COX-2 expression between these conditions or in comparison to the baseline expression prior to sera exposure. However, COX-2 expression continued to increase during 24 hours of incubation in SFM following sera removal, with the expression in cells exposed to OB patient sera increasing to a greater degree. At 24 hours, COX-2 expression in the MCF-7 cells that had been exposed to OB patient sera was 40% higher than N ($p<0.05$) (Figure 4.1b). This methodology corresponds to the conditions under which the MCF-7 CM was generated, with the 24 hour time point reflecting COX-2 expression at the time of CM collection. Intriguingly, the PGE2 concentration in MCF-7 cell OB-CM was almost 14-fold higher than N-CM ($p<0.05$) (Figure 4.1c). Celecoxib treatment was then used to determine whether the OB patient sera-induced COX-2 expression contributes to the OB-CM's enhancement of pre-adipocyte aromatase expression. COX-2 inhibition in MCF-7 cells during CM generation significantly decreased the pre-adipocyte aromatase expression levels induced by both OB and N-CM ($p<0.05$), eliminating the difference between the two conditions (Figure 4.1d). This indicates that one or more obesity-associated circulating factors stimulate greater breast cancer cell PGE2 production, resulting in the promotion of higher pre-

adipocyte aromatase expression through a paracrine interaction between the two cell types.

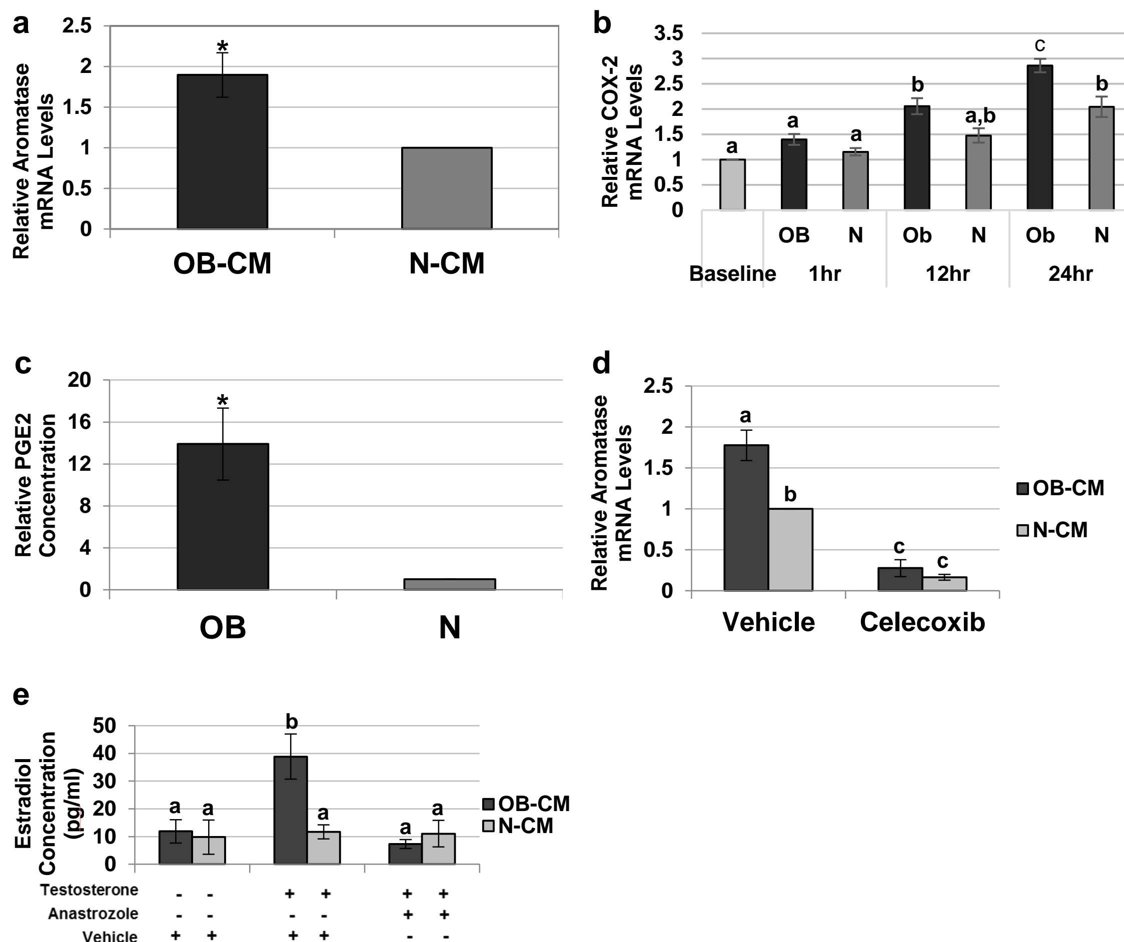

Figure 4.1 Obesity-associated circulating factors promote pre-adipocyte aromatase expression via stimulation of breast cancer cell COX-2 expression and PGE2 production. (a) Aromatase expression in pre-adipocytes incubated in MCF-7 cell conditioned media (CM) generated following obese versus normal weight sera exposure (OB-CM and N-CM). (b) COX-2 expression in MCF-7 cells after an 18-hour serum-starvation period (Baseline), 1 hr of sera exposure (OB and N, 1hr), 12 hr after removal of the sera (OB and N, 12hr), and 24 hr after removal of the sera (OB and N, 24hr). (c) Concentration of PGE2 produced by MCF-7 cells following exposure to OB or N sera. (d) Aromatase expression in pre-adipocytes incubated in MCF-7 cell CM generated following sera exposure (OB-CM, N-CM) with vehicle or celecoxib treatment. (e) Estradiol concentration in CM from MCF-7/pre-adipocyte co-culture exposed to sera (OB-CM, N-CM) followed by incubation in serum-free media with vehicle, testosterone, and/or anastrozole. Data shown represents the average of at least three independent experiments. Asterisks indicate significant differences in comparison to N or N-CM; different letters indicate significant differences, $p<0.05$.

4.3.2 Obesity-associated systemic IL-6 indirectly stimulates pre-adipocyte aromatase expression

We have previously shown that the serum IL-6, TNF-α, and leptin concentrations in our obese patient population were significantly elevated in comparison to normal weight patients. Serum insulin levels in the obese patients were also four-fold higher, a difference that approached significance, but there was no significant variance in free IGF-1 concentration (99). Our next aim was to determine whether any of these obesity-associated systemic factors contribute to the promotion of the aromatase-enhancing paracrine interaction between breast cancer cells and pre-adipocytes. To this end, we repeated our measurement of MCF-7 CM-stimulated pre-adipocyte aromatase expression with the addition of each factor to the pooled N patient sera during CM generation. For IL-6, TNF-α, leptin, and insulin, the amount of each factor added was determined by the difference in average concentration between the OB and N patients' serum samples. Though free IGF-1 was not elevated in our OB patient population, others have shown this growth factor to be increased with obesity (38-39), so we also included a condition with IGF-1 added to the N patient sera in an amount typically used for cell culture. Similar to our previous results, OB-CM stimulated approximately two-fold greater pre-adipocyte aromatase expression in comparison to N-CM ($p<0.05$). The addition of IL-6 or TNF-α to the N patient sera during MCF-7 CM generation significantly increased the resulting CM's effect on pre-adipocyte

aromatase expression, with IL-6 having the greatest effect ($p<0.05$). In fact, N-CM produced with IL-6 stimulated pre-adipocyte aromatase levels that were statistically equivalent to the OB-CM condition. The addition of both IL-6 and TNF-α or all five factors to the N sera did not produce CM that further enhanced aromatase expression above the addition of IL-6 alone (Figure 4.2a). To confirm that IL-6 was the primary factor in the OB patient sera mediating its indirect enhancement of pre-adipocyte aromatase expression, we next added an IL-6 depleting antibody to both the OB and N patient sera prior to their use, thereby neutralizing the variation in IL-6 content between the two. This sera was then utilized to generate MCF-7 CM. Aromatase expression in pre-adipocytes exposed to OB or N-CM produced with IL-6-depleted sera was significantly reduced in comparison to the OB or N-CM generated with untreated sera ($p<0.05$), and the IL-6 depletion eliminated the difference between the OB and N-CM conditions (Figure 4.2b). This demonstrates that IL-6, an inflammatory cytokine known to be systemically elevated with obesity, is the primary mediator of the OB patient sera's aromatase enhancing effects.

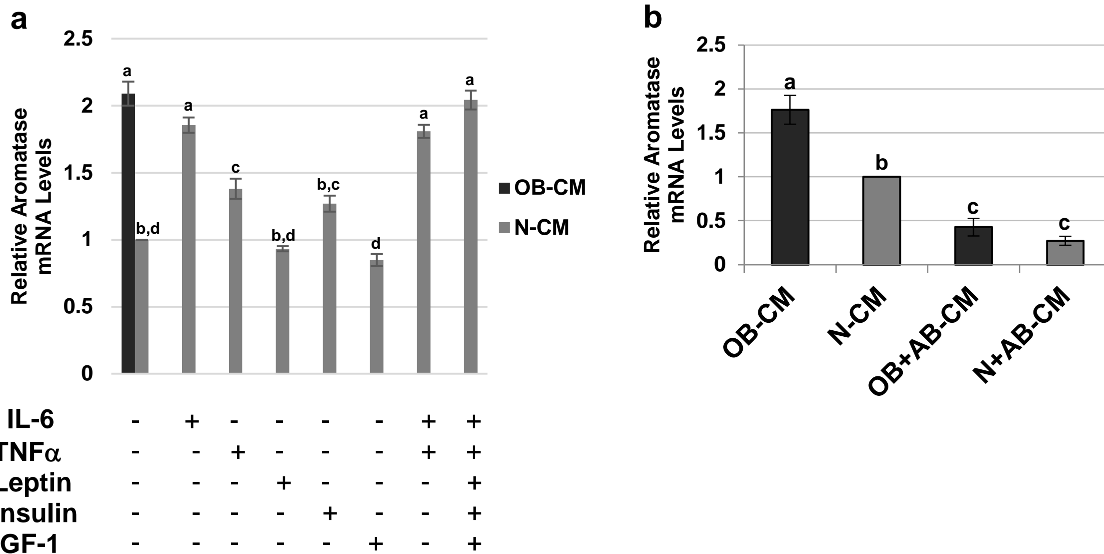

Figure 4.2. Pre-adipocyte aromatase expression is indirectly stimulated by obesity-associated systemic IL-6. (a) Aromatase expression in pre-adipocytes exposed to MCF-7 cell CM generated following patient sera exposure (OB-CM and N-CM) +/- five obesity-associated circulating factors, including IL-6, TNF-α, leptin, insulin, and IGF-1. (b) Aromatase expression in pre-adipocytes exposed to MCF-7 cell CM generated following exposure to untreated patient sera (OB-CM and N-CM) or patient sera pre-treated with an IL-6 depleting antibody (OB+AB-CM and N+AB-CM). Data shown represents the average of at least three independent experiments. Different letters indicate significant differences, $p<0.05$.

4.3.3 Obesity-associated pre-adipocyte aromatase expression enhances breast cancer cell ERα activity and proliferation

To examine whether the observed obesity-associated increase in pre-adipocyte aromatase expression could promote breast cancer progression, we first assessed whether it was associated with higher breast cancer cell ERα activity. CM from a co-culture of MCF-7 cells and pre-adipocytes was generated by exposing the cells to either OB or N patient sera, then incubating them in SFM plus testosterone, the latter serving as the substrate for aromatase. Expression of pS2,

a specific measure of ERα activity, was 76% and 80% higher ($p<0.05$) in MCF-7 and T47D breast cancer cells, respectively, cultured in the resulting OB versus N co-culture CM. Expression levels of cyclin D1, another ERα target gene, were also elevated ($p<0.05$) in the same cell lines following exposure to OB-CM versus N-CM (Figure 4.3a and b). Aromatase inhibition by anastrozole during CM generation reduced OB-CM-stimulated pS2 and cyclin D1 expression in the MCF-7 cells ($p<0.05$), neutralizing the difference between OB and N-CM in pS2, but not cyclin D1, expression. In the T47D cells, aromatase inhibition resulted in a similar decrease in the levels of OB-CM-induced pS2 and cyclin D1 expression ($p<0.05$), but the addition of anastrozole also significantly inhibited the N-CM's effect on these ERα target genes ($p<0.05$). Consequently, pS2 and cyclin D1 levels in the N-CM plus anastrozole condition were significantly lower than the levels in T47D cells exposed to either N-CM or OB-CM plus anastrozole, suggesting that aromatase inhibition may not be able to fully suppress the OB patient sera-induced estradiol production.

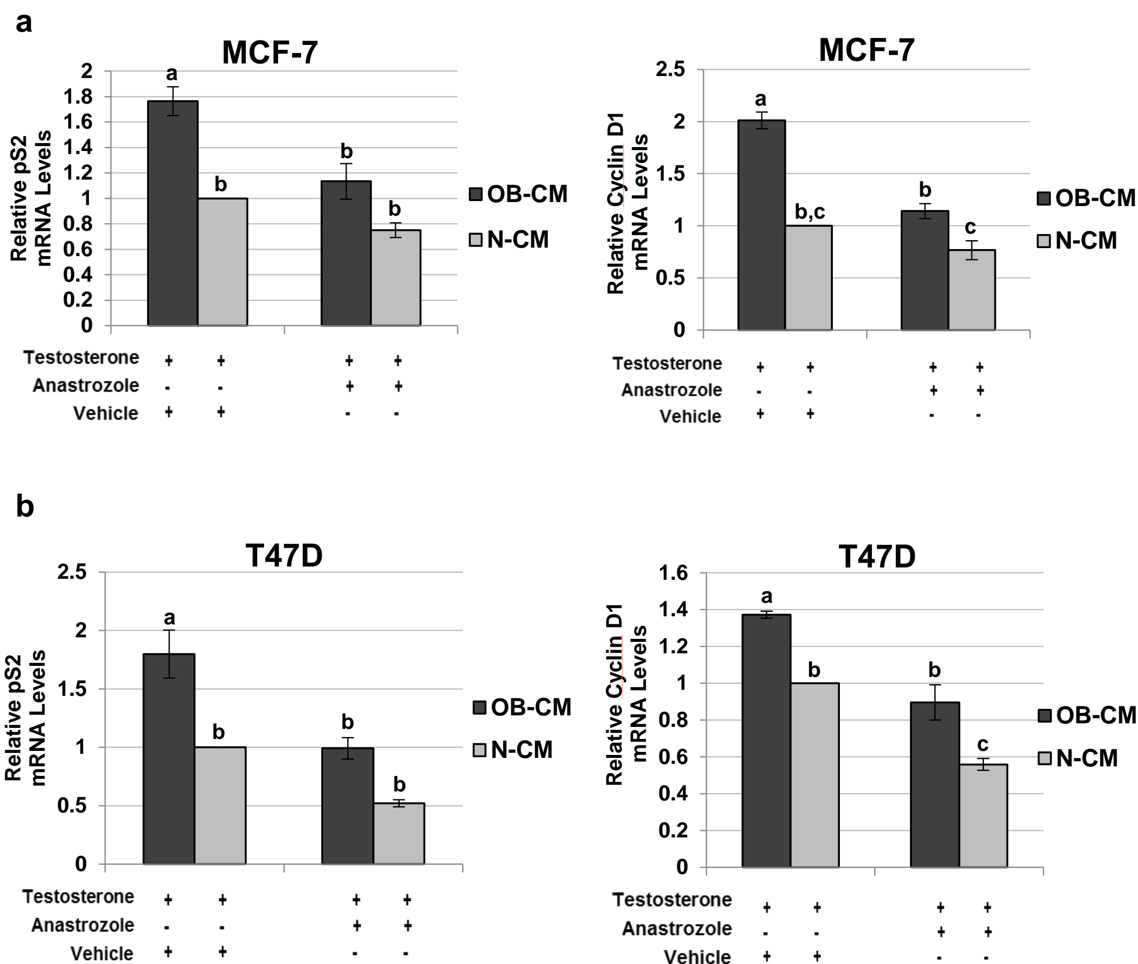

Figure 4.3. Obesity-associated pre-adipocyte aromatase expression enhances breast cancer cell ERα activity. The impact of MCF-7/pre-adipocyte co-culture CM on ERα activity in MCF-7 (a) and T47D (b) cells, as measured by qPCR analysis of pS2 and cyclin D1. Experimental conditions include CM from co-cultures exposed to patient sera (OB-CM, N_CM) followed by incubation in serum-free media with vehicle, testosterone, and/or anastrozole. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences, p<0.05.

These results closely correlated with the co-culture CM's effect on breast cancer cell proliferation (Figure 4.4a and b). MCF-7 and T47D cells cultured in OB versus N-CM demonstrated 45% and 68% greater proliferation levels (p<0.05).

The addition of anastrozole during CM generation reduced OB-CM-stimulated proliferation ($p<0.05$), but not to the level of N-CM without anastrozole. Aromatase inhibition was able to further decrease N-CM-stimulated cell proliferation ($p<0.05$).

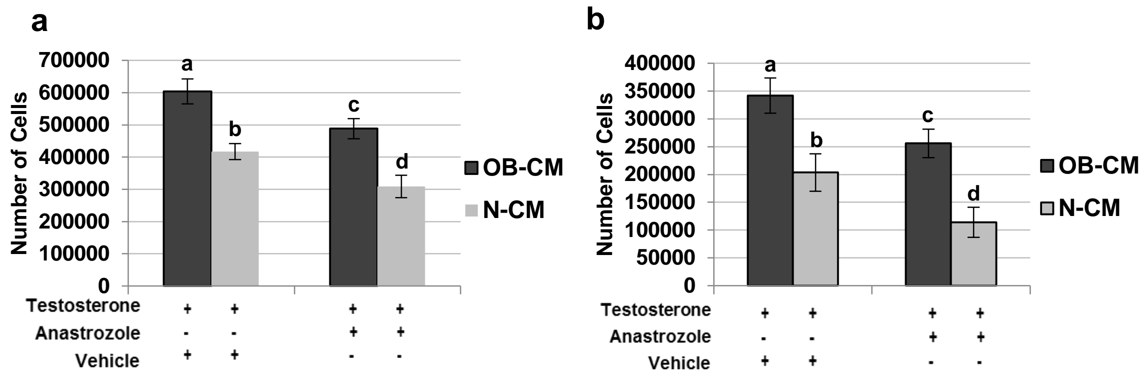

Figure 4.4. Breast cancer cell proliferation is increased by obesity-induced pre-adipocyte aromatase expression. MCF-7 (a) and T47D (b) breast cancer cell proliferation in response to culture in MCF-7/pre-adipocyte co-culture CM. Experimental conditions include CM from co-cultures exposed to patient sera (OB-CM, N-CM) followed by incubation in serum-free media with vehicle, testosterone and/or anastrozole. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences, $p<0.05$.

4.4 Discussion

Numerous studies have shown a positive association between obesity and a poor breast cancer prognosis, with the strongest effects frequently seen in postmenopausal, ERα positive patients (19-24). Obesity has also been linked to a reduced response to aromatase inhibitor therapy (48-49, 51), suggesting that it may promote breast cancer progression in this specific patient population via enhancement of local aromatase expression. In the current study, we establish that an obesity-associated elevation in systemic levels of the pro-inflammatory

cytokine IL-6 promotes a PGE2-mediated paracrine interaction between breast cancer cells and pre-adipocytes, resulting in greater pre-adipocyte aromatase expression.

Bulun et al. have previously demonstrated that pre-adipocyte aromatase expression is stimulated by a host of secretory products, including PGE2, found in MCF-7 breast cancer cell conditioned media. While PGE2 is known to be independently capable of stimulating aromatase expression in pre-adipocytes, the other breast cancer cell secretory products also make significant contributions to the conditioned media's effect. In fact, stimulation of pre-adipocyte aromatase expression via MCF-7 cell conditioned media exposure persists with inhibition of breast cancer cell COX-2 activity, clearly showing that these other factors are involved in this paracrine interaction (30-31). Breast cancer cells also release TNFα and interleukin-11 (IL-11), cytokines that inhibit the expression of adipogenic transcription factors and thereby prevent pre-adipocyte differentiation into mature adipocytes (114). Given that aromatase is expressed primarily in the undifferentiated fraction of adipose tissue (30, 115-116), this accrual of pre-adipocytes in the tumor-adjacent breast tissue also enhances local aromatase expression.

For this study, we focused on how obesity affects breast cancer cell production of secretory factors that directly stimulate pre-adipocyte aromatase expression. We were specifically interested in exploring whether obesity promotes

greater breast cancer cell PGE2 production because IL-6 has been shown to stimulate COX-2 expression in a number of cell types and concentrations of this cytokine were significantly elevated in our obese patient serum samples (35-37, 99). In addition, the pathways by which PGE2 stimulates pre-adipocyte aromatase expression have been well-characterized (117-118), while other aromatase-promoting breast cancer cell-derived factors remain largely unidentified. Consequently, we hypothesized that systemic factors known to be increased with obesity, including IL-6, stimulate greater MCF-7 cell COX-2 expression and PGE2 production, resulting in higher pre-adipocyte aromatase expression and a subsequent increase in breast cancer cell ERα activity and proliferation. Conditioned media from MCF-7 cells exposed to obese versus normal weight patient sera, which was shown to contain a higher concentration of PGE2, did stimulate greater pre-adipocyte aromatase expression. In support of an elevation in MCF-7 cell COX-2 expression and PGE2 production being responsible for this effect, we found that the difference in aromatase expression was eliminated by MCF-7 cell COX-2 inhibition during conditioned media generation. Thus, while other aromatase-promoting factors may be present in MCF-7 cell conditioned media, the production of these does not appear to vary with exposure to obese versus normal weight patient sera. In addition, our results indicate that an obesity-associated elevation in serum IL-6 is primarily responsible for the difference between obese versus normal weight MCF-7 CM-induced pre-adipocyte aromatase expression, as depletion of IL-6 from the pooled sera also neutralized

this difference. Much of the excess systemic IL-6 found with obesity is produced by the adipose tissue (34), so local breast tissue IL-6 levels are likely also increased with obesity and contribute to this effect.

Dannenberg et al. have also investigated the link between obesity and local aromatase expression, demonstrating that obesity is associated with elevated aromatase levels in the normal breast tissue of both disease-free women and breast cancer patients. Intriguingly, even stronger correlations were found between aromatase expression and levels of breast inflammation, defined by the number of macrophage-containing crown-like structures of the breast (CLS-B), as well as macrophage-secreted PGE2 (41-42). *In vitro* experiments further defined the pathways mediating this effect, showing that macrophage COX-2 expression and PGE2 production promote pre-adipocyte aromatase expression and ERα target gene expression (41, 43). The authors argue that this pathway may contribute to the increased breast cancer risk seen in obese postmenopausal women, but do not address how it might impact an established tumor.

We have recently submitted a manuscript detailing findings that complement the work of Dannenberg et al. and additionally examine whether this macrophage/pre-adipocyte paracrine interaction may promote breast cancer progression. Using an *in vitro* model of the obese patient's tumor microenvironment, we demonstrated that obesity-associated systemic factors further enhance macrophage COX-2 expression and PGE2 production, resulting

greater pre-adipocyte aromatase expression as well as higher breast cancer ERα activity, proliferation, and migration (119). However, while it is clear from our work and that of Dannenberg et al. that macrophages play a significant role in stimulating obesity-associated local aromatase expression, macrophages are not the only PGE2-producing cells within tumor-bearing breast tissue. If obesity does enhance breast cancer cell PGE2 secretion *in vivo*, then this would likely result in greater aromatase expression in the adjacent adipose tissue and a subsequent increase in breast cancer cell ERα activity and proliferation, as we have demonstrated here with our *in vitro* model. To confirm the relevance of our model, future studies of breast cancer patient tissue should examine whether correlations exist between the following: obesity, aromatase expression and IL-6 and PGE2 levels in the tumor and tumor-adjacent adipose tissue, and tumor ERα target gene levels.

Our assessment of the impact of this aromatase-promoting paracrine interaction on ERα target gene expression revealed a significant difference between the obese and normal weight conditions. While pre-adipocyte aromatase inhibition was able to significantly reduce the obese CM-induced ERα activity, it only eliminated the difference between the obese and normal weight conditions for MCF-7 cell pS2 expression. In addition, aromatase inhibition was not able to decrease obese CM-induced MCF-7 or T47D cell proliferation to the level of the normal weight CM without anastrozole. These results suggest that anastrozole

alone is unable to reduce the obese CM-induced pre-adipocyte aromatase activity down to the same level as the normal weight condition, resulting in significantly decreased but still elevated breast cancer cell ERα target gene and proliferation levels. Given that anastrozole was slightly more effective at inhibiting obese CM-induced ERα target gene expression versus proliferation, it is also possible that the obese patient sera promotes the production of additional proliferation-stimulating factors in the pre-adipocytes and/or breast cancer cells. However, it seems clear that pre-adipocyte aromatase expression is playing a role in the variation between the obese and normal weight CM in ERα activity and proliferation. Our findings reflect a possible mechanism to explain previous studies demonstrating a reduced response to aromatase inhibitors in obese women, as well as our recent study showing that obese and overweight ERα positive women receiving endocrine therapy have a higher recurrence rate and shorter time to recurrence (119). They also indicate that obese postmenopausal breast cancer patients may benefit from an aromatase inhibitor/COX-2 inhibitor combination treatment, as celecoxib treatment of the MCF-7 cells was able to significantly decrease MCF-7 cell CM-induced pre-adipocyte aromatase expression and neutralize the difference between the obese and normal weight conditions.

This study provides strong evidence that obesity may promote postmenopausal, hormone responsive breast cancer progression via an elevation in local aromatase expression. Obesity-associated IL-6 enhances breast cancer

cell COX-2 expression and PGE2 production, resulting in higher levels of pre-adipocyte aromatase expression and a subsequent increase in breast cancer cell ERα activity and proliferation. Based on these results, an assessment of the clinical benefit of an aromatase inhibitor/COX-2 inhibitor combination treatment for obese, postmenopausal breast cancer patients is warranted.

Chapter 5: Obesity enhances nongenomic estrogen receptor crosstalk with the PI3K/Akt and MAPK pathways to promote *in vitro* measures of breast cancer progression

5.1 Introduction

The prevalence of obesity in the United States has been climbing steadily for the past three decades, resulting in a current adult obesity rate of 35.7% (17). A similar trend is evident in other nations around the world and is no longer unique to wealthy, industrialized countries (18). This epidemic poses a dire threat to public health, as obesity can play a role in the pathogenesis of numerous diseases, including breast cancer. In postmenopausal women, obesity increases breast cancer risk by approximately 40% (3-5). A large body of evidence has also established that obesity is associated with a worse breast cancer prognosis for both pre- and postmenopausal women. One prospective study that followed a population of more than 900,000 U.S. adults over a 16 year period found that the mortality rate due to breast cancer was amplified with each successive increase in body mass index (BMI) category (19). Another study showed a significantly greater risk for disease recurrence within 10 years of diagnosis in breast cancer patients who were obese at the time of treatment in comparison to non-obese patients (20). These effects could be due to later diagnosis in the obese population, resulting in more advanced disease at the time of diagnosis. This hypothesis was initially supported by data from a large cohort of patients followed

for a 20 year period; Majed et al. (21) found that the obese patients presented with more advanced tumors, suggesting that diagnosis had been delayed. However, the authors ultimately found that multivariate analysis demonstrated an independent effect of obesity on breast cancer prognosis, regardless of tumor stage at time of diagnosis. Survival analysis revealed increased metastatic recurrence as well as decreased disease-free interval and overall survival in the obese patient population. While obesity has been shown to negatively impact prognosis for both pre- and postmenopausal patients, the most prominent effects are seen in estrogen receptor alpha (ERα) positive postmenopausal patients, a finding confirmed by a recent retrospective analysis of the German BRENDA-cohort (24).

Previous studies indicate that obesity may adversely impact prognosis in the ERα positive postmenopausal patient population in part by promoting endocrine therapy resistance (22). This theory is supported by an analysis of data from the ATAC trial by Sestak et al. (48), which found that obese breast cancer patients receiving anastrozole had a significantly greater risk of recurrence. In agreement with these findings, Schmid et al. (49) demonstrated that obese patients have a significantly reduced response rate to letrozole in comparison to lean (11 vs 35%). The ATAC trial also showed that while anatrozole treatment resulted in significantly greater recurrence-free survival in comparison to tamoxifen, this benefit was lost in the obese cohort (48). The primary site of aromatase expression and estrogen

production in postmenopausal women is the adipose tissue. Due to an abundance of this aromatase-expressing tissue, obese postmenopausal women typically have higher levels of circulating estradiol (25-27), and researchers have posited that this may contribute to the observed increase in breast cancer risk and worse outcome in this population. This hypothesis suggests that an adjustment of the aromatase inhibitor dosage may improve obese patient prognosis. However, that conclusion is confounded by two phase III clinical trials of anastrozole that found no overall benefit from a 10 mg dose (versus 1 mg), indicating that an increased dosage may not be effective in overcoming obesity-induced resistance to aromatase inhibitors (52-53).

The development of endocrine therapy resistance can be mediated by several mechanisms. Frequently, aberrant signaling from growth factor receptors, particularly the insulin-like growth factor 1 receptor (IGF-1R) and the HER family of receptors, is responsible. These receptors can engage in bidirectional crosstalk with ERα, leading to increased nongenomic ERα activity, ligand-independent activation of ERα, and abnormal regulation of cell cycle and apoptotic signaling. Nongenomic ERα activity results in the activation of the MAPK and PI3K/Akt signaling pathways, and these can in turn activate ERα via phosphorylation, leading to enhanced genomic ERα activity (54-55). Obesity is typically accompanied by elevated circulating levels of insulin, bioavailable IGF-1 and leptin, as well as a series of pro-inflammatory cytokines (34, 38-39, 65). All of

these obesity-associated circulating factors are able to activate the PI3K/Akt and/or MAPK pathways, potentially enhancing the ERα crosstalk pathways described above and leading to endocrine resistance and breast cancer progression (66-70). The metabolic alterations associated with obesity, including changes in insulin and insulin-like growth factor binding protein 1 (IGFBP-1) serum levels (which result in increased circulating free IGF-1 levels), are also significantly correlated with breast cancer recurrence and mortality (71). High serum concentrations of pro-inflammatory cytokines and leptin have been similarly linked to a worse breast cancer outcome (72-74). Overall, obesity creates a complex metabolic imbalance accompanied by chronic inflammation, enriching the blood with a number of signaling molecules that may promote breast cancer progression and adversely affect outcome.

This study utilized an *in vitro* model of obesity in which ERα positive breast cancer cells were exposed to pooled sera samples from normal weight or obese postmenopausal breast cancer patients. This model enabled us to examine the molecular pathways by which obesity-associated circulating factors in the blood stimulate greater ERα positive breast cancer cell viability and growth. Here we provide evidence that these physiological effects are mediated by enhanced crosstalk between nongenomic ERα signaling and the PI3K/Akt and MAPK pathways. These studies provide insight into one potential mechanism by which

obesity may promote postmenopausal ERα positive breast cancer progression and endocrine therapy resistance.

5.2 Materials and Methods

5.2.1 Serum Samples

Serum was collected from postmenopausal breast cancer patients under an Institutional Review Board (IRB) approved biorepository collection protocol at the Cancer Therapy and Research Center of the University of Texas Health Science Center at San Antonio (UTHSCSA). The collection and use of these biological samples was approved by the IRB of UTHSCSA (HSC20070684H) and conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained prior to participation, and all samples and data were deidentified prior to release to maintain patient confidentiality. Serum was pooled according to the BMI category of the patient (normal weight (N): 18.5-24.9 kg/m^2; obese (OB): ≥30 kg/m^2).

5.2.2 Cell Lines and Reagents

ERα positive MCF-7 and T47D cells (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) was purchased from Sigma. The drug treatments used in this study, which include PD 98,059 (a MEK1 inhibitor), LY 294,002 (a PI3K inhibitor), and 4-hydroxytamoxifen

(a selective estrogen receptor modulator), were also obtained from Sigma. The primary antibodies for pAkt (s473), tAkt, pERK1/2, tERK1/2, pERα(s167), pERα(s118), pIGF-1R(tyr1135/1136), and tIGF-1R were purchased from Cell Signaling (Beverly, MA). The tERα primary antibody was produced by Novacastra (Leica Microsystems, Buffalo Grove, IL).

5.2.3 MTT Assay

MCF-7 and T47D cells were seeded in IMEM supplemented with 10% FBS at a density of $8x10^3$ in 96 well plates. After 24 hours of growth in the 10% FBS media, the cells were exposed to 2% sera in serum-free media (SFM), with or without the addition of drug treatments, for 48 hours. MTT reagent in PBS (5 mg/ml) was then added to each well to a final concentration of 0.5 mg/ml. After two hours of incubation at 37°C, the media was removed and 50 ul DMSO added to each well to lyse the cells. Absorbance was read at 570 nm on a FLUOstar Omega Spectrometer (BMG Labtech, Offenberg, Germany). Relative cell viability was calculated by dividing each absorbance value by the absorbance for cells grown in N patient sera. Data shown represents the average of at least three independent experiments.

5.2.4 Colony Formation Assay

MCF-7 and T47D cells were seeded in IMEM supplemented with 10% FBS at a density of 500 and $1x10^3$, respectively, in six well plates. After 24 hours of growth in the 10% FBS media, the cells were continuously exposed to 2% sera in

SFM, with or without drug treatments, for nine days. On day five of the treatment period, the wells were aspirated and washed, and the media was replenished with the same concentration of sera and inhibitors. On day nine, the colonies were fixed and stained with 1% crystal violet in 70% acetic acid for 30 minutes and then counted. The relative number of colonies, a reflection of cell growth, was calculated by dividing each colony count by the count for cells grown in N patient sera and multiplying by 100. Data shown represents the average of at least three independent experiments.

5.2.5 Western Blot Analysis

The cells were grown to 80% confluence in IMEM supplemented with 10% FBS, then the growth medium was aspirated, the wells were washed, and the medium replaced with SFM overnight. After overnight serum-starvation to minimize the effect of growth factors and hormones in the growth medium, 2% OB or N patient sera was added directly to the overnight SFM with or without inhibitors for 15 minutes or one hour. Kinase lysis buffer or RIPA buffer was used for protein extraction. Protein content of the lysates was measured using the BCA Protein Assay kit from Thermo Scientific Pierce (Rockford, IL) or the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Images were acquired using a Syngene G:BOX Chemi (Frederick, MD). Relative protein levels were calculated by first standardizing phosphorylated protein to total protein levels for each experimental condition, then dividing the standardized protein level for each condition by that of cells grown in N patient sera. Data from at least three independent experiments

was compiled for each protein and cell line to calculate the average protein level, standard error of the mean and statistical significance, with one representative image for each protein shown.

5.2.6 ERE Luciferase Assay

A luciferase reporter gene driven by a 3X ERE-tk promoter was utilized to measure ERα transcriptional activity. Transient transfections were performed in triplicate wells three times. MCF-7 and T47D cell lines were seeded in IMEM supplemented with 10% FBS at a density of 1.5 x 10^4 in 24 well plates and concurrently transfected with the ERE luciferase and renilla plasmids after 24 hours of growth using Fugene 6 from Promega (Madison, WI) at a 1:3 ratio. The cells were serum-starved for six hours the following day, then exposed for 48 hours to 2% OB or N patient sera, added directly to the SFM. Luciferase activity was then measured using Promega's Dual Luciferase Reporter Assay System, with the fluorescence read on a FLUOstar Omega Spectrometer (BMG Labtech, Offenberg, Germany). Relative ERα activity was calculated by dividing the fluorescence value (standardized to renilla) from cells grown in OB patient sera by that from cells grown in N patient sera. Data shown represents the average of at least three independent experiments.

5.2.7 Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer

sequences are as follows: pS2: forward, 5'-GGTCGCCTTGGAGCAGA-3'; reverse, 5'-GGGCGAAGATCACCTTGTT-3'; cyclin D1: forward, 5'-TGGAGGTCTGCGAGGAACAGAA-3'; reverse, 5'-TGCAGGCGGCTCTTTTTCA-3'. The manufacturer's recommended cycling conditions for the QuantiFast SYBR Green PCR kit (Quiagen) were used. Data shown represents the average of at least three independent experiments.

5.2.8 Statistics

Differences between cells exposed to obese versus control sera were measured using Student's *t* test. One-way ANOVA was used to analyze differences between more than two experimental conditions. A difference of $p<0.05$ was considered significant.

5.3 Results

5.3.1 Obesity-Associated Circulating Factors Enhance Breast Cancer Cell Viability and Growth

In order to elucidate the potential mechanisms by which obesity promotes breast cancer progression, we first evaluated the effect of obesity-associated circulating factors on cell viability and growth, both *in vitro* parameters of cancer aggression. Viability of breast cancer cells in response to exposure to patient serum was measured by MTT assay. MCF-7 cells grown in 2% OB sera in SFM for 48 hours displayed a 43% increase in viability in comparison to cells grown in N sera

(p<0.01). OB sera also enhanced the viability of T47D cells by 32% versus N sera (p<0.01) (Figure 5.1a). Colony formation assay was utilized to assess the effects of patient sera on breast cancer cell growth. Both MCF-7 and T47D cells grew significantly better in OB sera, forming 63% (p<0.05) and 39% (p<0.01) more colonies, respectively, over a nine day exposure to the OB sera in comparison to N sera (Figure 5.1b). These results demonstrate that one or more circulating factors in the obese patient sera directly induces higher levels of ERα positive breast cancer cell viability and growth.

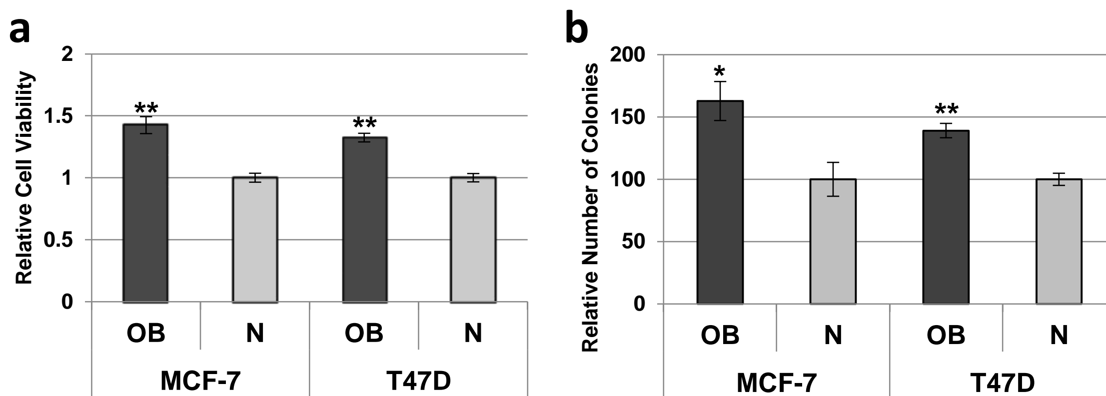

Figure 5.1 Obesity-associated circulating factors promote greater breast cancer cell viability and growth. (a) MCF-7 and T47D breast cancer cells were exposed to 2% obese (OB) or normal weight (N) patient sera for 48 hours; viability was then measured by MTT assay. (b) Colony formation assay was utilized to assess MCF-7 and T47D cell growth following a nine day exposure to 2% OB or N patient sera. Data shown represents the average of at least three independent experiments. *, p<0.05; **, p<0.01 in comparison to N.

5.3.2 PI3K/Akt, MAPK, and IGF-1R Pathway Activation is Stimulated by Obesity-Associated Circulating Factors in Breast Cancer Cells

The PI3K/Akt and MAPK pathways are both downstream targets common to many of the circulating factors typically upregulated with obesity (66-70). They are also involved in the regulation of cell proliferation and survival and can crosstalk with and ultimately activate ERα independent of estradiol (54-55). Consequently, we assessed the effects of OB and N sera on Akt and ERK1/2 activation. MCF-7 cells exposed to 2% OB sera for 15 minutes and one hour had 100% ($p<0.01$) and 55% ($p<0.05$) higher levels of pAkt (ser473), respectively, in comparison to cells exposed to N sera. pERK1/2 levels following 2% OB sera exposure were 79% and 33% ($p<0.05$) greater at the same time points in comparison to N (Figures 5.2a-b). A similar effect was observed in T47D cells exposed to OB versus N sera at these time points. OB sera exposure stimulated 53% and 64% ($p<0.01$) more Akt activation and 38% ($p<0.05$) and 72% ($p<0.01$) more ERK1/2 activation than N after 15 minute and 1 hour incubation periods (Figures 5.2c-d). These results suggest that the PI3K/Akt and MAPK pathways may both play a role in obesity-induced breast cancer progression. Despite finding no difference between the two patient groups in average serum free IGF-1, MCF-7 cells exposed to the OB sera had 20% ($p<0.01$) higher pIGF-1R (tyr1135/1136) levels in comparison to N (Figures 5.2e-f). This suggests that the OB sera-induced Akt and ERK1/2 activation described above may be at least partly mediated by IGF-1R signaling that is upregulated by a mechanism independent of bioavailable IGF-1 levels.

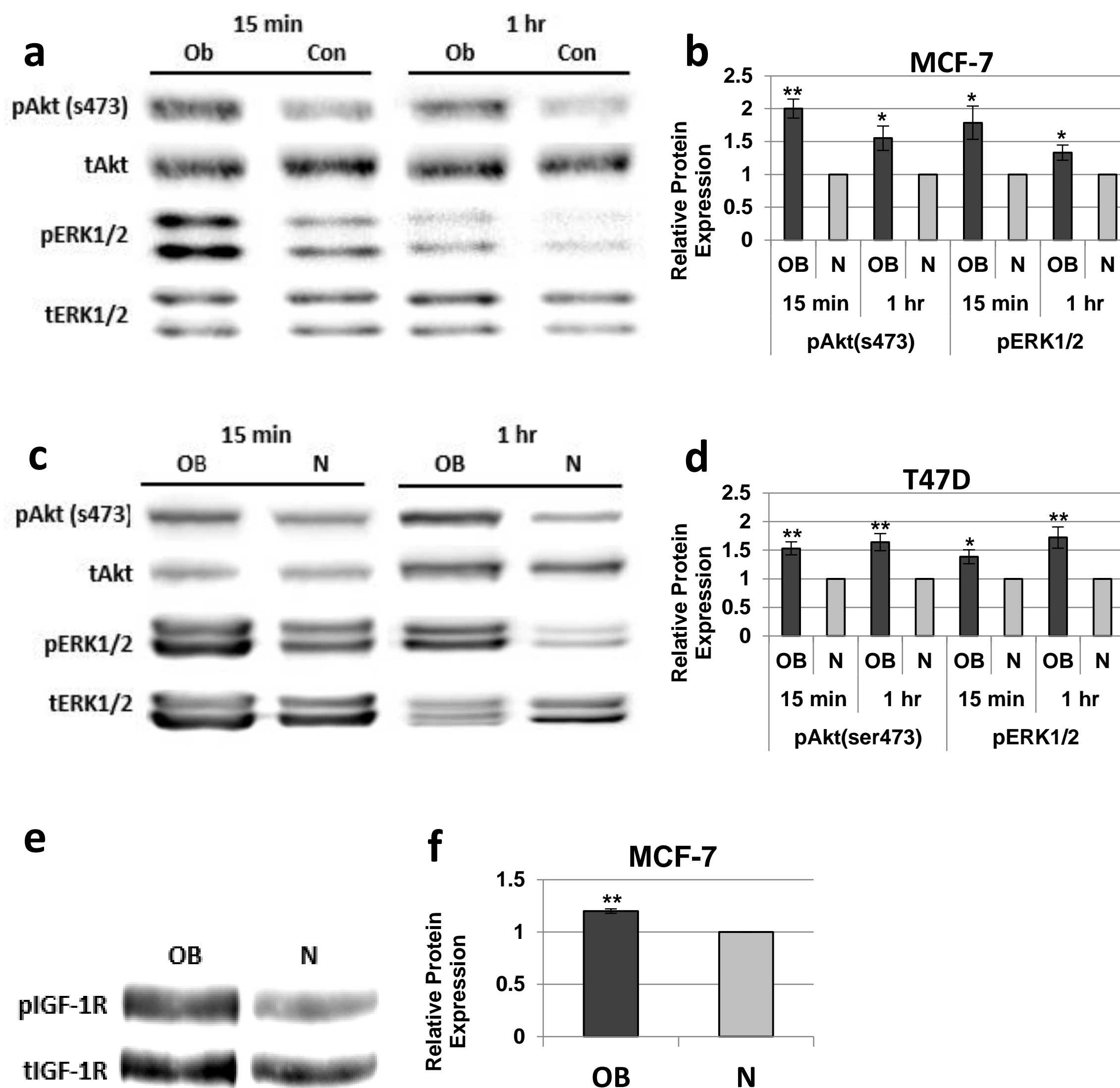

Figure 5.2. Breast cancer cell Akt, ERK1/2, and IGF-1R activation are enhanced by obesity-associated circulating factors. The effects of OB and N patient sera on Akt, ERK1/2, and IGF-1R activation were assessed by western blotting. MCF-7 (a and b) and T47D (c and d) cells were exposed to 2% OB or N patient sera for 15 minutes and one hour; pAkt (ser473) and pERK1/2 protein levels were then measured and standardized to tAkt and tERK1/2 protein levels, respectively. (e and f) pIGF-1R (tyr1135/1136) protein levels in MCF-7 cells were measured and standardized to tIGF-1R following a 15 minute incubation in 2% OB or N patient sera. Densitometry data from at least three independent experiments was compiled for each protein and cell line to calculate the average protein level, standard error of the mean and statistical significance, with one representative image for each protein shown. *, $p<0.05$; **, $p<0.01$ in comparison to N.

5.3.3 Genomic ERα activity in breast cancer cells is not directly enhanced by obesity-associated circulating factors

In addition to an elevation in circulating levels of growth factors, pro-inflammatory cytokines, and leptin, obesity in postmenopausal women is typically accompanied by higher levels of circulating estrogens (120). In ERα positive breast cancer cells, estradiol can bind ERα and activate its canonical signaling pathway, in which ERα acts as a nuclear transcription factor or cofactor, modulating the expression of its target genes in a manner that promotes cell proliferation and growth. This genomic ERα activity can also be induced via ligand-independent phosphorylation of the receptor's AF-1 domain by Akt and ERK1/2 (54-55, 121). To assess the effect of OB and N sera on genomic ERα activity, we measured relative ERE luciferase reporter activity in MCF-7 and T47D cells in response to these conditions. No significant difference was detected in the luciferase activity, suggesting that the factors in OB sera do not directly enhance genomic ERα activity (Figure 5.a). Expression of pS2, an ERα target gene, was also measured as another indicator of ERα transcriptional activity. qPCR analysis of the relative levels of pS2 mRNA showed no difference in pS2 expression in either the MCF-7 or T47D cell lines after growth in OB versus N sera (Figure 5.3b). In contrast, OB sera did induce significantly higher expression of cyclin D1, another ERα target gene, in both cell lines. MCF-7 cells expressed 34% more cyclin D1 following 24 hours of growth in OB sera versus N, while cyclin D1 mRNA levels

were 30% higher in T47D cells under these conditions ($p<0.05$) (Figure 5.3c). However, while pS2 expression is considered to be a very specific and reliable indicator of ERα activity, cyclin D1 expression is regulated by many signaling pathways, including PI3K/Akt and MAPK. Therefore, the upregulation of cyclin D1 expression following OB sera exposure is likely related to increased activity in these upstream pathways. Because cyclin D1 is involved in promoting progression through the cell cycle, these results are also supportive of our data demonstrating a significant difference in breast cancer cell growth following OB sera exposure.

One potential critique of our study design is the use of sera from breast cancer patients. Many of the patients that provided sera for this study were receiving aromatase inhibitor treatment at the time of serum collection, leading to a decrease in their circulating estradiol levels. The lack of difference in genomic ERα activity could be an artifact of the drug's effects. To address this issue, we repeated the ERE luciferase assay in MCF-7 cells with pooled sera from patients who had not been prescribed aromatase inhibitors (OB(-AI) versus N(-AI)) and again found no difference in genomic ERα activity (Figure 5.3d). Together, these studies strongly suggest that genomic ERα activity plays a minimal role in mediating obese sera-induced breast cancer cell viability and growth.

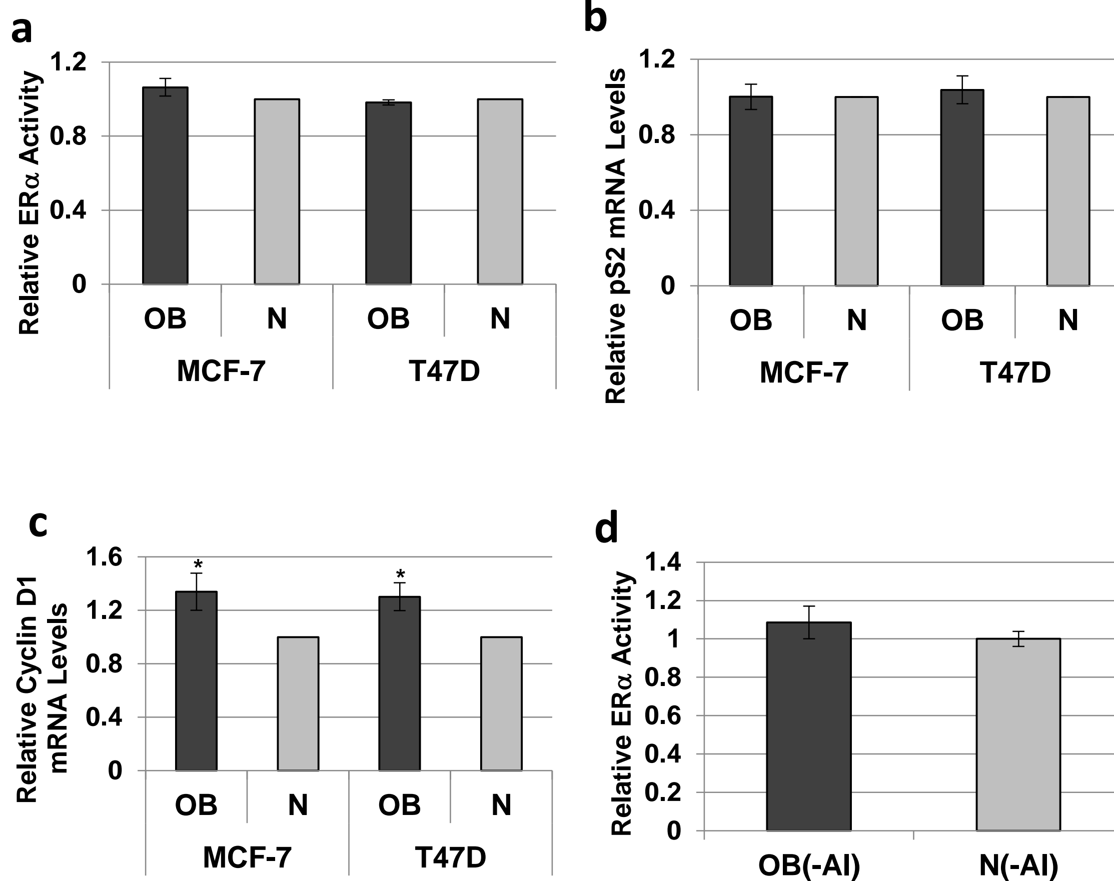

Figure 5.3. Genomic ERα activity in breast cancer cells is not directly enhanced by obesity-associated circulating factors. Genomic ERα activity in response to 2% OB or N patient sera exposure was measured in MCF-7 and T47D cells with an ERE luciferase reporter (a) and qPCR analysis of pS2 expression (b). Expression of cyclin D1, which is regulated by both ERα and the PI3K/Akt and MAPK pathways, was assessed by qPCR analysis in both cell lines following growth in 2% OB or N patient sera (c). The effect of 2% OB(-AI) and N(-AI) patient sera on genomic ERα activity in MCF-7 cells was also measured by ERE luciferase reporter (d). This pooled sera excluded breast cancer patients receiving aromatase inhibitors at the time of collection. Data shown represents the average of at least three independent experiments. *, $p<0.05$ in comparison to N.

5.3.4 Combined PI3K and ERα inhibition attenuates effects of obese patient sera on breast cancer cell viability and growth

After demonstrating that OB sera exposure directly increases PI3K/Akt and MAPK pathway activation, but not genomic ERα activity, we examined the contribution of these pathways to OB sera-induced MCF-7 cell viability and growth. Using the targeted inhibitors LY 294,002 (LY, a PI3K inhibitor), PD 98,059 (PD, a MEK1 inhibitor), and 4-hydroxytamoxifen (Tam, a selective estrogen receptor modulator), we established which factors were essential for the observed increase in viability and growth. While each drug was able to significantly decrease the viability of MCF-7 cells exposed to OB sera ($p<0.05$), LY/Tam inhibited viability by 54% and was the only treatment able to inhibit it to a level significantly less than cells grown in N sera ($p<0.05$). In addition, cells exposed to N sera and LY/Tam had a significantly lower viability level in comparison to all N sera-exposed cells ($p<0.05$) except those also treated with LY/Tam, suggesting that this drug combination is the most effective at neutralizing obesity-induced viability (Figure 5.4a). OB sera-induced MCF-7 cell growth was significantly decreased by all drug treatments except PD. However, the LY/Tam combination again proved to be the most effective inhibitor; it decreased OB sera-induced growth by 87%, inhibiting it to a level significantly lower than that produced by all other drug treatments ($p<0.01$). Intriguingly, PD alone significantly increased the number of colonies formed by MCF-7 cells grown in OB or N sera, but also inhibited OB sera-induced growth when administered in combination with Tam ($p<0.01$) (Figure 5.4b). These

results suggest that signaling from all three pathways, as well as enhanced crosstalk between them, contributes to the upregulation of breast cancer cell viability and growth by OB patient sera. However, because the most effective drug combination was LY/Tam, the data also indicates that the PI3K/Akt pathway and its interactions with ERα may play a more critical role than the MAPK pathway in mediating these effects.

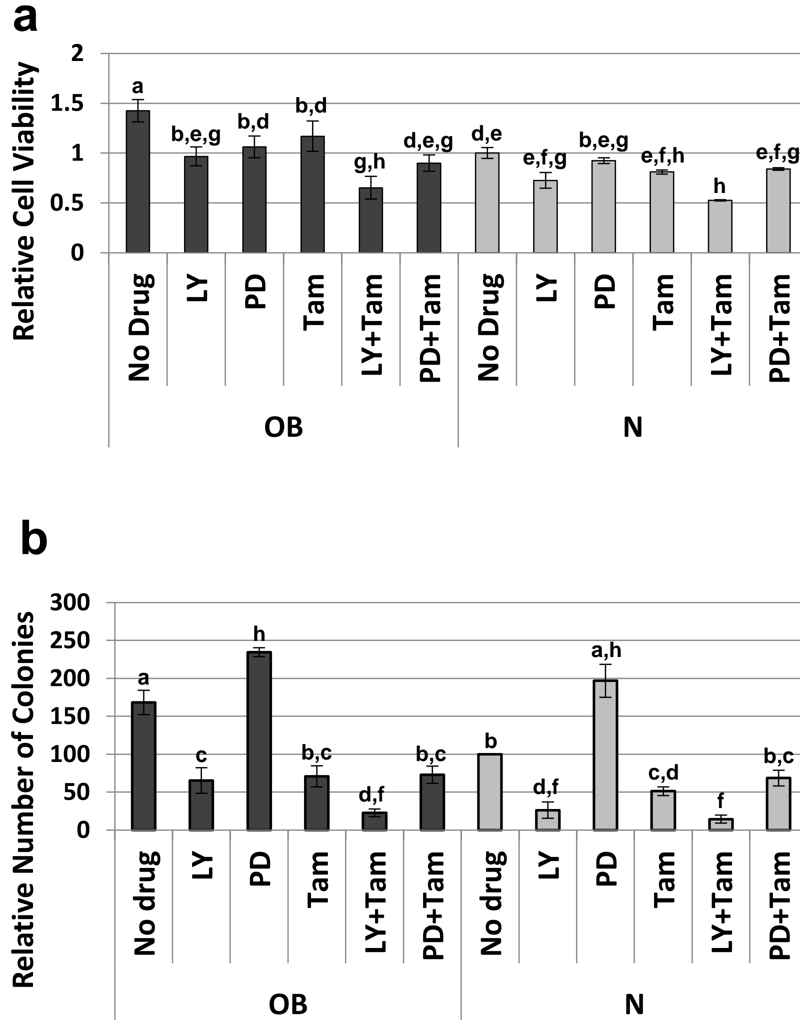

Figure 5.4. Combined PI3K/ERα inhibition attenuates effects of obesity on breast cancer cell viability and growth. The contribution of the PI3K/Akt, MAPK, and ERα pathways to Ob patient sera-induced cell viability and growth was examined via treatment of MCF-7 cells with the following inhibitors during sera exposure: LY 294,002 (LY, a PI3K inhibitor, 10uM), PD 98,059 (PD, a MEK1 inhibitor, 10uM), and 4-hydroxytamoxifen (Tam, a selective estrogen receptor modulator, 100nM). (A) MCF-7 cell viability was measured by MTT assay following a 48 hour exposure to 2% OB or N patient sera, with or without drug treatment. (B) Colony formation assay was used to assess MCF-7 cell growth over a nine day exposure to 2% OB or N patient sera, with or without drug treatment. Data shown represents the average of at least three independent experiments, and different letters indicate significant differences ($p<0.05$).

5.3.5 Obesity-Associated Circulating Factors Enhance Akt-mediated Activation of ERα and Nongenomic ERα activity

In addition to its transcriptional activity, ERα signaling also occurs at the plasma membrane and in the cytoplasm. Here, ERα can activate the PI3K/Akt and MAPK pathways when it forms complexes with other signaling molecules, including the IGF-1R and the regulatory subunit of PI3K, p85. Akt and ERK1/2 can in turn activate ERα in a ligand-independent manner by phosphorylation (54-55). Although there was no difference in genomic ERα activity following OB versus N sera exposure, our data demonstrated that LY/Tam is the most effective drug combination for the inhibition of OB sera-induced breast cancer cell viability and growth, indicating that ERα is indeed a critical player in mediating these effects. Consequently, we next examined whether nongenomic ERα activity is enhanced by obesity-associated circulating factors. We found that OB sera, in comparison to N, promotes 53% ($p<0.01$) and 52% ($p<0.05$) higher levels of ERα phosphorylation at the Akt target site (s167) in MCF-7 cells following a 15 minute or one hour exposure, respectively ($p<0.05$). No difference between OB and N was seen at the ERK1/2 target site (ser118) under the same conditions (Figures 5.5a and 5.5b). OB sera also stimulated an increase in Akt and ERK1/2 phosphorylation via ERα activity in the cytoplasm. This is demonstrated by the ability of Tam to inhibit OB sera-induced Akt and ERK1/2 activation in MCF-7 cells by 36% ($p<0.01$) and 33% ($p<0.05$), respectively. In contrast, Tam had no effect

on N sera-induced Akt and ERK1/2 activation (Figures 5.5c and 5.5d). ERα inhibition also eliminated the difference in Akt and ERK1/2 activation levels stimulated by OB and N sera exposure alone, suggesting that obesity-associated circulating factors are promoting greater nongenomic ERα activity. This enhanced crosstalk explains why the addition of Tam to either LY or PD results in greater inhibition of OB sera-induced breast cancer cell viability and growth in comparison to either drug alone.

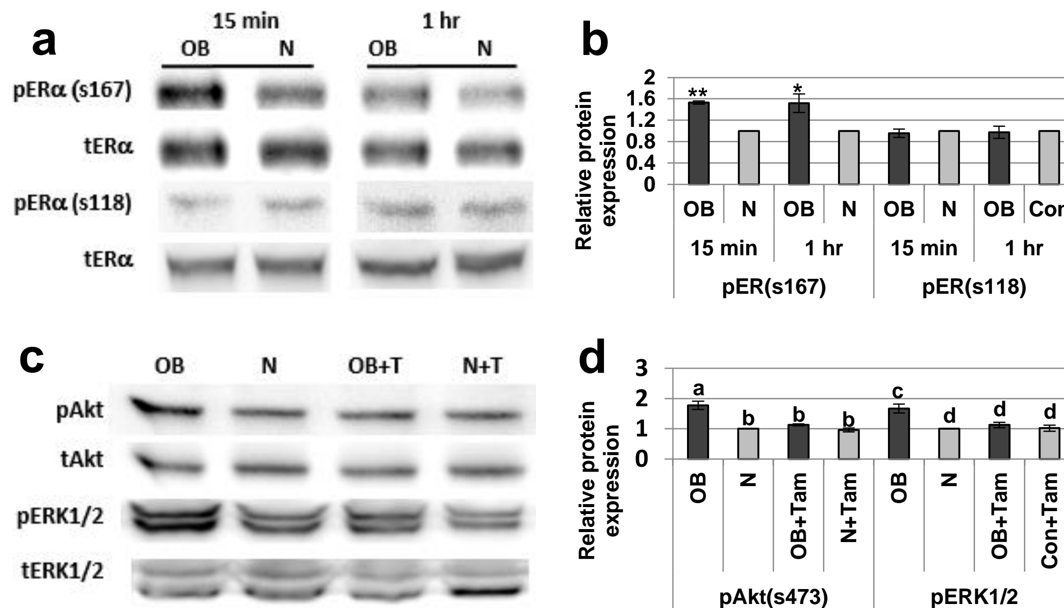

Figure 5.5 Obesity-associated circulating factors promote greater Akt-mediated ERα phosphorylation and nongenomic ERα activity. Phosphorylation of ERα at two different sites (ser167 and ser118, the Akt and MAPK target sites, respectively) following a 15 minute and one hour exposure to 2% OB or N patient sera was assessed in MCF-7 cells by western blot and standardized to tERα protein levels (a and b). The effect of tamoxifen (T) treatment on Akt and ERK1/2 activation in MCF-7 cells following a 15 minute exposure to 2% OB or N patient serum was also measured by western blot (c and d). Densitometry data from at least three independent experiments was compiled for each protein to calculate the average protein level, standard error of the mean and statistical significance, with one representative image for each protein shown. *, $p<0.05$; **, $p<0.01$ in comparison to N, and different letters indicate significant differences ($p<0.05$).

5.4 Discussion

Growth factor signaling is known to promote the development of endocrine resistance in breast cancer. However, while obesity has been shown to modulate growth factor signaling pathways, its impact on hormone independence remains relatively unexplored. We have previously reported that obese ovariectomized

mice implanted with syngeneic mouse mammary tumor cells displayed enhanced mammary tumor development and progression, and this was associated with elevated levels of bioavailable IGF-1 and downstream PI3K/Akt/mTOR signaling (122-123). Because elevated growth factor signaling can stimulate cytoplasmic ERα localization and nongenomic ERα activity (54), we investigated the role of bidirectional crosstalk among various growth factor pathways and ERα. Based on our current findings, we propose that obesity-induced systemic factors promote breast cancer progression and may increase resistance to aromatase inhibitor therapy by initiating crosstalk between nongenomic ERα activity and the IGF-1R, PI3K/Akt and MAPK signaling pathways.

Here we demonstrate that circulating factors associated with postmenopausal obesity increased ERα positive breast cancer cell viability and growth (Figure 5.1). This was coupled with greater breast cancer cell Akt and ERK1/2 phosphorylation, as well as enhanced IGF-1R activation (Figure 5.2). Intriguingly, we previously found that there was no difference between the obese and control patients in average serum free IGF-1 concentration. However, average insulin levels were non-significantly higher in the obese group, and insulin can also bind and activate the IGF-1R (Table 2.1). The lack of significant differences in these hormones may be due to the non-fasting status of the patients, as other studies examining their association with obesity have assessed fasting serum samples (38-39). Obese postmenopausal women are also known to have, on average, higher levels of

circulating estradiol (25-27). Consequently, we were surprised to find no difference in the genomic ERα activity of breast cancer cells grown in obese versus control patient sera, even with the exclusion of patients on aromatase inhibitors at the time of serum collection, suggesting that obesity-related circulating factors promote ERα positive breast cancer cell viability and growth independent of ERα transcriptional activity.

However, previous studies have demonstrated that ERα, in addition to its canonical genomic signaling pathway, is active outside the nucleus. Over the past decade, a number of researchers have successfully characterized several interactions between ERα and other signaling molecules that occur in the cytoplasm. For example, Song et al. (56-57) discovered that, in the presence of estradiol, ERα undergoes translocation to the plasma membrane and complexes with IGF-1R and the adaptor protein Shc, resulting in MAPK pathway activation. Down-regulation of IGF-1R prevents ERα translocation to the membrane, suggesting that IGF-1R signaling is necessary for nongenomic ERα activity. Ligand-bound ERα can also directly bind Src as well as p85, the regulatory subunit of PI3K, resulting in Akt activation downstream (58-59). In addition, p85 can bind IGF-1R, leading to speculation that ERα may complex with both of these molecules upon activation by estradiol (60-61). The receptor for leptin, an obesity-associated adipokine that was significantly elevated in our obese patient group (Table 2.1), has also been shown to crosstalk with IGF-1R, resulting in greater

IGF-1R activation and an upregulation of Akt and ERK1/2 phosphorylation (62). This interaction could potentially enhance IGF-1R/ERα crosstalk. Activated Akt and ERK1/2 can in turn activate ERα via phosphorylation at serine 167 and 118, respectively, within the receptor's AF-1 domain, leading to enhanced genomic ERα activity (63-64). Illustration 5.1 summarizes the different mechanisms of ERα activity. Because PI3K/Akt, MAPK, and IGF-1R activity were all upregulated with obese patient sera exposure, we next explored the effects of obesity-associated factors on nongenomic ERα activity.

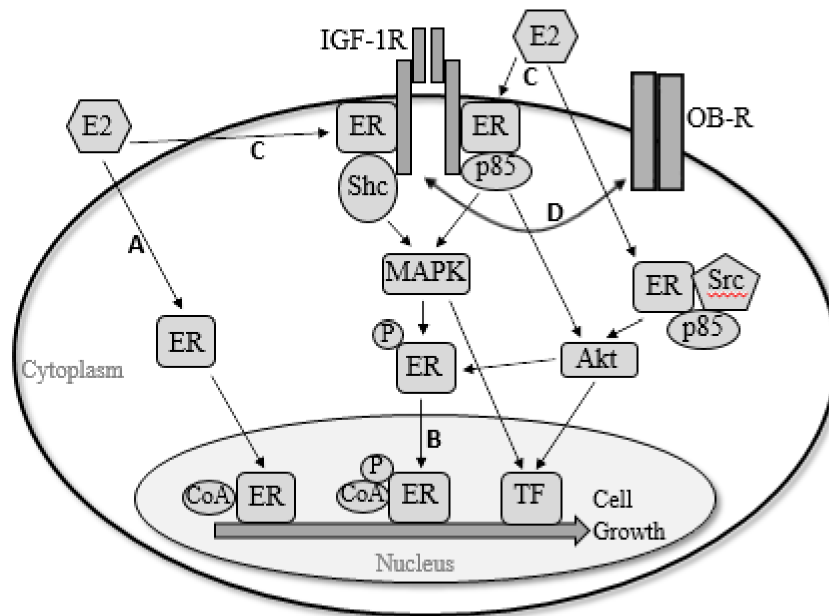

Illustration 5.1. Breast cancer cell estrogen receptor activity. Three general mechanisms of ERα signaling are depicted. First, ERα can bind estradiol (E2) and enter the nucleus, where it forms complexes with co-activators (CoAs) and regulates gene transcription by binding directly to estrogen response elements (EREs) specific to ERα or via interaction with other transcription factors (A). This genomic ERα activity can also be stimulated by Akt or MAPK-mediated phosphorylation of the receptor (B). Ligand-bound ERα can also remain outside the nucleus, where it complexes with the IGF-1R at the cell membrane or with other signaling molecules in the cytoplasm to activate the PI3K/Akt and MAPK pathways (C). IGF-1R crosstalk with the leptin receptor (OB-R) is also depicted (D). Each mechanism ultimately leads to the transcription of target genes that promote breast cancer cell growth.

To determine whether obese patient sera promotes this nongenomic ERα activity and cross-talk with growth factor signaling pathways, we first examined the contribution of the PI3K/Akt, MAPK, and ERα pathways to obese patient sera-induced breast cancer cell viability and growth. Intriguingly, we found that a combination of the PI3K inhibitor LY 294,002 (LY) with the ERα inhibitor tamoxifen

(Tam) most effectively mitigated the pro-growth effects of obese patient sera in the MCF-7 cells. The combination of PD 98,059 (PD) and Tam also demonstrated an attenuating effect on MCF-7 cell growth, so we were surprised that PD treatment alone stimulated significantly more cell growth than sera alone (Figure 5.4). This may be due to feedback upregulation of the PI3K/Akt pathway in response to MEK inhibition, as Hoeflich, et al. (91) has demonstrated that the selective MEK inhibitor PD0325901 enhances PI3K/Akt signaling in several breast cancer cell lines. Together, these data support the possibility that crosstalk between both the PI3K/Akt and MAPK pathways and nongenomic ERα signaling may be playing a role in obesity-induced postmenopausal breast cancer progression, though the PI3K/Akt pathway may be the more important mediator of these effects. Additional evidence to support this conclusion includes the observation that Tam alone is sufficient to decrease obese patient sera-induced Akt and ERK1/2 activation to the levels observed in breast cancer cells grown in control patient serum (Figure 5).

In addition to demonstrating that obesity-associated circulating factors increase ERα-mediated Akt and MAPK activation, we also found that they stimulated greater Akt-mediated phosphorylation of ERα at serine 167 in MCF-7 cells (Figure 5.5). In contrast, exposure to obese patient sera did not upregulate ERα phosphorylation at the MAPK target site (serine 118), but researchers have found that breast cancer cell MAPK activity does not always correlate with phosphorylation at this site (124). This ligand-independent activation of ERα via

its AF-1 domain is a purported mechanism by which endocrine resistance can develop (54-55). However, ligand-independent ERα activity is thought to be limited to the nucleus, where phosphorylated ERα acts as a transcription factor or co-factor (Illustration 5.1B). As we did not detect a difference in ERα genomic activity, it is unclear whether the obese patient sera-induced increase in pERα(s167) has any biological significance.

Given the lack of any detectable effect on genomic ERα activity, it is possible that the obese sera-induced breast cancer cell viability and growth may be independent of circulating estrogen levels. If this hypothesis is confirmed, it would suggest one mechanism by which obesity may contribute to the development of resistance to aromatase inhibitor therapy, a finding with potential clinical implications. This conjecture, as well as the proposed importance of the PI3K/Akt/mTOR pathway in mediating the effects of obesity-associated systemic factors, is supported by the literature on endocrine resistance. For example, Miller et al. (125) found that induction of hormone independence via long-term estrogen deprivation of ERα positive breast cancer cells was accompanied by an amplification of PI3K/Akt/mTor signaling linked to upstream IGF-1R/insulin receptor hyperactivation, similar to the effects of obese patient sera exposure. PI3K signaling was required for the induction of hormone independence, illustrating the key role this pathway plays in the development of endocrine resistance. An earlier study by Beeram et al. (126) demonstrated that MCF-7 cells

expressing a constitutively active Akt were refractory to treatment with letrozole, fulvestrant, and tamoxifen, providing further basis for our conclusions. Results indicated that the Akt-induced resistance was mediated by both ERα-dependent and independent mechanisms and that response to endocrine therapy in these cells was achieved only by combining letrozole with the mTOR inhibitor RAD001. Similarly, Cavazzoni et al. (127) found that letrozole-resistant, aromatase-overexpressing MCF-7/AROM cells displayed greater PI3K/Akt/mTOR and MAPK pathway activity. Further, mTOR inhibition with RAD001 was able to completely inhibit proliferation in this cell line. The authors correlated these results with an analysis of pathway activation in breast cancer patients that had progressed on letrozole, finding an upregulation of PI3KA, pAkt, and p-mTOR after three months on treatment in comparison to the patients' pre-treatment baseline. All of these studies suggest that the PI3K/Akt/mTOR pathway and its interaction with ERα are key mediators in the development of resistance to aromatase inhibitors. Consequently, it is probable that an upregulation of the crosstalk between these pathways, as seen in ERα positive breast cancer cells grown in obese patient sera, will lead to aromatase inhibitor resistance and disease progression.

The continuous rise in obesity rates around the world underscores the importance of identifying the molecular pathways by which obesity contributes to the pathogenesis and progression of numerous chronic diseases, including breast cancer. This study provides evidence that postmenopausal obesity enhances ERα positive breast cancer cell viability and growth via crosstalk between the ERα,

PI3K/Akt, and MAPK signaling pathways, suggesting that the addition of a PI3K/Akt/mTOR pathway inhibitor to aromatase inhibitor therapy may improve the clinical outcome of obese postmenopausal patients. Additional clarification of the crosstalk mechanisms responsible for the effects of obesity on postmenopausal breast cancer progression will be the goal of future studies.

Chapter 6: Obesity suppresses estrogen receptor beta expression in breast cancer cells via a HER2-mediated pathway

6.1 Introduction

The National Cancer Institute estimates that one in eight women born in the United States today will develop breast cancer at some point in their lives (1). A multitude of factors have been shown to affect an individual's risk of developing breast cancer as well as her breast cancer prognosis. One modifiable lifestyle factor that impacts both disease risk and outcome is obesity. In postmenopausal women, obesity has consistently been associated with an increased risk of breast cancer (3-5). A number of studies have also demonstrated that obesity negatively affects breast cancer prognosis for both pre- and postmenopausal women. Obese patients do not respond as well to chemotherapy and aromatase inhibitor treatment, and an elevated body mass index (BMI) has been associated with increased metastatic recurrence and decreased disease-free interval and overall survival (19-23, 48-50, 128-130). Our understanding of the mechanisms mediating this link between obesity and poor breast cancer outcome remains incomplete, hindering efforts to develop new chemotherapeutic regimens that may improve prognosis in the obese patient population.

In addition to modifiable lifestyle factors like obesity, breast cancer outcome is significantly affected by the non-modifiable intrinsic characteristics of a given tumor, including tumor expression of estrogen receptor alpha (ERα), progesterone

receptor (PR), and human epidermal growth factor receptor 2 (HER2). The presence or absence of these three protein receptors defines the four major breast cancer subtypes, listed here in increasing order of aggressiveness: luminal A (ERα+, PR+, HER2-), luminal B (ERα+, PR-, HER2-), HER2-overexpressing (ERα+/-, PR+/-, HER2+), and triple-negative (ERα-, PR-, HER2-) (13). While ERα expression is generally considered to be a positive prognostic marker (131), ERα activity does promote breast cancer cell proliferation, survival, and motility (132-133). This is in contrast with the activity of estrogen receptor beta (ERβ), a second estrogen receptor that was first identified in 1995. Multiple studies have demonstrated that higher tumor expression of ERβ is correlated with an improved prognosis in all breast cancer subtypes (134-140), suggesting that ERβ may act as a tumor suppressor. These findings are supported by *in vitro* models that show ectopic expression of ERβ in ERα positive breast cancer cells results in decreased growth, motility, and invasion (141-145). In addition, tumor formation and angiogenesis in mouse xenografts was reduced with ERβ overexpression (142, 146). Finally, the protective effect of ERβ expression is further evidenced by its decreased expression in breast cancer cells, particularly in higher versus lower-grade tumors (143, 146-150). In normal mammary tissue, ERβ is the predominant ER, but its expression is inhibited via promoter hypermethylation in breast cancer cells (151-152). However, approximately 76% of breast tumors still express ERβ,

leading a number of researchers to explore its potential as a therapeutic target (153).

While many studies have been devoted to the examination of whether increased ERβ activity can improve breast cancer outcome, relatively little attention has been paid to expanding our understanding of the factors affecting ERβ expression. In fact, the effects of obesity on breast cancer cell ERβ expression remain completely unknown, though a reduced ERβ expression may be a potentially targetable mechanism by which obesity promotes a worse breast cancer prognosis. In the current study, we utilized an *in vitro* model of obesity to investigate how obesity-associated circulating factors affect ERβ expression in multiple cell lines representing the four major breast cancer subtypes. We demonstrate that exposure to obesity-associated circulating factors reduces ERβ expression in HER2-overexpressing breast cancer cell lines via a HER2-mediated signaling pathway.

6.2 Materials and Methods

6.2.1 Serum samples

Serum was collected from 25 postmenopausal breast cancer patients under an IRB approved bio-repository collection protocol at the CTRC of UTHSCSA. The collection and use of these biological samples was conducted in accordance with

the Declaration of Helsinki and good clinical practice. Informed consent was obtained prior to participation, and all samples and data were de-identified prior to release to maintain patient confidentiality. Body mass index (BMI) was calculated, and serum was pooled according to the BMI category of the patient (normal weight (18.5-24.9 kg/m^2) or obese (≥30.0 kg/m^2).

6.2.2 Cell lines and reagents

SKBR3, MCF-7, ZR75, and MDA-MB-231 human breast cancer cell lines (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS). Mammary tumor cells isolated from MMTV-neu transgenic mice were cultured in DMEM (GIBCO Life Technologies) plus 10% FBS. Herceptin is manufactured by Genentech Inc. (San Francisco, CA). ON-TARGETplus SMARTpool siRNA to ERBB2 and scrambled siRNA were purchased from Thermo Fisher Scientific (Waltham, MA).

6.2.3 Quantitative RT-PCR

ERβ mRNA levels in all cell lines were measured following an 18 hour serum-starvation period and a one-hour exposure to a 2% concentration of pooled patient sera in serum-free media (SFM). To assess the role of HER2 receptor signaling, SKBR3 cells were pre-treated for one hour with Herceptin (1.0 ug/ml) prior to sera exposure. HER2 receptor expression was also silenced using siRNA (25 nM). Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer sequences are as follows: human ERβ: forward, 5'-ATGGAGTCTGGTCGTGTGAAGG-3';

reverse, 5’-TAACACTTCCGAAGTCG GAGG-3’; mouse ERβ: forward, 5’-GACTGTAGAACGGTGTGGTCATCAA-3’; reverse, 5’-CTGTGAGGTAGGAATG CGAAAC-3’; HER2: forward, 5’-GGAGCCGCGAGCACCGA AGT-3’; reverse, 5’-CGGCCAGGGCATAGTTGTCC-3’. The manufacturer’s recommended cycling conditions for the QuantiFast SYBR Green PCR kit (Qiagen) were used. Data shown represents the average of at least three independent experiments.

6.2.4 Statistical Analyses

Differences between cells exposed to two different experimental conditions were measured using Student’s *t* test. One-way ANOVA was used to analyze differences between more than two experimental conditions. A p value of <0.05 was considered significant.

6.3 Results

6.3.1 Obesity-associated circulating factors suppress ERβ expression levels in HER2/neu-overexpressing breast cancer cell lines

This project was initiated in order to further explore the mechanism(s) mediating the effects seen in a currently unpublished study that examined the impact of energy balance modification on ERβ expression in the mammary tissue of MMTV-neu transgenic mice. This study demonstrated that mice placed on a diet-induced obesity regimen had lower mammary tumor ERβ expression levels in comparison to mice receiving a calorie-restricted diet (Stephen Hursting, personal

communication). Consequently, we first measured the impact of patient sera exposure on mammary tumor cells isolated from MMTV-neu mice and found that ERβ expression was 67% higher ($p<0.05$) in cells exposed to normal weight (N) versus obese (OB) patient sera. Similarly, ERβ expression in the human HER2-overexpressing SKBR3 cell line was 41% greater ($p<0.05$) following incubation in N versus OB patient sera (Figure 6.1a). In contrast, there was no significant difference in ERβ expression in MCF-7, ZR75, or MDA-MB-231 cells after exposure to N versus OB patient sera (Figure 6.1b). These human cell lines are representative of the luminal A, luminal B, and triple negative breast cancer subtypes, respectively, and all express low levels of HER2.

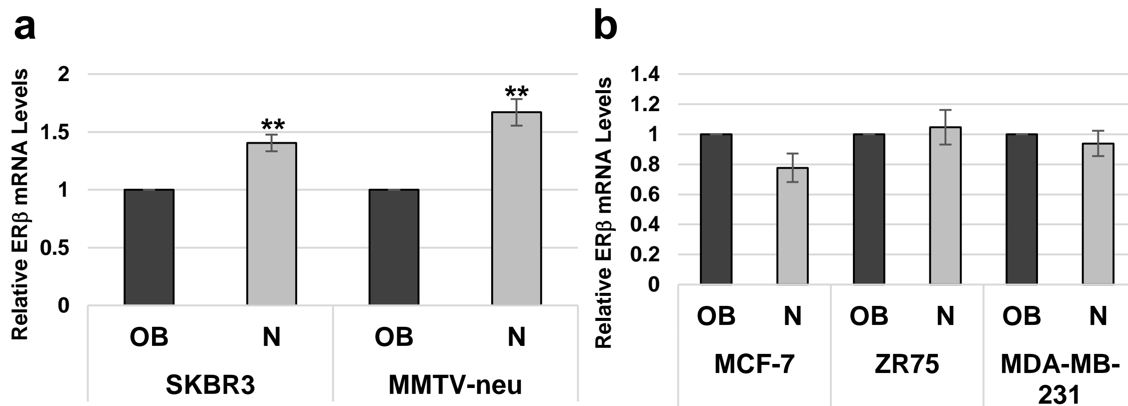

Figure 6.1 ERβ levels are suppressed by obesity-associated systemic factors in HER2-overexpressing breast cancer cells. (a) ERβ expression, measured by quantitative RT-PCR, in SKBR3 breast cancer cells and mammary carcinoma cells isolated from MMTV-neu mice following a 1 hr exposure to obese (OB) or normal weight (N) patient sera. (b) ERβ expression measured by quantitative RT-PCR, in MCF-7, ZR75, and MDA-MB-231 breast cancer cells following a 1 hr exposure to OB or N patient sera. Data shown represents the average of at least three independent experiments. **, $p<0.01$ relative to OB

6.3.2 Inhibition of HER2 signaling attenuates obesity-induced suppression of SKBR3 cell ERβ expression

Given that a difference in ERβ expression following N versus OB patient sera exposure was only observed in HER2-overexpressing cell lines, we next examined whether the effects of the OB sera may be mediated by HER2 signaling by utilizing the HER2-targeting monoclonal antibody Herceptin. SKBR3 cell ERβ expression increased by 2.79-fold with Herceptin treatment prior to and during OB sera exposure in comparison to OB sera alone ($p<0.05$). In contrast, HER2 inhibition in SKBR3 cells exposed to N patient sera resulted in a non-significant 39% increase in ERβ expression over N sera alone. Consequently, ERβ expression was 42% higher ($p<0.05$) in OB versus N sera-exposed SKBR3 cells when HER2 signaling was suppressed with Herceptin (Figure 6.2a). These results strongly suggest that the HER2 receptor plays a role in mediating the effects of OB patient sera on breast cancer cell ERβ expression.

6.3.3 HER2 receptor silencing reverses obesity-induced suppression of SKBR3 cell ERβ expression

To further assess the role of HER2 signaling in the OB patient sera-induced suppression of SKBR3 cell ERβ expression, we next silenced the HER2 receptor using siRNA. Transfection of SKBR3 cells with HER2-targeting siRNA resulted in a 52% decrease ($p<0.05$) in HER2 expression, while transfection with a scrambled siRNA sequence did not significantly alter HER2 expression (Figure 6.2b). HER2

silencing prior to OB patient sera exposure led to a 2.32-fold increase ($p<0.05$) in SKBR3 cell ERβ expression in comparison to non-transfected SKBR3 cells. However, in SKBR3 cells exposed to N patient sera, there was no significant change in ERβ expression with HER2 silencing (Figure 6.2c). This data provides additional evidence in support of the hypothesis that obesity-associated systemic factors suppress breast cancer cell ERβ expression via a HER2-mediated signaling pathway.

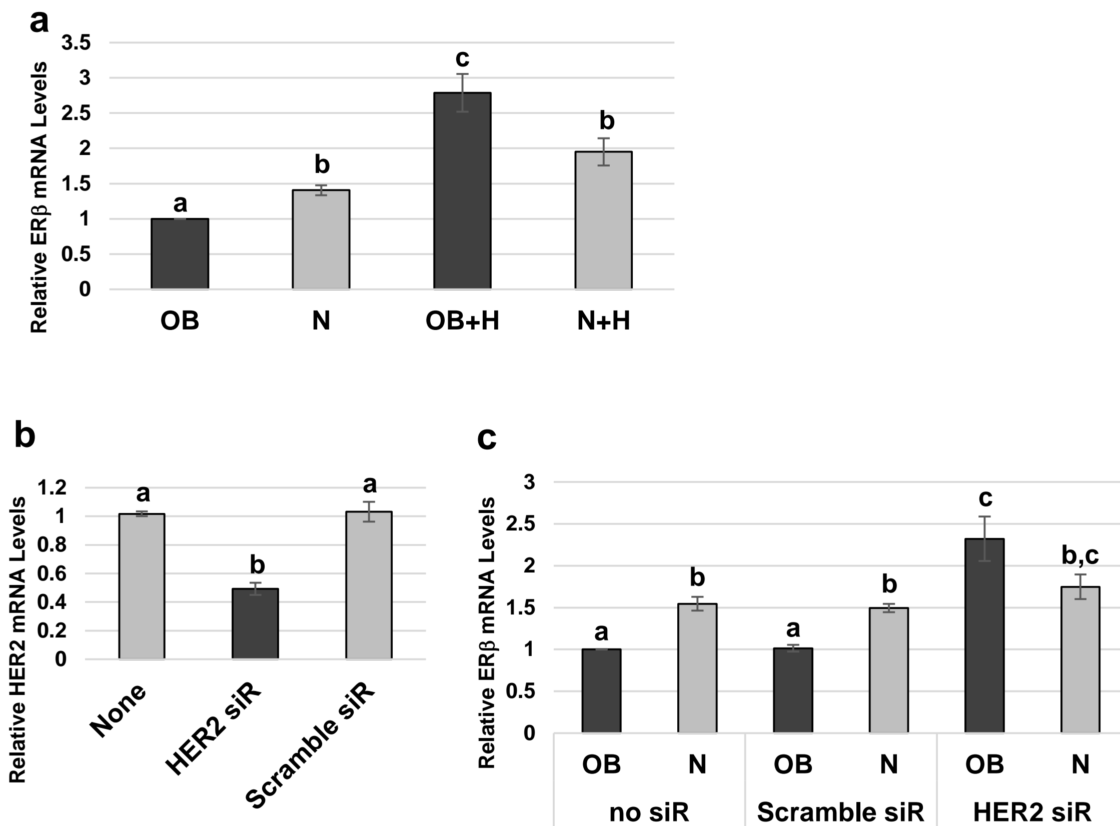

Figure 6.2 Obesity-associated suppression of ERβ expression is mediated by HER2 signaling. (a) ERβ expression, measured by quantitative RT-PCR, in SKBR3 cells exposed to obese (OB) or normal weight (N) patient sera for 1 hr +/- Herceptin (H). (b) HER2 expression, measured by quantitative RT-PCR, in SKBR3 cells without transfection (None) or following transfection with HER2 or Scramble siRNA (siR). (c) ERβ expression, measured by quantitative RT-PCR, in SKBR3 cells without siRNA (siR) transfection or following transfection with HER2 or Scramble siR, exposed to OB or N patient sera. Data shown represents the average of at least three independent experiments. Different letters indicate statistically significant differences, $p<0.05$.

6.4 Discussion

In the years following the discovery of ERβ, researchers have devoted substantial resources to exploring the functions of this second ER, with many

focused on defining how ERβ activity impacts breast cancer prognosis. The consensus among most investigators of this subject is that greater ERβ expression and activity is generally associated with an improved disease outcome, especially in patients with ERα positive disease (134-140). In contrast, few studies have examined the mechanisms by which ERβ expression is regulated. Human ERβ gene transcription is known to be controlled by at least two promoters, 0N and 0K, with transcripts from 0N predominating in both normal and transformed breast epithelial cells (154-155). Promoter 0N, cloned in 2000, was shown to have silencer elements in a region containing a putative AP-1 binding site (156). We have demonstrated in the current study that exposure to obesity-associated circulating factors suppresses ERβ expression in HER2-overexpressing breast cancer cells, and this effect is reversed by HER2 inhibition or silencing. Given that HER2 signaling is known to activate AP-1 (157), this binding site is a potential mechanistic link between elevated HER2 activity and a reduction in ERβ expression.

However, it is still unclear how obesity-associated circulating factors might activate this HER2-mediated ERβ-suppressing pathway. One possibility is via stimulation of the insulin-like growth factor-1 receptor (IGF-1R). Serum levels of insulin and free IGF-1 are typically elevated with obesity, and both of these factors can promote IGF-1R activation (38-39). Several studies have demonstrated cross-talk between HER2 and IGF-1R (158-160), suggesting that obesity-induced IGF-

1R signaling may further enhance HER2 activity. IGF-1R signaling may also independently impact ERβ expression, as silencing of this receptor in MCF-7 breast cancer cells has been shown to increase ERβ levels (161). Greater IGF-1R activity has not been linked to a reduction in ERβ, though, and an ERβ-IGF-1R link that is independent of HER2 would conflict with our results.

In conclusion, the current study demonstrates that obesity-associated circulating factors suppress ERβ expression in breast cancer cells that overexpress HER2. While HER2 inhibition and silencing were definitively shown to reverse this effect, the precise molecular mechanism(s) connecting obesity, HER2, and ERβ expression remains unclear. Elucidation of this signaling pathway, as well as an assessment of the biological relevance of the observed obesity-induced inhibition of ERβ levels for breast cancer progression, will be the focus of our future studies.

Chapter 7: Obesity Promotes Chemotherapy Resistance in Triple-Negative Breast Cancer Cells

7.1 Introduction

Based on current incidence rates, the National Cancer Institute estimates that one in eight women born in the United States will develop breast cancer at some point in their lives (1). Among those with invasive breast cancer, approximately 10-20% are diagnosed with the triple negative subtype of tumor (162), characterized by low expression of the estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (13). Triple-negative tumors, which have a 1.5 to 2-fold higher risk of mortality in comparison to those that express ERα/PR, are typically the most aggressive of the four major breast cancer subtypes (14). They are also the most difficult to treat because they lack the three receptors listed above, which makes them insensitive to endocrine and HER2-targeted therapies. Consequently, combination cytotoxic chemotherapy remains the standard of care for these patients (163).

Unfortunately, some patients are also resistant to these chemotherapeutic agents, and a number of factors can contribute to an individual's risk of chemotherapy resistance, including obesity. Several studies have linked an elevated body mass index (BMI) with a poor response to chemotherapy. For example, Fabbro et al (128) found that overweight (BMI≥25 kg/m^2) patients receiving neoadjuvant chemotherapy had a lower pathological complete response

(pCR) and shorter progression-free survival in comparison to normal weight patients. Others have demonstrated a similar correlation between overweight status and a decreased chance of achieving pCR with neoadjuvant chemotherapy (129-130). These results are supported by reports that obesity is associated with a worse breast cancer outcome, including lower disease-free survival and overall survival, for all breast cancer subtypes treated with taxane agents (164-165) and specifically for triple-negative breast cancer (12).

In the current study, we have demonstrated that obesity-induced chemotherapy resistance can be modeled in cell culture by growing triple-negative breast cancer cells in serum from breast cancer patients that has been pooled by BMI category. The chemotherapy drug docetaxel, a taxane agent, was less effective in its inhibition of cell viability when the cells were cultured in obese versus normal weight patient sera. Possible mechanisms mediating this effect have been explored, with preliminary data indicating that upregulation of the Akt/mTOR pathway by obesity-associated circulating factors may be the key mediator. Given that more than a third of adult women in the United States are currently obese (17), a greater understanding of this mechanism is imperative to improving breast cancer outcomes.

7.2 Materials and Methods

7.2.1 Serum samples

Serum was collected from postmenopausal breast cancer patients under an Institutional Review Board (IRB) approved biorepository collection protocol at the Cancer Therapy and Research Center of the University of Texas Health Science Center at San Antonio (UTHSCSA). The collection and use of these biological samples was approved by the IRB of UTHSCSA (HSC20070684H) and conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained prior to participation, and all samples and data were deidentified prior to release to maintain patient confidentiality. Serum was pooled according to the BMI category of the patient (normal weight (N): 18.5-24.9 kg/m^2; obese (OB): ≥30 kg/m^2).

7.2.2 Cell lines and Reagents

Triple-negative MDA-MB-231 breast cancer cells (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) was purchased from Sigma. The drug treatments used in this study, which include docetaxel, celecoxib, and rapamycin, were also obtained from Sigma. The primary antibodies for Bcl-2 and actin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).

7.2.3 MTT Assay

MDA-MB-231 cells were seeded in IMEM supplemented with 10% FBS at a density of $5x10^3$ in 96 well plates. After 24 hours of growth in the 10% FBS media, the cells were exposed to serum-free media (SF), 2% FBS, 2% OB, or 2% N sera (all sera was diluted in SF), with or without the addition of docetaxel (25 nM), for 24, 48, 72, or 96 hours. MTT reagent in PBS (5 mg/ml) was then added to each well to a final concentration of 0.5 mg/ml. After two hours of incubation at 37°C, the media was removed and 50 ul DMSO added to each well to lyse the cells. Absorbance was read at 570 nm on a FLUOstar Omega Spectrometer (BMG Labtech, Offenberg, Germany). This process was also done for an untreated plate of cells at the time the other plates were treated in order to establish a baseline absorbance value. Relative cell viability for each treatment condition was calculated by dividing the absorbance values for a given treatment at the different time points by the baseline absorbance value. Percent inhibition of cell viability achieved with docetaxel treatment of cells grown in each of the three sera conditions was also calculated for each time point. This experiment was repeated with celecoxib (30 uM) or rapamycin (10 nM) treatment, alone or in combination with docetaxel, for a 96 hour period only, and percent inhibition with each drug or drug combination under the different sera conditions was calculated.

7.2.4 Quantitative RT-PCR

Macrophage *PTGS2* (COX-2) mRNA levels were measured following a six-hour serum-starvation period and a 24-hour exposure to a 2% concentration of obese

or normal weight pooled patient sera in SF. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer sequences are as follows: *PTGS2*: forward, 5'-CCCTTGGGTGTCAAAGGTAA-3'; reverse, 5'-GCCCTCGCTTATGATCT GTC-3'; The manufacturer's recommended cycling conditions for the QuantiFast SYBR Green PCR kit (Qiagen) were used. Data shown represents the average of at least three independent experiments.

7.2.5 Western Blot Analysis

The cells were grown to 50% confluence in IMEM supplemented with 10% FBS, then the growth medium was aspirated, the wells were washed, and the medium replaced with SF overnight. After overnight serum-starvation to minimize the effect of growth factors and hormones in the growth medium, the cells were either left in SF or 2% FBS, OB or N patient sera was added directly to the overnight SF. The cells were incubated in these conditions for 72 or 96 hours. Laemli lysis buffer was used for protein extraction. Protein content of the lysates was measured using the BCA Protein Assay kit from Thermo Scientific Pierce (Rockford, IL). Images were acquired using a Syngene G:BOX Chemi (Frederick, MD).

4.2.8 Statistical analysis

Differences between cells exposed to two different experimental conditions were measured using Student's *t* test. Two-way ANOVA was used to analyze

differences in experiments with two independent variables. A p value of <0.05 was considered significant.

7.3 Results

7.3.1 Obesity-associated circulating factors induce docetaxel resistance

To examine the impact of obesity on breast cancer cell response to the chemotherapeutic agent docetaxel, we utilized an *in vitro* model of obesity in which MDA-MB-231 cells, a triple-negative breast cancer cell line, were exposed to sera from breast cancer patients that had been pooled by BMI category (obese (OB): ≥30 kg/m^2; normal weight (N): 18.5-24.9 kg/m^2). During incubation in SFM or 2% FBS, OB or N sera in SFM, the cells were treated with a vehicle or docetaxel. After a 72 hour incubation in N sera, the viability of cells exposed to the vehicle was significantly higher ($p<0.05$) than those treated with docetaxel. This difference also approached significance ($p=0.01$) at the 96 hour time point, and docetaxel significantly suppressed ($p<0.05$) the viability of cells grown in FBS at both time points. In contrast, docetaxel did not significantly inhibit the viability of cells grown in OB sera at any time point, suggesting that obesity-associated circulating factors may promote resistance to docetaxel treatment (Figure 7.1a). This conclusion is supported by the fact that there is a significant difference in the percent inhibition of cell viability achieved by docetaxel treatment of MDA-MB-231 cells grown for 96 hours in OB sera versus FBS or N sera (Figure 7.1b).

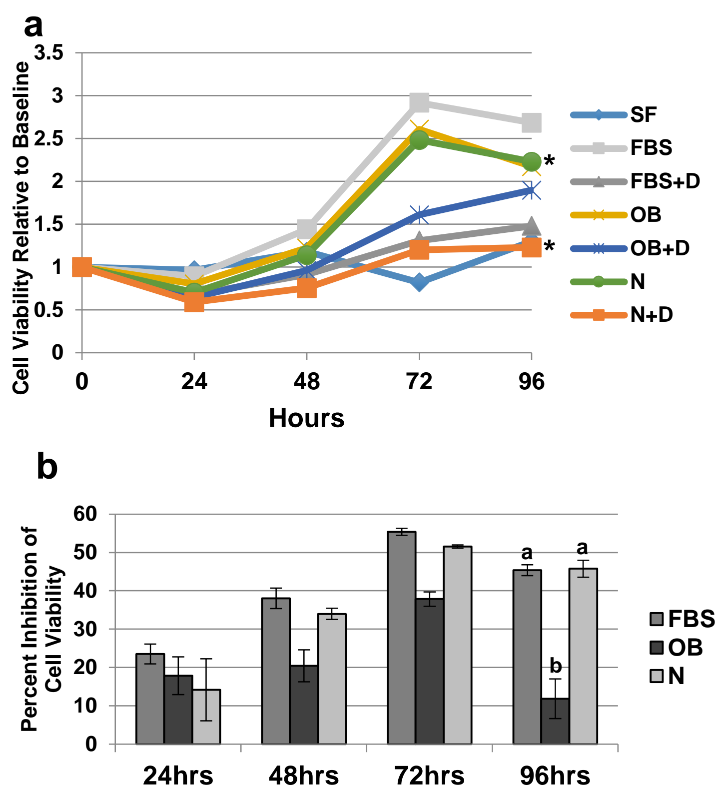

Figure 7.1 Obesity-associated circulating factors induce docetaxel resistance. The impact of obese (OB) versus normal weight (N) patient sera exposure on MDA-MB-231 cell response to docetaxel was measured via MTT assay assessment of cell viability. Serum-free media (SF) and fetal bovine sera (FBS) were utilized as controls. (a) Relative MDA-MB-231 cell viability following a 24, 48, 72, or 96 hour exposure to SF, FBS, OB, or N sera in the presence of a vehicle or docetaxel (D). (b) Percent inhibition of MDA-MB-231 cell viability achieved by docetaxel treatment while the cells were grown in FBS, OB, or N sera for 24, 48, 72, or 96 hours. *Indicates a significant difference from each other; different letters indicate significant differences, $p<0.05$.

7.3.2 Obesity-induced resistance to docetaxel is not mediated by COX-2 or Bcl-2

Resistance to chemotherapy has previously been shown to be mediated by upregulation of COX-2 and Bcl-2 signaling (166-174). Consequently, we next

examined whether either of these pathways may be responsible for the obesity-induced docetaxel resistance we observed. To assess whether COX-2 may be playing a role in this effect, we first measured the impact of OB versus N sera exposure on COX-2 expression in MDA-MB-231 cells. A 24 hour incubation in OB sera resulted in COX-2 mRNA levels that were two-fold ($p<0.05$) the level seen in cells grown in N sera (Figure 7.2a). We next inhibited COX-2 activity with celecoxib during a 96 hour sera exposure, with a vehicle or docetaxel treatment, to see if the drug combination could resolve the resistance and produce an additive or synergistic effect. We found that the celecoxib plus docetaxel combination did not result in a greater suppression of cell viability in comparison to either drug plus vehicle for any of the three sera conditions, indicating that COX-2 is not responsible for mediating obesity-induced docetaxel resistance (Figure 7.2b). To measure whether exposure to OB versus N sera increases MDA-MB-231 cell Bcl-2 expression, which would suggest that this protein could be involved in promoting obesity-associated docetaxel resistance, we utilized western blotting. Incubation in 2% OB sera for 72 and 96 hours did not stimulate greater Bcl-2 expression in comparison to N sera (Figure 7.2c). Consequently, we did not pursue any further investigation of this pathway.

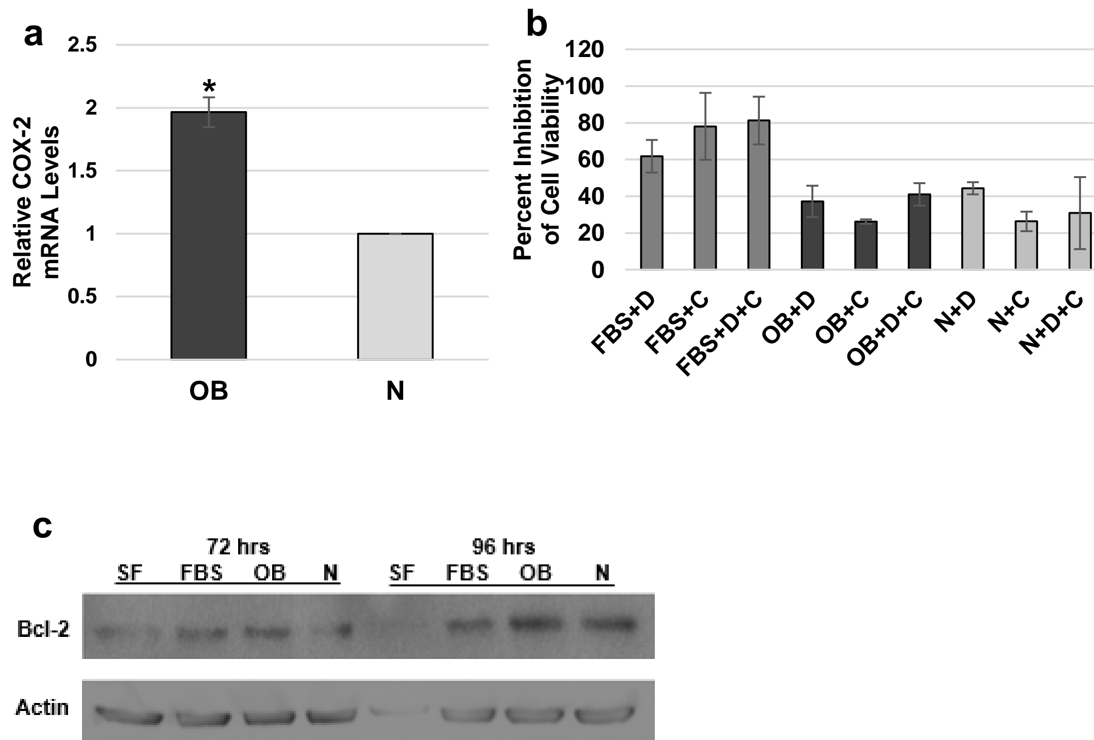

Figure 7.2 Obesity-induced resistance to docetaxel is not mediated by COX-2 or Bcl-2. (a) The effects of obese (OB) versus normal weight (N) sera exposure on MDA-MB-231 cell COX-2 expression were measured by qPCR. (b) The impact of COX-2 inhibition using celecoxib (C) on response to docetaxel (D) in MDA-MB-231 cells grown for 96 hours in the presence of 2% fetal bovine sera (FBS), OB, or N sera was assessed by MTT measurement of cell viability. (c) Bcl-2 protein expression following a 72 or 96 hour exposure to serum-free media (SF) or 2% FBS, OB, or N sera was measured by western blot. *, $p<0.05$

7.3.3 The Akt/mTOR pathway is a potential mediator of obesity-induced docetaxel resistance in triple-negative breast cancer

Several researchers have demonstrated that increased Akt/mTOR signaling confers resistance to chemotherapeutic agents, including docetaxel and other taxane agents (175-178). Numerous obesity-associated circulating factors, including interleukin-6 (IL-6) and insulin-like growth factor 1 (IGF-1), can activate

the Akt-mTOR pathway (34, 38-39, 68-69), and we have shown that exposure to OB patient sera promotes Akt activation in the ERα positive MCF-7 and T47D cells lines (Figure 5.2a-d). Consequently, we next utilized the mTOR inhibitor rapamycin to examine whether Akt/mTOR signaling may be mediating the observed obesity-induced docetaxel resistance. Preliminary data indicate that a combined docetaxel plus rapamycin treatment synergistically inhibits the viability of MDA-MB-231 cells grown in OB patient sera. That is, the combined treatment resulted in a 61% decrease in cell viability, while docetaxel and rapamycin plus vehicle suppressed cell viability by only 34% and 11%, respectively. The drug combination produced an additive, but not synergistic, effect in cells cultured in N sera. Unfortunately, there was no difference in the effect of docetaxel alone on the viability of cells grown in OB versus N sera in this preliminary experiment. However, our results suggest that Akt/mTOR signaling may be mediating the previously observed obese sera-induced docetaxel resistance.

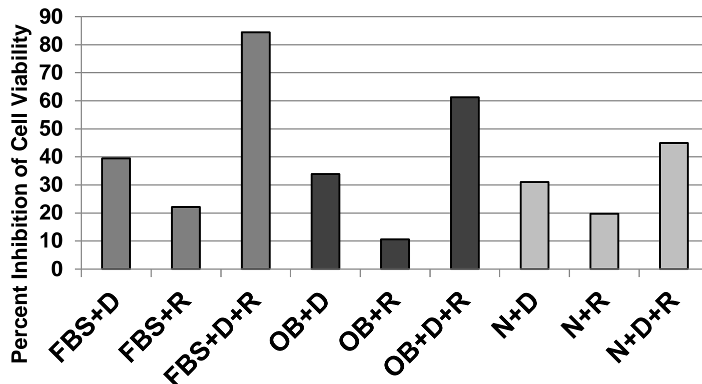

Figure 7.3 Akt/mTOR may mediate obesity-induced docetaxel resistance in triple-negative breast cancer. Percent inhibition of MDA-MB-231 cell viability was measured by MTT assay. Cells were exposed to 2% fetal bovine sera (FBS, used as a control), obese (OB), or normal weight (N) sera for 96 hours and treated with a vehicle, docetaxel (D), rapamycin (R), or a combination of the two drugs (D+R).

7.4 Discussion

Docetaxel is one of the most frequently prescribed chemotherapy drugs for the treatment of invasive breast cancer. This cytotoxic agent, like other members of the taxane family of drugs, triggers cell apoptosis by preventing cell cycle progression via impairment of the centrosome during mitosis. More specifically, it stabilizes the microtubules, causing the cells to be frozen in metaphase (179). Several studies have demonstrated that obesity is associated with a reduced response rate to chemotherapy drugs, including taxanes, leading to a worse disease outcome (12, 128-130, 164-165). However, while there has been ample research investigating the mechanisms mediating general chemotherapy

resistance, little attention has been paid to how obesity may specifically reduce patients' chemotherapy response rate.

The study described here is still ongoing, but we have shown that exposure to sera from obese versus normal weight breast cancer patients induces docetaxel resistance in a triple-negative breast cancer cell line, mimicking the effects of obesity in vivo. We have explored three possible mechanisms for this effect: upregulation of COX-2, Bcl-2, and Akt/mTOR signaling. While obese patient sera exposure did increase MDA-MB-231 cell COX-2 expression, suppression of COX-2 activity with celecoxib did not improve docetaxel response in cells grown in obese patient sera. Studies have previously linked increased COX-2 expression with a reduced response to various chemotherapies, including taxanes, and a worse disease outcome in breast, ovarian, and cervical cancer patients (167-170). Some researchers have attributed this association to COX-2-mediated enhancement of the MDR1 gene and its product, p-glycoprotein, which actively pumps substrates like chemotherapy drugs out of cells. Consequently, overexpression of MDR1 can lead to multi-drug resistance (167-168). However, despite the upregulation of MDA-MB-231 cell COX-2 expression with obese patient sera exposure, this mechanism does not appear to be mediating the obesity-associated docetaxel resistance seen here. Neither is Bcl-2, as obese patient sera does not modulate its expression in MDA-MB-231 cells. Overexpression of Bcl-2 can block taxane-induced apoptosis, resulting in resistance to these drugs, and Bcl-2 expression has been correlated with outcome in patients receiving taxanes (172, 174).

However, based on the results from our *in vitro* model, Bcl-2 cannot be mediating the effects of the obese patient sera on docetaxel response in the MDA-MB-231 cells.

We have previously demonstrated that breast cancer cell Akt activation is stimulated by exposure to obese patient sera (99), while pS6K1 levels have been shown to predict breast cancer patient response to neoadjuvant chemotherapy (175). In addition, mTOR inhibition with rapamycin has been shown to synergistically enhance the growth inhibitory effects of taxane treatment on breast cancer cells *in vitro* and *in vivo*. Researchers have speculated that activated mTOR may promote cell survival via its downstream targets, so inhibition of the mTOR pathway improves chemotherapy response by allowing apoptosis to proceed (23). Consequently, we recently began investigating whether the addition of rapamycin to docetaxel treatment would improve the taxane agent's inhibition of MDA-MB-231 cell viability. Preliminary data suggests that rapamycin synergistically enhances the inhibitory effects of docetaxel on the viability of cells grown in obese patient sera. Further experimentation will be necessary to confirm these results and explore the mechanism by which mTOR inhibition improves docetaxel response in the context of obesity. To demonstrate the biological relevance of our findings, it will then be necessary to conduct animal studies assessing whether obesity-induced docetaxel resistance occurs in a mouse model of TN breast cancer and, if so, does the addition of rapamycin improve docetaxel response. The results of these studies could provide highly relevant translational data to inform

the development of a more effective chemotherapeutic regimen for obese women with triple-negative breast cancer.

Chapter 8: The Impact of Obesity on Tumor Incidence and Characteristics in the MMTV-Wnt1 Mouse Model of Mammary Carcinogenesis

8.1 Introduction

Currently, 35.7% of adult women in the United States are obese, defined as a body mass index (BMI) of ≥30 kg/m^2. Among women aged 40-59 years, the time period in which menopause typically occurs, the obesity rate is 38.2% (17). These numbers are a significant public health concern given the association between both obesity and increasing age and an elevated risk of developing and dying from numerous diseases, including breast cancer. A large body of evidence has demonstrated that obesity is correlated with an increased risk of breast cancer in postmenopausal women (3-5) and enhanced breast cancer progression in both pre- and postmenopausal women. Studies have shown that obesity is associated with a higher breast cancer recurrence rate and lower disease-free interval (20-21). In addition, the breast cancer mortality rate escalates with each increase in BMI category (19). While obesity has been linked to a worse prognosis in patients of all ages and across breast cancer subtypes, the most prominent effects have been seen in postmenopausal, estrogen receptor alpha (ERα) patients (24).

The MMTV-Wnt-1 (Wnt-1) transgenic mouse model is predisposed to the development of mammary adenocarcinoma. The proto-oncogene Wnt-1 encodes a cysteine-rich glycosylated secretory protein that is normally expressed during mouse embryonic development but not expressed in the normal mammary gland (180). Extensive ductal hyperplasia occurs early in the life of Wnt-1 female mice,

and approximately 50% develop mammary tumors by six months of age (181-182). The tumors are heterogenous in ERα positive/negative status, but exhibit an ERα negative phenotype (183). In fact, ERα positive tumors arising in this model have been shown to be refractory to ovariectomy and tamoxifen treatment, suggesting that estrogen signaling is unnecessary for tumor growth in Wnt-1 mice, despite the presence of ERα (184). However, ovariectomy did delay tumor growth in C57BL/6 mice implanted subcutaneously with syngeneic Wnt-1 mouse mammary tumor cells, indicating that these cells may be estrogen-responsive. In addition, diet induced obesity (DIO) enhanced tumor growth in this syngeneic mouse model of breast cancer, but only in the absence of ovarian hormones (185). While the impact of obesity on Wnt-1 tumor progression in ovariectomized mice has been previously explored in several studies using this orthotopic transplant model, no one has analyzed how obesity affects tumor development and characteristics in the spontaneous Wnt-1 mouse model.

In the current study, we found that DIO enhances tumor incidence and growth rate, but does not decrease tumor latency or survival, in ovariectomized Wnt-1 mice. When examining possible mechanisms mediating these effects, we focused on how DIO impacts ERα expression and signaling at multiple time points in normal mammary fat pad (MFP) and tumor tissue. In addition, we measured ERβ expression, as estradiol also activates this second ER, and ERβ signaling can counteract the pro-growth effects of ERα. The ultimate goal of this study was to

improve our understanding of how obesity impacts tumor development and characteristics in the spontaneous Wnt-1 mouse model. With this enhanced knowledge, we will be better able to move forward with intervention studies designed to counteract the negative effects of obesity on postmenopausal breast cancer risk and progression.

8.2 Materials and Methods

8.2.1 Animals and Diets

Fifty female MMTV-Wnt-1 (Wnt-1) mice, a transgenic mouse model predisposed to the development of mammary carcinoma, were bred. Our Wnt-1 mouse strain was developed on a pure C57BL/6 background, resulting in a greater susceptibility to obesity versus the original mixed background strain. To model the postmenopausal state, the mice were ovariectomized at 12 weeks of age. Ovariectomized mice exhibit characteristics of the postmenopausal state in human females, including decreased levels of circulating estrogen, loss of bone mineral density, and cessation of estrous cycles (186). The mice were housed singly and allowed to recover for one week following ovariectomy.

To assess the effects of obesity on tumor development and characteristics in this mouse model, the mice were randomized at 13 weeks of age to a diet-induced obesity (DIO) or control (Con) regimen (n=25 in each group) consisting of ad libitum access to a 60% or 10% kcal from fat diet, respectively (D12492 or D12450B; Research Diets, Inc, New Brunswick, NJ). The DIO and Con diets were

stored at -20°C and thawed in aliquots sufficient for 3-4 days to prevent rancidity. The mice were further randomized to three diet time groups: 2 months (n=5/diet group), 4 months, (n=10/diet group), and 6 months (n=10/diet group) on diet. Body weights were measured weekly, and feed intake was measured biweekly. All animal protocols were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

8.2.2 Tumor Assessment and Tissue Collection

Mice were palpitated for tumors twice a week. Using calipers, the length and width of the tumors were measured. Mice were euthanized when a tumor reached a 1.5 cm diameter or, if no tumor developed, following the assigned number of months on diet. On the day of sacrifice, the mice were fasted for four to six hours. Mice were euthanized using carbon dioxide inhalation followed by cervical dislocation. Tumors and MFP were excised, with some of each tissue preserved for paraffin embedding and some flash frozen in liquid nitrogen. Lungs and liver were also removed and preserved for paraffin embedding in order to assess for metastases. Whole blood was collected by cardiac puncture and allowed to clot at room temperature for 30 minutes prior to centrifugation at 1000 x *g* for 10 minutes. The serum was removed and stored at -80° C for analyses. After euthanasia, carcasses were stored at -20° C for future body composition analysis.

8.2.3 Body Composition Analysis

Percent body fat was assessed on each mouse carcass by dual-energy X-ray absorptiometry (DEXA) (GE Lunar PIXImus II, Madison, WI) as described in

(187). The head of each mouse was eliminated from the scan by using the region of interest exclusion option provided in the software.

8.2.4 Quantitative RT-PCR

Gene expression in tumor and MFP was assessed by quantitative RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer sequences are as follows: ERα: forward, 5'-TGCACCATTGACAAGAACCGGA-3'; reverse, 5'-AGCACCCATTTCATTTCGGCCT-3'; ERβ: forward, 5'-GACTGTAGAACGGTG TGGTCATCAA-3'; reverse, 5'-CTGTGAGGTAGGAATGCGAAAC-3'; pS2: forward, 5'-CCACAATTTATCCTCTCCCG-3'; reverse, 5'-AAGGTGATCTGT GTCCTCGC-3'; progesterone receptor: forward, 5'-AGGTGATTCTCTCTGGCT CAGG-3'; reverse, 5'-ATGGTCCTTGGAGGTCGTAA-3'; cyclin D1: forward, 5'-GGGTGGGTTGGAAATGAACT-3'; reverse, 5'-CTTCCTCTCCAAAATGCCAG-3'; Bcl-2: forward, 5'-TTCTCTCGTCGCTACCGTCGTGACT-3'; reverse, 5'-AGAGAC AGCCAGGAGAAATCAAACA-3'. The manufacturer's recommended cycling conditions for the QuantiFast SYBR Green PCR kit (Qiagen) were used.

8.2.5 Statistical Analysis

Values are presented as mean ± standard error of the mean (SEM). Statistical analysis of the tumor-free survival curves was conducted using the Mantel-COX test. Comparisons between the DIO and control groups were analyzed using Student's *t* test. Two-way analysis of variance (ANOVA) was used to assess the

effects of diet type and time on diet on normal mammary tissue and tumor gene expression. A p value of <0.05 was considered significant.

8.3 Results

8.3.1 Obesity promotes greater tumor incidence and growth rate in Wnt-1 mice, but did not affect tumor latency or survival

Among the mice that were not euthanized due to tumor burden prior to the end the study, mean body mass was significantly higher ($p<0.05$) in the DIO versus Con group for all diet time groups (Figure 8.1a-c). For all mice, mean body mass (data not shown) and body fat percentage (Figure 8.1d) at the time of euthanization was also significantly greater ($p<0.05$) in the DIO versus Con group for all diet time groups. There was no difference in tumor latency between the DIO and Con diet groups for any of the three diet time points. In both the two-month (2mo) and four-month (4mo) groups, tumor incidence was higher among the DIO mice. The growth rate was faster in DIO mice in the 4mo and 6mo groups, but the differences did not reach statistical significance (Table 8.1). There was no statistically significant variance in the survival curves of the DIO versus Con groups for any diet time point (Figure 8.2)

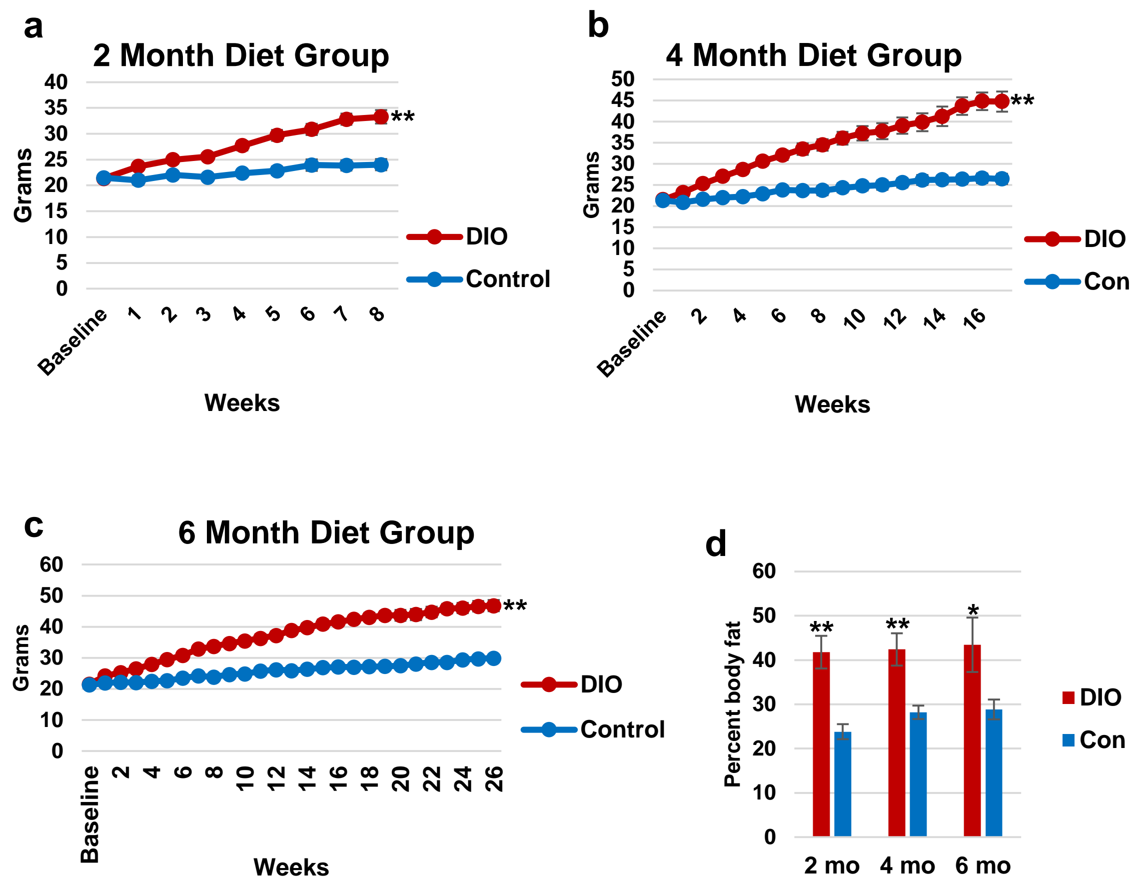

Figure 8.1 DIO diet promotes elevated body mass and body fat percentage in Wnt-1 mice. Change in body mass (grams) in Wnt-1 mice over 2 (a), 4 (b), and 6 (c) months on a high-fat diet-induced obesity (DIO) or low-fat Control diet is shown. Significant differences indicated are between DIO and Control mice that were not euthanized prior to the end of the diet time point due to tumor burden. (d) Percent body fat at the time of euthanization in Wnt-1 mice on a DIO or Control (Con) diet for 2, 4 , or 6 months. **, p<0.01; *, p<0.05 in comparison to Control.

Table 8.1 Impact of obesity on tumor formation in Wnt-1 mice

	2 Month		4 Month		6 Month	
	DIO	Con	DIO	Con	DIO	Con
Tumor Incidence	2	0	5	2	4	4
Tumor Latency in days	119 (17.0)	NA	172 (18.4)	182 (7.0)	157 (24.1)	168 (34.8)
Growth Rate in mm/day	1.75 (0.39)	NA	2.19 (0.51)	1.16 (0.09)	3.93 (1.43)	0.75 (0.09)

Numbers for Tumor Latency and Growth Rate indicate mean (standard error of the mean). DIO (diet-induced obesity), Con (control).

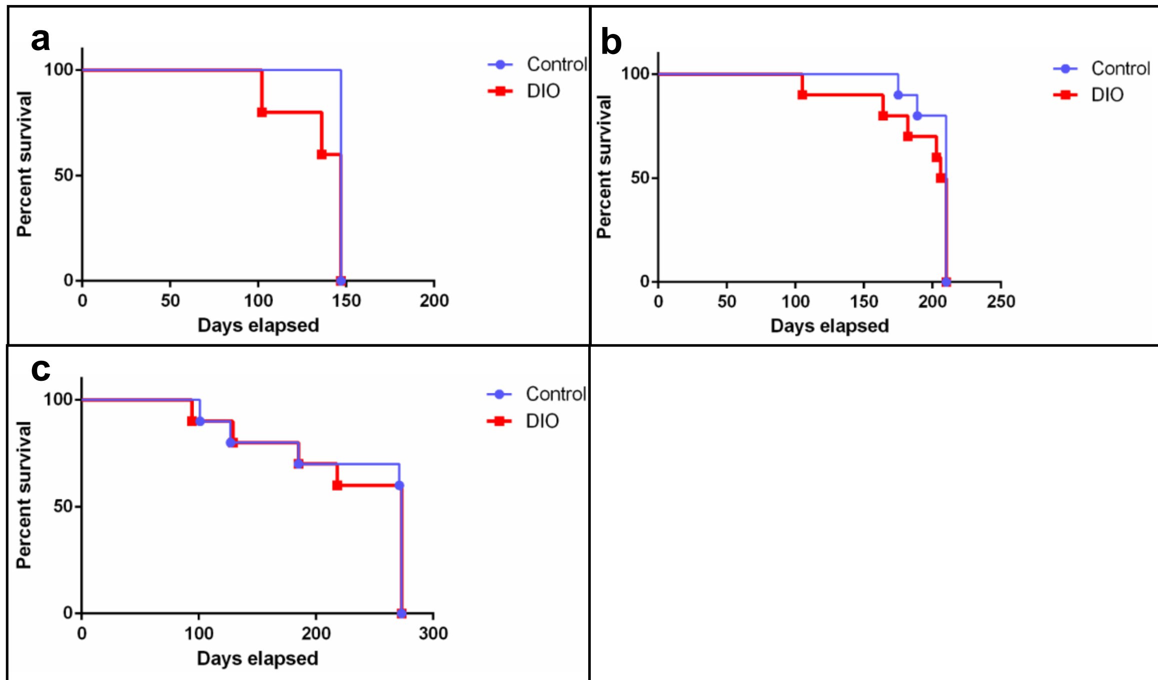

Figure 8.2 Obesity does not adversely impact tumor survival in Wnt-1 mice. The effect of DIO vs Control diet on survival in Wnt-1 mice receiving diet for 2 (a), 4 (b), or 6 (c) months.

8.3.2 Obesity is associated with higher Wnt-1 mouse tumor ERα expression, but not ERα activity

After menopause and the cessation of ovarian estrogen synthesis, the primary site of estrogen production becomes the adipose tissue, where the aromatase enzyme catalyzes the conversion of testosterone to estradiol (25-27). Due to an abundance of this aromatase-expressing tissue, obesity is associated with increased circulating levels of estradiol in postmenopausal women (28). It has also been correlated with higher mammary tissue aromatase expression (41-42). Given that DIO enhanced tumor incidence and growth rate in our Wnt-1 mice, we next examined whether these effects may be mediated by an obesity-induced increase in tumor ERα expression and/or activity. Obesity resulted in 2.27-fold higher ($p<0.05$) tumor ERα expression (Figure 8.3a). There was a trend towards higher ERα levels specifically in the six month (6mo) DIO group, but this variance did not reach statistical significance (Figure 8.3b). There was no difference between DIO and Con in tumor expression levels of four ERα target genes, including pS2, PR, cyclin D1, and Bcl-2 (Figure 8.3c). Stratifying the data by diet and diet time point groups did not change this result or reveal any clear trend in the expression of the four genes (data not shown).

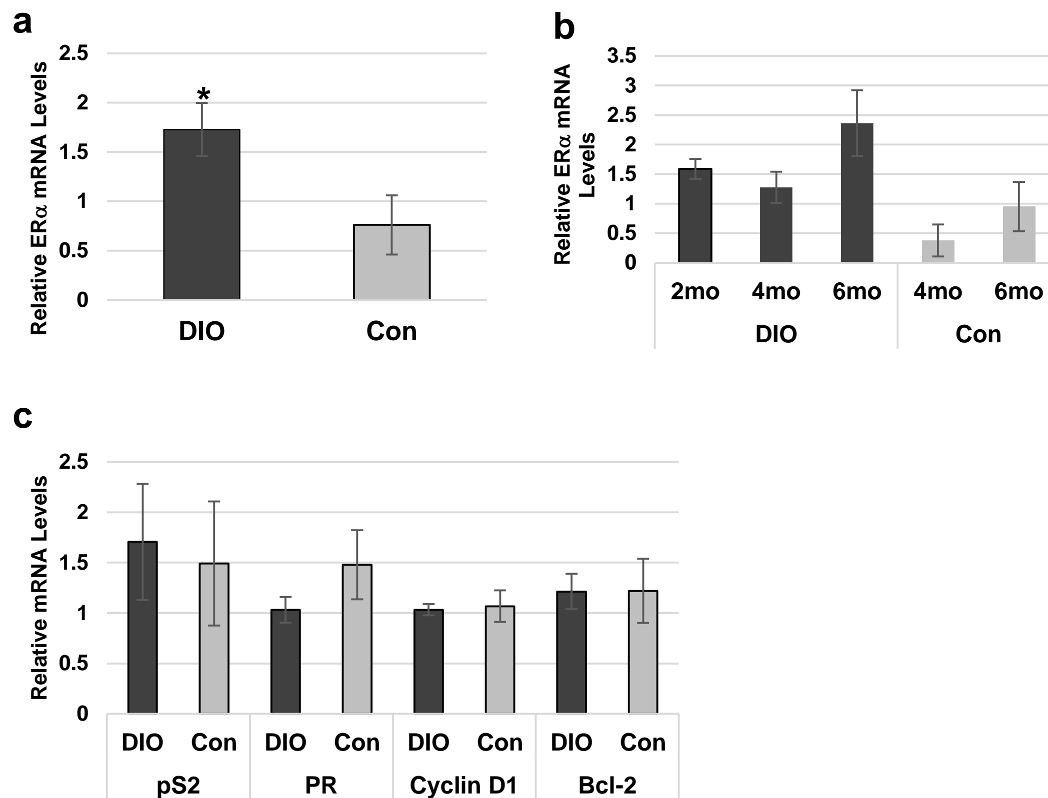

Figure 8.3 Diet-induced obesity promotes elevated tumor ERα expression levels in Wnt-1 mice. (a) ERα expression in the tumors of Wnt-1 mice maintained on a high-fat diet-induced obesity (DIO) or low-fat Control (Con) diet. (b) Wnt-1 mouse tumor ERα expression, stratified by diet (DIO or Con) and months on diet (2mo, 4mo, or 6mo). (c) Tumor expression of four ERα target genes (pS2, progesterone receptor (PR), Cyclin D1, and Bcl-2) in Wnt-1 mice maintained on the DIO or Con diet. *, $p<0.05$

8.3.3 Wnt-1 mouse mammary fat pad (MFP) ERα expression and activity decrease over time and have variable associations with obesity and tumor development

While obesity did not appear to be promoting greater tumor ERα activity in the Wnt-1 mice, ERα signaling in the normal MFP could still be playing a role in

promoting greater tumor incidence. Consequently, we next assessed the impact of diet-induced obesity on ERα expression and activity in Wnt-1 mouse MFP. At each diet time point, levels of ERα expression were similar between the DIO and Con groups, but they clearly decreased over time in mice on both diets. In mice receiving the DIO diet, there was a 71% decrease ($p<0.05$) between the 2mo and 6mo groups, while ERα expression decreased by 65% ($p<0.05$) in the Con group (Figure 8.4a). In the 2mo DIO group, there was a trend towards higher ERα expression in the mice that developed a tumor, but the 2mo Con group had zero tumor incidence while also having relatively high ERα expression (Figure 8.4b). The expression of pS2, an ERα target gene, also seemed to decrease over time, but only for the DIO group. In addition, expression was highest in the 2mo DIO group, but not statistically different from the levels in the other groups (Figure 8.4c). When we stratified the data further by tumor incidence, pS2 expression was greatest in the 2mo DIO with tumor group, and may be higher among the Con mice that developed tumors (Figure 8.4d). Finally, the expression of another ERα target gene, PR, was highest in the 2mo Con group and appeared to decrease over time, with levels significantly lower at 6mo versus 2mo for the Con group (Figure 8.4e). In the Con group, higher PR expression was associated with a lack of tumor development, though the differences between groups were not statistically significant (Figure 8.4f). There was no clear pattern of PR expression in the DIO group by time point or tumor incidence.

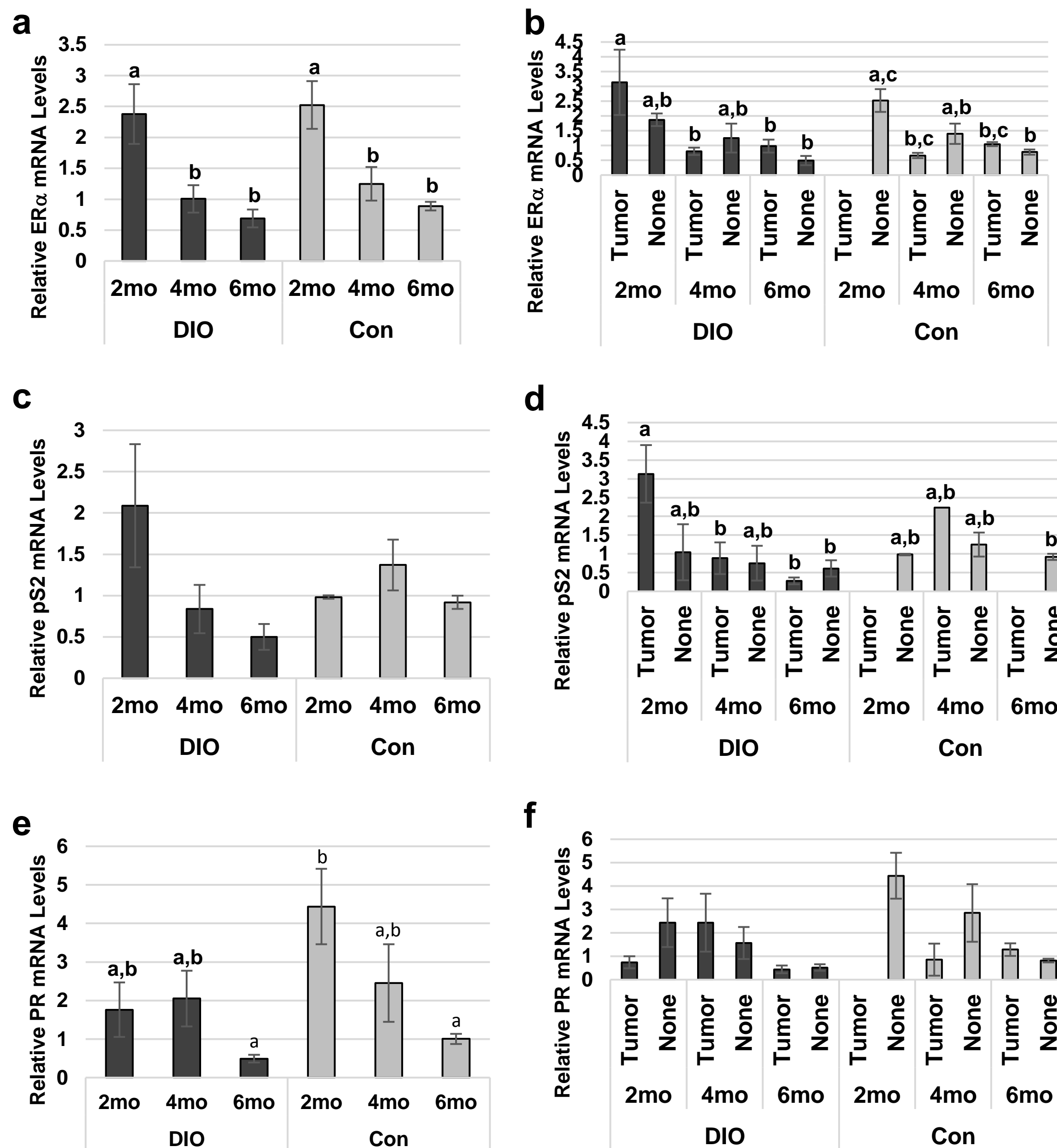

Figure 8.4 MFP ERα expression and activity in Wnt-1 mice decreases over time. ERα (a), pS2 (c), and PR (e) expression in the MFP of Wnt-1 mice maintained on a high-fat diet-induced obesity (DIO) diet or low-fat Control (Con) diet for different time periods (2mo, 4mo, or 6mo). Data from a, c, and e was further stratified by tumor incidence (b, d, f). Mice were classified as tumor-bearing (Tumor) or lacking any tumor formation (None) during their prescribed time on experimental diet. Different letters indicate significant differences, $p<0.05$.

8.3.4 ERβ expression decreases over time in Wnt-1 mouse MFP and is associated with lower tumor incidence in lean mice

As there was no clear association between MFP ERα expression or activity and obesity-induced tumor incidence in the Wnt-1 mice, we next examined whether tumor or MFP ERβ expression or the ratio of ERα/ERβ may be playing a role in the effects of obesity in this model. ERβ is typically considered to be anti-proliferative and protective against tumor formation (142-145, 149-152). Obesity had no effect on tumor ERβ levels (Figure 8.5a), and this did not change when the data was further stratified by diet time point (data not shown). The tumor ERα:ERβ expression level ratio was over two-fold higher in the DIO versus Con group (Figure 8.5b), but this was entirely due to the difference in ERα expression between the two groups (Figure 8.3a). When the data was stratified by diet time point, the ERα:ERβ also largely followed the same pattern as ERα expression (data not shown). Intriguingly, ERβ expression in the MFP of the 2mo DIO Wnt-1 mice was 3.5-fold greater ($p<0.05$) than 2mo Con mice. In both the DIO and Con groups, ERβ expression decreased over time, and there was no significant difference between the two groups at the 4mo and 6mo time points (Figure 8.6a). ERβ expression in the DIO mice did not vary by tumor incidence within the three time point groups, but there was a non-significant trend towards higher ERβ expression in the 2mo and 4mo Con mice that did not develop tumors (Figure 8.6b). The ERα:ERβ expression level ratio in MFP increased over time for both the

DIO and Con diet groups. This was largely due to the steep decline in ERβ levels over time, as ERα also decreases with time but to a smaller degree. At the 4mo and 6mo time points, the ratio in the DIO group was higher in comparison to Con, which could contribute to tumor development in the DIO mice (Figure 8.6c). However, when we stratified the data by tumor incidence, the ERα:ERβ ratio in the DIO tumor bearing mice was greater only in the 6mo group (Figure 8.6d).

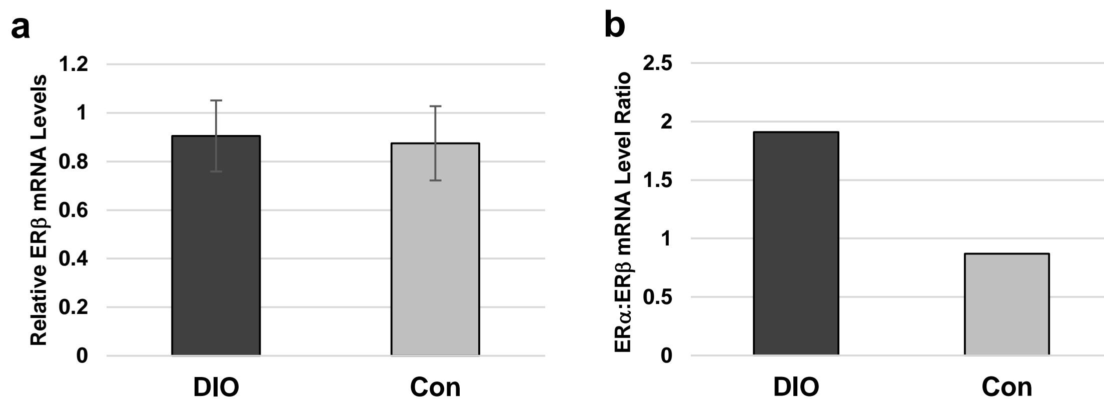

Figure 8.5 Diet-induced obesity does not modulate tumor ERβ expression in Wnt-1 mice. (a) ERβ expression in tumors from Wnt-1 mice maintained on a high-fat diet-induced obesity (DIO) or low-fat Control (Con) diet. (b) The ratio of ERα to ERβ expression in tumors from Wnt-1 mice fed a DIO or Con diet.

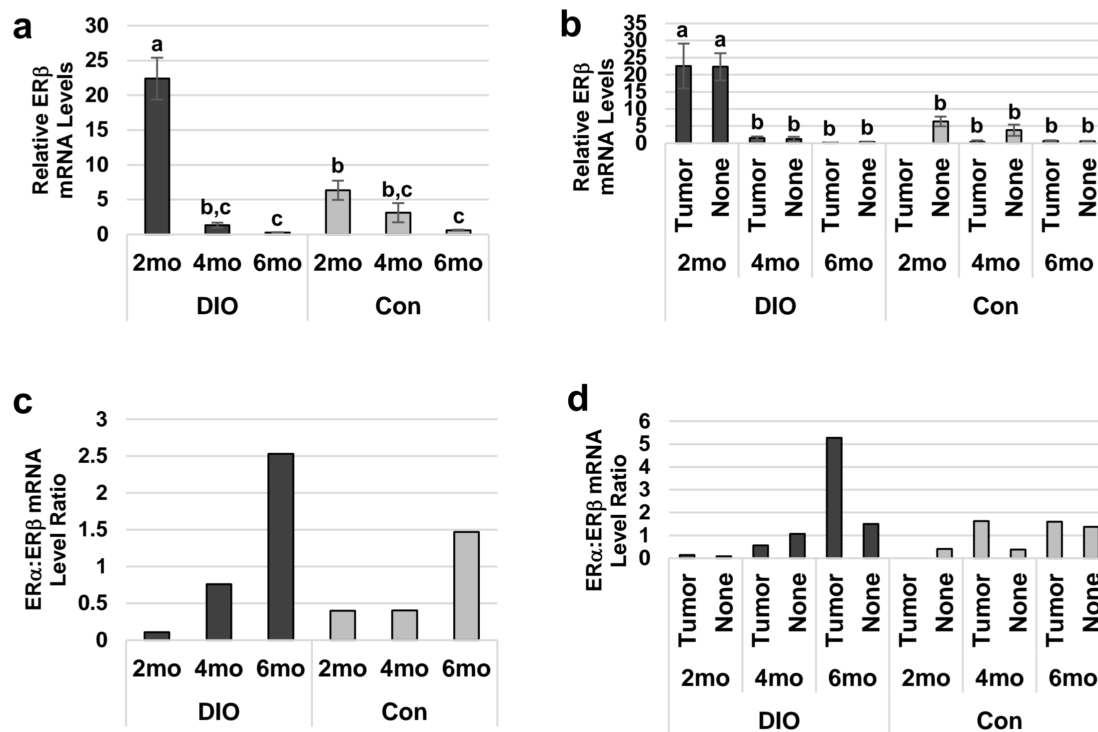

Figure 8.6 Obesity promotes elevated early MFP ERβ expression and a high MFP ERα:ERβ ratio is associated with increased late tumor development in DIO mice. (a) ERβ expression in the mammary fat pad (MFP) of Wnt-1 mice receiving a high-fat diet-induced obesity (DIO) or low-fat Control (Con) diet for one of three time periods (2mo, 4mo, or 6mo). (b) Data from (a) further stratified by tumor incidence. Mice were classified as tumor-bearing (Tumor) or lacking any tumor formation (None) during their prescribed time on experimental diet. (c) Ratio of ERα to ERβ expression in the MFP of Wnt-1 mice maintained on the DIO or Con diet for the three different time points. (d) Data from (c) further stratified by tumor incidence as described above. Different letters indicate significant differences, $p<0.05$.

8.4 Discussion

The majority of breast cancer patients, particularly in the postmenopausal population, have ERα positive tumors. These tumors tend to be less aggressive and such patients generally have a better prognosis in comparison to those lacking ERα (13-14). However, there is still a subset of patients that have an intrinsic or

acquired resistance to endocrine therapies targeting estrogen signaling due to a number of factors, including obesity, and this negatively affects their prognosis (48-49, 54-55). Consequently, it is imperative that we develop animal and cell culture models of ERα positive breast cancer that can be used to examine how factors like obesity promote a more aggressive and drug-resistant disease in these patients.

The MMTV-Wnt-1 transgenic murine model of mammary carcinogenesis develops tumors that are heterogenous in their ERα status (183) and is thus not the ideal model for the study of ERα positive disease. However, there are very few widely available mouse models that develop ERα positive tumors. Other ERα positive models also tend to have a relatively lower tumor incidence and/or longer tumor latency (188), making studies more expensive and time-consuming. For these reasons we chose to examine the impact of obesity on ERα positive tumor development and characteristics in the Wnt-1 model. To our knowledge, this is the first study to investigate the effects of obesity on the spontaneous Wnt-1 model. We found that obesity promoted an increase in tumor incidence and growth rate, but no decrease in tumor latency or survival. Previous studies have assessed the effects of obesity on Wnt-1 tumor growth and characteristics, but they have used an orthotopic transplant model in which cells isolated from a Wnt-1 tumor were subcutaneously injected into mouse MFPs. The results from these studies differ slightly from our findings. While they demonstrated a similar increase in growth rate with obesity (123, 185, 189-190), two also reported a reduction in tumor

latency (123, 189). This suggests that there may be differences in how the spontaneous model responds to obesity, perhaps due to the heterogeneity of the tumors that develop in this model in comparison to those that arise from orthotopic injection of cells derived from a single Wnt-1 tumor.

Because we were primarily interested in improving our understanding of how obesity promotes postmenopausal, ERα positive tumor risk and progression, we initially focused on the impact of obesity on ERα and ERβ expression and ERα signaling in the Wnt-1 tumor and MFP tissue. Intriguingly, we found that obesity was associated with increased tumor ERα expression, but not ERα activity. We actually expected to find the opposite result, given that obesity is known to promote greater estrogen production both systematically and locally in the breast via an increase in adipose tissue aromatase expression in humans (25-27, 41-42). These results are also in contrast with a previous report that obesity suppresses tumor ERα expression in orthotopically injected Wnt-1 cells (189). However, in addition to the differences in the animal model methodology used, the study by Ford et al (189) measured ERα expression by immunohistochemistry (IHC), while we used qPCR. IHC is the more accepted method for examination of tumor ERα levels, and we plan to measure tumor and MFP ERα and ERβ expression by IHC in the future to validate our qPCR results. Using qPCR, we found no difference between the DIO and Con groups in tumor ERβ expression.

We next investigated whether obesity may be modulating ERα expression and activity as well as ERβ expression in the Wnt-1 mouse MFP, as this could play a role in the observed obesity-induced increase in tumor incidence. There was a trend towards greater tumor incidence with increased MFP pS2 expression (a measure of ERα activity) in the 2mo DIO group, which had the highest pS2 expression overall. However, pS2 did not seem to correlate with tumor incidence in the other groups, particularly at the 6mo time point, suggesting that ERα activity may promote early but not later tumor growth. PR expression, typically used as another measure of ERα activity, was actually higher in the Con group and seemed to be associated with reduced incidence of early tumors. The literature regarding the impact of progesterone on postmenopausal breast cancer risk is mixed, with some studies indicating no effect and others suggesting an increase in risk (191-194). However, we did not find any studies supporting a protective effect of increased PR expression.

While there were no clear trends in the MFP expression of ERα between diet groups, the 2mo DIO group had significantly greater MFP ERβ expression in comparison to all other groups. MFP ERβ expression decreased precipitously in the DIO mice at later time points, though, and did not correlate with tumor development in any of the DIO groups. In contrast, there was a trend towards a protective effect with higher MFP ERβ expression in the 2mo and 4mo Con groups. This suggests that obesity may confer resistance to the protective effects of ERβ

expression. Since ERβ expression is often considered in the context of ERα levels due to their opposing effects on cell proliferation and other tumor-promoting factors (132-133, 138, 140-146), we also calculated the ERα:ERβ ratios for each group. The 6mo DIO group, followed by the 6mo Con group, had the highest ERα:ERβ ratios, likely due to the very low ERβ levels found in these groups. When stratified by tumor incidence, a higher ratio was associated with tumor incidence in the 6mo DIO group and 4mo Con group. These results suggest that obesity may promote a higher ERα:ERβ ratio that contributes to increased late tumor incidence. However, the 6mo time point was the only one at which the DIO group did not have a greater tumor incidence in comparison to Con, making the biological relevance of ERβ and the ERα:ERβ ratio in this model questionable.

In sum, ERα activity and ERβ expression do not appear to be playing a role in the obesity-induced elevation in tumor growth rate in the Wnt-1 model. An increase in MFP ERα activity may be contributing to the obesity-associated increase in tumor incidence, though. Obesity may also be somehow conferring resistance to the protective effects of MFP ERβ expression. Further experimentation will be necessary to provide a better characterization of the mechanisms by which obesity promotes tumor incidence and growth in this model. This will include measurement of ERα and ERβ expression by IHC and examination of additional factors known to mediate the pro-carcinogenic effects of obesity, including growth factor and inflammatory cytokine signaling pathways.

Others have demonstrated that mTOR signaling clearly plays a role in obesity's promotion of tumor growth in the Wnt-1 orthotopic injection model (123, 190). In addition, characterization of cells isolated from Wnt-1 tumors have demonstrated that these tumors contain both basal-like and claudin-low subtype cell populations (195). Given that claudin-low breast cancer cells are known to be more aggressive than the other breast cancer subtypes, obesity could also be promoting tumor growth in the Wnt-1 model via an escalation in the claudin-low cell population. Consequently, we will also be measuring the tumors for markers of these subtypes. With an enhanced understanding of the mechanisms by which obesity promotes tumor incidence and growth in the Wnt-1 spontaneous model, we hope to be better equipped to design future intervention studies that target the identified signaling pathways to prevent and improve the treatment of breast cancer in obese postmenopausal women.

Chapter 9: Concluding Remarks

9.1 Conclusions

Over the past 30-40 years, a growing epidemic of obesity that began in the United States and other Western countries has now spread across the globe (18). In the United States, over a third of the adult population is now obese, defined as a body mass index (BMI) of ≥30 kg/m^2 (17). The rise in obesity rates has had alarming effects on public health, as the metabolic abnormalities that accompany excess adiposity can increase individuals' risk of and mortality from numerous diseases, including breast cancer. Obesity has been shown to enhance the risk of postmenopausal breast cancer and also promote breast cancer progression in women of all ages (3-5, 19-21). However, an elevated BMI is most strongly associated with a worse prognosis in postmenopausal, estrogen receptor alpha (ERα) positive breast cancer (24). Obese women in this patient population do not respond as well to aromatase inhibitors (48-49), the standard endocrine therapy for postmenopausal patients. Consequently, the studies detailed here, which examine the mechanisms by which obesity promotes breast cancer progression, have primarily focused on investigating the role of increased aromatase expression in the link between obesity and postmenopausal, ERα positive breast cancer. Our goal has been to identify relevant signaling pathways that may be targeted in order to develop a more effective chemotherapeutic regimen for this patient population.

Building on previous studies from Dannenberg et al that demonstrated a correlation between obesity, elevated breast tissue inflammation, and higher levels of aromatase expression in the breast (41-43), we utilized an *in vitro* model of obesity to determine whether this local aromatase expression could be contributing to obesity-associated breast cancer progression via its production of excess estrogen. To model obesity *in vitro*, we used serum collected from postmenopausal breast cancer patients and pooled by BMI category (normal weight: 18.5-24.9 kg/m^2; obese: ≥30 kg/m^2). Breast cancer cells exposed directly to the obese versus normal weight patient sera had higher aromatase expression levels as well as $ER\alpha$ activity and viability in the presence of testosterone, the substrate for aromatase. These effects were mediated by an increase in Akt and MAPK pathway signaling. Exposure to the obese patient serum also caused pre-adipocytes to produce more interleukin 6 (IL-6), which led to greater breast cancer cell aromatase expression, $ER\alpha$ activity, and viability. We did not pursue further investigation of obesity's impact on breast cancer cell aromatase expression, though, because we found that the pre-adipocytes themselves were actually a substantially greater source of aromatase expression. Given that pre-adipocytes are found in abundance in the breast tumor microenvironment and tumor-adjacent tissue (28), exploration of how obesity impacts pre-adipocyte aromatase expression seemed to be the more biologically relevant question.

Direct exposure to obese versus normal weight serum had no differential effect on pre-adipocyte aromatase expression. However, macrophages and breast

cancer cells both express cyclooxygenase 2 (COX-2), which catalyzes the production of the aromatase-stimulating eicosanoid prostaglandin E2 (PGE2) from arachidonic acid (30, 43). Consequently, we next conducted a retrospective analysis of the medical records of ERα positive breast cancer patients that received endocrine therapy to determine whether recurrence rates differed according to patient use of COX-2-targeting non-steroidal anti-inflammatory drugs (NSAIDs). We found that daily NSAID use decreased recurrence by approximately 50% and delayed time to recurrence by over two years. The fact that the vast majority of the patients in this pilot study were overweight or obese and had a higher risk of recurrence suggested that perhaps the strong effect of NSAID use in this patient population was due to the predominance of excess adiposity. Unfortunately, we did not have adequate power in this study to test this hypothesis.

Based on these results and other researchers' (41-43), we next examined whether obese patient sera exposure enhanced macrophage and breast cancer cell PGE2 production. We found that obese patient sera did increase these cells' COX-2 expression and PGE2 secretion, leading to greater pre-adipocyte aromatase expression and estradiol production. This estradiol stimulated greater breast cancer cell ERα activity and proliferation, suggesting that targeting macrophage and breast cancer cell COX-2 expression and PGE2 production may improve obese patients' response to aromatase inhibitor therapy.

Another possible mechanism mediating obesity-induced aromatase inhibitor resistance and breast cancer progression in postmenopausal women is an

enhancement of the cross-talk between cytoplasmic ERα and the PI3K/Akt and MAPK signaling pathways. This cross-talk has been associated with resistance to endocrine therapy (54-55), and obesity is typically accompanied by increased circulating insulin, insulin-like growth factor 1 (IGF-1), leptin, and inflammatory cytokines that can all activate the PI3K/Akt and MAPK pathways (34, 38-39. 65, 68-70). We found that breast cancer cells grown in obese versus normal weight patient sera demonstrated higher cell viability and growth, effects mediated by PI3K/Akt, MAPK, and nongenomic ERα signaling. Combined inhibition of PI3K and ERα produced the greatest reduction in obesity-induced breast cancer cell viability and growth, while ERα inhibition significantly reduced the Akt activation stimulated by obese patient sera exposure. These results suggest that obesity-associated circulating factors may promote postmenopausal breast cancer progression, and perhaps aromatase inhibitor resistance, by enhancing PI3K/Akt and MAPK signaling and their cross-talk with cytoplasmic ERα.

The last three studies described here are still ongoing, but have produced promising initial results. We assessed the effects of obesity on estrogen receptor beta (ERβ) expression in breast cancer cells because activation of this second ER counterbalances the pro-growth effects of ERα, and higher ERβ expression has been associated with an improved prognosis (137-146). We have demonstrated that exposure to obese versus normal weight patient sera suppresses ERβ expression in HER2 overexpressing breast cancer cells, but not in cells

representative of the other three breast cancer subtypes. Further experimentation revealed that the obese patient sera's effects on ERβ expression are mediated by HER2 signaling. In agreement with clinical data indicating that obesity is associated with a reduced response to chemotherapy treatment (128-130, 164-165), we have also shown that obese patient sera exposure confers resistance to docetaxel treatment in triple-negative breast cancer cells. Preliminary data suggests that this effect may be mediated by activation of the Akt/mTOR pathway. Finally, we have initiated an analysis of obesity's effects on tumor development and characteristics in the MMTV-Wnt-1 transgenic mouse model, which is predisposed to the spontaneous development of mammary adenocarcinoma (181). Diet-induced obesity promotes tumor incidence and faster tumor growth in this model, but no decrease in tumor latency or survival. Assessment of tumor and mammary fat pad (MFP) ERα expression and activity as well as ERβ expression suggests that an obesity-induced increase in MFP ERα activity could be playing a role in promoting tumor incidence. However, ERα and ERβ signaling do not appear to be substantial contributors to obesity's promotion of mammary tumorigenesis in this model.

9.2 Future Directions

The aromatase-focused studies described here produced some compelling results suggesting that obesity may promote postmenopausal, ERα positive breast cancer progression and aromatase inhibitor resistance via an increase in PGE2-

induced pre-adipocyte aromatase expression. However, the biological relevance of our data remains uncertain without *in vivo* studies to support these findings. Consequently, the next step should be to utilize a transgenic mouse model of mammary carcinogenesis to test whether COX-2 inhibition improves response to aromatase inhibitor therapy in the context of obesity. Our preliminary data from the MMTV-Wnt-1 mouse study suggests that this model is probably not a good choice for the study of ERα positive breast cancer. Another option is the $Pik3ca^{H1047R}$ transgenic mouse, which has a conditional knock-in of this common activating mutation of the *PIK3CA* gene that results in 100% penetrance of ERα positive tumors by approximately 12 months of age (196). Due to the long tumor latency in this model, an orthotopic injection model in which cells isolated from one of these tumors are subcutaneously injected into the MFP of non-transgenic mice of the same background strain could also be utilized. Because we have not previously used this mouse model, a pilot study to assess whether obesity promotes tumor development and mammary tissue aromatase expression will be necessary prior to proceeding with any drug intervention studies. If this proves to be a suitable model for further studies, the next step would be to choose the most appropriate COX-2 targeting drug. While we employed the selective COX-2 inhibitor celecoxib for our *in vitro* studies, this drug has been linked to an increase in major vascular events in humans (197). Thus, an over-the-counter NSAID, like aspirin, or an omega-3 fatty acid supplement, which can shift COX-2 activity away from PGE2 production and towards the production of anti-inflammatory eicosanoids (198),

should be used for this study. Based on the animal study results, a pilot clinical trial examining the efficacy of this drug combination in obese postmenopausal ERα positive breast cancer patients could follow.

Confirmation of the essential role that we believe increased aromatase expression plays in obesity-induced postmenopausal ERα positive breast cancer progression will also require an animal study. Researchers at the University of Texas Health Science Center at San Antonio have developed a Cre-inducible aromatase knock-out transgenic mouse. This mouse could be bred with the *Pik3ca*^{H1047R} transgenic mouse, then a mouse line with inducible, Cre-dependent recombination in adipocytes to ultimately produce a triple-transgenic mouse with inducible aromatase knock-out in the adipocytes and a predisposition for ERα positive mammary carcinogenesis. The impact of diet-induced obesity on tumor development in mice with and without the aromatase knock-out induced would then be assessed to determine whether aromatase over-expression is an important mediator of obesity's effects.

Based on our determination that exposure to obesity-associated circulating factors enhances cross-talk between ERα and the PI3K/Akt and MAPK signaling pathways in breast cancer cells, further investigation into the importance of this effect in obesity-induced breast cancer progression is warranted. The focus of future studies regarding this effect should be an assessment of the role of PI3K/Akt activation and cross-talk with cytoplasmic ERα in obesity-related aromatase

inhibitor resistance, as inhibition of these two pathways produced the greatest suppression of obese patient sera-mediated breast cancer cell viability and growth. Such an assessment could best be accomplishment with another animal study in which aromatase inhibitor therapy is combined with a PI3K inhibitor in the context of diet-induced obesity to determine if this improves aromatase inhibitor response. Given its activating PIK3CA mutation, the *Pik3ca*^{H1047R} transgenic mouse model would not be a good choice for this study, so another transgenic mouse model of ERα positive mammary carcinogenesis would have to be utilized.

The final three studies described here remain in progress. To complete our analysis of the impact of obesity on breast cancer cell ERβ expression, the biological relevance of obesity-induced ERβ suppression must first be assessed. We will transfect the cells with an ERβ-overexpressing plasmid to prevent this suppression, then measure whether the obese patient sera is still able to promote greater breast cancer cell viability. If ERβ suppression does appear to be biologically relevant, studies designed to further define the HER2-mediated pathway by which obesity inhibits ERβ expression should be planned. In addition, the efficacy of an ERβ agonist in counteracting the effects of the obese patient sera on breast cancer cell viability could be tested. Finally, depending on the results of the above *in vitro* experiments, an *in vivo* study utilizing the MMTV-neu transgenic mouse model of HER2-overexpressing breast cancer could be

designed to determine whether an ERβ agonist can counteract the negative effects of obesity on tumor progression in this breast cancer subtype.

Our analysis of how obesity promotes resistance to docetaxel treatment in triple-negative breast cancer cells should continue by first confirming our finding that mTOR inhibition is able to reverse this resistance. If this result holds up, the next steps should include an exploration of how obesity confers docetaxel resistance and why the addition of the mTOR inhibitor rapamycin results in synergistic inhibition of cell viability. One possibility is that obesity is promoting cell survival via mTOR activation, so an analysis of the induction of cell apoptosis under these different treatment conditions should be conducted. Future studies should also include confirmation of these effects in an animal model of triple-negative breast cancer, like the C3(1)/SV40 T-antigen transgenic mouse model. Positive results from an animal study would provide a strong rationale for the development of a pilot clinical trial testing a docetaxel/rapamycin combination treatment in obese women with triple-negative breast cancer.

Finally, our characterization of how obesity impacts tumor development in the MMTV-Wnt-1 mouse model will continue with measurement of ERα and ERβ expression by IHC and examination of additional factors known to mediate the pro-carcinogenic effects of obesity, including growth factor and inflammatory cytokine signaling pathways. In addition, given that Wnt-1 mouse tumors have been shown to contain populations of cells representative of both the basal-like and claudin-low breast cancer subtypes (195), we will also examine whether obesity affects tumor

expression of the various subtype markers. This will demonstrate whether obesity may be promoting tumor growth in the Wnt-1 model via an escalation in the highly aggressive claudin-low cell population.

All of these studies have enhanced our understanding of the mechanisms by which obesity promotes breast cancer progression. Hopefully, this improved knowledge will lead to the development of future studies in our laboratory and others' that build on the findings reported here in order to achieve the overall goal of this project. That goal has been to identify relevant, targetable obesity-induced signaling pathways that will inform the design of improved chemotherapeutic regimens for the treatment of breast cancer in obese women.

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