Fatty Acid Synthase Expression Under Obese Conditions in Breast Cancer Cells

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ABSTRACT

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Purpose: In women, breast cancer is the second leading cause of cancer death, and one in eight will develop breast cancer during their lifetime (Siegel et al., 2016). Furthermore, studies have shown that obesity has been associated with worse prognosis in some breast cancer patients. These facts led to this study on the effects of obesity on the fatty acid synthase (FASN) in breast cancer. FASN catalyzes the formation of long-chain fatty acids such as palmitate. Palmitate is commonly used as a component of the phospholipid membrane and for energy. Other types of cancer have been shown to harness the activity of the FASN in order for increased proliferation and energy. This study has two aims: 1) To determine if obesity modulates FASN expression in breast cancer cells, and 2) To determine the mechanism behind how obesity modulates FASN.

Experimental Design: Sera was obtained from women and pooled by Body Mass Index (Normal weight: 18.5 – 24.9 kg/m^2, Obese: ≥ 30 kg/m^2). MCF-7 cells, an estrogen receptor and progesterone receptor positive breast cancer cell line, was grown in 2% obese or 2% normal women sera to model cell growth in vivo. The effect of obese and normal women sera on FASN expression was measured through quantitative PCR.

Afterwards, MCF-7 cells were grown in normal growth media or normal growth media with 0.5 mM of H_2O_2 in order to see the effects of hypoxia on cell expression of FASN. Again, FASN expression was measured through quantitative qPCR.

Results: Treatment with 2% obese women sera on MCF-7 cells increased FASN expression by approximately 2.5-fold. Hypoxic conditions on MCF-7 cells increased FASN expression by approximately 4-fold. The effect of obesity on FASN in breast cancer cells is still being investigated.

Conclusions: Exposure to sera from obese women increases the presence of FASN for energy and for membrane formation in breast cancer cells. Furthermore, cells produce various factors that increase FASN expression under hypoxic conditions.

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INTRODUCTION

Cancer plagues our country as the second leading cause of death, and experts have estimated that there will be almost 250,000 new cases of breast cancer this year alone (Siegel et al., 2016). Additionally, more and more Americans are gaining weight and becoming obese every year. When looking at the relationship between breast cancer and obesity, studies showed that obesity has been associated with a worse prognosis in breast cancer patients, and obesity is considered an established risk factor for the development of postmenopausal breast cancer (Sinicrope and Dannenberg, 2011). This is partially explained because obesity induces changes in cellular metabolism and causes hypoxic conditions. Since hypoxic conditions lead to rapid proliferation in cancer cells partially through increased expression of the fatty acid synthase (FASN), it is hypothesized that obesity increases expression of FASN. Overexpression of FASN has been shown in different types of cancer including breast, prostate, and ovarian (Flavin et al., 2010). Furthermore, FASN plays an integral part in tumor development and progression (Duan et al., 2016). The expression of FASN and its significance in breast cancer remains vague and led to this study. Obese breast cancer patients are more likely to experience resistance to treatment. Defining the role and mechanism FASN has in obese breast cancer patients would be integral in determining whether FASN is a target to go after for cancer treatment.

The fatty acid synthase is a multi-enzyme complex composed of two identical monomers. Each monomer contains six catalytic activities necessary for

function. However, fatty acid synthase functions only in dimer form (Chirala and Wakil, 2004).

Mechanisms by which FASN Promotes Cancer Progression

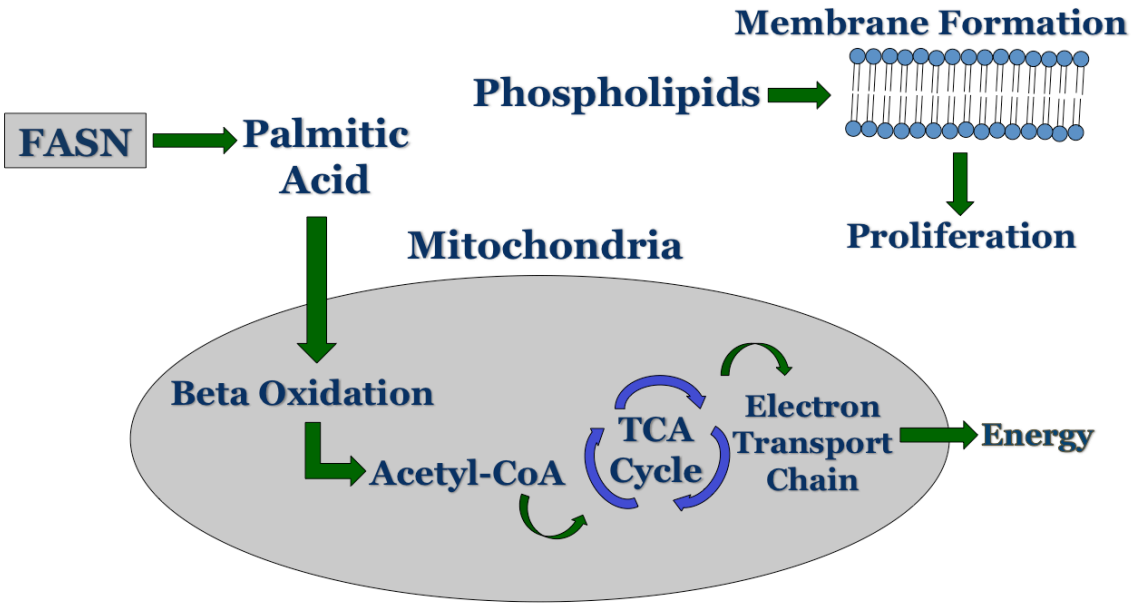

Figure 1: The primary role of FASN is to catalyze the synthesis of palmitate from free fatty acids. Palmitate is a long chain fatty acid that could go through two processes in the cell. On one hand, palmitate can be used to form phospholipids necessary for membrane formation and ultimately, proliferation. On the other hand, palmitate can go into the mitochondria, and through beta-oxidation, the TCA cycle, and the electron transport chain, produce ATP for energy processes (DeBardinis et al., 2008).

Cancer cells utilize FASN to form the phospholipid membrane needed during cell division and to derive energy. Since cancer cells are rapidly proliferating, they require more energy. Normal cells do not express high levels of

FASN unless during cell division. The use of FASN to produce the fatty acids required for energy and proliferation allows cancer cells to rapidly divide.

Obesity associated hypoxic conditions are induced in a variety of ways. For instance, expanding adipose tissue, due to obesity, disrupts capillary function and creates hypoxic microenvironments (Ye, 2012).

Hypothesized Mechanism by which Obesity Upregulates FASN

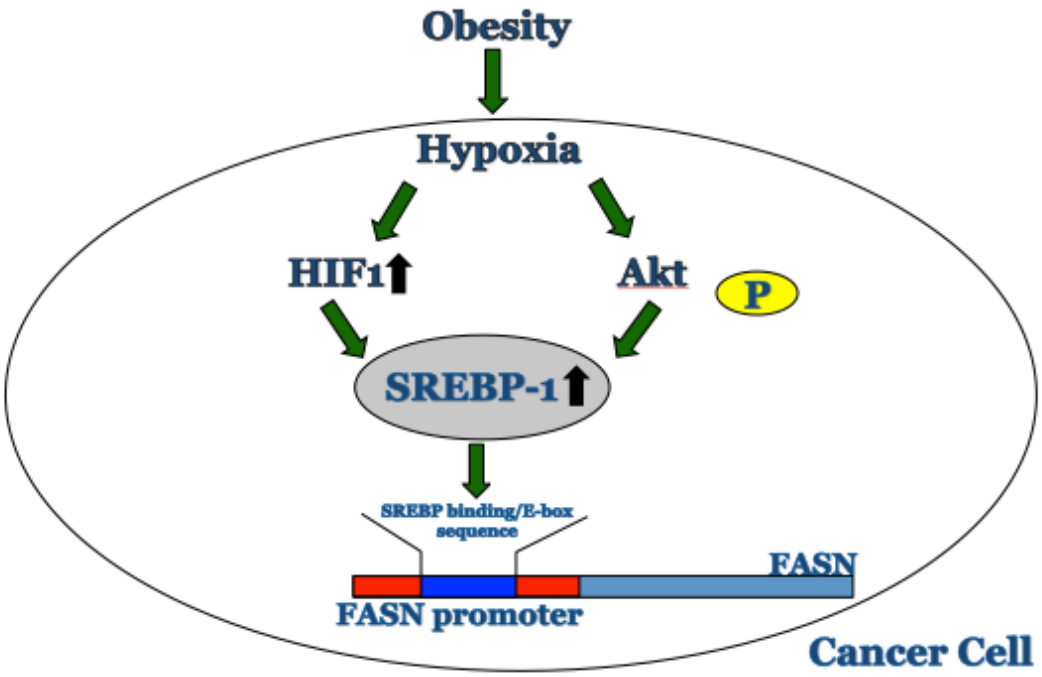

Figure 2: Obesity induces hypoxic environments. Hypoxia significantly increases phosphorylation of Akt and activation of hypoxia-inducible factor 1 (HIF1). This leads to upregulation of sterol regulatory-element binding protein (SREBP-1). SREBP-1 is a transcription factor that binds to the SREBP binding site/E-box sequence located in the FASN promoter. Under hypoxic conditions, SREBP-1 binds more strongly to this area. SREBP-1 binding to the FASN promoter results in increased expression of FASN. Therefore, hypoxia ultimately

upregulates the gene expression of FASN through the HIF1/Akt pathway (Furuta et al., 2008). Cancer cells harness FASN and go through the processes for both proliferation and energy. Therefore, it is hypothesized that obesity affects FASN expression by inducing hypoxic conditions in the tumor microenvironment, and increased levels of free fatty acids in obese patients contribute to production of palmitate.

Hypothesis: Obesity induces hypoxic conditions, which ultimately upregulates FASN

For this project, specific aims were developed in order to explore the relationship between obesity and breast cancer.

Specific Aim 1a – Determine if exposure to obese conditions induces upregulation of FASN expression levels in breast cancer cells

Obesity promotes cancer progression, but the specific mechanisms by which this occurs are unclear. FASN has been shown to promote cancer cell proliferation and malignant progression through generating fatty acid precursors required for cell proliferation, altering membrane fluidity and activating oncogenic signaling pathways. Therefore, it is possible that circulating factors in sera from obese individuals might lead to increased expression of FASN in cancer cells.

Specific Aim 1b – Determine the mechanism by which obesity modulates FASN expression in breast cancer cells

Obesity is associated with elevated levels of numerous circulating growth factors, adipokines, and inflammatory cytokines that can activate the Akt

signaling pathways. It is hypothesized that obesity associated hypoxic conditions increase the presence of these factors, and they are present in sera obtained from obese women. Therefore, it is hypothesized that subjecting the cells to hypoxic conditions, or subjecting them to the circulating factors contained in this sera, will activate the HIF1/Akt signaling pathway and increase expression of FASN.

Discovering the answers to these questions would be useful in clinical settings. Therefore, this translational research laboratory started collaborating with the University of Texas Health Science Center at San Antonio for this project. The results of this project will lead to future experiments regarding a specific fatty acid synthase inhibitor, TVB-3166, currently in a Phase 1 clinical drug trial for treating glioblastomas. This particular inhibitor was chosen because it presents with fewer side effects in patients with glioblastomas. The use of other fatty acid synthase inhibitors in breast cancer patients has resulted in unintended, dramatic weight loss. Therefore, it is hypothesized that TVB-3166 could be a better potential treatment for obese breast cancer patients, who are particularly prone to obesity related cancer therapy resistance due to the factors produced in obese conditions.

MATERIAL AND METHODS

Cell Lines

MCF-7 (ER/PR +), a human breast carcinoma cell line, was maintained in IMEM supplemented with 10% fetal bovine serum, 5% penicillin/streptomycin, and 0.01 mg/ml human recombinant insulin. The cell line was subjected to four conditions: normal growth media (10% FBS), normal growth media with hydrogen peroxide (10% FBS + 0.5 mM H_2O_2), 2% obese donor sera (SFM+ 2% obese donor sera), and 2% normal donor sera (SFM + 2% normal donor sera). Human sera were obtained from normal weight and obese postmenopausal women from Equitech Enterprises. The collected sera were pooled into two groups by Body Mass Index (Normal weight: 18.5 – 24.9 kg/m^2, Obese: ≥ 30 kg/m^2).

RNA Extraction

Total RNA was isolated from the cells with TRIzol® Reagent (Life Technologies, 2012).

Reverse Transcription

cDNA was reverse transcribed from previously extracted RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 2006).

qPCR

FASN mRNA expression was measured through quantitative Polymerase Chain Reaction and quantified by SYBR green dye binding. The cDNA was amplified with a pair of forward and reverse primers for the following genes: FAS (5′-CATCCAGATAGGCCTCATAGAC-3′ and 5′-CTCCATGAAGTAGGAGTGGAAG-3′) and β-actin (5′-TGAGACCTTCAACACCCCAGCCATG-3′ and 5′-CGTAGATGGGCACAGTGTGGGTG-3′). qPCR reactions were performed using SYBR® Select Master Mix (Life Technologies, 2013). The qPCR gene expression was normalized to human β-actin. All experiments were executed in triplicates and will be repeated three independent times.

Statistical analysis

Results were expressed as the mean±SEM. Statistical tests included paired, one-tailed Student test. P-values of 0.05 or less were considered to denote significance.

RESULTS

The MCF-7 cell line was utilized for this experiment. MCF-7 human breast cancer cell line was derived from a pleural effusion from a patient with metastatic breast cancer (Levenson and Jordan, 1997). MCF-7 has estrogen receptors and progesterone receptors, and can be classified as Luminal A breast cancer in clinical terms. Luminal A breast cancer is the most common subtype of breast cancer. Luminal A represents 50-60% of all breast cancers, so performing these studies with the MCF-7 breast cancer cell line would be the most relevant to the clinical setting (Yersal and Baructa, 2014). The traditional way of executing these experiments are through a monolayer two-dimensional culture, therefore this method was implemented.

The first step to fulfill Specific Aim 1a was to see if cells treated with sera from obese women would increase or decrease the expression of FASN (Figure 3). The various factors that are elevated in obese conditions could be activating the HIF1/Akt pathway that leads to upregulation of FASN. The results showed an approximately 2.5-fold increase in FASN mRNA levels in cells treated with obese donor sera for 24 hours. The data suggests that obesity causes cells to secrete various factors that will be present when sera from obese women is collected. This experiment was done three times, and the results are statistically significant at a 5% confidence level with a P-value of 0.03.

Circulating Factors from Obese Women Induce FASN Expression

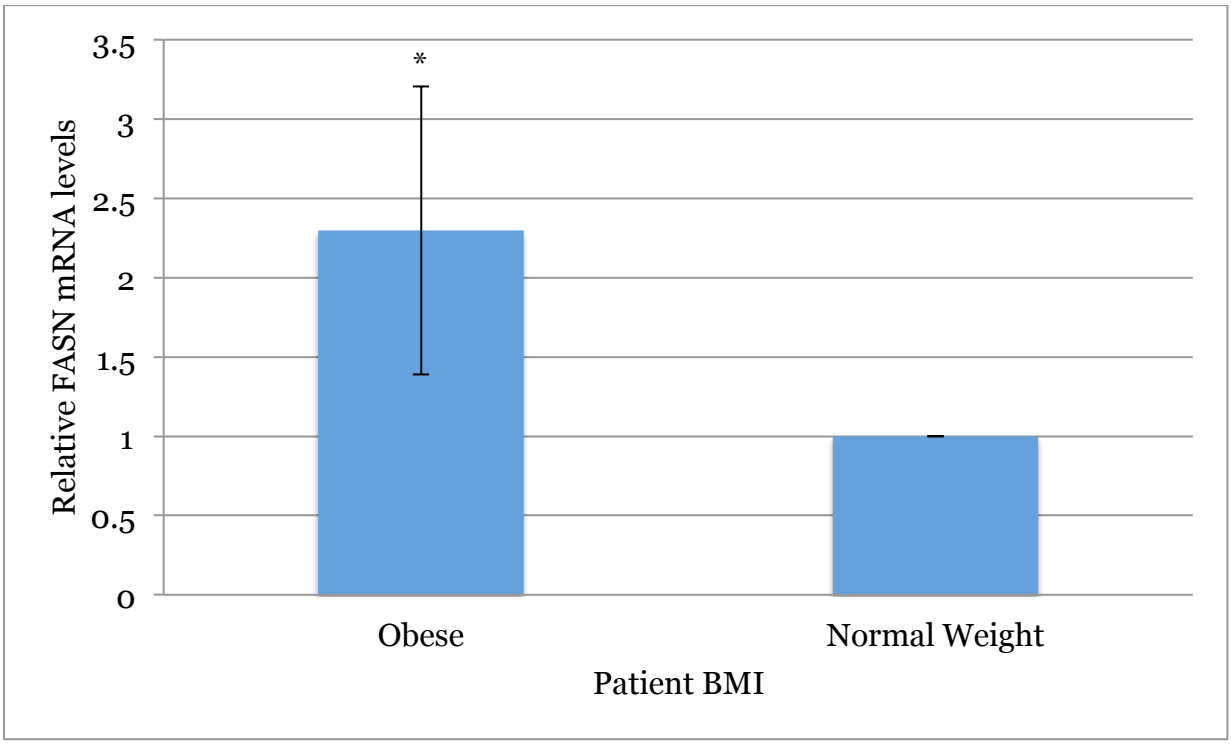

Figure 3: Quantitative PCR was used to measure the FASN mRNA levels in MCF-7 breast cancer cells after a 24 hour exposure to 2% sera from normal weight (BMI = 18.5 – 24.9 kg/m^2) or obese (BMI ≥ 30 kg/m^2) postmenopausal women. There is approximately a 2.5-fold increase in FASN mRNA levels when cells are exposed to sera from obese women. (P-value = 0.03)

Treating cells with hydrogen peroxide was the next step to fulfill Specific Aim 1b in order to elucidate the mechanism by which obesity is inducing increased FASN expression. It was hypothesized that hypoxic conditions caused by obesity would stress the cell into inducing the production of various factors involved in the HIF1/Akt pathway. Since obesity induces hypoxia, using hydrogen peroxide is a good model for the hypoxic environment induced by obesity without the circulating factors to see if the cell will still induce expression of FASN (Furuta et al., 2008).

The results from this experiment (Figure 4) showed that cells treated with 0.5 mM of hydrogen peroxide in normal growth media for 24 hours had a 4-fold increase in FASN mRNA expression levels. When cells were treated with hydrogen peroxide, they would become stressed and produce factors that would contribute to increased FASN mRNA expression. Cells treated in normal growth media would not be stressed, and would use the nutrients in the media for growth and energy purposes instead of FASN. The results suggests that inherent factors produced when the cells are stressed also upregulate FASN expression levels. Therefore, the data proposes that circulating factors as well as inherent factors induce expression of FASN. This experiment was carried out once, and further experiments are currently in progress.

Inherent Factors Increase FASN Expression Under Hypoxia

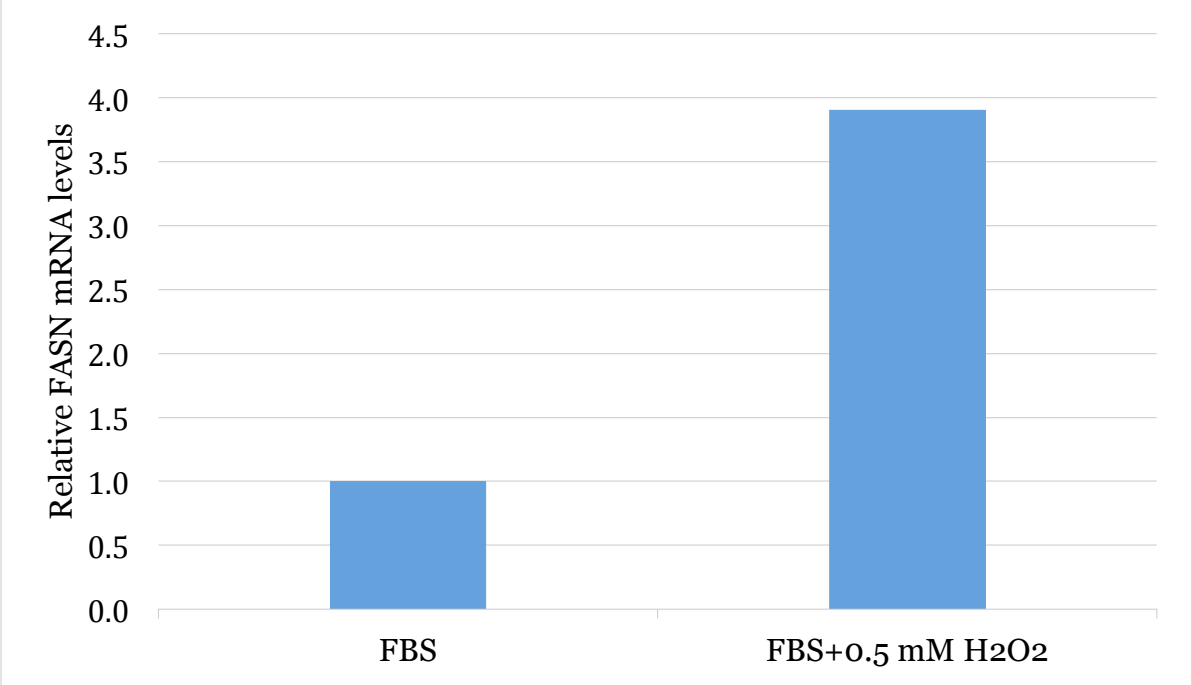

Figure 4: Quantitative PCR was used to measure the FASN mRNA levels in MCF-7 breast cancer cells after a 24 hour exposure to 10% FBS or 10% FBS + 0.5 mM H_2O_2. There is an approximately 4-fold increase in FASN mRNA levels when cells are exposed to hydrogen peroxide. This experiment is in the process of being replicated, so statistics cannot be performed at this point.

Troubleshooting by mixing and matching different doses of hydrogen peroxide with different time points was a long process. Doses of 0 mM, 0.5 mM, and 1 mM of hydrogen peroxide were used, and 24 and 48 hour time points were tested. Furthermore, serum starving the cells overnight before treating them for 24 or 48 hours was tested as well. Serum starving was quickly ruled out as unnecessary because the cells needed to be at their baseline before measuring FASN expression levels. Starving the cells before adding the treatment would skew results since serum starving itself could affect FASN expression levels. Treating for 24 hours would yield results, while treating for 48 hours would kill the cells. Finally, treating the cells with 1 mM of hydrogen peroxide would also kill the cells. Using a 0.5 mM dose of hydrogen peroxide was also confirmed through other published works (Furuta et al., 2008).

One of the major obstacles in this experiment was obtaining uncontaminated RNA. RNA extraction was a difficult process that needed troubleshooting. Using the TRIzol® Reagent protocol (Life Technologies, 2012), under homogenizing samples, on the third step of the adherent cells (monolayer) section, it was best to use cell scrapers to aid the lysing and homogenizing process in addition to pipetting up and down the solution. In the second step of phase separation, it was found that chloroform is prone to evaporation. Therefore, wetting the pipet tip before loading chloroform into the tip would give the accurate amount of chloroform needed. Completely removing as much liquid as possible in step 4 of RNA wash was crucial in giving clean, uncontaminated RNA. Removing the last drops of liquid could be achieved by collecting the liquid

at the bottom of the tube through centrifuging. These extra additions would yield higher quality RNA. Higher quality RNA would yield a qPCR reaction with more reliable results. High quality RNA could be determined through Nanodrop. High quality RNA would have a 260/280 absorbance value close to 2.0, and a 260/230 absorbance value ranging from 2.0-2.2. Since RNA is extremely unstable and prone to degradation, it was discovered that running a Reverse Transcriptase PCR (RT-PCR) right after extracting RNA from the cells, instead of freezing it back for storage and then thawing to continue the experiment, would give quality cDNA.

cDNA obtained from (RT-PCR) is stable, but it was discovered that mixing cDNA through vortexing would break the cDNA strands. The optimal way to mix cDNA was to gently flick the tube.

According to the SYBR® Select Master Mix protocol, the optimal primer concentration range is 150 nM – 400 nM (Life Technologies, 2013). After testing 300 nM, 350 nM, and 400 nM of FASN primers in the qPCR reaction, we established that a 400 nM FASN primer reaction was the most efficient. The qPCR ran more effectively and yielded the most reliable results at this primer concentration. Precise pipetting was achieved by using master mixes containing primer, cDNA and SYBR® Select Master Mix. The standard cycling mode was used for qPCR, and it gave the most reliable results (Life Technologies, 2013).

DISCUSSION

Cells treated with sera from obese women showed an almost 2.5-fold increase in FASN mRNA expression levels. This result was statistically significant and suggests that factors in the sera from obese women induce increased FASN mRNA levels, and obesity affects the expression of FASN, which promotes cancer progression. Since obesity is associated with elevated levels of numerous circulating growth factors, adipokines, and inflammatory cytokines that can activate the Akt signaling pathways, we hypothesize that these increased levels of different factors will activate the HIF1/Akt signaling pathway which increases expression of FASN (Bowers, 2014). Furthermore, cells themselves are affected by hypoxic conditions and will produce factors that increase expression of FASN. Factors secreted by cells in combination with factors produced by the cell itself will promote expression of FASN and ultimately drives cancer progression.

A future step this study could take is to investigate if the HIF1/Akt pathway is truly being upregulated. This would be accomplished by treating the cells with obese and control sera and using a Western Blot with an antibody for Akt.

The specific microenvironment of the breast also has many factors that contribute to the survival and progression of breast cancer. Since the breast is made up of adipose tissue, pre-adipocytes are the next target to look at. Another step this project could go in is to measure FASN mRNA expression levels in pre-adipocytes after being treated with sera from either obese or non-obese women.

Pre-adipocytes are the undifferentiated form of adipocytes. They are pro-inflammatory and more bioactive than adipocytes, making them an attractive target for the next step. The results from this will show whether FASN mRNA levels are increased in pre-adipocytes treated with sera from obese women or not. Ultimately, it is hypothesized that palmitate from FASN in pre-adipocytes can be secreted out of the pre-adipocyte. Cancer cells can then use the exogenous free fatty acids as an additional source of palmitate for proliferation and/or energy (Louie et al., 2008).

Since it has been shown that obesity confers increased resistance in different types of therapies for breast cancer (Hursting et al., 2015), the last step of this project would be to test a fatty acid inhibitor (TVB-3166), currently in a phase 1 clinical drug trial for glioblastoma, on breast cancer cells. The results from this would show if this fatty acid inhibitor would be an effective treatment for obese breast cancer patients. This fatty acid inhibitor could potentially be a more beneficial treatment specifically for obese breast cancer patients.

Further aims currently planned out for this project to explore after Specific Aim 1a and 1b are completed are listed as follows:

Specific Aim 2 – Determine if exposure to obese conditions upregulate FASN expression in pre-adipocytes

In addition to altered expression in epithelial cells, FASN levels in adipocytes have been shown to be altered in the obese individual, potentially contributing to increased circulating levels of free fatty acids that can fuel cancer cell growth and proliferation. Replicating the qPCR studies in Specific Aim 1

using human pre-adipocytes would determine if exposure to sera from obese women upregulates expression of FASN in the pre-adipocytes.

This experiment will determine if obesity induces release of free fatty acids from the pre-adipocytes. Next would be to generate conditioned media from pre-adipocytes exposed to sera from either obese or lean women for 24 hours and measure the free fatty acid content using Abcam's Free Fatty Acid Quantification Kit. The pre-adipocytes would briefly be exposed to the same conditions as in Specific Aim 1a: 2% obese donor sera (SFM+ 2% obese donor sera) and 2% normal donor sera (SFM + 2% normal donor sera). After 24 hours, media would be removed, cells would be washed with PBS, and fresh SFM would be added to the cells. This conditioned media would then be collected after 24 hours, and the free fatty acid content would be measured. This experiment is currently in progress.

Specific Aim 3 – Determine if use of a FASN inhibitor suppresses obesity-induced proliferation in vitro

Previous studies using less specific FASN inhibitors have demonstrated that FASN is a viable target for therapeutic intervention in other cancer cell types. These early inhibitors also induced significant weight loss, limiting use for the cancer patient. Newer, more targeted and reversible FASN inhibitors such as TVB-3166 have not demonstrated similar weight loss complications in early clinical studies, suggesting that these newer compounds may provide a highly effective, less toxic alternative for the treatment of certain breast cancers (Ventura et al., 2015). It is hypothesized that since obesity promotes worse

outcome in all breast cancer tumor types, and in both menopausal and postmenopausal women, TVB-3166 may be effective at suppressing disease progression in several clinical subtypes. Future steps this project would take would be to use in vitro modeling to assess the efficacy of TVB-3166 at limiting obesity-induced parameters of progression. Concentrations of TVB-3166 (50 nM) are based upon previous studies done by 3-V Biosciences (Ventura et al., 2015). This experiment is also currently in progress.

These pending experiments will determine the role of FASN in breast cancer cells and if TVB-3166 is effective at limiting obesity-induced changes in cellular behavior associated with progression. MTT and Ki67 assays could be used to determine changes in proliferation, and wound healing assays could be used to determine changes in motility, and invasion chambers to determine changes in invasive potential.

Currently, MTT assays are being utilized to measure cell viability of cells treated with different doses of TVB-3166 in 2% FBS, 2% obese donor sera, and 2% normal donor sera.

The last direction this project could go in is to repeat these experiments in other breast cancer cell line subtypes. This would show if there is a difference in how obesity impacts other types of breast cancer, and if so, how TVB-3166 efficacy would be affected.

CONCLUSIONS

This project has shown that obesity upregulates FASN. This is potentially due to a combination of obesity-associated hypoxic conditions and increased levels of circulating factors. Further research is necessary to conclude if these results are specific to this particular cell type, if other cells in the tumor microenvironment have the same effects on breast cancer, and if FASN inhibitors could potentially reduce cancer cell proliferation and improve breast cancer prognosis.

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