6-1-2013

Role of IGF-II, PRL, and FABPs in the Breast Cancer Disparity among AA Women

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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Role of IGF-II, PRL, and FABPs in the Breast Cancer Disparity among AA Women

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Biochemistry

June 2013

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Daisy De León, for her guidance and support, and the members of her laboratory who assisted me in the technical aspects of the research. I would also like to thank the Center for Health Disparities and Molecular Medicine for their funding of my research, as well as the National Institutes of Health. I would like to thank my parents, Mattahais and Irene Patrick, my siblings, Matthias and Tenesha Patrick, and my extended family for their love and support. Thanks to my best friend, Fredricka Cassandra McKnight, for her steadfast support. And lastly, I thank God for His support, constant encouragement, and everlasting love.

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ABBREVIATIONS

IGF-II	Insulin-like Growth Factor II
PRL	Prolactin
FABP	Fatty Acid-Binding Protein
AA	African-American
CA	Caucasian-American
MDGI	Mammary-Derived Growth Inhibitor
LCFA	Long Chain Fatty Acid
PPAR	Peroxisome Proliferator-Activated Receptor
CoA	Coenzyme A
HIF1α	Hypoxia Inducible Factor 1α
pVHL	Von Hippel-Lindau tumor suppressor
VEGF	Vascular Endothelial Growth Factor
MCF-7	Michigan Cancer Foundation-7
CRABP-2	Cellular Retinoic Acid Binding Protein 2
IGF-IR	Type I IGF Receptor
IR-A	Insulin Receptor A
IGF-IIR	Type II IGF Receptor
mIGF-II	Mature IGF-II
proIGF-II	Precursor IGF-II
PRLR	Prolactin Receptor
MAP kinase	Mitogen-activated Protein kinase
PKC	Protein Kinase C

PI3 kinase	Phosphoinositide-3-kinase
MCF10A	Michigan Cancer Foundation-10 Adherent
SF	Short Form (of PRLR)
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal Growth Factor
MEBM	Mammary Epithelial Cell Basal Medium
MEGM	Mammary Epithelial Cell Growth Medium
RIPA	Radioimmunoprecipitation Assay
PBS	Phosphate-Buffered Saline
BSA	Bovine Serum Albumin
SDS	Sodium Dodecyl Sulfate
PVDF	Polyvinylidene Difluoride
HRP	Horseradish Peroxidase
RT-PCR	Real Time Polymerase Chain Reaction
dNTP	Deoxynucleotide Triphosphates
WST-1	Water Soluble Tetrazolium salt-1
SEM	Standard Error of the Mean
ANOVA	Analysis Of Variance
IDV	Integrated Density Values
AAM	African-American Malignant
CAM	Caucasian-American Malignant
AAN	African-American Normal
CAN	Caucasian-American Normal

ABSTRACT OF THE DISSERTATION

Role of IGF-II, PRL, and FABPs in the Breast Cancer Disparity among AA Women

by

Teleka Cassandra Patrick

Doctor of Philosophy, Graduate Program in Biochemistry
Loma Linda University, June 2013
Dr. Daisy D. De León, Chairperson

It is well documented that African-American (AA) patients present with more advanced states of breast cancer and have lower survival rates than Caucasian-Americans (CA). Epidemiological data have shown that pregnancy and lactation at an early age in humans reduce the risk of breast cancer. Studies in mice and rats have shown that lactation stimulates the expression of intracellular fatty-acid binding protein 3 (FABP3). FABP3 is detected in human breast tissue and is known as mammary-derived growth inhibitor (MDGI) because it has a strong inhibitory effect on cell proliferation. FABP5 is also expressed in the breast, and has been associated with metastasis and angiogenesis. Thus we hypothesized that increased expression of FABP3 and decreased expression of FABP5 in the normal breast would be associated with protection against carcinogenesis, and that these changes would be regulated by prolactin (PRL) and IGF-II, which are major mediators of breast differentiation. We also hypothesized that differential expression of these FABPs could provide insight into the breast cancer health disparity, particularly since AA women have lower lactation rates relative to CA women. Therefore, we assessed the expression of these FABPs in paired normal/tumor breast cancer tissues from AA and CA, and used AA and CA-derived cell lines to characterize

potential regulatory mechanisms involving PRL and IGF-II. Our studies have provided much needed information about the mechanisms involved in the protective effect lactation induces in the breast, and potentially offer new tools to prevent breast cancer.

CHAPTER ONE

INTRODUCTION

Breast cancer is the number one cancer affecting women, taking first place in incidence, and second only to lung cancer in mortality. Recently, health disparities between African-American (AA) and Caucasian-American (CA) breast cancer patients have emerged. AA and Hispanic patients present with more advanced stages of breast cancer and have lower survival rates than CA patients [Li et al., 2003].

Some of this disparity may be due to socioeconomic factors [Adams et al., 2009]. In other cases, certain therapies, such as cancer-directed surgery for invasive breast cancer or radiation therapy, are recommended to AA women less frequently [Shavers et al., 2003]. Apart from socioeconomic factors, however, there may be demonstrable biological differences between AA and CA patients, as demonstrated in a recent study. Yancy and coworkers [2007] discovered that there were five metastasis-related genes (Atp1b1, CARD 10, KLF4, Spint2, Acly) whose expression significantly differed between AA and CA breast cancer cell lines. One of these genes, Acly, plays a critical role in fatty acid biosynthesis. Differences in levels of fatty acid binding proteins (FABPs) may be yet another biological factor, which may help to explain the differences in breast cancer presentation and survival between AA and CA patients.

Role of Fatty Acid-Binding Proteins in the Cell

Intracellular fatty acid-binding proteins (FABPs) are about 15 kDa in size, and are encoded by multiple genes. FABPs bind to and increase the solubility of long chain fatty acids (LCFAs) and other hydrophobic ligands. High levels of unbound long-chain fatty acids have adverse effects on the cell, including increased reactive oxygen species production due to mitochondrial uncoupling and inhibition of electron transport within the respiratory chain [Glatz and van der Vusse, 1996; Zimmerman and Veerkamp, 2002; Schonfeld and Wojtczak, 2008]. As shown in Figure 1, FABPs transport LCFAs from the plasma membrane to various sites of fatty acid oxidation, to sites of fatty acid esterification into triacylglycerols or phospholipids, to cytosolic or membrane-bound proteins for metabolic activation, or to the nucleus, to promote transcription of genes by binding nuclear receptors [Hertzel and Bernlohr, 2000; Zimmerman and Veerkamp, 2002; Coe and Bernlohr, 1998]. LCFAs bind to peroxisome proliferator-activated receptors (PPARs) α, β/δ, and γ, while acyl CoA-modified LCFAs (LCFA-CoAs) bind to the PPARs as well as to hepatocyte nuclear receptor-4α [Schroeder et al., 2008]. In this dissertation, we will focus on FABP3 and FABP5, because FABP3 is highly expressed in differentiated mammary tissue, and FABP5 has been shown to be upregulated in pathologic states in various tissues [De León et al., 1996]. The role of FABPs in binding hydrophobic ligands is very critical. Unbound LCFAs may have adverse effects on the cell, such as disturbance of membrane structure and function, inhibition of enzymes, modulation of receptor binding, activation of soluble protein kinase C, activation of brown adipose tissue uncoupling protein, and impairment of ion channels [Glatz and van der Vusse, 1996; Zimmerman and Veerkamp, 2002].

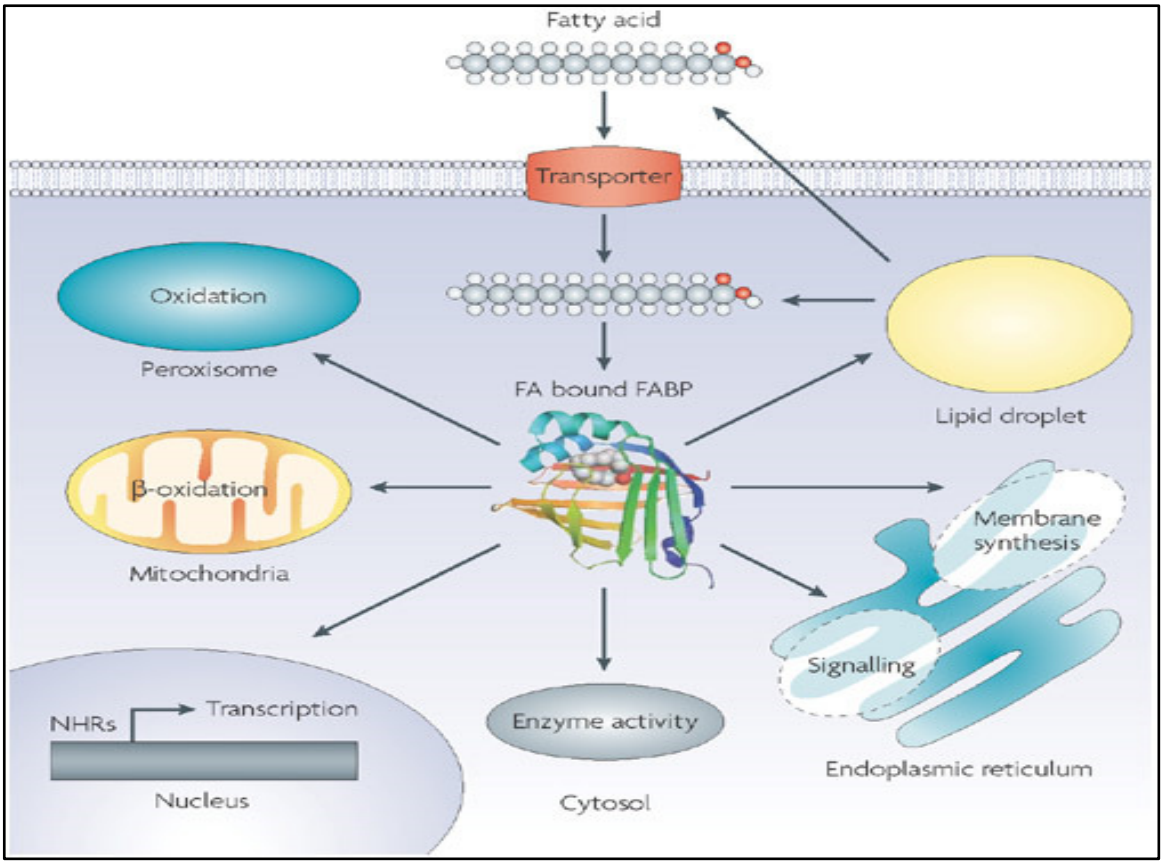

Fatty acid
Transporter
Oxidation
Peroxisome
FA bound FABP
Lipid droplet
β-oxidation
Mitochondria
Membrane synthesis
Signalling
NHRs
Transcription
Nucleus
Enzyme activity
Cytosol
Endoplasmic reticulum

Figure 1. Cellular Function of Fatty Acid-Binding Proteins. FABPs have several roles within the cell, including transporting fatty acids to the nucleus to regulate transcription of target genes. From Furuhashi M and Hotamisligil GS, 2008, Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets, Nat Rev Drug Discov, 7(6), 489-503.

FABP3 Associated with Protection Against Carcinogenesis

FABP3 is also known as mammary derived growth inhibitor (MDGI). It is the most widely distributed of the FABPs, and is expressed in heart, skeletal and smooth muscle, mammary epithelial cells, aorta, distal tubules of the kidney, lung, brain, placenta, and ovary [Zimmerman and Veerkamp, 2002]. FABP3 is known to signal through the nuclear receptor PPARα [Schroeder et al., 2008] by transporting long chain fatty acids to this receptor. Omega-3 fatty acids, which are known to exert antitumor effects, bind to PPARα [Bocca et al., 2008]. PPARα activation has been shown to inhibit tumor growth and angiogenesis in malignant mammary epithelial cells, by enhancing the binding of hypoxia inducible factor 1α (HIF1α) to von Hippel-Lindau tumor suppressor (pVHL). pVHL then causes degradation of HIF-1α through the ubiquitin-proteasome pathway [Zhou et al., 2012], which decreases secretion of vascular endothelial growth factor (VEGF), and inhibits tube formation by endothelial cells. The tube formation assay, which looks at the ability of endothelial cells to form tubes on a gelled basement membrane matrix, is considered to be an *in vitro* assay of angiogenesis [Arnaoutova and Kleinman, 2010]. Thus, FABP3 signaling in the nucleus results in inhibition of angiogenesis, which limits metastasis. Apart from nuclear signaling, FABP3 also binds to integrin α-subunits within the cell to inhibit integrin activity, thus reducing integrin adhesion to type I collagen and fibronectin, and inhibiting cell migration and invasion [Nevo et al., 2010].

Pregnancy and lactation before the age of 24 has been shown to be protective against breast cancer [Russo et al., 2005], and the induction of FABP3 expression in the

breast may help to explain this. According to Russo and coworkers, pregnancy and lactation stimulate the stem cells ("Stem Cells 1") of Lobule 1, which is the cell population that is left after menopause, to differentiate and to express a different genomic signature ("Stem cells 2"). "Stem cells 1" highly express estrogen receptor I, IGF-II receptor, IGF binding proteins 4 and 7, and progesterone receptor. "Stem cells 2" highly express a different set of proteins, including FABP3 and are thought to be refractory to carcinogenesis [Russo et al., 2005]. Studies indicate that AA women have lower breastfeeding rates compared to all other ethnic groups, and therefore the induction of FABP3 expression may not be as significant as in CA women [Bentley et al., 2003]. This difference in lactation rates may be due to lack of education regarding the positive benefits of breastfeeding for mother and child, cultural norms, and individual beliefs [Ibid.]. FABP3 has been shown to regulate growth of normal mouse mammary epithelial cells, to promote normal mammary gland differentiation, and stimulate milk protein synthesis [Yang et al., 1994; Specht et al., 1996; Borchers et al., 1997]. In breast cancer tissues, levels of FABP3 are downregulated [Hertzel and Bernlohr, 2000], and the gene may even be silenced in many human breast cancers [Huynh et al., 1996]. FABP3 has a well-demonstrated tumor-suppressing role: when the gene is introduced into MCF-7 breast cancer cells, they exhibit characteristics of more differentiated cells [Borchers et al., 1997; Huynh and Beamer, 2008].

FABP5 Associated with Tumor Aggressiveness

FABP5 is also expressed in multiple tissues. It can be found in skin, lens, adipose tissues, endothelial cells, lung, mammary cells, stomach, tongue (especially stratified epithelia), stratified epithelia of epidermis, stomach, placenta, heart, brain, liver, spleen, skeletal muscle, intestine, testis, urothelium, and retina [Hertzel and Bernlohr, 2000]. In nerve cells FABP5 has been shown to be involved in stress response and regulation of neurite growth and neuronal differentiation [De León et al., 1996; Liu et al., 2008; Allen et al., 2000; Allen et al., 2001]. Similarly to FABP3, FABP5 binds to long chain polyunsaturated fatty acids and translocates them to the nucleus, to bind to PPARβ/δ. This then causes upregulation of VEGF [Kannan-Thulasiraman et al., 2010; Schug et al., 2007]. VEGF is overexpressed in a significant percentage of breast tumors, and this expression has been associated with a worse prognosis in patients with invasive breast disease [Bajo et al., 2004; Heffelfinger et al., 1999; Gieseler et al., 2007]. FABP5 also competes with cellular retinoic acid binding protein 2 (CRABP-2) for retinoic acid. FABP5 binds retinoic acid, keeping it in the cytoplasm and preventing it from being translocated to the nucleus by CRABP-2, thus inhibiting tumor growth [Liu et al., 2011].

FABP5 has been shown to be upregulated in a number of altered states with disturbed lipid profiles. The gene has been shown to induce metastasis, and the levels of mRNA are 6.5-fold higher in malignant breast cell lines compared to Huma 121 benign lines [Jing et al., 2000]. A high correlation has been demonstrated between elevated FABP5 mRNA and protein levels and estrogen receptor/progesterone receptor-negative status, high tumor grade, and poor prognosis [Liu et al., 2011]. Another study

by Hammamieh and colleagues [2005] demonstrated that FABP5 levels were decreased in malignant breast cell lines compared to normal human mammary epithelial cells. Within tissues, FABPs have a compensatory relationship. For example, in FABP5-gene knockout mice, FABP3 was elevated in the liver [Owada et al., 2002] and in FABP4 (adipocyte type)- knockout mice, FABP5 mRNA was upregulated in adipocytes 20- to 40-fold [Hertzel and Bernlohr, 2000]. Therefore, it is reasonable to believe that levels of FABP5 would be increased in the breast cancer state, since levels of FABP3 are decreased.

Significance of Studying FABP Levels in Breast Tissues

The literature describing differential expression of FABPs in benign and malignant breast tissues/cells is very scarce. Additionally, levels of FABPs 3 and 5 have not been specifically measured in AA and CA tissues/cell lines. Therefore, measuring the levels of FABPs 3 and 5 in normal and malignant tissues from AA and CA women, and in normal and malignant cell lines established from AA and CA women, will add to this body of literature. Establishing differential expression between these ethnic groups may help to elucidate the breast cancer health disparity. For example, if FABP3 is significantly lower in AA normal tissues compared to CA normal tissues/cell lines due to decreased lactation and induction of FABP3 expression in the former, this may indicate a lack of protection against cancer in normal AA breast tissue. Furthermore, we will be able to determine if the patterns of expression differ among age groups (ages below 50, and ages 50 and older), and if certain age groups may be at higher risk for breast cancer, based on FABP3 expression, relative to other age groups.

Role of IGF-II in Normal Breast Differentiation and in Carcinogenesis

The expression of FABP3 and FABP5 may be mediated through insulin growth factor II (IGF-II). PRL regulation of IGF-II mRNA has been demonstrated, and since PRL and FABP3 are associated with mammary differentiation, it is feasible that IGF-II may also regulate FABP expression [Brisken et al., 2002]. IGF-II, a major mediator of proliferation in breast cancer, is expressed at high levels in cancer tissues due to a loss of parental imprinting. Figure 2 shows the ligands and receptors of the IGF family. IGF-II signals classically through the type 1 IGF receptor (IGF-IR), resulting in growth promoting effects through activation of the PI3/Akt pathway and induction of cyclin D1 protein [Sachdev and Yee, 2006]. IGF-II also signals through other receptors, including insulin receptor A (IR-A) for metabolic effects, and IGF-IIR for IGF-II degradation [O'Dell and Day, 1998; Scott and Firth, 2004; Pandini et al., 1999; Belfiore et al., 2009]. IGF-II has short and long forms, known as mIGF-II (mature IGF-II) and proIGF-II. ProIGF-II and mIGF-II bind the IGF-1R with the same affinity, but proIGF-II is highly expressed in cancer, and has been shown to be more potent than mIGF-II in activating the PI3/Akt pathway [Singh et al., 2007; Singh et al., 2008].

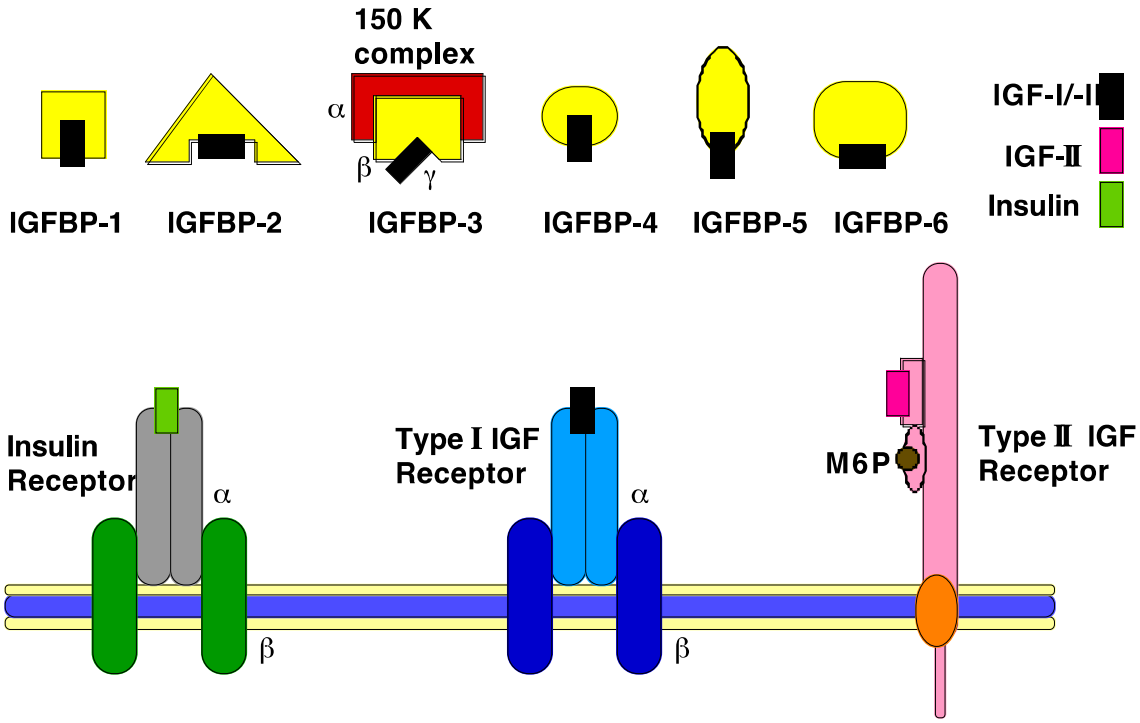

150 K
complex
α
β
γ
IGF-I/-II
IGF-II
Insulin
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
IGFBP-6
Insulin
Receptor
α
β
Type I IGF
Receptor
α
β
M6P
Type II IGF
Receptor

Figure 2. The IGF Family- Ligands and Receptors. IGF-II signals classically through the type 1 IGF receptor (IGF-IR) resulting in growth promoting effects, through the insulin receptor for metabolic effects, and through the type II IGF receptor for IGF-II degradation.

Role of PRL in Normal Breast Differentiation and in Carcinogenesis

Another major proliferative mediator in breast cancer is PRL, which is produced by the pituitary gland and locally in breast tissue. The Jak2-Stat5 pathway is the best studied. When PRL binds to the long form of the PRL receptor (PRLR), there is a conformational change in and activation of preformed PRLR dimers. This causes Jak2 trans-phosphorylation, and eventual activation of Stat5 proteins by Jak2. Stat5 proteins become either homo- or heterodimers with other transcription factors, and then go to the nucleus, where they regulate gene transcription of beta-casein, cyclin D1, and other genes [Chen et al., 2009; Oakes et al., 2008; Clevenger et al., 2009]. PRL also activates MAP kinase, PKC delta, c-Src, and PI3-kinase, estrogen receptor alpha, and potassium channels in addition to the Jak2-Stat5 pathway [Chen et al., 2009; Gonzalez et al., 2009]. Though the PRLR long form is prototypical and has the entire spectrum of PRLR signaling capabilities, there are also short forms of the receptor which attenuate signaling [Swaminathan et al., 2008; Tan and Walker, 2009]. In the normal breast state, PRL is important in proliferation and differentiation of normal mammary epithelium and in stimulation of lactation (Figure 3). Mean basal daily levels of PRL are 9-10 ng/ml in plasma. Levels increase during pregnancy (15 to 25 times basal) and lactation (up to 30 times basal) and lead to full lobuloalveolar development of the breast [Tworoger and Hankinson, 2006; Bercovich and Goodman, 2009]. As aforementioned, IGF-II mediates the differentiating effect of PRL in the breast through upregulation of cyclin D1 [Brisken et al., 2002]. Pregnancy and lactation, especially before the age of 24, has been shown to reduce pregnancy risk [Russo et al., 2005]. This may be due to the long-lasting reduction in PRL levels after

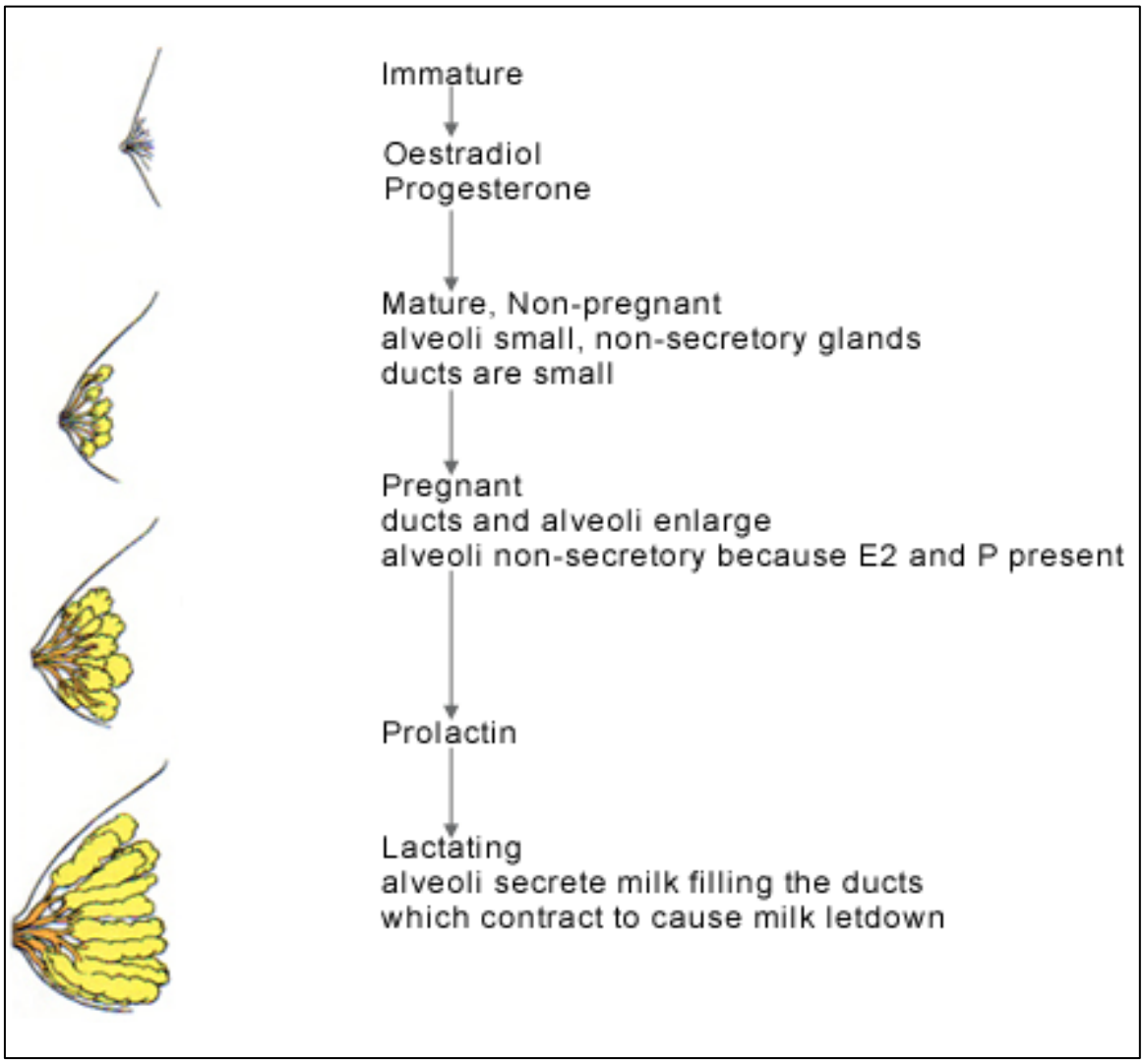

Immature
Oestradiol
Progesterone
Mature, Non-pregnant
alveoli small, non-secretory glands
ducts are small
Pregnant
ducts and alveoli enlarge
alveoli non-secretory because E2 and P present
Prolactin
Lactating
alveoli secrete milk filling the ducts
which contract to cause milk letdown

Figure 3. Hormonal Regulation of Human Breast Development. PRL acts on the breast tissue during pregnancy and lactation to induce terminal development of the milk glands. From http://www.elu.sgul.ac.uk/rehash/guest/scorm/228/package/content/index.htm

a first pregnancy that is consistently observed in both premenopausal and postmenopausal women [Tworoger and Hankinson, 2008]. In the breast cancer state, PRL fails to trigger differentiation and instead enhances breast epithelial survival by preventing the apoptosis of cancer cells, enhancing cell motility and invasive growth, and possibly supporting tumor vascularization [McHale et al., 2008; Howell et al., 2008; Abdelmagid and Too, 2008; Plotnikov et al., 2009; Tworoger and Hankinson, 2006]. Also, there is a decrease in the ratio of short forms to PRLR in tumor tissue and breast cancer cell lines, and thus, a loss of regulation [Swaminathan et al., 2008]. Since FABP3 is strongly expressed in breast tissues during the breast differentiation that is associated with pregnancy [Russo et al., 2005], it is possible that PRL may induce FABP3 expression.

Significance of Studying IGF-II and PRL Regulation of FABP Expression

The roles of IGF-II and PRL in breast cancer have been more than adequately investigated and demonstrated. However, a possible relationship between PRL/IGF-II and FABPs 3 and 5 has not been studied. Also, there have been no studies performed to date regarding the protective effect of significantly increased FABP3 levels, induced by PRL, in mammary epithelial cells. Determining the effect of PRL and IGF-II on FABP3 levels in normal breast cell lines, established from AA and CA women, would possibly elucidate additional regulatory mechanisms for FABPs 3 and 5, and would uncover therapeutic targets through which FABP3 could be upregulated. If we can demonstrate that FABP3 levels are regulated by IGF-II, then IGF-II levels could potentially be lowered in breast cancer patients so as to increase FABP3 levels and

thus cause tumor tissue to revert to more normal behavior. If we can demonstrate that FABP3 levels are regulated by PRL, then PRL levels could potentially be increased in normal women, short-term, so as to induce protective FABP3 expression. Furthermore, examining the differential expression of FABP3 in response to IGF-II and PRL in AA and CA normal breast cell lines allows us to determine the potency of response between the ethnic groups, and is an exciting, groundbreaking study.

Therefore, we proposed to complete the following: assess expression of FABPs 3 and 5 in paired normal/tumor breast tissues from AA and CA patients (from Human Cooperative Tissue Network) to determine if lower expression of FABP3/higher expression of FABP5 among AA women is associated with the disparity in breast cancer progression observed between AA and CA patients; assess expression of FABPs 3 and 5 in normal and malignant breast epithelial cell lines established from CA and AA patients; and simulate breast differentiation by treating normal breast epithelial cell lines established from AA and CA patients with PRL to determine and compare effects on expression of IGF-II, FABP3, and FABP5. Our studies have provided much needed information about the mechanisms involved in the protective effect lactation induces in the breast and will potentially offer new tools for preventive breast cancer treatment.

CHAPTER TWO

PRL AND IGF-II REGULATION OF FATTY ACID-BINDING PROTEINS 3 AND 5 IN NORMAL BREAST EPITHELIAL CELLS

Abstract

Epidemiological studies have shown that lactation reduces the risk of breast cancer in young women. Lactation's protective effect is in part mediated by fatty-acid binding protein 3 (FABP3), which is associated with breast differentiation. FABP5, a member of the same family, is also expressed in the breast and stimulates angiogenesis, essential for lactation. The effects of prolactin (PRL), which stimulates breast differentiation and lactation, are mediated by IGF-II. Thus, we propose that PRL treatment of normal epithelial breast cell lines increases IGF-II to promote differentiation, regulating FABP3 and FABP5. We treated MCF10A and AG11132 normal breast epithelial cell lines with recombinant human PRL (0-200 ng/ml) for 24 hours. Cell lysates were examined for protein and mRNA levels of IGF-II, FABP3, and FABP5. In MCF10A cells, PRL treatment decreased FABP5 protein ($p=0.044$). IGF-II mRNA levels significantly increased when cells were treated with 100 and 200 ng/ml PRL (2- and 2-fold, respectively). In AG11132 cells, phosphorylated FABP3 protein increased, and FABP5 protein decreased, with increasing PRL concentration ($p=0.037$, 0.028, respectively). IGF-II mRNA levels increased at 10 ng/ml PRL (34-fold). Though both cell lines responded to PRL with decreased FABP5 protein and increased IGF-II mRNA, only AG11132 displayed increased FABP3 protein. The dissimilar responsiveness of

these cell lines to PRL may suggest decreased protection against breast carcinogenesis in AA patients, and may help us to further understand this health disparity.

Introduction

Breast cancer is the number one cancer affecting women, taking first place in incidence, and second only to lung cancer in mortality. Recently, health disparities between African-American (AA) and Caucasian-American (CA) breast cancer patients have come to the forefront. AA and Hispanic patients present with more advanced stages of breast cancer and have lower survival rates than Whites [Li et al., 2003]. Additionally, the age specific breast cancer incidence rate for AA women under age 35 is more than twice the rate for white women of similar age, and the mortality rate is more than three times higher [Shavers et al., 2003].

Socioeconomic factors play a predominant role in this disparity [Adams et al., 2009]. In other cases, certain therapies, such as cancer-directed surgery for invasive breast cancer or radiation therapy, are recommended to AA women less frequently [Shavers et al., 2003]. Apart from socioeconomic factors, however, there is evidence of biological differences between AA and CA patients, as demonstrated in a recent study. Yancy and coworkers discovered that there were five metastasis-related genes (Atp1b1, CARD 10, KLF4, Spint2, Acly) whose expression significantly differed between AA and CA breast cancer cell lines [Yancy et al., 2007]. Further, we have reported that AA cell lines and breast tissues contain higher levels of IGF-II relative to CA cell lines and tissues [Kalla Singh et al., 2010]. Differences in levels of fatty acid binding proteins (FABPs) may be yet another biological factor, which may help to explain the differences in breast cancer presentation and survival between AA and CA patients.

Intracellular fatty acid-binding proteins (FABPs) are about 15 kDa in size, and are encoded by multiple genes. FABPs increase fatty acid solubility and transport them from the plasma membrane to various sites within the cell, including to the nucleus, to promote transcription of genes by binding peroxisome proliferator-activated receptors (PPARs) [Hertzel and Bernlohr, 2000; Zimmerman and Veerkamp, 2002; Glatz and van der Vusse, 1996]. Several FABPs are expressed in mammary tissue, including FABP3 and FABP5. FABP3 has been shown to be highly expressed in differentiated mammary tissue, while FABP5 has been shown to be upregulated in pathologic states in various tissues throughout the body [De León et al., 1996].

FABP3, also known as mammary derived growth inhibitor (MDGI), has been shown to regulate growth of normal mouse mammary epithelial cells, promote normal mammary gland differentiation, and stimulate milk protein synthesis [Yang et al., 1994; Specht et al., 1996; Borchers et al., 1997]. FABP3 binds to the nuclear receptor PPARα, and inhibits production of VEGF, which promotes angiogenesis and metastasis [Schroeder et al., 2008]. Additionally, stimulation of PPARα and PPARγ causes FABP3 upregulation [Motojima, 2000; Lindegaard and Nielsen, 2008; Keen et al., 2004]. In breast cancer tissues, levels of FABP3 are downregulated, and the gene may even be silenced in many human breast cancers [Hertzel and Bernlohr, 2000; Huynh et al., 1996]. FABP3 has a well-demonstrated tumor-suppressing role: when the gene is introduced into MCF-7 breast cancer cells, they revert to more normal behavior [Huynh et al., 1996; Huynh and Beamer, 1998]. Pregnancy and lactation has been shown to be protective against breast cancer development in rats and in humans [Russo et al., 1982; Sinha et al., 1988; Russo et al., 2005], and the induction of FABP3 expression in the breast may help

to explain this. Pregnancy and lactation alter the genetic profile of the mammary cells so as to render them refractory to carcinogenesis, and FABP3 is the only FABP that is a part of this protective genetic profile [Russo et al., 2005]. Studies indicate that AA women have lower lactation rates compared to all other ethnic groups, and therefore the induction of FABP3 expression may not be as significant in this group as a whole, relative to CA women [Bentley et al., 2003]. FABP3 levels may be regulated by PRL and IGF-II, as PRL is the major hormonal influence during pregnancy and lactation, and IGF-II mediates PRL's effects [Oakes et al., 2008; Brisken et al., 2002]. During pregnancy, PRL levels in the plasma increase to 15 to 25 times the basal level; during lactation, levels increase up to 30 times the basal level. This increased expression leads to full lobulo-alveolar development of the breast [Tworoger and Hankinson, 2006; Bercovich and Goodman, 2009]. PRL binds to its receptor (PRLR) to effect these changes. There are several forms of the PRLR, including the long form (85-95 kDa), the delta S1 form and S2 forms (both 70 kDa), the intermediate form (50 kDa), the short form 1b (SF1b; 29 kDa), and the short form 1a (SF1a; 39 kDa). The delta S1 form has the full signaling capabilities of the long form but requires more ligand, the delta S2 form prevents constitutive activation of the PRLR, and the intermediate form enhances cell survival but has limited proliferative effects. The short forms are dominant negatives to the long form, with SF1b being stronger than SF1a [Clevenger et al., 2009; Swaminathan et al., 2008; Tan and Walker, 2010; Tan et al., 2008].

FABP5 is upregulated in a number of states with disturbed lipid profiles. The literature shows that FABPs have a compensatory relationship within tissues [Zimmerman and Veerkamp, 2002; Shaughnessy et al., 2000]. For example, in FABP5-

gene knockout mice, FABP3 was elevated in the liver [Owada et al., 2002] and in FABP4 (adipocyte type)- knockout mice, FABP5 mRNA was upregulated in adipocytes 20- to 40-fold [Hertzel and Bernlohr, 2000]. The *fabp5* gene is an inducer of metastasis, and its mRNA levels are 6.5-fold higher in malignant breast cell lines compared to HUMA 121 benign lines [Jing et al., 2000]. It is thought that FABP5 transports nucleic acids to the nuclear receptor PPAR β/δ, where they bind and cause upregulation of vascular endothelial growth factor (VEGF) [Kannan-Thulasiraman et al., 2010; Schug et al., 2007]. VEGF is overexpressed in a significant percentage of breast tumors, and this expression has been associated with a worse prognosis in patients with invasive breast disease [Bajo et al., 2004; Heffelfinger et al., 1999; Gieseler et al., 2007]. Though FABP5 has a metastasis-promoting role in cancer cells, it has a beneficial role in breast differentiation. Bovine studies have shown that both FABP3 and FABP5 mRNA increase upon pregnancy and lactation (which corresponds with breast differentiation); however, FABP3 increases to a greater extent than FABP5, and stays elevated for a longer period of time [Bionaz and Loor, 2008]. Thus, PRL, the main hormone that induces breast differentiation during pregnancy and lactation, may also regulate FABP5 levels, though this has not yet been shown in the literature. Due to FABPs' compensatory relationship, it is also possible that a large increase in FABP3 could be associated with a FABP5 decrease.

To determine if response to PRL was a factor in the differential FABP3 levels expressed in AA and CA benign tissues in ages below 50, we treated AA and CA benign cell lines with recombinant human PRL and measured the change in FABP3 protein and mRNA. We also measured FABP5 levels to determine if there would be a compensatory

relationship between FABP5 and FABP3 levels in these cells, and if decreased FABP5 would be associated with differentiation in these cells. Since IGF-II is the mediator of PRL's effects, we measured IGF-II levels, and thus assessed for IGF-II regulation of FABP changes. Lastly, we measured PRL receptor (PRLR) levels to account for potential differences in forms of PRLR expressed, and in the responses of the cell lines to PRL.

Materials and Methods

Cell Culture

MCF10A normal breast epithelial cell line was obtained from the American Type Culture Collection (Manassas, VA). This non-tumorigenic, estrogen and progesterone receptor negative line was established from a 36 year old Caucasian-American patient with fibrocystic disease. MCF10A cells were maintained in a 5% CO_2 incubator at 37 ^{o}C, using Dulbecco's modified eagle's medium (DMEM)/F12 50:50 media (Cellgro) supplemented with 5 ml of penicillin/streptomycin (10,000 I.U./ml penicillin, 10,000 µg/ml streptomycin sulfate; Cellgro), 2 mM L-glutamine (Cellgro), 10 µg/ml insulin (Sigma, St. Louis, MO), 0.5 µg/ml hydrocortisone (Sigma), murine epidermal growth factor (EGF) 20 ng/ml (Sigma), cholera toxin 100 ng/ml (Sigma), and 5% bovine serum albumin (HyClone). AG11132 normal breast epithelial cell line was obtained from the Coriell Institute (Camden, NJ). This non-tumorigenic, estrogen receptor poor line was established from a 16 year old African-American patient who underwent reduction mammoplasty. AG11132 cells were maintained in a 5% CO_2 incubator at 37 ^{o}C, using Clonetics Mammary Epithelium Basal Medium (MEBM) media (Lonza) supplemented with 70 µg/ml bovine pituitary extract, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, and 5

ng/ml human epidermal growth factor (EGF) from the MEGM BulletKit; 10 µM isoproterenol (Sigma); and 5 µg/ml transferrin (Sigma). Recombinant human PRL was purchased from R&D Systems (Minneapolis, MN). MCF10A and AG11132 cells were seeded at a density of 2 x 10^5 cells/well in six-well plates; one plate per cell line to allow for triplicate assays. Cells were incubated at 37 ^oC in a 5% CO_2 incubator. At 80% confluence, media on both cell lines was changed to serum-free DMEM supplemented with 5 ml penicillin/streptomycin, and cells were incubated for 6 hours prior to treatment. PRL treatment was then performed for 24 hours in fresh serum-free DMEM supplemented with 5 ml penicillin/streptomycin; cells were incubated for the duration of treatment. Cell lysates for protein analysis were prepared using radioimmuniprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology, Santa Cruz, CA), according to manufacturer protocol (http://www.cellsignal.com/products/9806.html) and stored at -20 ^oC until assayed. Cell lysates for RNA analysis were prepared using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer protocol (http://www.mrcgene.com/tri.htm) and stored at -20 ^oC until assayed.

PRL Treatment

MCF10A and AG11132 cells were seeded at a density of 2 x 10^5 cells/well in six-well plates. Recombinant human PRL (R&D Systems, Minneapolis, MN) was diluted in vehicle of 1X PBS w/0.1% BSA. Various concentrations of PRL—0 (vehicle control), 10, 50, 100, and 200 ng/ml were added to the cells followed by 24 h incubation at 37 ^oC in a 5% CO_2 incubator. Concentrations were chosen to mimic physiological basal PRL concentration (9-10 ng/ml), as well as concentrations during pregnancy and lactation

(150-300 ng/ml). Treatments were performed in triplicate. Cell lysates for protein analysis were prepared using RIPA buffer according to manufacturer protocol and stored at -20 ^{o}C until assayed. Cell lysates for RNA analysis were prepared using TRI Reagent according to manufacturer protocol and stored at -20 ^{o}C until assayed.

Western Blot Analysis

Total protein (30 µg) of cell lysates were collected after 24 h of PRL treatment and used to load polyacrylamide sodium dodecyl sulfate (SDS) gradient gels (4-12%), transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) using a X-Cell SureLock ® electrophoretic transfer module (Invitrogen). Protein concentration was measured using the Coomassie Plus Protein Assay Reagent ™ (Pierce Biotechnology, Rockford, IL). PVDF membranes were then blocked with 5% non-fat milk or 5% bovine serum albumin (Sigma) in PBS/0.05% Tween for 1 h. Membranes were then incubated with IGF-II monoclonal antibody (Amano, Mitsubishi, Troy, VA), FABP3 monoclonal antibody (Abcam, Cambridge, UK), FABP5 polyclonal antibody (Abcam, Cambridge, UK), or PRL receptor monoclonal antibody (Invitrogen), followed by overnight incubation at 4 ^{o}C. The blots were also probed with ß-actin antibody (Sigma) as a protein loading control. After 3 x 10 min washes in PBS/0.05% Tween, the corresponding secondary antibodies (1:1000, GE Healthcare, Piscataway, NJ) were added to the membranes (1 h at room temperature), followed by 3 x 10 min washes and incubation with horseradish peroxidase (HRP) complexes (1:2000, GE Healthcare). Protein visualization was achieved by using enhanced chemiluminescence (ECL) and autoradiography with Blue Lite Autorad film (GeneMate from BioExpress, Kaysville,

UT). The signals on the X-ray films were quantified using QuantityOne 1-D Analysis Software v. 4.5.0 (BioRad, Hercules, CA).

Real-Time PCR

Two-step SYBR real-time-PCR (RT-PCR) was performed to assess gene expression in PRL- treated cells at 24 h, using the primers FABP3-forward (5'-CAC TAT GGT GGA CGC TTT CC-3'), and FABP3-reverse (5'-GTG GTA GGC TTG GTC ATG CT-3'); FABP5-forward (5'-ACA GAT GGT GCA TTG GTT CA-3'), and FAB5-reverse (5'-GAT CCG AGT ACA GGT GAC ATT G-3'); IGF-II-forward (5'-GAC CGC GGC TTC TAC TTC AG-3'), and IGF-II reverse (5'-AAG AAC TTG CCC ACG GGG TAT-3'). Primers for FABP3 utilized NCBI RefSeq NM_004102.3 and resulted in a 126 bp-length product; primers for FABP5 utilized NCBI RefSeq NM_001444.2 and resulted in a 123 bp-length product. Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu/) was used to generate primers, and NCBI PrimerBlast was used to test specificity. The iScript cDNA synthesis kit (Bio-Rad) and GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA) were used to synthesize cDNA; 400 ng of RNA was used per synthesis reaction. RT-PCR was performed using the iQ5 (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad). Reactions were performed in a mixture consisting of a 25 µl volume solution containing SYBR Green supermix PCR buffer (Bio-Rad; 2x reaction buffer with dNTPs, iTaq DNA polymerase, 6 mm $MgCl_2$, SYBR Green I, fluorescein, and stabilizers), 1 µl of synthesized cDNA, 400 nM of each primer, and sterile water. Forty cycles of denaturation (95 ^{o}C, 15 s), annealing (60 ^{o}C, 30 s), and elongation (72 ^{o}C, 10 s) were performed. Fluorescence was detected at the end of every

72 ^{o}C extension phase. Melting curve analysis was done to exclude contamination by non-specific PCR products.

Cell Viability Assay

Cell viability was measured by the colorimetric water soluble tetrazolium salt-1 (WST-1) assay (Roche, Mannheim, Germany), according to manufacturer protocol. Cells (5000 cells/well) were seeded in 96-well plates and grown in culture media as described above. Cell viability was assessed at 24 h post-treatment with PRL by reading the absorbance at 450 nm.

Statistical Analysis

Values are expressed as the mean ± SEM of three replicate experiments. For protein values, statistical differences were determined by independent samples t-test (to compare 2 means) or one-way ANOVA (to compare greater than 2 means), SPSS 19.0 software (IBM, Armonk, New York). A level of $p < 0.05$ was considered significant. For mRNA values, fold change was calculated, and statistical significance was set at a fold change of 2 or greater.

Results

Untreated Cells

Protein Levels

As shown in Figure 4, FABP3 and 5 protein levels in untreated AG11132 and MCF10A cells were significantly higher in the MCF10A cell line relative to the

AG11132 line ($p < 0.001$ for both FABPs). Figure 5 demonstrates that protein levels of PRL and of IGF-II were significantly higher in MCF10A cells ($p = 0.003$, 0.002, respectively) relative to AG11132 cells. FABP3 mRNA was detectable in AG11132 cells but not in MCF10A cells. FABP5 was 6052-fold higher than FABP3 in AG11132 cells.

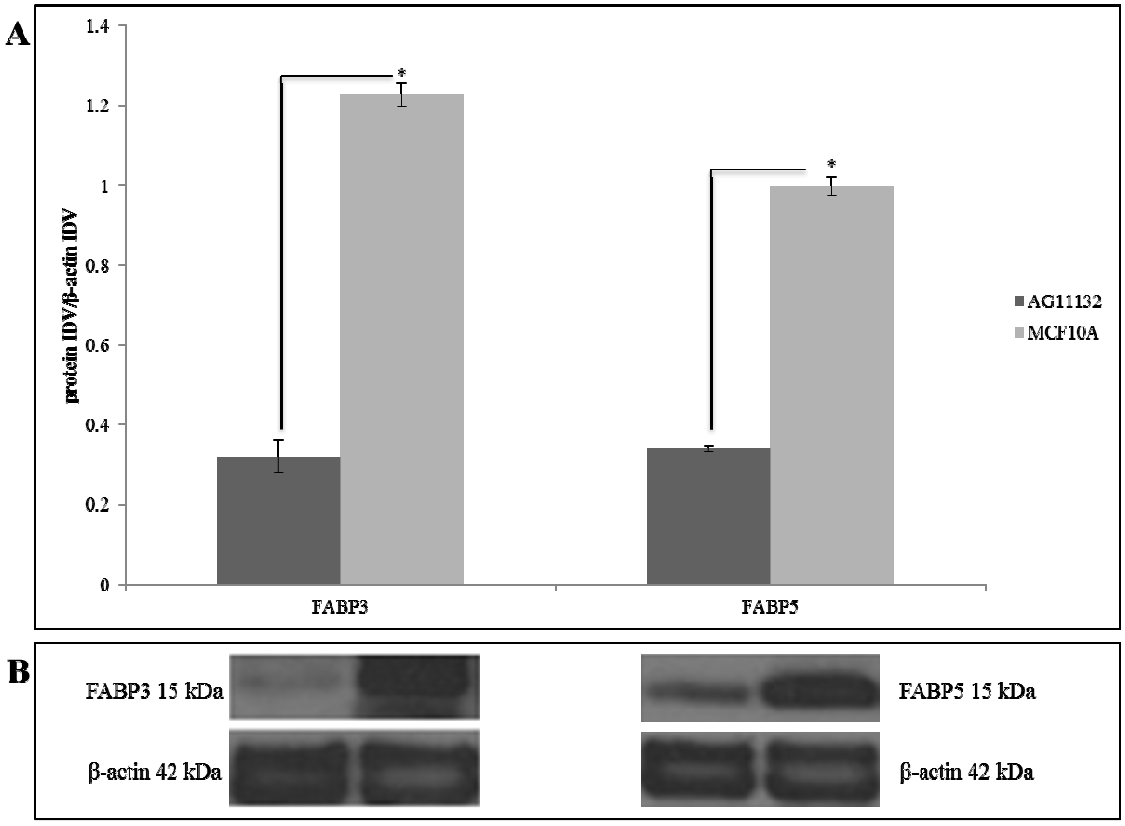

A
1.4
1.2
1
0.8
0.6
0.4
0.2
0
protein IDV/β-actin IDV
*
*
AG11132
MCF10A
FABP3
FABP5
B
FABP3 15 kDa
β-actin 42 kDa
FABP5 15 kDa
β-actin 42 kDa

Figure 4. Western Blot of FABPs 3 and 5 in Nontransformed Breast Epithelial Cells. A. Levels of FABPs 3 and 5 in AG11132 and MCF10A cells. FABP3 and FABP5 were significantly increased in MCF10A cells relative to AG11132 cells ($p < 0.001$ for both FABPs). IDV- integrated density values. B. Representative Western blots of FABPs 3 and 5 in AG11132 and MCF10A cells.

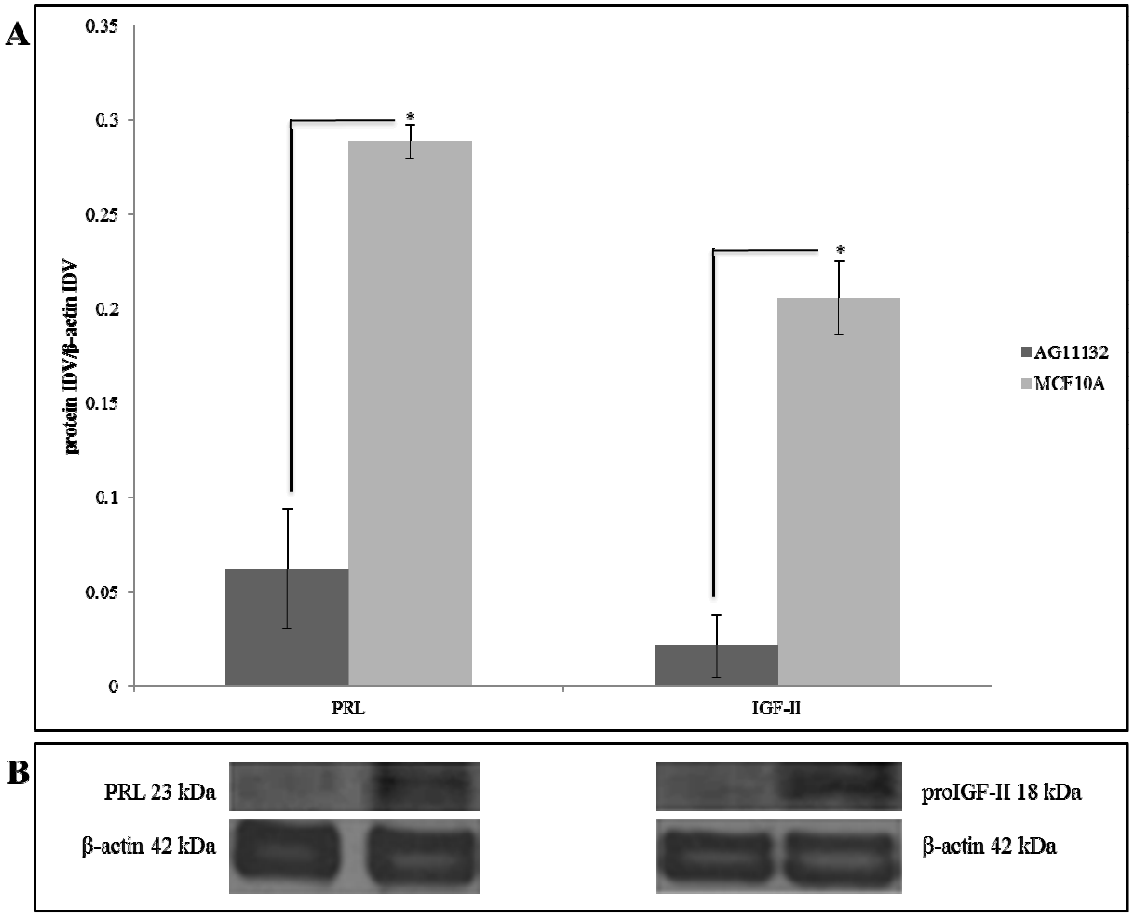

A
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
protein IDV/β-actin IDV
*
*
PRL
IGF-II
AG11132
MCF10A
B
PRL 23 kDa
β-actin 42 kDa
proIGF-II 18 kDa
β-actin 42 kDa

Figure 5. Western Blot of PRL and IGF-II in Nontransformed Breast Epithelial Cells. A. Levels of PRL and IGF-II in AG11132 and MCF10A cells. PRL and IGF-II were significantly increased in MCF10A cells relative to AG11132 cells ($p = 0.003$ for PRL, $p = 0.002$ for IGF-II). IDV- integrated density values. B. Representative Western blots of PRL and IGF-II in AG11132 and MCF10A cells.

Messenger RNA Levels

As shown in Figure 6, FABP5 mRNA was 2-fold higher in MCF10A cells relative to AG11132 cells. Conversely, PRL and IGF-II mRNA were higher in AG11132 cells relative to MCF10A cells (2-fold and 43-fold, respectively).

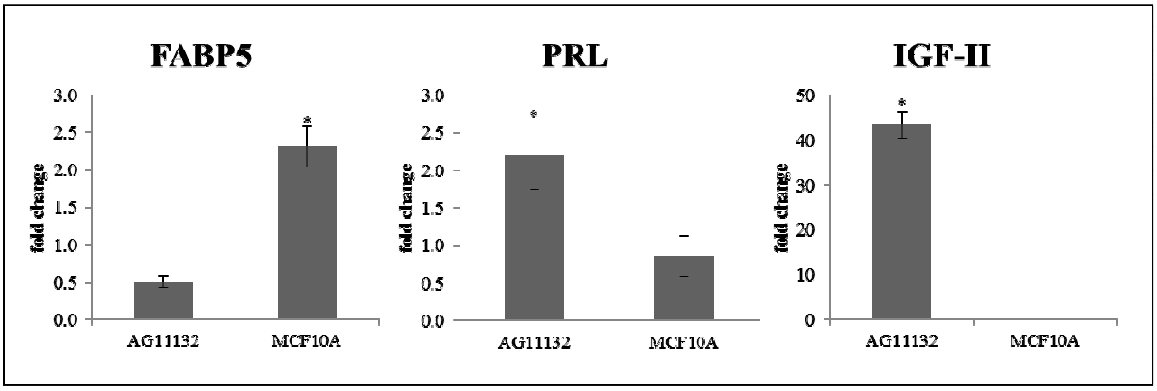

FABP5
PRL
IGF-II
fold change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
40
30
20
10
0
*
AG11132
MCF10A

Figure 6. Messenger RNA Levels of FABP5, IGF-II, and PRL in Nontransformed Breast Epithelial Cells. FABP5 mRNA was 2-fold higher in MCF10A cells relative to AG11132 cells. PRL and IGF-II mRNA were higher in AG11132 cells relative to MCF10A cells (2-fold and 43-fold, respectively). FABP3 mRNA was not detected in MCF10A, thus a comparison could not have been made.

Protein Response

Figure 7 shows the protein response of AG11132 cells to PRL treatment. FABP3 (18 kDa form) at 200 ng/ml was significantly increased relative to control ($p=0.037$). FABP5 was significantly decreased at 200 ng/ml relative to level at 10 ng/ml ($p=0.028$). No significant change was noted in IGF-II protein. Figure 8 shows the protein response of MCF10A cells to PRL treatment. FABP3 (18 kDa form) was significantly decreased at 200 ng/ml relative to levels at 0, 10, 50, and 100 ng/ml ($p=0.002$, 0.004, <0.001, 0.001, respectively). FABP5 at 200 ng/ml was significantly decreased relative to level at 50 ng/ml ($p=0.044$). No significant change was noted in IGF-II protein.

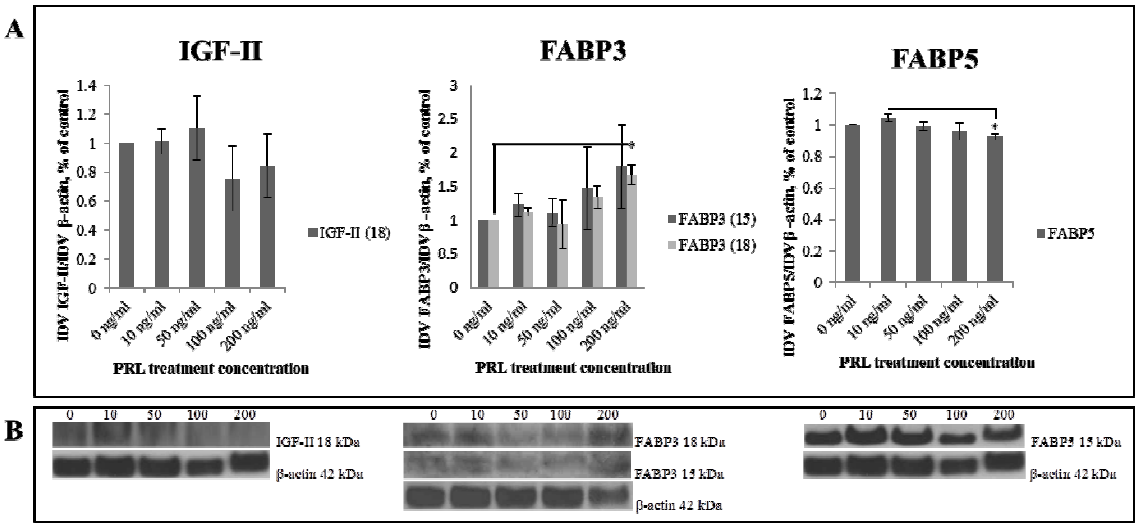

A
IGF-II
FABP3
FABP5
IDV IGF-II/IDV β -actin, % of control
IDV FABP3/IDV β -actin, % of control
IDV FABP5/IDV β -actin, % of control
IGF-II (18)
FABP3 (15)
FABP3 (18)
FABP5
0 ng/ml
10 ng/ml
50 ng/ml
100 ng/ml
200 ng/ml
PRL treatment concentration
B
IGF-II 18 kDa
β-actin 42 kDa
FABP3 18 kDa
FABP3 15 kDa
FABP5 15 kDa

Figure 7. Western Blot of IGF-II, FABP3, and FABP5 in PRL-Treated AG11132 Cells. A. Levels of IGF-II, FABP3, and FABP5 protein at various PRL treatment concentrations. FABP3 (18 kDa form) was significantly increased at 200 ng/ml ($p=0.037$) relative to control. FABP5 was significantly decreased at 200 ng/ml relative to level at 10 ng/ml ($p=0.028$). IDV- integrated density values. B. Representative Western blots of IGF-II, FABP3, and FABP5 in PRL-treated AG11132 cells.

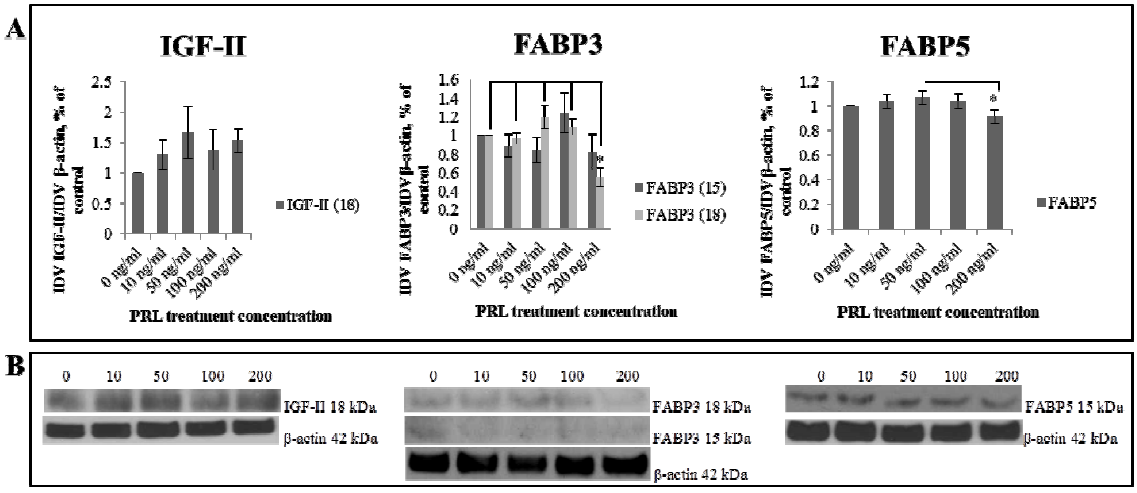

A
IGF-II
FABP3
FABP5
IDV IGF-II/IDV β-actin, % of control
IDV FABP3/IDV β-actin, % of control
IDV FABP5/IDV β-actin, % of control
IGF-II (18)
FABP3 (15)
FABP3 (18)
FABP5
0 ng/ml
10 ng/ml
50 ng/ml
100 ng/ml
200 ng/ml
PRL treatment concentration
B
0
10
50
100
200
IGF-II 18 kDa
β-actin 42 kDa
FABP3 18 kDa
FABP3 15 kDa
FABP5 15 kDa

Figure 8. Western Blot of IGF-II, FABP3, and FABP5 in PRL-Treated MCF10A Cells. A. Levels of IGF-II, FABP3, and FABP5 protein at various PRL treatment concentrations. FABP3 (18 kDa form) was significantly decreased at 200 ng/ml relative to levels at 0, 10, 50, and 100 ng/ml ($p = 0.002, 0.004, <0.001, 0.001$, respectively). FABP5 was significantly decreased at 200 ng/ml relative to level at 50 ng/ml ($p=0.044$). IDV- integrated density values. B. Representative Western blots of IGF-II, FABP3, and FABP5 in PRL-treated MCF10A cells.

Messenger RNA Response

Figure 9 shows that for AG11132 cells, levels of IGF-II mRNA were significantly increased at 10 ng/ml and 50 ng/ml (fold change 34 and 2, respectively). IGF-II mRNA levels decreased with further increase in PRL treatment concentration. No significant changes were seen in FABP3 or FABP5 mRNA levels. The mRNA response of MCF10A cells is shown in Figure 10. IGF-II levels were significantly increased at 100 and 200 ng/ml (fold change 2 and 2, respectively). No significant changes were seen in FABP3 or FABP5 mRNA levels.

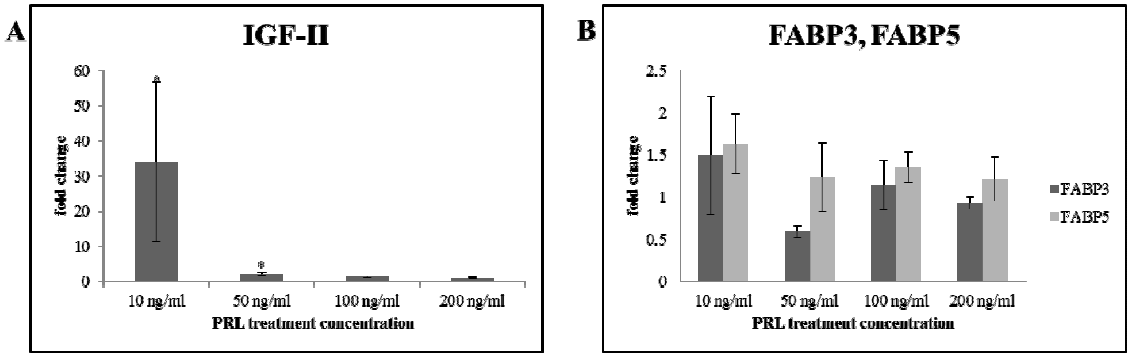
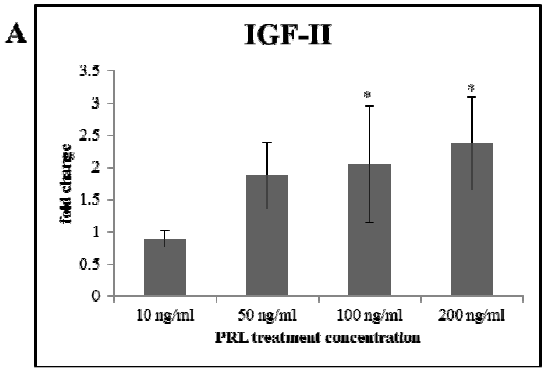

A
IGF-II
fold change
60
50
40
30
20
10
0
*
10 ng/ml
50 ng/ml
100 ng/ml
200 ng/ml
PRL treatment concentration
B
FABP3, FABP5
fold change
2.5
2
1.5
1
0.5
0
10 ng/ml
50 ng/ml
100 ng/ml
200 ng/ml
PRL treatment concentration
FABP3
FABP5

Figure 9. Messenger RNA Levels of IGF-II, FABP3, and FABP5 in PRL-Treated AG11132 Cells. A. Levels of IGF-II mRNA at various PRL treatment concentrations. IGF-II mRNA levels were significantly higher at 10 ng/ml (34-fold) and 50 ng/ml (2-fold) of PRL treatment, relative to control. B. Levels of FABP3 and FABP5 mRNA at various PRL treatment concentrations. No significant differences were seen in FABP mRNA levels with treatment. Note difference in scale between graphs of IGF-II and FABP mRNA levels.


A
IGF-II
fold change
3.5
3
2.5
2
1.5
1
0.5
0
*
*
10 ng/ml
50 ng/ml
100 ng/ml
200 ng/ml
PRL treatment concentration

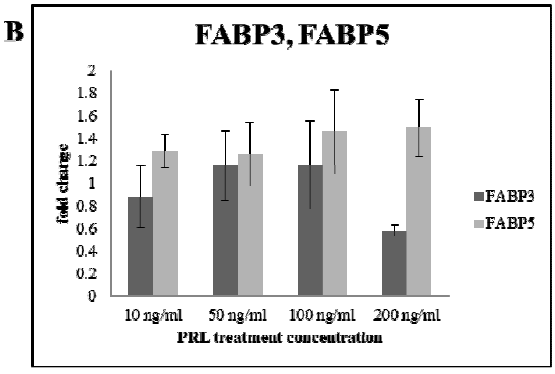

B
FABP3, FABP5
fold change
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10 ng/ml
50 ng/ml
100 ng/ml
200 ng/ml
PRL treatment concentration
FABP3
FABP5

Figure 10. Messenger RNA Levels of IGF-II, FABP3, and FABP5 in PRL-Treated MCF10A Cells. A. Levels of IGF-II mRNA at various PRL treatment concentrations. IGF-II levels were significantly increased at 100 (2-fold) and 200 ng/ml (2-fold) of PRL treatment. B. Levels of FABP3 and FABP5 mRNA at various PRL treatment concentrations. No significant differences were seen in FABP mRNA levels with treatment.

Discussion

The higher levels of both FABP3 and FABP5 protein in untreated MCF10A cells, relative to untreated AG11132 cells, may suggest a more differentiated cell status. Bovine studies have shown that both FABP3 and FABP5 mRNA increase upon pregnancy and lactation (which corresponds with breast differentiation); however, FABP3 increases to a greater extent than FABP5, and stays elevated for a longer period of time [Bionaz and Loor, 2008]. FABP5 mRNA was also higher in MCF10A cells than AG11132 cells. However, FABP3 mRNA was undetectable in MCF10A cells, and detected at low levels in AG11132 cells. This may indicate that FABP5 has a greater role in normal cell functioning which includes intracellular fatty acid transport, while FABP3 may have a more differentiation-specific role (i.e., FABP3 mRNA levels only increase when the cells receive a differentiation stimulus). FABP5-mediated upregulation of VEGF may lead to appropriate proliferation in normal cells.

Untreated MCF10A cells also had higher levels of PRL and IGF-II protein than untreated AG11132 cells. Levels of PRL and IGF-II mRNA were significantly higher in AG11132 cells, particularly IGF-II levels, which were over 40-fold higher. This finding is consistent with previous results from our laboratory, which demonstrated higher IGF-II mRNA levels in AA normal breast tissue relative to CA normal tissue, with the difference most pronounced in women below 45 years of age [Kalla Singh et al., 2010]. The higher IGF-II levels in AG11132 normal cells and in AA normal breast tissue may indicate a greater potential for carcinogenic transformation. IGF-II is expressed at high levels in cancer tissues due to a loss of parental imprinting, and is a major mediator of proliferation in breast cancer [Sachdev and Yee, 2006].

AG11132 cells responded to PRL treatment with increased FABP3 protein (18 kDa form) and decreased FABP5 protein at 200 ng/ml treatment, both changes associated with protection against carcinogenesis [Schonfeld and Wojtczak, 2008; Ishii, 2007; Rohan et al., 2010]. The increase in FABP3 was likely due to the lower levels of FABP3 in untreated AG11132 cells, which may represent a lower baseline level of protection against carcinogenesis in these cells. The dominant PRLR form in the AG11132 cell line was the 70 kDa form, representing delta S1 and/or S2, and the long form of the PRLR was not expressed (data not shown). With regard to mRNA levels, IGF-II mRNA increased several-fold with 10 ng/ml PRL, increased modestly with 50 ng/ml PRL, and then was further decreased at higher PRL concentrations. This may have been due to negative feedback, as IGF-II mRNA was several-fold higher in untreated AG11132 cells relative to MCF10A cells. This may also indicate that PRL's effects were mediated through IGF-II at lower treatment concentrations, but not at higher concentrations. Though IGF-II has been reported to mediate PRL's differentiation effects through upregulation of cyclin D1, there is some evidence that IGF-II may not be required for these effects in all cases [Brisken et al., 2002; Hovey et al., 2003]. For example, Carver and Schuler demonstrated that in the MCF-7 breast cancer cell line, PRL was able to induce cyclin D1 expression prior to upregulation of IGF-II transcript levels, suggesting that though PRL caused IGF-II increase, this was not required for signaling [Carver and Schuler, 2008].

Since MCF10A cells had higher FABP3, PRL, and IGF-II protein levels prior to PRL treatment, relative to AG11132 cells, it followed that FABP3 protein levels were maintained with PRL treatment, and then decreased at the highest PRL concentration.

The dominant PRLR form in this cell line was the 70 kDa form; however, MCF10A cells also expressed the 85-90 kDa long form of PRLR, though not as strongly (data not shown). Thus the decrease in FABP3 protein may represent negative feedback due to PRLR overstimulation at high PRL concentrations. FABP5 protein levels significantly decreased with 200 ng/ml PRL treatment, likely reflecting decreased carcinogenic potential of the cells. With regard to mRNA levels, significant increases were seen in IGF-II mRNA at 100 ng/ml and 200 ng/ml, pointing to IGF-II signaling in maintenance of FABP3 levels. These increases were modest relative to increases seen in the AG11132 cell line; this may have been due to higher initial levels of IGF-II protein in MCF10A cells.

In summary, the AG11132 cell line displayed several differences compared to the MCF10A cell line, which may help us understand the increased mortality in AA breast cancer patients. The differing growth conditions of the cell lines is a potential drawback of this study; however, serum-free media was placed on both cell lines for 6 hours prior to and also during the treatment, to enable comparison of results. AG11132 cells displayed decreased FABP3 protein at baseline, in comparison to MCF10A cells, likely indicative of lower protection against carcinogenesis. FABP3 protein increased in AG11132 cells with increasing PRL treatment concentration, but decreased in MCF10A cells, likely due to higher initial FABP3 protein levels and expression of the PRLR long form in the latter. Interestingly, the significant FABP3 changes in both cell lines are seen in the 18 kDa form, which is most likely tyrosine-phosphorylated. FABP3 from rat heart has been shown to be phosphorylated on Tyr19 in response to insulin stimulation, and according to PhosphoSite Plus, an NIH-supported site which predicts post-translational

protein modifications, human FABP3 is tyrosine-phosphorylated on Tyr-20 [Nielsen and Spener, 1993; Cell Signaling, 2012]. The role of phosphorylation in FABP3 function is as yet unknown, but has been proposed to be related to signal transduction and/or regulation [Huynh et al., 1996; Nielsen and Spener, 1993]. Decreased FABP5 protein was observed with treatment in both cell lines, which is protective against future carcinogenesis. PRL treatment of AG11132 cells at 10 ng/ml was associated with a several-fold increase in IGF-II mRNA; however, levels decreased with subsequent increases in PRL concentration, indicating that PRL signaling in AG11132 cells may not involve IGF-II at higher treatment concentrations. PRL treatment of MCF10A cells did result in increased IGF-II mRNA at highest PRL concentrations, suggesting that PRL is mediating its differentiation changes through IGF-II in this cell line.

Understanding possible molecular mechanisms underlying the breast cancer health disparity is crucial to its resolution, along with addressing socioeconomic and access-to-care concerns. In order to apply these findings to the larger population, PRL treatment would need to be done in other CA and AA normal cell lines in order to determine if these findings are unique to the particular cell lines studied, or if they represent common patterns of PRL signaling and FABP3 expression in cell lines derived from CA and AA patients. The studies described here provide preliminary insights into possible mechanisms undergirding the survival health disparity observed among AA women affected with breast cancer.

CHAPTER THREE

DIFFERENTIAL EXPRESSION OF FATTY ACID-BINDING PROTEINS 3 AND 5 IN CAUCASIAN-AMERICAN AND AFRICAN-AMERICAN BREAST TISSUES

Abstract

While Caucasian-American (CA) women have a higher overall breast cancer incidence relative to African-American (AA) women, AA women have a higher mortality rate. Fatty acid-binding protein (FABP) 3 is a protective tumor suppressor that is downregulated in breast cancer tissues. FABP5 is associated with metastasis, and is upregulated in breast cancer tissues. We hypothesized that AA women would express lower levels of FABP3 and higher levels of FABP5, as shown by analysis of 94 paired (normal/tumor) breast tissues. Contrary to expectations, FABP3 protein in AA malignant (AAM) samples was higher than in CAM samples in ages below 50. FABP5 protein was higher in AAM samples relative to CAM samples when data were not split by age. In ages 50 and older, FABP3 mRNA was significantly higher in CAM, while FABP5 mRNA was significantly higher in AAM. These findings demonstrate that differential FABP expression may be implicated in the breast cancer health disparity.

Introduction

Breast cancer is a significant cause of female mortality, second only to lung cancer. In recent years, significant emphasis has been put on cancer health disparities among ethnic groups. African-American (AA) women are less likely than Caucasian-American (CA) women to develop breast cancer. However, AA women are more likely to present with advanced disease, and have a higher mortality rate from breast cancer, as compared to CA women [Li et al., 2003]. Several studies have indicated that socioeconomic factors are clearly related to the disparities problem [Adams et al., 2009; Shavers et al., 2003]. In recent years, investigators have begun to examine biological factors that may be implicated as well [Kalla Singh et al., 2010; Yang et al., 1994]. One such factor may be differential expression of fatty acid binding proteins (FABPs).

FABPs, approximately 15 kDa in size, are proteins which shuttle long chain fatty acids throughout cells, similar to albumin's role in the circulation [Glatz and van der Vusse, 1996]. FABPs move fatty acids to sites of oxidation and esterification, as well as into the nucleus, where the fatty acids signal through peroxisome proliferator-activated receptors (PPARs) to affect gene transcription [Coe and Bernlohr, 1998; Hertzel and Bernlohr, 2000; Zimmerman and Veerkamp, 2002]. FABPs are expressed in various tissues of the body, including the breast, and have been shown to have a compensatory relationship. For example, in FABP5-gene knockout mice, FABP3 was elevated in the liver [Owada et al., 2002] and in FABP4 (adipocyte type)- knockout mice, FABP5 mRNA was upregulated in adipocytes 20- to 40-fold [Hertzel and Bernlohr, 2000]. Breast tissue/cell lines have been shown to express FABP1, FABP3, FABP4, FABP5, and FABP7 [Hammamieh et al., 2005; Huynh et al., 1996; Liu et al., 2011].

FABP3 expression in breast tissue is of particular interest since it is has been shown to regulate growth of normal human and some malignant mouse and human mammary epithelial cell lines [Borchers et al., 1997; Huynh and Beamer, 1998; Lehmann et al., 1989]. Previous literature reports downregulation or silencing of FABP3 levels in breast cancer tissues [Hertzel and Bernlohr, 2000; Huynh and Beamer, 1998]. FABP3 has been shown to bind to the nuclear receptor PPARα, and thus inhibit production of VEGF, which promotes angiogenesis and metastasis [Schroeder et al., 2008]. Additionally, stimulation of PPARα and PPARγ causes FABP3 upregulation [Motojima, 2000; Lindegaard and Nielsen, 2008; Keen et al., 2004]. Pregnancy and lactation protects against future breast cancer development in rats and in humans, and the induction of FABP3 expression in the breast may help to explain this [Russo, Moral et al., 2005; Russo et al., 1982; Sinha et al., 1988]. During pregnancy and lactation, mammary cells attain maximal development, and their genetic profile is altered to render them refractory to carcinogenesis, and FABP3 is the only FABP that is upregulated in this new genetic profile [Borchers et al., 1997; Russo, Moral et al., 2005; Specht et al., 1996; Yang et al., 1994]. AA women have lower lactation rates compared to all other ethnic groups [Bentley et al., 2003], and therefore FABP3 levels may not be induced as highly as in CA women, leading to decreased protection.

Another FABP that is very pertinent to breast carcinogenesis is FABP5. The *fabp5* gene has been linked to breast cancer metastasis, and mRNA levels were found to be 6.5-fold higher in malignant breast cell lines relative to HUMA 121 benign lines [Jing et al., 2000]. FABP5 expression has also been determined to be a negative prognostic factor for breast cancer patients, particularly those with triple-negative breast cancer [Liu

et al., 2011; Münz et al., 2005]. FABP5 is thought to transport nucleic acids to the nuclear receptor PPAR β/δ, where they bind and cause upregulation of vascular endothelial growth factor (VEGF) [Kannan-Thulasiraman et al., 2010; Schug et al., 2007]. VEGF is overexpressed in a significant percentage of breast tumors, and this expression has been associated with a worse prognosis in patients with invasive breast disease [Bajo et al., 2004; Gieseler 2007; Heffelfinger et al., 1999]. Though the literature on the role of FABP5 in breast is heavily focused on the association of FABP5 with tumor aggressiveness and metastasis, FABP5 has also been shown to have a beneficial role in breast differentiation. Bovine studies have shown that both FABP3 and FABP5 mRNA increase upon pregnancy and lactation; however, FABP3 increases to a greater extent than FABP5, and stays elevated for a longer period of time [Bionaz and Loor, 2008]. Given the decreased lactation rate in AA women, FABP3 may be lower in AA normal women relative to CA normal counterparts. Given that AA women tend to have more aggressive cancer at time of diagnosis, the FABP5 levels may be higher in AA breast cancer patients relative to CA counterparts. Furthermore, FABPs have a compensatory relationship in the tissues of the body, and have been shown to increase to compensate for deficits in another type of FABP [Hertzel and Bernlohr, 2000; Owada et al., 2002; Shaughnessy et al., 2000; Zimmerman and Veerkamp, 2002]. Therefore, this may be an alternate rationale for increased FABP5 levels in AA breast cancer patients relative to CA counterparts, as FABP3 levels may likely be decreased in the former.

To determine if expression of FABP3 and FABP5 differed between AA and CA breast cancer patients, we obtained frozen normal and malignant breast tissues, and

analyzed protein levels by immunoprecipitation, and mRNA levels by RT-PCR. Findings were compared by age, race, and by normal/malignant status.

Materials and Methods

Sample Preparation

Frozen, paired samples, AA and CA, were obtained from the Cooperative Human Tissue Network. Portions of each sample (0.2 mg) were cut on dry ice under sterile conditions. Tissues (0.1 mg) were homogenized in Tri Reagent (Molecular Research Center, OH) for mRNA extraction, according to standard Tri Reagent protocol (http://www.mrcgene.com/tri.htm). Tissues (0.1 g) were also homogenized in RIPA Buffer (Cell Signaling Technology, Beverly, MA) for protein extraction, according to standard RIPA Buffer protocol (http://www.cellsignal.com/products/9806.html).

Western Blot Analysis

RIPA buffer cell lysates containing 30 µg of protein were loaded onto polyacrylamide sodium dodecyl sulfate (SDS) gradient gels (4-12%), transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) using a X-Cell SureLock ® electrophoretic transfer module (Invitrogen). Protein concentration was measured using the Coomassie Plus Protein Assay Reagent ™ (Pierce Biotechnology, Rockford, IL). PVDF membranes were then blocked with 5% non-fat milk or 5% bovine serum albumin (Sigma) in PBS/0.05% Tween for 1 h. Membranes were then incubated with FABP3 mouse monoclonal antibody (Abcam, Cambridge, UK) or FABP5 rabbit polyclonal antibody (Abcam), followed by overnight incubation at 4 ^{o}C. The blots were

also probed with cytokeratin 18 antibody (Sigma) as a protein loading control. After 3 x 10 min washes in PBS/0.05% Tween, the corresponding secondary antibodies (1:1000, GE Healthcare, Piscataway, NJ for FABP5; 1:1000, Vector, Burlingame, CA for FABP3) were added to the membranes (1 h at room temperature), followed by 3 x 10 min washes and incubation with horseradish peroxidase (HRP) complexes (1:2000, GE Healthcare). Protein visualization was achieved by using enhanced chemiluminescence (ECL) and autoradiography with Blue Lite Autorad film (GeneMate from BioExpress, Kaysville, UT). The signals on the X-ray films were quantified using QuantityOne 1-D Analysis Software v. 4.5.0 (BioRad, Hercules, CA).

Real-Time PCR

Two-step SYBR real-time-PCR (RT-PCR) was performed to assess gene expression in PRL treated cells at 24 h, using the primers FABP3-forward (5'-CAC TAT GGT GGA CGC TTT CC-3'), and FABP3-reverse (5'-GTG GTA GGC TTG GTC ATG CT-3'); FABP5-forward (5'-ACA GAT GGT GCA TTG GTT CA-3'), and FAB5-reverse (5'-GAT CCG AGT ACA GGT GAC ATT G-3'). Primers for FABP3 utilized NCBI RefSeq NM_004102.3 and resulted in a 126 bp-length product; primers for FABP5 utilized NCBI RefSeq NM_001444.2 and resulted in a 123 bp-length product. Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu/) was used to generate primers, and NCBI PrimerBlast was used to test specificity. The iScript cDNA synthesis kit (Bio-Rad) and GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA) were used to synthesize cDNA; 200 ng of RNA was used per synthesis reaction. RT-PCR was performed using the iQ5 (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad).

Triplicate reactions were performed in a mixture consisting of a 25 µl volume solution containing SYBR Green supermix PCR buffer (Bio-Rad; 2x reaction buffer with dNTPs, iTaq DNA polymerase, 6 mm $MgCl_2$, SYBR Green I, fluorescein, and stabilizers), 1 µl of synthesized cDNA, 400 nM of each primer, and sterile water. Forty cycles of denaturation (95 ^{o}C, 15 s), annealing (60 ^{o}C, 30 s), and elongation (72 ^{o}C, 10 s) were performed. Fluorescence was detected at the end of every 72 ^{o}C extension phase. Melting curve analysis was done to exclude contamination by non-specific PCR products.

Statistical Analysis

For protein values, statistical differences were determined by one-way ANOVA, SPSS 19.0 software (IBM, Armonk, New York). A level of $p < 0.05$ was considered significant. For mRNA values, fold change was calculated, and statistical significance was set at a fold change of 2 or greater.

Results

FABP Protein Analysis

As shown in Figure 11, when both age groups were combined, AA malignant (AAM) had higher FABP5 protein relative to AA normal (AAN; p=0.030) and CA malignant (CAM; p=0.012). No differences were observed when the data were split by age.

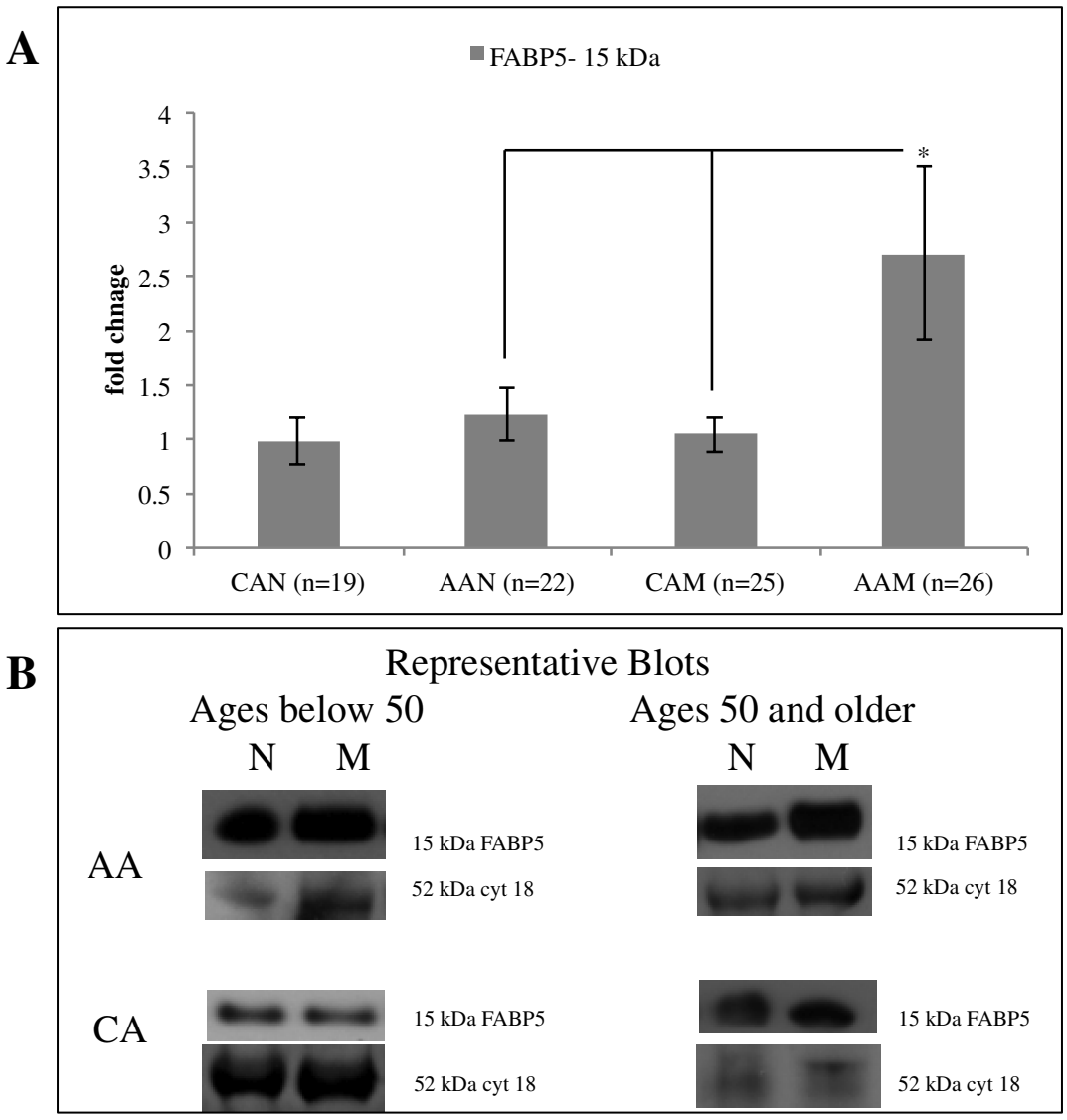

A
FABP5- 15 kDa
fold chnage
4
3.5
3
2.5
2
1.5
1
0.5
0
*
CAN (n=19)
AAN (n=22)
CAM (n=25)
AAM (n=26)
B
Representative Blots
Ages below 50
Ages 50 and older
N
M
N
M
AA
15 kDa FABP5
52 kDa cyt 18
15 kDa FABP5
52 kDa cyt 18
CA
15 kDa FABP5
52 kDa cyt 18
15 kDa FABP5
52 kDa cyt 18

Figure 11. FABP5 Protein Levels, All Ages. A. Graphed results. AAM had higher FABP5 protein relative to AAN ($p=0.030$) and CAM ($p=0.012$). IDV- integrated density values. B. Representative Western blots of FABP5 and cytokeratin 18 internal standard from both age groups.

Figure 12 shows the results of FABP3 protein analysis. When both age groups were combined, FABP3 (15 kDa) was significantly higher in CAM relative to CA normal (CAN; $p=0.028$), and in AAM relative to AAN ($p<0.001$) and to CAM ($p=0.012$). FABP3 (18 kDa) was significantly higher in CAM relative to CAN ($p<0.001$) and in AAM relative to AAN ($p<0.001$). FABP3 (total) was significantly higher in CAM relative to CAN ($p=0.006$), and in AAM relative to AAN ($p<0.001$) and to CAM ($p=0.017$). For ages below 50, FABP3 (15 kDa) was significantly higher in AAM relative to AAN ($p=0.001$) and to CAM (0.045). FABP3 (18 kDa) was significantly higher in AAM relative to AAN ($p=0.001$). FABP3 (total) was significantly higher in AAM relative to AAN ($p=0.001$) and to CAM ($p=0.045$). For ages 50 and older, FABP3 (15 kDa) was significantly higher in CAM relative to CAN ($p=0.028$) and in AAM relative to AAN ($p<0.001$). FABP3 (18 kDa) and FABP3 (total) were also significantly higher in CAM relative to CAN ($p<0.001$ and $p=0.006$, respectively), and in AAM relative to AAN ($p<0.001$ for both).

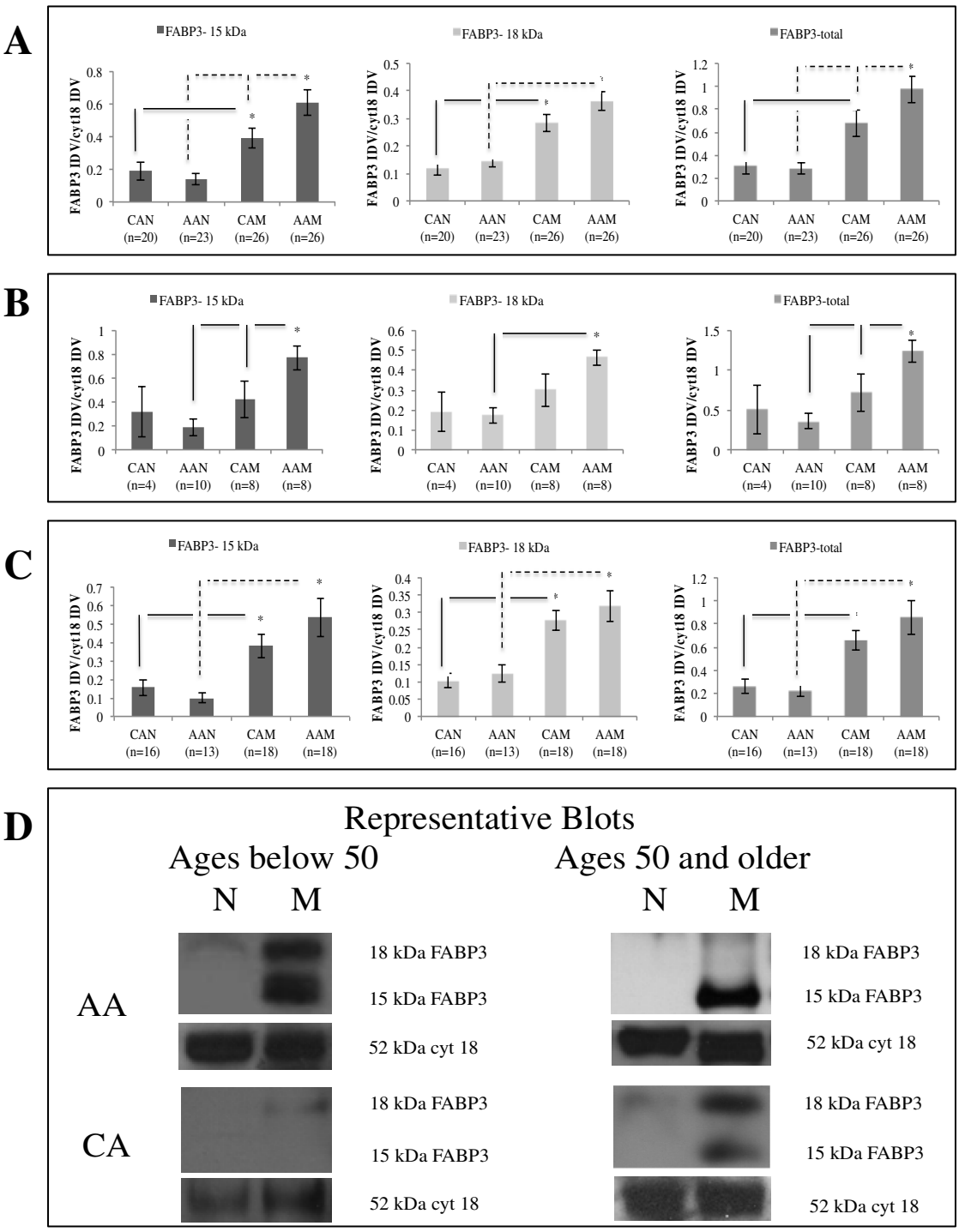
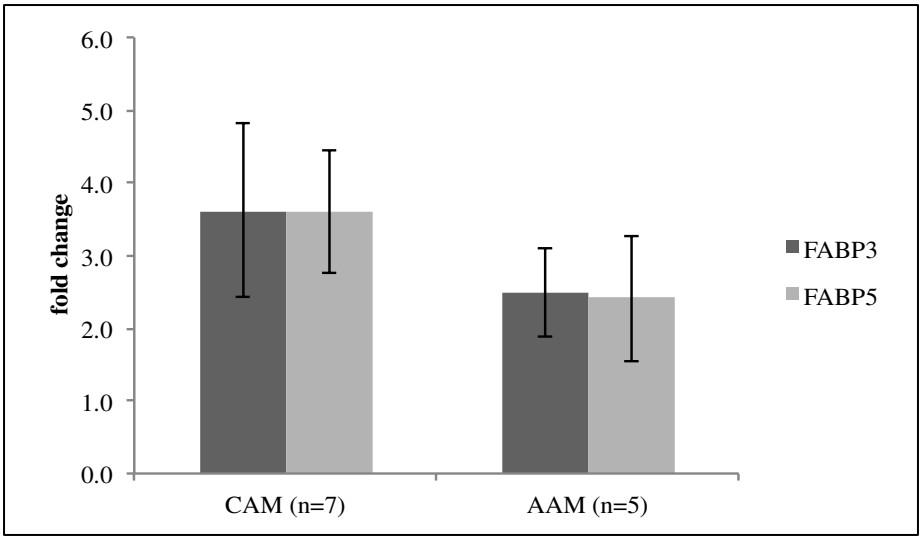

A
FABP3- 15 kDa
FABP3- 18 kDa
FABP3-total
FABP3 IDV/cyt18 IDV
CAN (n=20)
AAN (n=23)
CAM (n=26)
AAM (n=26)
B
CAN (n=4)
AAN (n=10)
CAM (n=8)
AAM (n=8)
C
CAN (n=16)
AAN (n=13)
CAM (n=18)
AAM (n=18)
D
Representative Blots
Ages below 50
Ages 50 and older
N
M
AA
CA
18 kDa FABP3
15 kDa FABP3
52 kDa cyt 18

Figure 12. FABP3 Protein Levels, All Ages and Split By Age Group. A-C. Graphed results. A. All ages- FABP3 (15 kDa) was higher in CAM and AAM relative to normal ($p=0.028$ and $p<0.001$, respectively), and in AAM relative to CAM ($p=0.012$). FABP3 (18 kDa) was higher in CAM and AAM relative to normal ($p<0.001$ for both). FABP3 (total) was higher in CAM and AAM relative to normal ($p=0.006$ and $p<0.001$, respectively), and in AAM relative to CAM ($p=0.017$). B. Ages below 50- FABP3 (15 kDa) was higher in AAM relative to AAN ($p=0.001$) and to CAM (0.045). FABP3 (18 kDa) was higher in AAM relative to AAN ($p=0.001$). FABP3 (total) was higher in AAM relative to AAN ($p=0.001$) and to CAM ($p=0.045$). C. Ages 50 and older- FABP3 (15 kDa) was higher in CAM and AAM relative to normal ($p=0.028$ and $p<0.001$, respectively). FABP3 (18 kDa) and FABP3 (total) were also higher in CAM relative to CAN ($p<0.001$ and $p=0.006$, respectively), and in AAM relative to AAN ($p<0.001$ for both). D. Representative Western blots of FABP3 and cytokeratin 18 internal standard from both age groups.

FABP Messenger RNA Analysis

As shown in Figure 13, in ages below 50, levels of both FABPs were not significantly different between CAM and AAM. In ages 50 and older, FABP3 mRNA was significantly higher in CAM relative to AAM (87-fold). Conversely, FABP5 mRNA was significantly higher in AAM relative to CAM (9-fold).

A


6.0
5.0
4.0
3.0
2.0
1.0
0.0
fold change
FABP3
FABP5
CAM (n=7)
AAM (n=5)

B

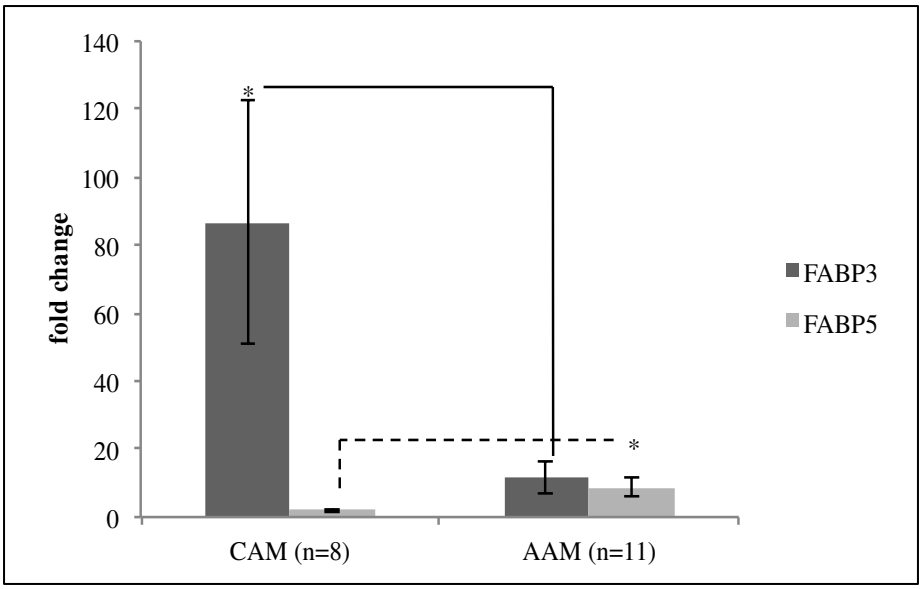

140
120
100
80
60
40
20
0
fold change
*
*
FABP3
FABP5
CAM (n=8)
AAM (n=11)

Figure 13. FABP Messenger RNA Levels, Split by Age Group. A. FABP mRNA levels, ages below 50. Levels of both FABPs were not significantly different between CAM and AAM. B. FABP mRNA levels, ages 50 and older. FABP3 mRNA was higher in CAM relative to AAM (87-fold). FABP5 mRNA was higher in AAM relative to CAM (9-fold).

Discussion

Prior to initiation of the study, we hypothesized that levels of FABP5 protein would be significantly higher in malignant tissues, and particularly in AAM tissues relative to CAM tissues, which would reflect increased tumor aggressiveness. Regarding FABP3 protein and mRNA levels, we hypothesized that levels would be significantly higher in normal tissues, and particularly in CAN tissues relative to AAN tissues, due to differences in lactation rates between the ethnic groups. Our findings for FABP5 were as hypothesized; however findings for FABP3 differed from those hypothesized.

Analysis of FABP5 protein levels showed that in AA tissues, malignant samples had significantly higher FABP5 protein compared to normal samples. Moreover, the AAM samples had higher FABP5 relative to CAM, indicative of increased tumor aggressiveness and metastatic potential in the former. Notably, the CAM and CAN samples had similarly low levels of FABP5 protein, consistent with decreased aggressiveness. RNA analysis in ages below 50 showed no significant FABP5 difference between AAM and CAM; however, analysis in ages 50 and older showed that AAM samples expressed higher levels of FABP5 mRNA relative to CAM, again, indicative of increased tumor aggressiveness in this group.

FABP3 protein was expressed in 3 bands- 14, 15, and 18 kDa. A recently published study analyzing FABP3 protein levels in the mouse heart indicated a similar immunoprecipitation banding pattern [Lewis et al., 2010]. The 14 kDa and 15 kDa bands were merged in some samples, and thus we analyzed the total of these two bands (referred to as 15 kDa). The 18 kDa band represents modified FABP3, likely tyrosine-phosphorylated, as predicted in the literature [Nielsen and Spener, 1993]. In this study,

increased FABP3 was consistently associated with malignancy, in both CA and AA samples, and in both age groups; to our knowledge, this association has not been previously described in the literature. There did not appear to be a difference in this trend among the various molecular weights. Of note, FABP3 was higher in AAM relative to CAM in ages less than 50 (14,15 kDa, total). When age was not used to split the data, FABP3 was also higher in AAM relative to CAM (14,15 kDa, total). RNA analysis indicated that CAM tissues expressed much higher levels of FABP3 mRNA relative to AAM.

The FABP3 mRNA levels do not reflect protein levels, as in the FABP5 analysis; the significance of this is yet unknown. In a recent study which attempted to define the genetic profile of tumors that were at high risk of local recurrence after breast conserving therapy, the top scoring gene was *fabp3* [Kreike et al., 2006]. This supports our findings that increased expression of FABP3 is highly associated with the malignant state, in addition to being associated with differentiation as supported by several studies in the literature.

Thus, this study demonstrates that increased FABP3 and FABP5 are both associated with breast malignancy, and that there is differential expression of these proteins in AA and CA tissues. This finding is particularly significant for FABP3, as previous literature generally described this protein as a tumor suppressor and as decreased in the cancer state. Both proteins are increased in AAM patients relative to AAN and CAM counterparts. In ages 50 and older, significant mRNA changes are also seen, showing increased FABP5 mRNA in AAM relative to CAM and thus increased aggressiveness and metastatic potential. Further work involves determining the post-

translational modifications present in the FABP3 protein expressed in the tissue samples, and further elucidating the FABP3 signaling pathway. Clarification of the function and signaling mechanisms of FABPs 3 and 5 would aid in a better understanding of their role in the breast cancer health disparity.

Acknowledgements

Thanks to Qianwei Tan for her assistance in tissue processing.

CHAPTER FOUR

OVERALL DISCUSSION

Role of FABPs in Breast Tissue

Our studies have shown that, regardless of age group, FABP3 protein is more highly expressed in malignant breast tissues, relative to normal tissues. In ages below 50, FABP3 protein is more highly expressed in AAM tissues relative to CAM tissues. This finding is a new one, in light of previous studies showing that FABP3 mRNA and protein are decreased in malignant breast tissues [Hertzel and Bernlohr, 2000; Huynh et al., 1996]. In a clinical study, which attempted to define the genetic profile of breast tumors at high risk of recurrence after breast conserving therapy, the top scoring gene was *fabp3* [Kreike et al., 2006]. Thus far, this study is the only published study, in addition to our studies, regarding FABP3 and increased risk of breast carcinogenesis. Furthermore, our study is the first, to our knowledge, to demonstrate that increased FABP3 protein levels are associated with malignancy, in addition to the existing association of increased FABP3 levels with differentiation. It is possible that FABP3 has protective effects in the normal state, as repeatedly demonstrated in the literature, and then in the malignant state, is markedly upregulated in an attempt to halt cancer progression through its signaling effects. To determine if FABP3 expression in the breast is higher in pregnancy or in the malignant state, one could sample breast tissue while women are pregnant and/or lactating; however, this would obviously be unethical. We were also able to see that FABP3 is expressed in an 18 kDa form in addition to the 15

kDa form. A precedent for this finding was seen in the literature, in a study of FABP3 found in mouse cardiac tissue [Lewis et al., 2010]. The 18 kDa form may be tyrosine-phosphorylated, as predicted in the literature [Nielsen and Spener, 1993], and may relate to proper FABP3 signaling and function. Both forms of FABP3 are increased in the malignant state. When looking at mRNA levels, we found that in ages below 50, FABP3 was not significantly different between CAM and AAM patients. However, in ages 50 and older, FABP3 mRNA had almost 90-fold greater expression in CAM relative to AAM. This is interesting given that as it relates to protein expression, FABP3 is significantly higher in AAM relative to CAM in ages below 50, and not significantly different between AAM and CAM in ages 50 and older. The significance of this is as yet unknown. It could be that the FABP3 mRNA in AAM tissues is translated or degraded at a higher rate, thus causing the mRNA to be significantly decreased.

In AA patients, FABP5 protein is more highly expressed in malignant tissues; however, this does not hold true in CA patients. Additionally, FABP5 is more highly expressed in AAM relative to CAM. For ages 50 and older, the FABP5 mRNA levels are consistent with the protein findings; levels in AAM are 9-fold higher than in CAM. Since FABP5 is associated with metastasis and carcinogenesis [Jing et al., 2000], this implies greater aggressiveness of the AA tumors.

Role of IGF-II and PRL in Breast Differentiation and Regulation of FABPs

Studies of two untreated ER negative normal breast epithelial cell lines, MCF10A and AG11132, demonstrated that the CA MCF10A cell line had significantly higher levels of FABP3 and FABP5 protein relative to the AA AG11132 cell line. FABP5

mRNA was also significantly higher in MCF10A cells, relative to AG11132 cells. However, FABP3 mRNA was undetectable in MCF10A cells, and expressed at very low levels in AG11132 cells. This may indicate that FABP5 has a greater role in normal cell functioning which includes intracellular fatty acid transport, while FABP3 may have a more differentiation-specific role (i.e., FABP3 mRNA levels only increase when the cells receive a differentiation stimulus). Untreated MCF10A cells also had higher levels of PRL and IGF-II protein relative to AG11132 cells. Conversely, the mRNA levels of these two proteins were increased in AG11132 cells, with IGF-II mRNA levels being over 40-fold higher. This finding is consistent with previous results from our laboratory, which demonstrated higher IGF-II mRNA levels in AA normal breast tissue relative to CA normal tissue, with the difference most pronounced in women below 45 years of age [Kalla Singh et al., 2010].

Studies of PRL-treated MCF10A and AG11132 cell lines demonstrated that FABP3 protein increased in response to PRL treatment in the AG11132 cell line, and remained steady in the MCF10A cell line with a decrease at highest treatment concentration. This was mostly likely due to differences in PRLR and initial FABP3 protein levels between the two cell lines. MCF10A cells expressed the long form of PRLR, and had high levels of FABP3 protein in the untreated state, while AG11132 cells did not express the PRLR long form, and had significantly lower levels of FABP3 protein. In both cell lines, PRL treatment resulted in decrease in FABP5 protein, which is associated with decreased metastatic potential, and was in accordance with previous findings in the literature. Response of IGF-II to PRL signaling differed somewhat between the two cell lines. In the MCF10A cell line, PRL treatment resulted in increases

in IGF-II mRNA. This was to be expected as IGF-II has been reported to mediate PRL's differentiation effects [Brisken et al., 2002]. However, in the AG1132 cell line, PRL treatment resulted in increased IGF-II mRNA at lower concentrations, and decreased IGF-II mRNA at higher concentrations. Additionally, maximal levels of FABP3 were associated with low IGF-II mRNA levels in this cell line.

Thus, it can be seen that PRL treatment of both cell lines induced a favorable profile as it relates to decreased risk of carcinogenesis—increased or maintained protective FABP3 and decreased FABP5, which is associated with metastasis. Interestingly, in AG11132 cells, IGF-II mRNA levels only increased at low PRL concentration, while FABP3 protein levels increased with increasing PRL concentration. This indicates that IGF-II may mediate increased/maintained FABP3 protein levels in MCF10A cells, but not in AG11132 cells, and that PRL signaling may not involve IGF-II in the latter cell line at higher treatment concentrations. Though IGF-II has been reported to mediate PRL's differentiation effects through upregulation of cyclin D1 [Brisken et al., 2002; Hovey et al., 2003], there is some evidence that IGF-II may not be required for these effects in all cases. For example, Carver and Schuler [2008] demonstrated that in the MCF-7 breast cancer cell line, PRL was able to induce cyclin D1 expression prior to upregulation of IGF-II transcript levels, suggesting that though PRL caused IGF-II increase, this was not required for signaling.

Conclusions and Future Directions

In summary, we have seen that though FABP3 is associated with differentiation, both FABP3 and FABP5 proteins increase in the malignant state, and that AAM breast

tissues express higher levels of these proteins, relative to CAM tissues. In ages above 50, the FABP3 mRNA is significantly increased in CAM, pointing to a possible greater protection, or reserve against carcinogenesis, while the FABP5 mRNA is significantly increased in AAM, pointing to greater tumor aggressiveness. Cell studies demonstrate that FABP3 and FABP5 are expressed in normal mammary epithelial cells, and that treatment of these cells with PRL causes increased/maintained FABP3 levels, with the AA cell line showing a more robust response likely due to decreased initial FABP3 and PRLR levels. If the finding of lower PRLR levels were to hold true in AA breast tissue, it would provide a rationale for decreased protection against carcinogenesis, as higher levels of PRL would be required to induce protective differentiation changes in the breast. In both cell lines, decreased FABP5 is also seen, which may be protective against carcinogenesis. In the CA cell line, levels of FABP protein appear to be regulated by increased IGF-II, while in the AA cell line, IGF-II appears to only be involved at lower treatment concentrations.

The demonstration that PRL treatment of AG11132 cells, normal mammary epithelial cells with low levels of FABP3, may simulate the protective effect of pregnancy, and cause a protective increase in FABP3, may be utilized for protective short-term pretreatment of human breast tissue in the future. Russo and colleagues [2005] have demonstrated that short-term pretreatment of breast tissue with human chorionic gonadotropin is able to induce a protective genomic signature, mimicking that of breast tissue that has undergone pregnancy and lactation-induced differentiation. Further PRL treatment experiments in normal mammary epithelial cell lines and animal breast tissue may validate a protective pretreatment effect at the genetic level, which

would include increased FABP3 expression. In the breast cancer state, PRL levels are dysregulated, and high levels are associated with increased proliferation [Swaminathan et al., 2008]. However, increasing PRL levels in normal breast cells for short-term treatment should not have the same effect. As aforementioned, in both premenopausal and postmenopausal women, PRL levels are markedly upregulated after the first pregnancy, followed by a long-lasting reduction in levels, which is protective against future carcinogenesis [Tworoger and Hankinson, 2008].

To be able to apply our initial findings to the larger population, further studies are needed. Some of these studies would include: determining the post-transcriptional modifications present in FABP3 protein in breast tissues from AA and CA women, measuring PRLR levels in AA and CA breast tissues, PRL treatment of other CA and AA normal cell lines to determine if the findings above are consistent, and elucidating the FABP3 nuclear signaling pathway in further detail. At present, we can state that our studies have been able to preliminarily elucidate the differences in FABP3 and FABP5 expression between AA and CA breast tissues and cell lines, as well as differences in the responses of the cell lines to PRL treatment, and have shed light on possible biological mechanisms undergirding the breast cancer health disparity.

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