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# IGF2 Promotes Activation of Estrogen Receptors in Basal-like Breast Cancer Cells

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

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IGF2 Promotes Activation of Estrogen Receptors in Basal-like Breast Cancer Cells

by

Angelique E. Richardson

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A Dissertation submitted in partial fulfillment of  
the requirements for the degree of  
Doctor of Philosophy in Microbiology and Molecular Genetics

---

March 2011

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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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## ABBREVIATIONS

|              |  |
|--------------|--|
| AA           | African-American                             |
| BLBC         | Basal-like Breast Cancer                     |
| ER- $\alpha$ | Estrogen Receptor alpha                      |
| ER- $\beta$  | Estrogen Receptor beta                       |
| ERE          | Estrogen Response Element                    |
| FBS          | Fetal Bovine Serum                           |
| DMEM         | Dubelcco's Modified Eagle Medium             |
| IGF          | Insulin-like Growth Factor                   |
| IGF1         | Insulin-like Growth Factor 1                 |
| IGF2         | Insulin-like Growth Factor 2                 |
| IGFBP        | Insulin-like Growth Factor Binding Proteins  |
| IGF1R        | IGF-1 Receptor                               |
| IGF2R/M-6-P  | IGF-2 Receptor /Mannose-6-Phosphate Receptor |
| IR           | Insulin Receptor                             |
| Kb           | Kilobase                                     |
| Kda          | Kilodalton                                   |
| ProIGF2      | Precursor IGF2                               |
| MIGF2        | Mature IGF2                                  |
| PAGE         | Polyacrylamide Gel Electrophoresis           |
| PBS          | Phosphate Buffered Saline                    |
| SDS          | Sodium Dodecyl Sulfate                       |

## ABSTRACT OF THE DISSERTATION

IGF2 Promotes Activation of Estrogen Receptors in Basal-like Breast Cancer Cells

by

Angelique Ellerbee Richardson

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics  
Loma Linda University, March 2011  
Daisy D. De León, Chairperson

The autocrine-paracrine effects of IGF2 are important in the growth and differentiation of normal breast. In breast cancer (BC), IGF2 is initially stimulated by estrogen, progesterone and prolactin to regulate proliferation and cancer progression. These actions are mediated by the IGF-1R and insulin receptor A (IR-A) both members of the tyrosine-kinase receptors family. The activation of Estrogen Receptor (ER) is also very important in BC growth and progression.

As BC progresses to estrogen-independent growth, the IGF-1R and the estrogen receptor (ER) interact in crosstalk mechanisms that are synergistic and results in enhanced activation of both receptors signaling cascades. This mechanism plays a central role in the transition of estrogen-dependent to estrogen-independent breast cancer (BC) progression.

Basal-like BC (BLBC) is a sub-group of estrogen-independent tumors that have a very aggressive clinical behavior and are resistant to hormone-based therapy resulting in reduced disease-free survival period and increasing the mortality of breast cancer (BC) patients. Our BC research team has elucidated how IGF2 crosstalk signaling results in the activation of ER pathways independent of estrogen. Central to our investigation is how this mechanism is associated to the survival disparity observed among African American

(AA) BC patients. BLBC accounts for nearly 15-20% of all breast cancers, however it represents 45% of all BC observed in AA patients.

Analyses of subcellular compartments, Western-Blot and siRNA demonstrated that IGF2 activates ER- $\alpha$  and ER- $\beta$  in ER negative BLBC cells Hs578t and CRL-2335. Our studies show that both IGF-1R and IR crosstalk with ER- $\alpha$  and ER- $\beta$  promoting BLBC progression. This novel mechanism offers new therapeutic targets that will significantly impact treatment and diagnosis of BLBC patients.

## CHAPTER ONE

### INTRODUCTION

#### **Disparities in Breast Cancer**

African-Americans (AA) diagnosed with all cancer types have a higher death rate and lower survival when compared to other ethnic groups in the United States (Ward et al. 2004). This health disparity is also present in breast cancer. Breast cancer is the second most common form of cancer in women, however, it is the number one diagnosed cancer in African-American (AA) women (Ward et al. 2004). AA women are more likely to have highly aggressive tumors when compared to their Caucasian (CA) counterparts (Chlebowski et al. 2005).

Reasons for this survival health disparity in AA women are complex. Some factors are social (inequalities in work, wealth, education, standard of living) and some factors are biological such as loss of tumor suppressor gene expression, loss imprinting of insulin like growth factor 2 gene, and BRCA 1 and 2 mutations (Baker, Metzler, and Galea 2005; Fine, Ibrahim, and Thomas 2005; Woolf 2004). This study will evaluate the role of biological factors in the breast cancer survival disparities seen in AA women. There are several findings which suggest that the biological factor IGF2 is a contributor in the survival health disparity seen among breast cancer patients (Elledge et al. 1994)(Fu et al. 2003) (Poola et al. 2005).

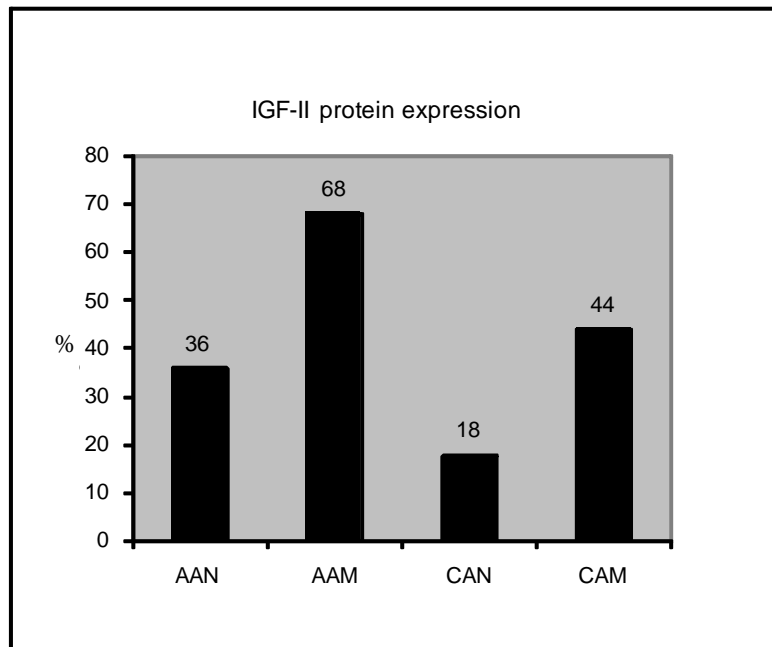


Figure 1. Bar graph of Western Blot Analysis Showing IGF2 Expression in Normal and Malignant Breast Tissue Samples.

Kalla Singh et al. 2010. Differential Insulin-like Growth Factor (IGF-II) Expression: A Potential Role for Breast Cancer Survival Disparity. *Growth Hormone & IGF Research*, 20:162-170.

### **Role of IGF-II in Breast Cancer**

There is a higher expression of IGF2 in both normal and cancerous breast tissues of AA women when compared to normal and tumor breast tissues from Caucasian women (Figure 1). AA women overexpress several factors that lead to an increased expression of IGF2 which in turn promotes breast cancer cell survival (Singh, et al. 2008)(Singh, et al. 2007). One such factor is mutated p53. Mutated p53 has been shown to lead to an increase in IGF2 expression and promote the upregulation of cell survival pathways leading to increased tumor aggressiveness (Zhang, et al. 1996) (Kanashiro, et al. 2003) (Carey et al. 2006) (Carey et al. 2006). This evidence suggests that there may be a direct correlation between an increased expression of IGF2 and breast tumor

aggressiveness in AA women which may, in part, explain the decrease in survival seen in these women.

Multiple steps occur as a cell progresses from its normal cell phenotype to its cancerous phenotype. The cancer cell develops the ability to avoid apoptosis, maintain a limitless replicative potential, and provide its own growth signals (Hanahan and Weinberg 2000). Interestingly, IGF2 has been shown to regulate all of the steps needed in carcinogenesis. The overexpression of IGF2 is involved in the development of various cancers, including breast cancer (Haruta et al. 2008; Honda et al. 2008; Wilkin et al. 2000)(Vu et al. 2003; Wise and Pravtcheva 2006).

Our lab has shown that IGF2 plays a role in promoting breast tumor progression towards aggressiveness. The study demonstrated that the expression of IGF-II promoted tumor growth and metastasis without the requirement of estrogen in nude and Severe Combined Immunodeficient (SCID) mice. This data suggests that IGF2 promotes tumor progression towards aggressiveness by allowing the tumor to grow independent of estrogen in these mice models.

### **Insulin-like Growth Factor Family**

IGF2, a member of the insulin-like growth factor (IGF) family, plays an important role in the development and maintenance of normal body function (LeRoith et al. 1995). Also, included in the IGF family is the IGF1 ligand, cell membrane receptors insulin-like growth factor receptor 1 (IGF1R), IGF2R and the Insulin Receptor (IR), as well as six IGF-binding proteins (IGFBP-1 through IGFBP-6) (Yu and Rohan 2000). Notably, the IGF family ligands are highly homologous (62%) and both IGF1 and IGF2 are able to bind to each of the IGF family receptors and promote mitogenic and



antiapoptotic actions affecting cell proliferation, differentiation, and transformation (Corps and Brown 1991; Wood et al. 2000; Yu and Rohan 2000).

## IGF2

The *igf2* gene is located on chromosome 11 (Brissenden, Ullrich, and Francke 1984). From four promoters (P1-P4) multiple mRNA transcripts are produced and alternatively spliced (Sara and Hall 1990)(Yu and Rohan 2000). In the adult, the P1 promoter is responsible for the transcription of IGF2 whereas in the fetus P3 and P4 regulates IGF2 transcription (Sara and Hall 1990). Importantly, P3 and P4 promoters are also activated in breast cancer (Yu and Rohan 2000). Activation of P3 and P4 promoters in cancer cells is a result of the cell reverting back to a less differentiated fetal phenotype.

IGF2 is produced as a prohormone (pre-proIGF2) and it is cleaved post-translationally by proprotein convertase 4 (PC4), a serine protease, to yield several isoforms including proIGF2 (1-156 amino acids, aa) and (1-104 aa) and mature IGF2 (1-67 aa) (Figure 2) (Qiu et al. 2005). ProIGF2 and mature IGF2 are glycosylated to yield isoforms of varying molecular weights ranging from 21 kDa (proIGF2) to 7.5 kDa (mature IGF2) (Vyas, Asmerom, and De Leon 2005). The various IGF2 isoforms have different biological activities. ProIGF2, also known as “big” IGF2, is the predominate form expressed in breast cancer and it has a different mechanism of action when compared to mature IGF2 (mIGF2) (Singh et al. 2008; Singh et al. 2007) (Ellis et al. 1998; Ishida et al. 1995; Rasmussen and Cullen 1998; Singh et al. 2007; Vyas, Asmerom, and De Leon 2005).

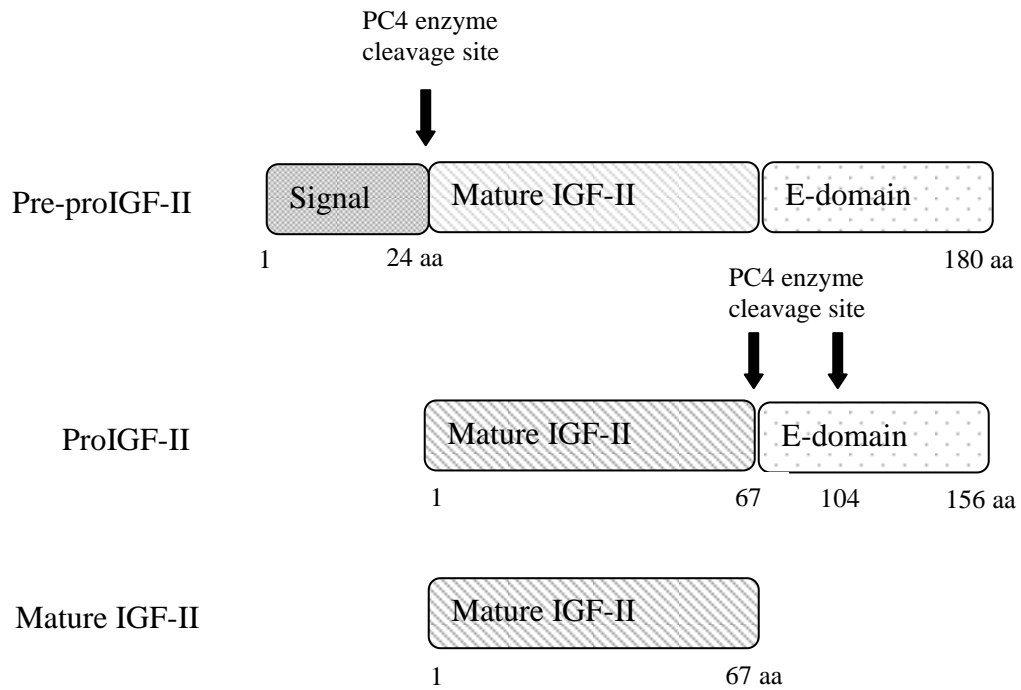


Figure 2. Schematic Representation of pre-proIGF2, proIGF2, and mature IGF2. This figure shows the cleavage of pre-proIGF2 into the proIGF2 (1-156 amino acids, aa) and (1-104 aa) and mature IGF2 (1-67 aa) by proprotein convertase 4 (PC4).

Adapted from Miraki-Moud F. et al. J Clin Endocrinol Metab 2005; 90:3819-3823

### Insulin-like Growth Factor 1 Receptor

Insulin-like growth factor 1 Receptor (IGF1R) is activated by both IGF1 and IGF2 (Ellis et al. 1998). IGF1R is a tetrameric glycoprotein located on the cell membrane. The expression of IGF1R is upregulated by estrogens, growth hormone (eg. IGFs), follicle-stimulating hormone (FSH) and glucocorticoids, and downregulated by wild-type p53 and the Wilms tumor protein (Sepp-Lorenzino 1998)(Werner 1998; Werner et al. 1996; Werner et al. 1993). Once IGF1 or IGF2 binds to the IGF1R a conformational change occurs resulting in its autophosphorylation at tyrosine residues in

the juxtamembrane and flanking regions which serve as docking sites for insulin receptor substrates (IRS) and Shc adaptor proteins. IRS and Shc recruit other proteins, such as Grb2/SOS and phosphatidylinositol 3'kinase (PI3 Kinase), which can then activate the mitogen-activated protein kinase (MAP kinase) and PI3 kinase pathways. The MAP kinase and PI3 kinase pathways regulate transcription factors that alter gene expression, as well as, control cell proliferation, differentiation, and apoptosis (LeRoith et al. 1995; Yu and Rohan 2000).

The *igf1r* mRNA expression has been found to be upregulated in most breast cancer cell lines and in over 90% of human breast tumors (Papa et al. 1993; Pekonen et al. 1988). IGF1R expression is important for the inhibition of apoptosis in tumor cells and it has been postulated that the primary role of IGF1R signaling in tumor cells is to maintain tumor cell survival and protection from apoptosis (Baserga et al. 1997) (Dunn et al. 1997; Resnicoff et al. 1995).

### Insulin-like Growth Factor 2 Receptor

IGF2 also binds the insulin-like growth factor 2 receptor (IGF2R), and though it is highly debated, evidence suggests that the IGF2R may mediate signaling via a G protein-coupled mechanism (Baserga 1995; LeRoith et al. 1995)(Hawkes et al. 2007). The IGF2R is monomeric and it contains three ligand binding regions in the extracellular domain: one site is for IGF2 binding and the other two sites are for the binding of mannose-6-phosphate (M-6-P) containing proteins (Brown, Jones, and Forbes 2009; Morgan et al. 1987; Oshima et al. 1988) (Oates et al. 1998) (Jones and Clemmons 1995).

Thus, the IGF2R is sometimes referred to as the IGF2R/ mannose-6-phosphate (M-6-P) receptor (Kiess et al. 1989; Vignon and Rochefort 1992).

The IGF2R regulates the bioavailability of IGF2 by leading to its degradation and thus it has been considered as a tumor suppressor (Hankins et al. 1996)(LeRoith et al. 1995; Oates et al. 1998). Thus, loss of this receptor may contribute to various aspects of tumor pathophysiology (Oates et al. 1998). Little is known about the regulation of the IGF2R but it has been suggested that IGFs, epidermal growth factor (EGF) and M-6-P may lead to an increase in its expression (Hoeflich et al. 1996; Jones and Clemmons 1995).

### Insulin Receptor

The IR is a cell surface glycoprotein with a heterotetrameric structure made up of two alpha and two beta subunits. The extracellular alpha-subunits consist of the insulin binding domains, and the transmembrane beta-subunits consist of the tyrosine kinase and the phosphorylation sites. The binding of ligand to the IR activates its tyrosine kinase signaling pathway leading to autophosphorylation of the IR and its associated endogenous substrates such as insulin receptor substrate proteins (IRS-1, -2, -3, and -4), and Shc (White 1998; White and Kahn 1994).

Insulin and IGF2 bind to and activate the insulin receptor (IR) (LeRoith et al. 1995). An IR-isoform, IR-A, has been shown to display high affinity for IGF2. IR-A is encoded by the mRNA lacking exon 11. The binding of IGF2 to IR-A mediates proliferative and anti-apoptotic effects. In contrast, IGF-II binding to IR-B, the IR

isoform which includes exon 11, elicits primarily metabolic effects and cell differentiation (Frasca et al. 1999).

IR and IGF1R are structurally homologous and activated by tyrosine phosphorylation. These receptors are implicated in the regulation of cellular differentiation, mitosis and metabolism (Moxham and Jacobs 1992; White and Kahn 1994). Moreover, the IR and IGF1R both share certain substrates such as Shc and members of the IRS family. Once IRS is phosphorylated then it forms a signaling complex with growth factor receptor binding protein (GRB2), Syp (SH PTP2) phosphotyrosine phosphatase, and phosphatidylinositor kinase (PI3K) (Lowenstein et al. 1992; Skolnik et al. 1993). IRS-1 couples GRB2 to IR and IGF1R. Shc functions in a similar manner. The coupling of GRB2 to the IR and IGF1R leads to association of the complex with son of sevenless (SOS) Ras GEF/GTP exchanger. This leads to the activation of Ras/mitogen-activated protein kinase (MAPK) pathway. Activation of MAPK pathway regulates cell growth, differentiation, and proliferation in response to insulin, IGF1 and IGF2. The increased expression of IR in breast cancer and breast cancer cell lines has been shown to promote tumor development (Papa et al. 1990) (Milazzo et al. 1992).

## **Estrogen Receptors**

### Estrogen Receptors in Cancer

Normal estrogen receptor signaling is necessary for the development and maturation of the mammary gland in contrast aberrant ER signaling is implicated in breast carcinogenesis and progression (Huseby, Maloney, and McGrath 1984; Mueller et

al. 2002) (Herynk and Fuqua 2004). Estrogen receptors are steroid hormone receptors and members of a nuclear superfamily whose subgroup consists of the estrogen receptor alpha (ER- $\alpha$ ) and estrogen receptor beta (ER- $\beta$ ) and its natural ligand estrogen (Griekspoor et al. 2007)(Arpino et al. 2008; Kuiper et al. 1997; Webb et al. 1999).

ER- $\alpha$  is located on human chromosome 6 and ER- $\beta$  is found on human chromosome 14 (Enmark et al. 1997; Gosden, Middleton, and Rout 1986; Gustafsson 1999; Ponglikitmongkol, Green, and Chambon 1988). Though they are different gene products, ER- $\alpha$  and ER- $\beta$ , exhibit 97% and 60% of homology in the DNA- and ligand-binding domains, respectively. Thus, ER- $\alpha$  and ER- $\beta$  interact with identical DNA estrogen response elements (EREs) and exhibit a similar binding affinity for estrogen, but they appear to respond to ligands in a receptor-specific manner (Lazennec et al. 2001)(Kuiper et al. 1997)(Driscoll et al. 1998).

Because of their high degree of homology, initially ER- $\beta$  was thought to represent a level of redundancy in estrogen signaling (Couse and Korach 1999; Ogawa et al. 1999; Ogawa et al. Molecular cloning and characterization of human estrogen receptor  $\beta$ -cx: A potential inhibitor of estrogen action in human 1998). Current studies indicate the unique but highly controversial role of ER- $\beta$  in breast cancer development and progression (Flynn et al. 2008). Some studies suggest that ER- $\beta$  behaves like a tumor suppressor in the breast and is related to a favorable outcome (Speirs 2002). Other studies suggest that ER- $\beta$  expression is associated with a poorer prognosis (Iwao et al. 2000; Knowlden et al. 2000; Roger et al. 2001; Speirs et al. 1999).

The estrogen receptor subgroup also includes three orphan receptors, estrogen related receptors  $\alpha$  (ERR  $\alpha$ ), ERR  $\beta$ , and ERR  $\gamma$ , and estrogen receptor variants

(Griekspoor et al. 2007)(Poola et al. 2005; Vladusic et al. 1998). In this dissertation, the expression and activity of the variant ER- $\beta$ 5 is studied because data suggests that this variant plays a significant role in the development of estrogen-independent breast cancer, and it is expressed at higher levels in African-American women. This increased expression has been linked to an increase in tumor aggressiveness (Inoue et al. 2000; Peng et al. 2003)(Poola et al. 2005; Vladusic et al. 1998)(Poola, Abraham, and Liu 2002).

The subcellular localization of the ERs is important when considering its role in tumor progression and must be taken into account. Studies demonstrate that when ER- $\beta$  localizes to the mitochondria, in breast cancer cells, it then binds to the mitochondrial genome at ERE sequences leading to the regulation of estrogen's effects at this organelle, including modulation of calcium influx, ATP production, apoptosis, and free radical species generation (Demonacos et al. 1996)(Chen et al. 2004; Demonacos et al. 1996; Nilsen and Diaz Brinton 2003; Richards et al. 1996; Sekeris 1990; Wang, Green, and Simpkins 2001; Yang et al. 2004). This dissertation shows the ability of IGF-II to promote ER- $\alpha$  and ER- $\beta$  translocation to the mitochondria in breast cancer cells providing a mechanism in which IGF-II can regulate the mitochondria.

### Estrogen Receptor Signaling

There are two signaling mechanisms for the estrogen receptor. The first mechanism discovered is called the classical ER signaling pathway or the genomic/nuclear-initiated signaling pathway (Hall, Couse, and Korach 2001) (Rosenfeld and Glass 2001) (Rosenfeld and Glass 2001). This mechanism involves the passive diffusion of estrogen into the cell and its binding to the ER, leading to receptor

dimerization and translocation into the nucleus. Once inside of the nucleus the receptor binds to the estrogen responsive element (ERE) in the promoters of target genes (Bjornstrom and Sjoberg 2005)(Hall, Couse, and Korach 2001) (Rosenfeld and Glass 2001)(Rosenfeld and Glass 2001).

Traditionally, the genomic or nuclear-initiated ER signaling has been considered the primary action of the ERs. However, recently plasma membrane estrogen receptors have been discovered (Moriarty, Kim, and Bender 2006). These rapid, membrane-initiated signaling cascades via plasma membrane-associated ERs are referred to as nonclassical or nongenomic/membrane-initiated estrogen receptor signaling (Bjornstrom and Sjoberg 2005; Razandi et al. 2003). This alternate pathway involves the activation of cell surface receptors that lead to the rapid activation of signaling cascades resulting in the regulation of target genes (Kousteni et al. 2001; Levin 2005; Mendelsohn 2000; Xu et al. 2004; Yang et al. 2004)(Rosenfeld and Glass 2001)(Moriarty, Kim, and Bender 2006).

It is important to note that the nuclear-initiated and membrane-initiated signaling pathways do not function independently of one another. Instead, the two pathways converge in the cell to regulate ER-regulated gene expression. Therefore, the distinction between the two signaling pathways is blurred (Bjornstrom and Sjoberg 2005; Moriarty, Kim, and Bender 2006).

### **Growth Factor Receptor and Estrogen Receptor Cross-talk**

Interactions between growth factor receptors and estrogen receptors are important in the development and progression of breast cancer (Arpino et al. 2008). Growth factor receptors have been shown to activate the estrogen receptors (ER) through a mechanism



known as cross-talk which is bidirectional (Hall, Couse, and Korach 2001)(Fagan and Yee 2008; Kahlert et al. 2000; Shou et al. 2004). There are studies showing cross-talk between the ER- $\alpha$  and the Human epidermal growth factor receptor (HER2/neu), the Epidermal Growth Factor Receptor (EGFR) and the IGF-1R (Kato et al. 1995; Lee et al. 1999; Richards et al. 1996; Shou et al. 2004). As the complexities of ER and growth factor signaling cross-talk are being discovered, it is becoming increasingly clear that the treatment of breast cancer will have to involve more than just endocrine therapy that only targets the ER.

### **Breast Cancer Subtypes**

Breast cancer patients are usually grouped together based on their tumor receptor status using immunohistochemistry. Treatment is based on the presence or absence of estrogen receptor (ER) and/or human epidermal growth factor receptor (HER2) (Walker 2008). Patients who have ER/PR positive tumors receive therapy directed towards blocking the activity of estrogen (Tamoxifen and Raloxifen) or stopping estrogen production (aromatase inhibitors) (Carpenter and Miller 2005; Jordan 2007; Reis-Filho and Tutt 2008; Robson and Offit 2007). If the tumor expresses HER2 then a HER2 directed therapy (Trastuzumab/Herceptin) is given to the patient (Albanell et al. 2003; Brenton et al. 2005). Those individuals whose tumors do not express ER, PR or HER2 are usually treated with chemotherapy.

Alternatively, breast cancers may be divided into subgroups using gene expression profiles (Brenton et al. 2005; Dawood and Cristofanilli 2007; Jumppanen et al. 2007). The subgroups are normal breast-like, luminal, basal-like, and HER2 (ERBB2)

overexpressing (Dawood and Cristofanilli 2007; Jumppanen et al. 2007). These subgroups display distinct differences in biology, behavior, prognosis and response to therapy (Brenton et al. 2005). The normal breast-like subtype consists of normal breast samples and fibroadenomas (benign tumors) (Ihemelandu et al. 2007). The luminal subgroup is mainly comprised of ER positive tumors and the ER negative tumors are in the HER2 overexpressing and basal-like subtypes (Table 1) (Brenton et al. 2005).

Basal-like breast cancers (BLBC) account for nearly 15-20% of all breast cancers (Kobayashi 2008). Forty-five percent of those diagnosed with BLBC are young (<50 years old), African-American women (Ihemelandu et al. 2007; Reis-Filho and Tutt 2008). There appears to be a positive correlation between Breast Cancer 1 gene (BRCA 1) mutations, increased expression of mutated p53 and the development of BLBC in AA women (Brenton et al. 2005; Sorlie et al. 2001)(Reis-Filho and Tutt 2008; Turner, Tutt, and Ashworth 2004).

Table 1. Typical Immunohistochemical Classification of Breast Cancer Subtypes

|                 |                  |       |
|-----------------|------------------|-------|
| Luminal A       | ER + and/or PR+, | HER2- |
| Luminal B       | ER+ and/or PR+,  | HER2+ |
| Basal cell-like | ER-, PR-,        | HER2- |
| HER-2/neu       | ER-, PR-,        | HER2+ |

Adapted from Ihemelandu et al. 2007. Journal of Surgical Research. Vol 143, Issue 1, p.109-118.

The majority of BLBC present with an ER, PR and HER2/neu negative (triple negative) phenotype (Brenton et al. 2005; Jumppanen et al. 2007)(Reis-Filho and Tutt 2008). BLBC has a poor prognosis due to the absence of targeted therapies and its intrinsically aggressive biology (Turner, Tutt, and Ashworth 2004). There is a need to develop targeted therapies for women diagnosed with these tumors.

### **Significance of the Studies**

Breast cancer is the second most common cancer in women (Ward et al. 2004). Basal-like breast cancer (BLBC) accounts for approximately 15-20% of all breast cancers in women, however 45% of these women are AA and this may partly explain why AA women overall have a higher mortality rate from breast cancer (Chlebowski et al. 2005; Finn et al. 2007; Munzone et al. 2006; Szepeshazi et al. 1992)(Ward et al. 2004).

Basal-like breast cancer has a poor prognosis due to the absence of targeted therapies and its intrinsically aggressive biology (Turner, Tutt, and Ashworth 2004). Currently, targeted therapies for breast cancer are aimed at preventing estrogen dependent growth which is not effective for the treatment of BLBC, because it has developed the ability to grow independent of estrogen. Little is known about how these cells grow independent of estrogen. This dissertation addresses this lack of knowledge by elucidating a mechanism in which these BLBC grow independent of estrogen via growth factor and estrogen receptor cross-talk mechanisms driven by IGF-II.

This study is highly significant and transformational because several observations made in this study will lead to major changes in clinical outcomes for AA women with BLBC.

## Approach of Studies

This study was designed to demonstrate the ability of IGF2 to circumvent the requirement for estrogen in breast cancer cells by acting through the IGF1R and IR to phosphorylate/activate ER- $\alpha$  and ER- $\beta$ . Central to this study is the association of this mechanism with survival health disparities in AA women with breast cancer. An appropriate cell model had to be selected for demonstrating the role of IGF2 in estrogen-independent activation/phosphorylation of ER- $\alpha$  and ER- $\beta$  in breast cancer cells.

The desirable cell model had to have several features important to the study. The cell model had to express ER- $\alpha$  and ER- $\beta$  in order to demonstrate the ability of IGF2 to phosphorylate both receptors. The breast cancer cell line, MCF-7, was chosen. MCF-7 is derived from a Caucasian woman and it is an ER positive cell line. This cell line expresses both ER- $\alpha$  and ER- $\beta$ . Hs578T cell line is also derived from a Caucasian woman, but it is an ER negative breast cancer cell line, and it was selected to serve as a control.

In addition, this study was designed to elucidate the role of IGF2 in the survival health disparity seen in AA women with breast cancer. Therefore, an ER positive and ER negative breast cancer cell line derived from AA women were selected and compared to the MCF-7 and Hs578T cell lines. CRL-2335 (ER-) and CRL-2315 (ER+) AA cell lines were chosen for this study.

The MCF-7, Hs578T, CRL-2315 and CRL-2335 cells were characterized using Real-time Polymerase Chain Reaction (RT-PCR) to determine the expression of ER- $\alpha$  and ER- $\beta$ . RT-PCR revealed that the ATCC designated ER-negative breast cancer cell lines (Hs578T and CRL-2335) express ER- $\alpha$  and ER- $\beta$ . Studies show that ER

designation is given to a breast cancer by immunohistochemistry. Breast tumors that express less than 10% of ER- $\alpha$  in the nucleus are designated as ER-negative (Nadji et al. 2005; Swain 2001).

Based on these observations focus shifted to the “ER-negative” breast cancer cell lines, and the subcellular localization of ER- $\alpha$  and ER- $\beta$ . First, the effect of IGF2 on the activation and subcellular localization of ER- $\alpha$  and ER- $\beta$  in these breast cancer cells needed to be determined. ER activation was measured by ER phosphorylation and translocation within the subcellular compartments. In order to determine the role of IGF2 in estrogen-independent activation of ER in the CRL-2335 and Hs578T cells had to be treated with both proIGF2 and mIGF2. CRL-2335 and Hs578T cells were treated with both proIGF2 and mIGF2 at varying concentrations 25, 50 and 100 ng/mL at different time points (0, 10, 15, 20 and 30 minutes) and analyzed for ER activation by measuring ER phosphorylation and translocation. The proIGF2 (100ng/mL) treatment for 20 minutes showed the most effect in the CRL-2335 and Hs578T cell lines. The experiments in this dissertation used this time point and concentration of proIGF2.

The cell lines used for this study were basal-like breast cancer cell lines, CRL-2335 and Hs578T. Molecular gene expression profiling data has subgrouped the CRL-2335 and Hs578T cell lines into the basal-like breast cancer subgroup (Finn et al. 2007). These cell lines were the ideal models for elucidating estrogen independent growth mechanisms in breast cancer cells. Estrogen independent growth is the primary mechanism of growth in basal-like tumors. Moreover, basal-like tumors are more common in AA women. Currently, there is little knowledge regarding how these basal-like tumors grow independent of estrogen. There is an even larger gap in knowledge

concerning what predisposes AA women to these tumors. In this study we have proposed a novel mechanism for how these basal-like breast cancers promote estrogen independent signaling mechanisms in AA women.

This dissertation study was designed to demonstrate the ability of IGF2 to activate the IGF-1R and IR promoting the phosphorylation of ER- $\alpha$  and ER- $\beta$  via cross-talk mechanisms. This proposed mechanism was based on studies showing cross-talk between the ER- $\alpha$  and IGF-1R (Kato et al. 1995; Lee et al. 1999; Richards et al. 1996). Although many breast cancers express both ER- $\alpha$  and ER- $\beta$  there is no published study demonstrating a cross-talk between ER- $\beta$  and IGF-1R. Also, there are no studies in the literature showing a cross-talk between ER- $\alpha$  and/or ER- $\beta$  and the IR. Nevertheless, we believe there is evidence supporting a crosstalk between the ER and IR based on the following observations. IGF-1R and the IR are 70% homologous, structurally and functionally, and ER may cross-talk with IR in a similar manner as IGF1R (Morrione et al. 1997). This study provides a more comprehensive understanding of this cross-talk pathway thus providing more insight into how to appropriately development more targeted therapies.

## CHAPTER TWO

### INSULIN-LIKE GROWTH FACTOR-2 (IGF-2) ACTIVATES ESTROGEN RECEPTOR- $\alpha$ AND - $\beta$ VIA THE INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR AND THE INSULIN RECEPTOR IN BASAL-LIKE BREAST CANCER CELLS.

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**Growth Factors (In Print)**

## **Abstract**

The estrogen receptor (ER) is a primary target for breast cancer (BC) treatment. As BC progresses to estrogen-independent growth, the IGF-1R and the ER interact in synergistic crosstalk mechanisms which results in enhanced activation of both receptors signaling cascades. Insulin-like growth factor 2 (IGF-2) is critical in BC progression and its actions are mediated by the IGF-1R. Our previous studies showed that IGF-2 regulates survival genes that protect the mitochondria and promote chemoresistance. In this study, we analyzed BC cells by subcellular fractionation, Western-Blot, qRT-PCR and siRNA analysis. Our results demonstrate that IGF-2 activates ER- $\alpha$  and ER- $\beta$  and modulates their translocation to the nucleus, membrane organelles and the mitochondria. IGF-2 actions are mediated by the IGF-1R and the insulin receptor (IR). This novel mechanism of IGF-2 synergistic crosstalk signaling with ER- $\alpha$  and ER- $\beta$  can promote estrogen-independent BC progression and provides new therapeutic targets for the treatment of breast cancer patients.

## **Introduction**

Estrogen signaling is mediated through two receptors, ER $\alpha$  and ER $\beta$  (Arpino, 2008; Kuiper, 1997; Webb, 1999) When the ER- $\beta$  was discovered it was thought that it represented a splice variant of ER $\alpha$ , however current studies showed that ER- $\alpha$  and ER- $\beta$  are different genes with distinct physiological functions (Flynn, 2008). While ER $\alpha$  mediates its effects in the nucleus, ER- $\beta$  is predominantly localized in the mitochondria and regulates mitochondrial gene expression and membrane potential as well as ATP production (Chen, 2004; Demonacos, 1996; Nilsen, 2003; Richards, 1996; Sekeris, 1990;



Wang, 2001; Yang, 2004). Activation of either ER- $\alpha$  or ER- $\beta$ , promotes BC cell growth and survival, however, estrogen dependent tumors eventually develop mechanisms to activate ER mediated pathways without the requirement of estrogen (Szepeshazi, 1992).

The development of estrogen-independent BC involves cross-talk between several growth factors and the estrogen receptors (Gee, 2005; Shou, 2004). Insulin-like growth factor-2 (IGF-2) is a growth factor that plays a critical role in organ development, differentiation and metabolism by signaling via both the IGF-1R and the IR-A (Abbas, 2007). Signaling through both receptors also regulates the growth and differentiation of normal breast epithelial cells (Ishida, 1995; Rasmussen 1998; Singh, 2007; Vyas, 2005; Yballe, 1996).

The expression of the IGF-II gene is tightly regulated and inhibited by tumor suppressor genes such as p53, Pten, WT1, GC factor (GCF) and several other oncogenes (Zhang *et al.* 1996, Toretsky and Helman 1996, Kitadai *et al.* 1993). Mutations in tumor suppressors contribute to higher IGF-II expression not only for lack of suppression but also for gain of function as mutated p53 that stimulates IGF-II (Zhang *et al.*, 1998). The hormonal regulation of IGF-II is positively regulated by E<sub>2</sub>, progesterone, prolactin and GH, all important hormones in normal breast development and in breast cancer progression (Brisken, *et al* 2002; Goldfine *et. al.*, 1992; Hamelers *et.al.*, 2003). Thus, IGF-II expression is important in normal breast development and increased IGF-II in the mammary gland contributes to tumor formation. In addition to hormonal stimulation and tumor suppressor inhibition of IGF-II, there are many other critical pathways that activate IGF-II that are also important in breast cancer development. The most significant include IGF-II stimulation by oxidative stress, the Wnt-pathway (disrupts eCadherin- $\beta$ catenin),

integrins and mTOR (Erbay et al, 2003;Goel et.al., 2006, Morali et.al., 2001). Mutations in the IGF-II receptor (Byrd, et al., 1999) and pTEN also regulate IGF-II levels and IGF-II regulates pTEN (Sukmi Kang-Park et al, 2003, Perks et.al. 2007). The extensive regulatory mechanisms involved in IGF-2 expression also includes miRNAs, methylation, imprinting and other epigenetic alterations present in early development of cancer (Chao et al, 2008, Ge and Chen, 2011)

IGF-2 is highly expressed in breast cancer patients and plasma levels of free IGF-2 directly correlates to BC tumor size (Singer, 2004). Furthermore, transgenic animal models expressing high levels of IGF-2 develop aggressive breast cancer tumors (Pravtcheva, 1998; Pravtcheva, 2003; Bates, 1995).

We have shown that IGF-2 also promotes proliferation, inhibit apoptosis and stimulate the transformation of breast cancer cells (Singh, 2007; Singh, 2008). Our studies also showed that IGF-2 can activate estrogen-regulated genes like survivin, BCL-xl and BCL-2 independent of estrogen through IGF-1R and Insulin receptor. Thus, our particular interest in IGF-2 effects in breast cancer is based on our original observations that IGF-1 was not present in these breast cancer cells and that published studies about IGF-1 analysis by RIA reflected interference with IGF-BPs and not IGF-I (De León et al, 1988,1989, 1992). Furthermore, IGF-1 is mainly regulated by GH while IGF-2, as discussed above, is tightly regulated by a wide range of tumor suppressors and hormones that are critical for breast cancer development. Thus, IGF-2 plays a critical role in normal breast differentiation and in the development and progression of breast cancer, demonstrating that IGF-2 have a broader range of biological functions at the cell or tumor level.

Since IGF-1 activation of the IGF-1R can cross-talk and activate the ER- $\alpha$  signaling pathway (Fagan and Yee, 2008) we propose that likewise, IGF-2 can activate the ER- $\alpha$ . We also propose that IGF-2 may activate ER- $\beta$  signaling pathway via cross-talk with the IGF-1R. Moreover, since IGF-2 not IGF-1 binds the insulin receptor-A, also a member of the IGF-1R family, we thought that IGF-2 activation of the IGF-1R and IR-A signaling pathways results in the phosphorylation/activation of ER- $\alpha$  and ER- $\beta$  in BC cells. Thus, the present study aims to elucidate if IGF-2 binding to both, IGF-1 receptor and the insulin receptor-A results in activation and translocation of the estrogen receptors.

## **Materials and Methods**

### **Cell Culture**

CRL-2335, HS578T, and MCF-7 cell lines were obtained from the American Type Culture Collection (ATCC). The CRL-2335 cell line was derived from a 60-year old African-American (AA) woman and the HS578t cell line was derived from a 74-year old Caucasian (CA) woman and both cell lines are triple negative (ER-,PR-,Her2-). MCF-7 cells were derived from a pleural effusion of a CA woman (69 yo breast cancer patient) and it is used as a positive control for ER- $\alpha$  and ER- $\beta$  expression. Cultures were maintained at 37C in a 5% CO<sub>2</sub> incubator. CRL-2335 cell cultures were maintained in RPMI 1640 media (ATCC) supplemented with 10mL of penicillin/streptomycin (10,000 units/mL penicillin and 10,000 units/mL streptomycin sulfate, Cellgro), 3ug/ml B-amphotericin, and 10% fetal bovine serum (Hyclone). HS578T cell cultures were maintained in DMEM/F12 media (Cellgro) with 4mM L-glutamine, 1.5g/L sodium bicarbonate, 4.5g/L glucose, 0.01mg/ml bovine insulin (Sigma), 10mL of

penicillin/streptomycin (10,000 units/mL penicillin and 10,000 units/mL streptomycin sulfate, Cellgro), and 10% fetal bovine serum (Hyclone). MCF-7 cells were maintained in DMEM/F12 media (Cellgro) supplemented with 10 ml of 5000 U penicillin/streptomycin (100 U/ml penicillin and 100 U/ml streptomycin sulfate, Cellgro), 4 mM L-glutamine (Cellgro), 3 µg/ml β-amphotericin, and 5% fetal bovine serum (Hyclone).

### Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted using the Aurum<sup>TM</sup> Total RNA Mini Kit (BioRad). RNA integrity was evaluated by UV spectroscopy and RNA Quality Indicator (RQI) values were obtained using the Experion<sup>TM</sup> Automated Electrophoresis System (BioRad). Total RNA samples were stored at -80C until assayed. Quantitative reverse-transcription PCR (qRT-PCR) was performed to determine ER-α and ER-β mRNA expression in MCF-7, CRL-2335 and HS578T cells.

The primer pairs used for ER-α (SA Biosciences) are:

ER-α forward – 5'-ATCCTGATGATTGGTCTCGTCT-3'

ER-α reverse - 5'-TCTGGAAGAGAAGGAACCATATCC-3'

The primer pairs used for ER-β (SA Biosciences) are:

ER-β forward - 5'-GTCATCTTTGCTCCAGATCTTG-3'

ER-β reverse - 5'-GATGCTTTGGTTTGGGTGATTG-3'

cDNA synthesis was performed using the iScript<sup>TM</sup> cDNA synthesis kit (Bio-Rad) with 400ng total RNA in a 20 µL reaction. The reaction protocol is as follows: 5 min 25C, 30 min 42C, and 5 min 85C. Each 50 µL qRT-PCR reaction contained 1 µl of

cDNA product from the first-strand synthesis, 25  $\mu$ l of iQ™ SYBR® Green Supermix (Bio-Rad:100mM KCL, 6 mM MgCl<sub>2</sub>, 40mM Tris-HCL, pH 8.4, 0.4mM of dATP, dCTP, dGTP and dTTP, iTaq DNA Polymerase 50 U/mL, SYBR Green I, 20mM Fluorescein), and 300nM of each forward and reverse primer. Before PCR amplification, iTaq DNA polymerase was activated at 95C for 10 minutes. This was followed by forty cycles of PCR amplification: 30 sec denaturation at 95C, 15 sec annealing at 57C, and 90 sec elongation at 72C. Fluorescence was detected at the end of every 72C extension phase. To confirm the presence of specific PCR products, melting curve analyses were performed on the PCR products after the cycling protocol.

#### Subcellular Fractionation

HS578T, CRL-2335 and MCF-7 cells were plated at a density of  $1 \times 10^6$  cells/ml and grown to 80% confluency in 10mm culture plates. Cultures were treated with 100ng/ml proIGF-2 (GroPep) or mIGF-2 (PeproTech) in phenol-red free and serum free media for 20 minutes. MCF-7 cells were left untreated. The Proteo-Extract Subcellular Proteome Extraction Kit (Calbiochem) was used according to manufacturer's protocol to obtain cytosolic, membrane/organelle (mitochondria) and nuclear fractions. All fractions were stored at -80C until assayed.

#### Western Blot Analysis

The Coomassie Plus Protein Assay Reagent (Pierce Biotechnology) was used to assess protein concentration. Forty micrograms of each protein sample were loaded into a 4-12% polyacrylamide-SDS gradient gel then transferred onto a PVDF membrane

(Invitrogen) using an X-Cell SureLock electrophoretic transfer module (Invitrogen). Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in 1X PBS/0.05% Tween. Phosphorylated ER- $\alpha$  was detected by incubating membranes at 4C overnight with either rabbit anti-human pER- $\alpha$  (Tyr 537) (Santa Cruz Biotechnology) diluted 1:500 in 3% w/v Bovine Serum Albumin (BSA) or mouse anti-human pER $\alpha$  (Ser 118) (Cell Signaling Technology) diluted 1:500 in 5% w/v nonfat dry milk in 1X PBS/0.05% Tween. Phosphorylated ER- $\beta$  was detected by incubating membranes with rabbit anti-human pER $\beta$  (Ser 87, 1:1000, Santa Cruz Biotechnology). Unphosphorylated ERs were detected by incubating membranes at 4C overnight in 3% w/v non-fat dry milk in 1X PBS/0.05% Tween with either mouse anti-human ER- $\alpha$  (1:1000, Cell Signaling Technology) or mouse anti-human ER- $\beta$  (1:1000, GeneTex). Proteins specific for the membrane/organelle (mitochondrial) and nuclear fractions were used to confirm the integrity of the subcellular compartments: membrane/organelle (mitochondrial) fraction - MnSOD (1:500, BD Biosciences) and nuclear fraction- LEDGF (1:1000, BD Biosciences). Membranes were then washed 3 times for 5 minutes in 10 ml of 1X PBS/0.05% Tween. The appropriate biotinylated secondary antibody was diluted in 1X PBS/0.05% Tween and incubated with each membrane for 1 hour (anti-rabbit IgG & anti-mouse IgG 1:5000, Amersham), followed by streptavidin-horseradish peroxidase (HRP, 1:2000, Amersham) for 1 hour. Each membrane was washed 4 times for 10 minutes in 10ml of 1X PBS/0.05% Tween. Protein visualization was achieved using enhanced chemiluminescence (ECL) (Pierce) followed by autoradiography with Hyperfilm ECL (Amersham). Band density obtained from WB analysis was quantified using the ChemiImager 4000 (Alpha Innotech Corporation).

### siRNA Analysis

CRL-2335 and HS578T cells were plated in 6-well plates at a density of  $1 \times 10^5$  cells/well and allowed to attach overnight in a 37C 5% CO<sub>2</sub> incubator. Cells were grown to 60 -80% confluency then treated with 60 pmols of either IR (sc-29370, Santa Cruz Biotechnology), IGF-1R (sc-29358, Santa Cruz Biotechnology) or scrambled (sc-Santa Cruz Biotechnology) siRNA in the supplied transfection media and incubated for 9 hours at 37C in a 5% CO<sub>2</sub> incubator. Complete media containing 20% FBS was added to the serum-free media so that the final serum concentration was 10% FBS. The cells were then incubated for 24 hours in a 37C 5% CO<sub>2</sub> incubator. Media was replaced with fresh media followed by an additional 24-hour incubation period in a 37C 5% CO<sub>2</sub> incubator. Afterwards, cells were incubated for 6 hours in serum-free, phenol-free media and then treated with either 100ng/ml of mIGF-2 or proIGF-2 for 20 minutes. Cells treated with scrambled siRNA did not receive treatment with IGF-2. Cellular fractions were prepared as previously described and stored at -20<sup>0</sup>C until assayed.

### Statistical Analysis

The data for rtPCR was analyzed by One-way ANOVA with Tukey's Multiple Comparison Post Test using GraphPad Prism software version 5.02 for Windows (GraphPad Software, San Diego, CA). \*\*\* -  $p < 0.001$ , \*\* -  $p < 0.01$ .

Statistical analysis for the Western blots scans was performed using one-way ANOVA, SPSS 11.0 software (SPSS, Inc.). Values are expressed as the mean  $\pm$  SEM of three or more replicate experiments performed in triplicate. Values of  $p < 0.05$  were considered statistically significant.

## Results

### ER- $\alpha$ and ER- $\beta$ mRNA in Breast Cancer Cells

ER status is the golden test to determine whether a breast cancer tumor is sensitive to endocrine therapy. Tumors are designated as ER- when there is less than 10% staining for ER- $\alpha$  in the nuclei as assessed by immunohistochemistry (IHCC). Likewise, when cell lines are established from tumors their ER status is assigned by IHCC. Figure 3 depicts the expression of ER- $\alpha$  and ER- $\beta$  mRNA in MCF-7 cells (positive control for ER- $\alpha$  and ER- $\beta$ ) and in the ER- cell lines CRL-2335 and Hs578T assessed by qRT-PCR.

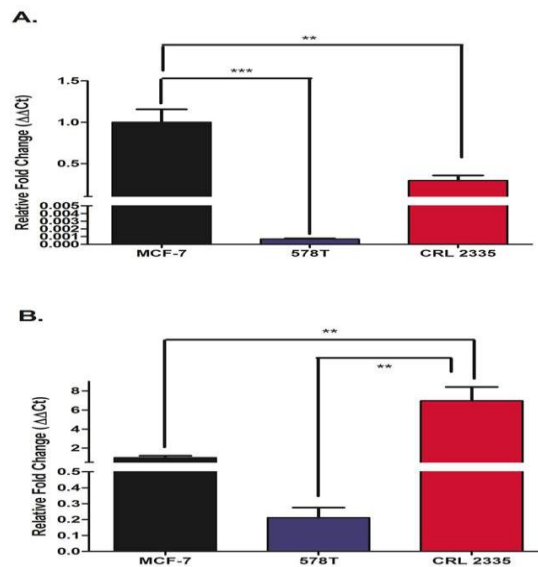


Figure 3. ER- $\alpha$  and ER- $\beta$  mRNA expressed as fold change of mRNA expression in MCF-7, CRL-2335 and Hs578T cells. Figure 3A depicts the expression of ER- $\alpha$  in MCF-7, CRL-2335 and Hs578T cell lines. Figure 3B shows the expression of ER- $\beta$  in these same cell lines. Results are expressed as relative fold change ( $\Delta\Delta Ct$ ). A p-value of less than 0.01 is represented by \*\* and a p value of less than 0.001 is represented by \*\*\*.



As expected, MCF-7 cells (ER+) expressed significantly higher levels of ER- $\alpha$  mRNA (Fig. 3A) compared to the ER- cell lines CRL-2335 and Hs578T. Of note, ER- CRL-2335 cells expressed 30% of the ER- $\alpha$  mRNA detected in ER+ MCF-7 cells. Fig.3 B shows the expression of ER- $\beta$  mRNA. The highest levels of ER- $\beta$  mRNA were detected in the CRL-2335 cells (6 fold higher than MCF-7 cells) while the ER- Hs578t cells expressed the lowest levels of ER- $\beta$  mRNA amongst all 3 cell lines studied.

#### Phosphorylation and Subcellular Translocation of ER- $\alpha$ and ER- $\beta$

Since IGF-2 stimulates estrogen regulated genes, we chose two ER- cell lines, CRL-2335 and Hs578T to determine whether IGF-2 treatment regulates the expression or localization of ER- $\alpha$  and ER- $\beta$  in the absence of estrogen. Figure 4 depicts the basal levels of ER- $\alpha$  and ER- $\beta$  in MCF-7 cells.

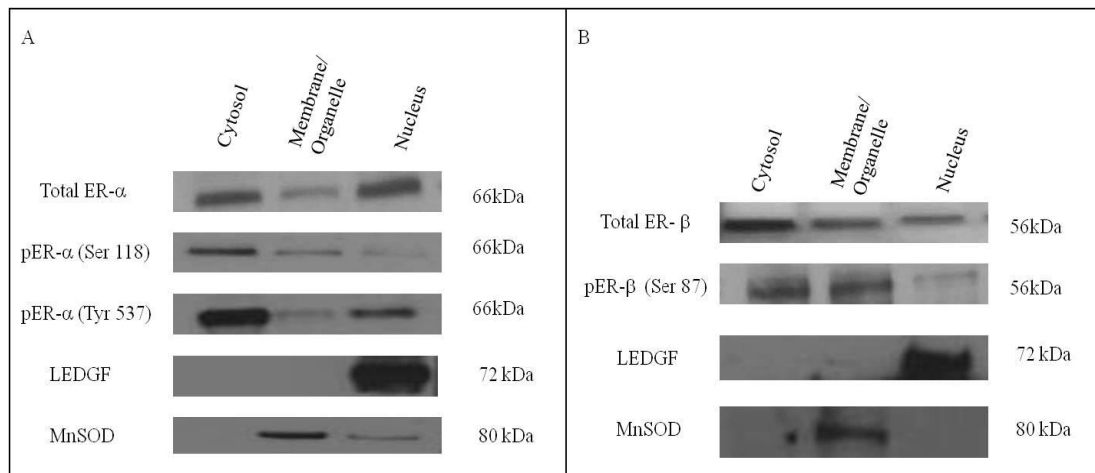


Figure 4. Western Blot of Subcellular localization of Total and Phosphorylated ER- $\alpha$  and ER- $\beta$  in MCF-7 cells. The basal expression of total and phosphorylated ER- $\alpha$  and ER- $\beta$  was detected in MCF-7 cells. In order to verify separation and quantify loading of the subcellular compartments LEDGF and MnSOD were used as controls. Western blot is representative of three independent experiments in triplicate.

As expected, there is significant expression of ER- $\alpha$  and ER- $\beta$  protein in the nuclear compartment of MCF-7 ER+ cells. MCF-7 cells were grown in serum-free, phenol-free media without estrogen, yet, ER- $\alpha$  and ER- $\beta$  are present in cytosolic, membrane/organelle (mitochondrial) and nuclear fractions. Thus, in MCF-7 cells we can detect activated nuclear and organelle ER- $\alpha$  and ER- $\beta$  receptors without the requirement of estrogen.

Likewise, the expression of ER- $\alpha$  and ER- $\beta$  in Hs578T and CRL-2335 cell lines was evaluated by Western blot analysis (WB). Fig. 5A and Fig. 5C shows the levels of total and phosphorylated ER- $\alpha$  (Fig. 5A) and ER- $\beta$  (Fig. 5C) in the cell compartment of ER- Hs578t cells. The basal expression of total ER- $\alpha$  and ER- $\alpha$  (Ser 118) in ER- Hs578t cells is localized in the cytosol (Fig. 5A). In contrast, ER- $\beta$  is also detected in the membrane/organelle (mitochondrial) fraction in ER- Hs578t cells. No pER- $\beta$  (Ser 87) was detected in the membrane/organelle, suggesting that the ER- $\beta$  present in the organelle/mitochondrial fraction is not phosphorylated. Interestingly, when ER- Hs578t cells were treated with proIGF-2, ER- $\alpha$  translocates from the cytosol to membrane/organelle (mitochondrial) fraction and to the nucleus. (Fig.5B). Of great significance, proIGF-II treatment to ER- Hs578t cells stimulates the translocation of ER- $\beta$  from the cytosol to the membrane/organelle (mitochondriafraction and the nucleus (Fig.5D). ProIGF-2 also stimulated the phosphorylation of the ER- $\beta$  receptor present in the membrane/organelle (mitochondria) fraction pER- $\beta$  (Ser 87). Thus, in ER- Hs578t cells, the basal expression of total and phosphorylated ER- $\alpha$  (Ser 118) is localized in the

cytosol. When ER- Hs578t cells are treated with proIGF-II, ER- $\alpha$  translocates to the membrane/organelle (mitochondria) fraction and nucleus while pER- $\alpha$  (Ser 118) is localized in the mitochondrial/membrane fraction. Of note, treatment with mIGF-2 did not stimulate any changes in ER- $\alpha$  or ER- $\beta$  subcellular localization in CRL-2335 and Hs578T BC cells (Data not shown).

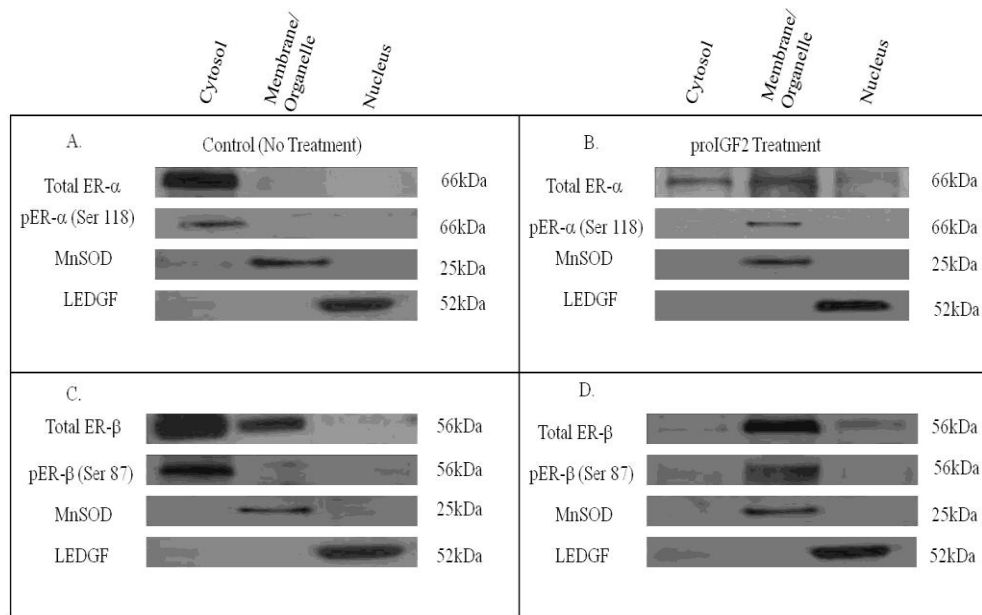


Figure 5. Western Blot of Subcellular localization of Total and Phosphorylated ER- $\alpha$  and ER- $\beta$  in Hs578T cells in response to proIGF2 (100ng/mL) treatment. Figure 5A and 5C shows the basal expression of total and phosphorylated ER- $\alpha$  and ER- $\beta$ . Figure 5B and 5D shows the subcellular translocation of total and phosphorylated ER- $\alpha$  and ER- $\beta$  in cells treated with proIGF2. Western blot is representative of three independent experiments in triplicate.

Figure 6 shows a western blot of the subcellular localization of total and phosphorylated ER- $\alpha$  and ER- $\beta$  in ER- CRL-2335 cells. Figs.6A and 6C shows that in basal conditions, total and phosphorylated ER- $\alpha$  and ER- $\beta$  are localized in the cytosol,

while ER- $\alpha$  is also localized in the membrane/organelle (mitochondria) fraction. Treatment with proIGF-2 stimulated cytosolic ER- $\alpha$  translocation to the membrane/organelle (mitochondria) fraction (Fig.6B) where all of the pER- $\alpha$  (Ser 118) was localized. In contrast, treatment with proIGF-II in CRL-2335 cells only partially stimulated ER- $\beta$  and pER- $\beta$  translocation to the membrane/organelle (mitochondria) fraction (Fig.6D). Thus, both cell lines designated as estrogen negative cells, CRL-2335 and Hs578T, express phosphorylated ER- $\alpha$  and ER- $\beta$  and treatment with proIGF-2 stimulates translocation of both receptors. These data suggests that ER- $\alpha$  and ER- $\beta$  play an important extranuclear function in estrogen negative breast cancer cells and also shows that both receptors can be regulated by proIGF-2.

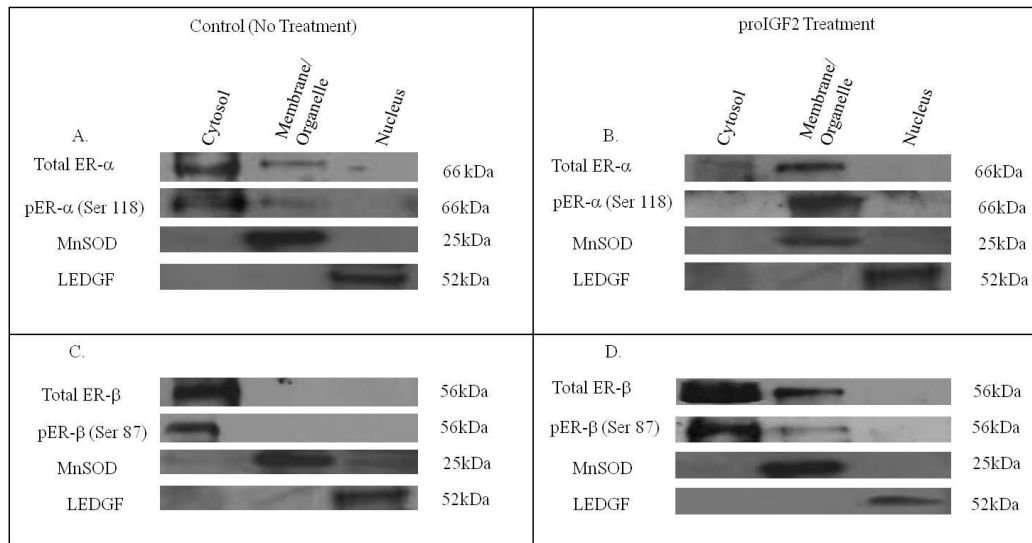


Figure 6. Western Blot of Subcellular localization of Total and Phosphorylated ER- $\alpha$  and ER- $\beta$  in CRL-2335 cells in response to proIGF2 (100ng/mL) treatment. Figure 6A and 6C shows the basal expression of total and phosphorylated ER- $\alpha$  and ER- $\beta$ . Figure 6B and 6D shows the subcellular translocation of total and phosphorylated ER- $\alpha$  and ER- $\beta$  in cells treated with proIGF2. WB is representative of at least three separate experiments.

## IGF-2 Stimulates the Translocation of the ERs through IGF-1R and IR

Since IGF-2 actions are mediated by binding to the insulin-like growth factor-1 receptor (IGF-1R) and the insulin receptor-A (IR-A) we assessed whether proIGF-2 mediates the activation and translocation of the estrogen receptors through IGF-1R, the IR-A or both. Hs578T and CRL-2335 cell lines were treated with IGF-1R and IR-A siRNA to determine which of these receptors were mediating IGF-2 actions. Figure 7 A shows a Western Blot of Hs578t cells treated with IGF-1R siRNA, scrambled siRNA and control and demonstrates that siRNA successfully blocked the expression of the IGF-1R.

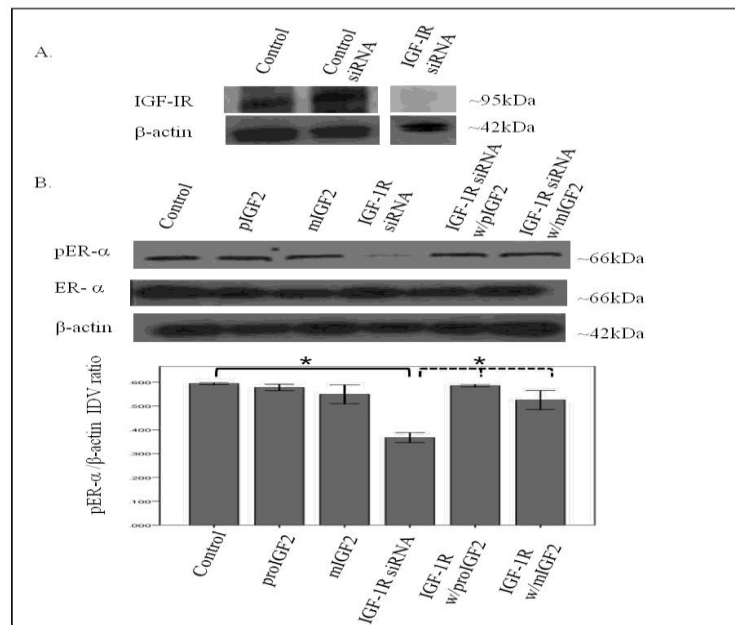


Figure 7 A&B. Western Blot of Total and Phosphorylated expression of ER- $\alpha$  in Hs578T cells treated with IGF-1R siRNA. Figure 7A shows a WB of Hs578T cells untreated (Control), treated with “scrambled” siRNA (siRNA control) and IGF-1R siRNA. Figure 7B shows WB of ER- $\alpha$  expression following proIGF2 and mIGF2 treatment of IGF-1R siRNA treated Hs578T cells. The bar graphs show the results of the densitometry analysis of the WBs phosphorylated ER- $\alpha$  normalized to  $\beta$ -actin and represent the mean  $\pm$  SE of three separate experiments. Solid brackets and \* represents values significantly different from control (\* $p$ <0.05). Dashed brackets and \* represents values significantly different from values between IGF-1R siRNA only treated cells and IGF-1R siRNA with proIGF2 and/ or IGF1R siRNA with mIGF2 treated cells.

Knock-down of the IGF-1R (Fig. 7B) in the Hs578T cell line significantly ( $p < .05$ ) reduced the phosphorylation of both pER $\alpha$  and pER $\beta$  (Fig. 7B & 7C). Interestingly, when siRNA transfected cells were treated with IGF-2 the levels of pER $\alpha$  and pER $\beta$  were restored comparable to the control cells (Fig. 7B & C). Thus, IGF-2 rescued the phosphorylation of pER $\alpha$  and pER $\beta$  when IGF-1R was reduced or knocked-down, suggesting that another receptor(s) were mediating IGF-2 signaling in the absence of the IGF1R.

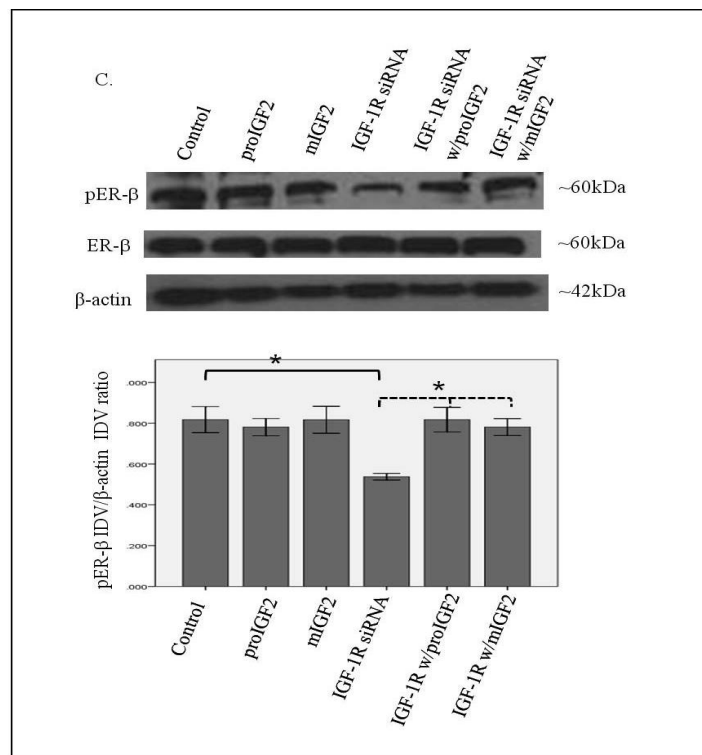


Figure 7 C. Western Blot of Total and Phosphorylated expression of ER- $\beta$  in Hs578T cells treated with IGF-1R siRNA . Figure 7C shows WB of ER- $\beta$  expression following proIGF2 and mIGF2 treatment of IGF-1R siRNA treated Hs578T cells. The bar graphs show the results of the densitometry analysis of the WBs phosphorylated ER- $\beta$  normalized to  $\beta$ -actin and represent the mean  $\pm$  SE of three separate experiments. Solid brackets and \* represents values significantly different from control ( $*p < .05$ ). Dashed brackets and \* represents values significantly different from values between IGF-1R siRNA only treated cells and IGF-1R siRNA with proIGF2 and/ or IGF1R siRNA with mIGF2 treated cells.

In contrast, no effect in the phosphorylation of ER $\alpha$  or ER $\beta$  was observed when the IR was successfully knocked-down in these same cells (Fig. 8A-C).

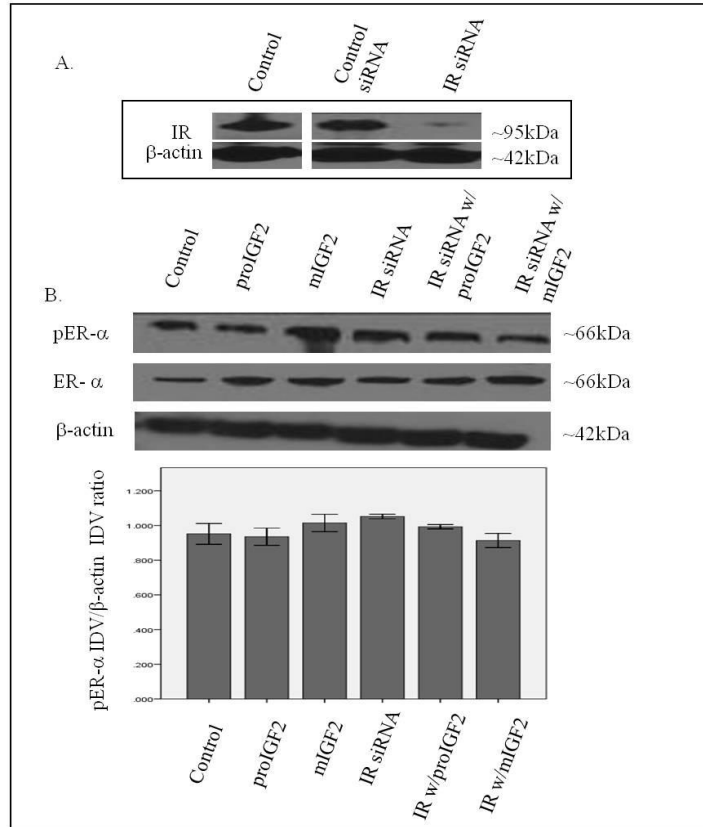


Figure 8 A & B. Western Blot of Total and Phosphorylated expression of ER- $\alpha$  in Hs578T cells treated with IR siRNA. Figure 8A shows a WB of Hs578T cells untreated (Control), treated with “scrambled” siRNA (Control siRNA) and IR siRNA. Figure 6B shows a WB of ER- $\alpha$  following proIGF2 and mIGF2 treatment of IR siRNA transfected Hs578T cells. The bar graphs show the results of the densitometry analysis of the WBs of phosphorylated ER- $\alpha$  normalized to  $\beta$ -actin and represent the mean  $\pm$  SE of three separate experiments.

These results suggest that IGF-2 mediated phosphorylation and translocation of ER $\alpha$  and ER $\beta$  is dependent on activation of the IGF-1R but in its absence IGF-2 can activate the IR or other receptor to restore ER- $\alpha$  and ER- $\beta$  phosphorylation.

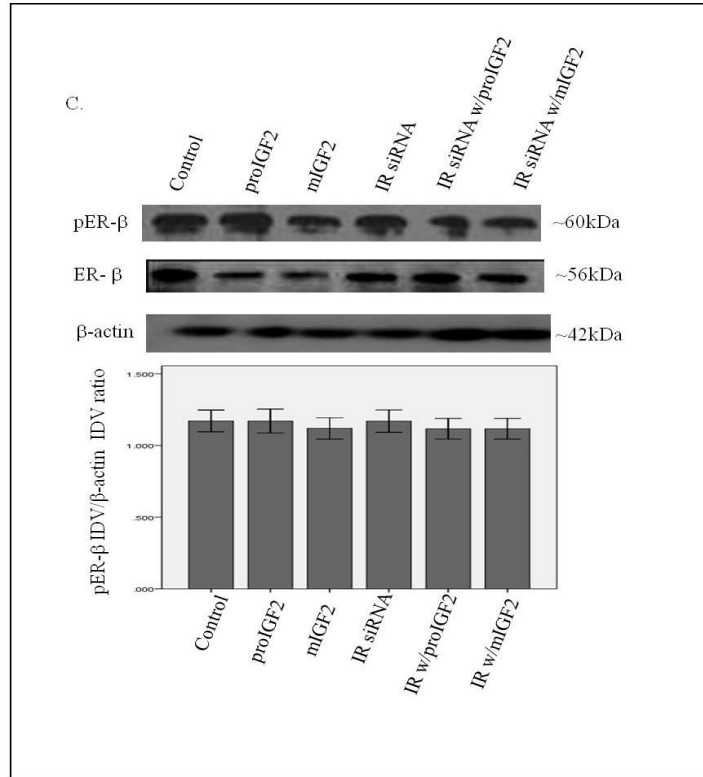


Figure 8 C. Western Blot of Total and Phosphorylated expression of ER- $\beta$  in Hs578T cells treated with IR siRNA. Figure 8C shows a WB of ER- $\beta$  following proIGF2 and mIGF2 treatment of IR siRNA transfected Hs578T cells. The bar graphs show the results of the densitometry analysis of the WBs of phosphorylated ER- $\beta$  normalized to  $\beta$ -actin and represent the mean  $\pm$  SE of three separate experiments.

Figure 9 A-C shows that successful knockdown of IGF-1R in the CRL-2335 breast cancer cells had no effect on the phosphorylation of ER- $\alpha$  and ER- $\beta$ 1. In contrast, a significant reduction in the phosphorylation of ER- $\beta$ 5 (53kDa) variant (Fig.9C) was observed when the IGF-1R was blocked with siRNA. The phosphorylation of the ER- $\beta$ 5 variant was not restored with IGF-2 treatment suggesting that it requires the activation of the IGF-1R signaling. This is significant because ER- $\beta$ 5 is expressed in breast tumors from African-American women and CRL-2335 cells were established from an AA breast cancer patient.



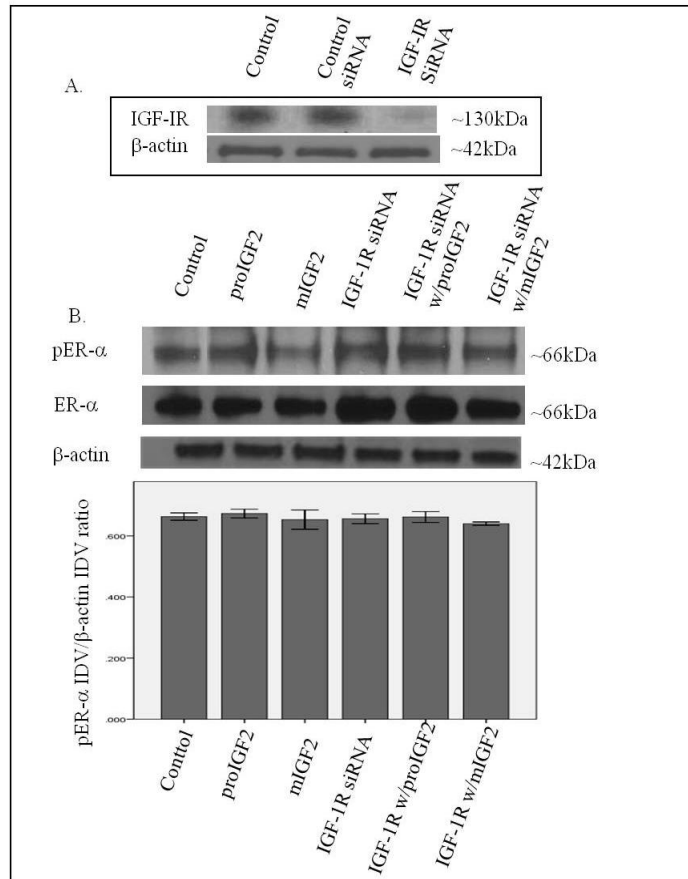


Figure 9 A & B. Western Blot of Total and Phosphorylated expression of ER- $\alpha$  in CRL-2335 cells treated with IGF-1R siRNA. Figure 9A shows a WB of CRL-2335 cells untreated (Control), transfected with “scrambled” siRNA (Control siRNA) and IGF-1R siRNA. Figure 9B shows a WB of ER- $\alpha$  in CRL-2335 cells treated with IGF-1R siRNA. The bar graphs show phosphorylated ER- $\alpha$  normalized to  $\beta$ -actin and represent the mean  $\pm$  SE of three separate experiments.

Notably, when CRL-2335 cells were treated with IR siRNA a significant reduction in the phosphorylation of ER- $\alpha$ , ER- $\beta$ 1 and ER- $\beta$ 5 (Fig.10 A-C) was observed. In contrast to the Hs578t BC cells, phosphorylation of ER- $\alpha$  and ER- $\beta$  in the CRL-2335 cells is more dependent on the activation of the IR signaling. Also distinct in the CRL-2335 cells is the expression of the ER- $\beta$ 5 variant which is not detected in the Hs578t BC cells.

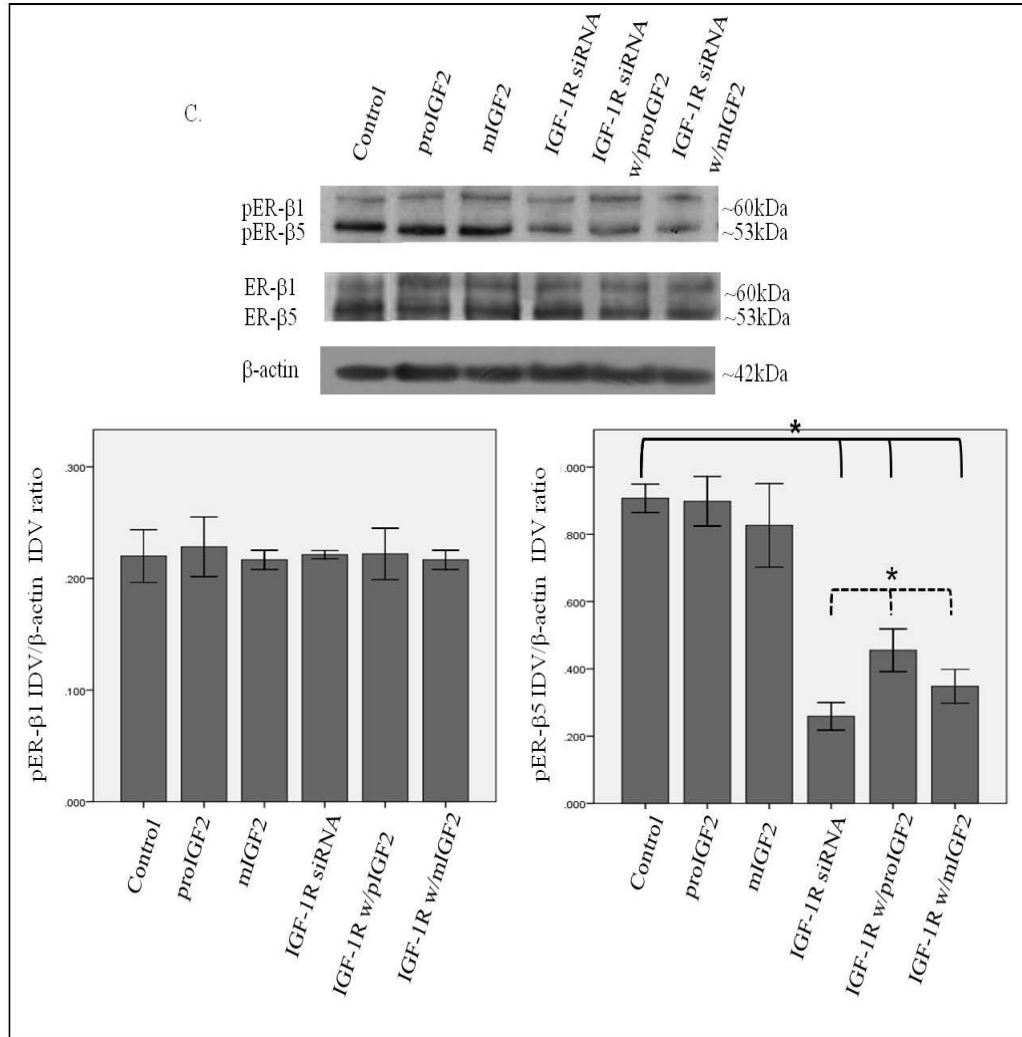


Figure 9 C. Western Blot of Total and Phosphorylated expression of ER-β in CRL-2335 cells treated with IGF-1R siRNA . Figure 9C shows a WB of ER-β in CRL-2335 cells treated with IGF-1R siRNA. The bar graphs show phosphorylated ER-β normalized to β-actin and represent the mean +/- SE of three separate experiments. Bar graphs with solid brackets and \* represents values significantly different from control (\*p<0.05). Bar graphs with dashed brackets and \* show significantly different (\*p<0.05) values between IGF-1R siRNA only treated cells and IGF-1R siRNA with proIGF2 and/ or IGF1R siRNA with mIGF2 treated cells.

Treatment with IGF-2 restored phosphorylation of ER-α, ER-β1 and ER-β5 in the CRL-2335 BC cells (Fig. 10 B&C). Simultaneous knockdown of IR and IGF-1R was lethal (Data not shown).

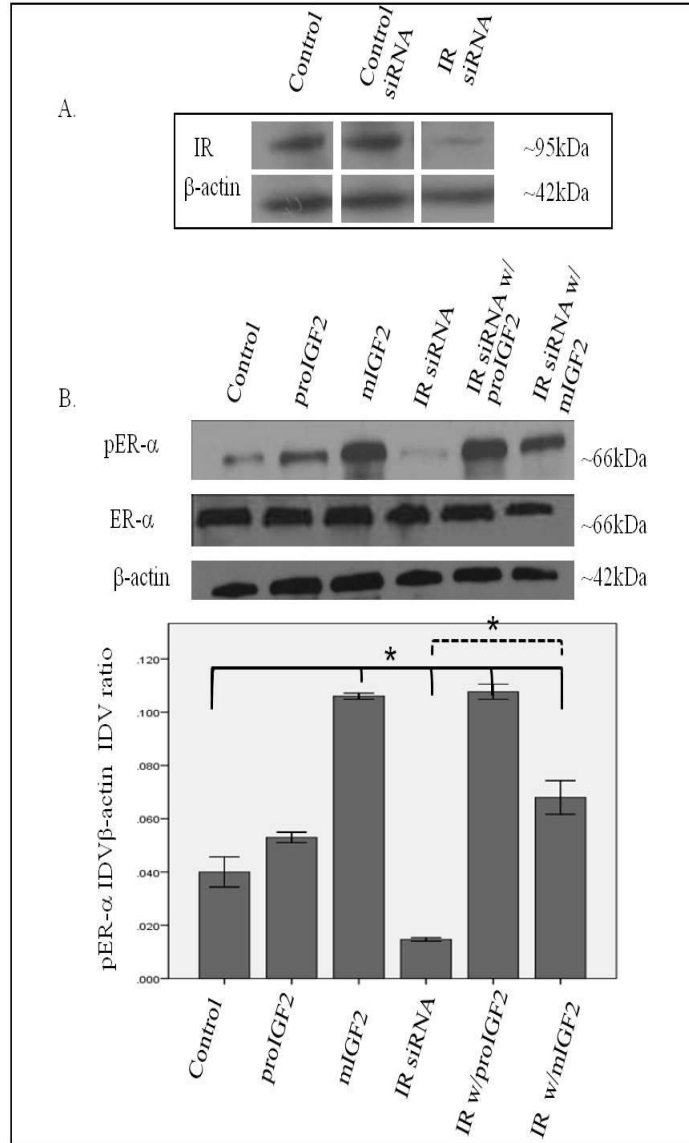


Figure 10 A & B. Western Blot of Total and Phosphorylated expression of ER- $\alpha$  in CRL-2335 cells treated with IR siRNA. Figure 10A shows a WB of CRL-2335 cells untreated (Control), transfected with “scrambled” siRNA (Control siRNA) and IR siRNA. Figure 10 B shows a WB of total and phosphorylated ER- $\alpha$  following proIGF2 and mIGF2 treatment and/or IR siRNA treatment in CRL-2335 cells. The bar graphs show phosphorylated ER- $\alpha$  normalized to  $\beta$ -actin and represent the mean  $\pm$  SE of three separate experiments. Bar graphs with solid brackets and \* represents values significantly different from control (\* $p$ <0.05). Bar graphs with dashed brackets and \* significantly different (\* $p$ <0.05) values between IR siRNA only treated cells and IR siRNA with proIGF2 and/ or IGF1R siRNA with mIGF2 treated cells.

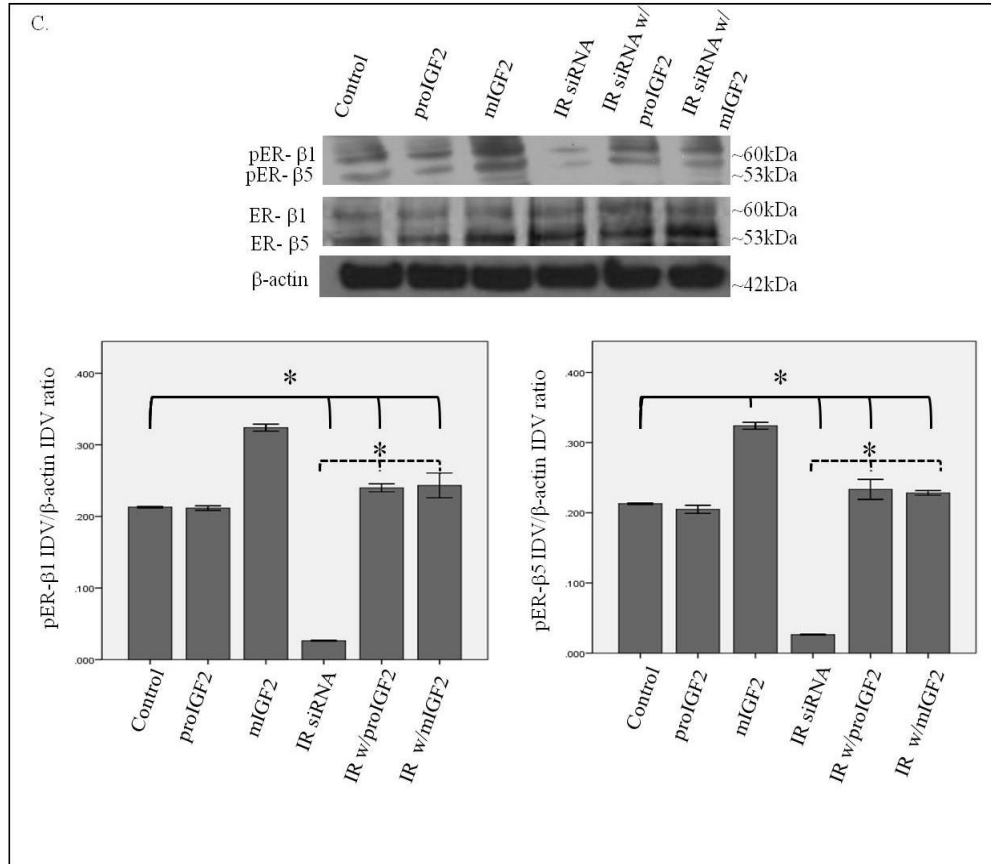


Figure 10 C. Western Blot of Total and Phosphorylated expression of ER-β in CRL-2335 cells treated with IR siRNA. Figure 10C shows a WB of total and phosphorylated ER-β following proIGF2 and mIGF2 treatment and/or IR siRNA treatment in CRL-2335 cells. The bar graphs show phosphorylated ER-β normalized to β-actin and represent the mean  $\pm$  SE of three separate experiments. Bar graphs with solid brackets and \* represents values significantly different from control (\* $p < 0.05$ ). Bar graphs with dashed brackets and \* significantly different (\* $p < 0.05$ ) values between IR siRNA only treated cells and IR siRNA with proIGF2 and/or IGF1R siRNA with mIGF2 treated cells.

## Discussion

The acquired ability of hormone refractory breast cancer cells to avoid cell death in the presence of anti-estrogen therapy means that the cells have developed the ability to maintain cell survival signaling pathways without the requirement of estrogen. The

mechanism(s) used by breast cancer to acquire this ability are not well-defined. A general consensus in the field is that “cross-talk” mechanisms between growth factor receptors (IGF-1R and EGFR) and estrogen receptors (ER- $\alpha$ ) facilitate the progression of breast cancer tumors that become hormone insensitive and refractory (Schiff, 2004). In fact, activation of the IGF-1R (Kato, 1995; Lannigan, 2003) can lead to the activation of ER- $\alpha$  through activation of the mitogen-activated protein (MAP) kinase pathway resulting in the phosphorylation of ER- $\alpha$  at Ser 118. Since IGF-2, not IGF-I, is the growth factor expressed in breast cancer cells (Pezzino, 1996), we propose that IGF-2 can maintain survival signals in an autocrine fashion by binding to the IGF-1R and possibly to the IR (LeRoith, 1995; Sciacca, 1999). Furthermore, since many breast cancers express both ER- $\alpha$  and ER- $\beta$ , we deduced that IGF-2 can bind and activate the IGF-1R and IR leading to the activation of both ER- $\alpha$  and ER- $\beta$  in BC cells. This IGF-1R and IR/ER cross-talk allows the cells to activate/phosphorylate the ERs without the need of estrogen. Thus, current anti-estrogen therapies would not be able to effectively prevent ER activation in these BC cells.

Binding of IGF-2 to the IGF-1R and IR activates different signaling pathways (Valentinis, 2001; Chen, 2009). Our study suggests that the activation of these different signaling pathways give each receptor a unique role in the phosphorylation of ER- $\alpha$  and ER- $\beta$ . In ER- Hs578T cells, IGF-1R knockdown decreased the phosphorylation of both ER- $\alpha$  and ER- $\beta$ . However, treatment with IGF-2 was able to increase the phosphorylation of both, ER- $\alpha$  and ER- $\beta$ , back to control levels in IGF-1R siRNA transfected Hs578T cells. Thus, IGF-1R appears to be important in the phosphorylation of ER- $\alpha$  and ER- $\beta$  in Hs578T cells, however IGF-2 treatment was able to restore ER

phosphorylation presumably by acting through the IR-A. Indeed, Hs578t was the first breast cancer cell line shown to express high levels of IR-A (Sciacca, 1999). In contrast, knocked down expression of the IGF-1R in the ER- CRL-2335 cells had no effect on the phosphorylation of ER- $\alpha$  or ER- $\beta$ 1 but it decreased the activation of the ER- $\beta$ 5 variant. The ER- $\beta$ 5 variant is overexpressed in African-American (AA) women with aggressive BC (Poola, 2005) and this study shows that ER- $\beta$ 5 variant is expressed in the ER- CRL-2335 cells derived from an AA breast cancer patient (Gazdar, 1998). Treatment with IGF-2 was unable to restore ER- $\beta$ 5 phosphorylation in the IGF-1R siRNA transfected ER- CRL-2335 cells. These results suggest that IGF-1R expression is required for the phosphorylation of the ER- $\beta$ 5 variant. In contrast, ER- $\beta$ 5 variant is not expressed in the Hs578T cell line derived from a Caucasian breast cancer patient (Hackett, 1977).

IR knockdown further demonstrated the different roles of IGF-1R and IR in the phosphorylation of ER- $\alpha$  and ER- $\beta$ . In contrast to the inhibition of the IGF-1R, IR knockdown in Hs578T cells had no effect on the phosphorylation of either estrogen receptor. Of note, IR siRNA transfected CRL-2335 cells showed a decrease in the phosphorylation of ER- $\alpha$ , ER- $\beta$  and ER- $\beta$ 5. IGF-2 treatment restored the phosphorylation of all, ER- $\alpha$ , ER- $\beta$  and ER- $\beta$ 5, possibly thorough the IGF-1R. These findings are very significant because they show the unique ability of IGF-2 to activate both the IGF-1R and IR to enhance ER activation and signaling in BC cells. Thus, both, IGF-1 and IR are important targets in the treatment of estrogen independent breast cancers.

Our study also shows that IGF-2 treatment not only phosphorylated ER- $\alpha$  and ER- $\beta$  but it also stimulated the sub-cellular translocation of both estrogen receptors from the cytosol to the organelles/membrane (mitochondria) fraction. The organelles/membrane

(mitochondria) fraction contains the plasma membrane where the estrogen receptors activate a unique signaling cascade that results in rapid changes that do not require estrogen receptor translocation to the nucleus (Bjornstrom, 2005). The translocation of the extranuclear ER to the plasma membrane allows for cross-talk between the growth factor receptors and ER which then promotes the activation of cell survival pathways such as the MAP kinase pathway (Kahlert, 2000; Klotz, 2002).

These data also demonstrates that IGF-2 stimulated extranuclear ER translocation to the mitochondria where it can regulate mitochondrial function (Bjornstrom, 2005; Pietras, 2007; Singh, 2007; Song, 2006; Xu, 2004). We have previously shown that IGF-2 regulation of the mitochondria allows breast cancer cells to prevent apoptosis and provide energy for cell growth, thus, promoting cell survival (Singh, 2007).

This paper shows that IGF-2 is able to promote breast cancer cell survival by activating the IGF-1R and the IR-A which promotes the activation of cell survival pathways. We also demonstrate that IGF-2 is able to regulate the translocation of ER- $\alpha$  and ER- $\beta$  to the mitochondria and plasma membrane in order to promote cell survival (Anders, 2008). Our study presents compelling evidence that IGF-2 plays an important role in promoting growth factor and estrogen receptor cross-talk mechanisms thus, activating estrogen signaling pathways without the requirement of estrogen.

In summary, IGF-2 promotes the activation of estrogen cell survival pathways without the requirement for estrogen. The novel proposed mechanism is that IGF-2 is able to bind to the IGF-1R and IR-A to promote ER- $\alpha$  and ER- $\beta$  phosphorylation and translocation to the nucleus, plasma membrane and mitochondria leading to the activation of cell survival pathways. Breast cancers that grow independent of estrogen currently

have no targeted treatments due to a gap in knowledge of cell survival mechanisms in these cells. The mechanism proposed in this paper may lead to the development of targeted treatments for these breast cancers.

#### **Declaration of Interest**

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No conflicts of interest are declared by the authors.



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## CHAPTER THREE

### EXTRACELLULAR EXPRESSION OF ERS IN BREAST CANCER TISSUES

#### **Introduction**

One major finding in this dissertation was the extranuclear expression of ER- $\alpha$  and ER- $\beta$  in two cell lines, CRL-2335 and Hs578T, designated by ATCC as ER negative. These cells were designated as ER negative based on the staining patterns of the cells nuclei for the presence of ER- $\alpha$  using immunohistochemistry (IHC) (Nadji et al. 2005; Swain 2001). The extranuclear expression of the ERs was not taken into account. The experiments presented in this chapter show that the extranuclear expression of ER- $\alpha$  and ER- $\beta$  in breast cancers designated as ER negative is not unique to breast cancer cell lines. The extranuclear expression of ER- $\alpha$  and ER- $\beta$  in ER negative designated tumor tissue samples was analyzed using IHC.

#### **Materials and Methods**

##### Breast Cancer Tissue Specimens

Frozen and parafilm AA women breast cancer tissue specimens were obtained from the Cooperative Human Tissue Network (CHTN). The tumors were designated as ER negative or ER positive in pathology reports received along with the tumor tissue samples.

## Immunohistochemistry Analysis

A four micron tumor slice of frozen tissue or paraffin block sections were slice, mounted on slides and stored at 4° C. Slides were de-waxed in CitriSolv solution (Fischer Scientific) three times for 5 minutes. The slides were then hydrated in 100%, 95% and 80% ethanol sequentially for 3 minutes then immersed in tap water for 5 minutes. Each slide was heated for 12 minutes in a 95°F water bath in 1x Reveal solution (Biocare Medical) then allowed to cool to room temperature and washed with 1x PBS for 1 minute. To quench endogenous peroxidase activity each slide was immersed in a 3% hydrogen peroxide solution (Sigma) for 10 minutes followed by a 1x PBS wash. Following a 1 hour incubation with blocking serum, a 1:50 dilution of ER $\alpha$  or ER $\beta$  antibody was prepared in blocking serum and 1x PBS following manufacturer's recommendations (Mouse or rabbit ABC Staining System, Santa Cruz Biotechnology). Each slide was incubated overnight at 4°C. The primary antibody was removed with 3 washes of 1x PBS. The biotinylated secondary antibody was diluted in 1x PBS and blocking serum according to manufacturer protocol (Mouse or rabbit ABC Staining System, Santa Cruz Biotechnology) and added to each slide. This incubation occurred at room temperature for 30 minutes. After washing each slide three times with 1x PBS streptavidin-peroxidase and its substrate were added according to manufacturer's recommendations (Mouse or Rabbit ABC Staining System, Santa Cruz Biotechnology) for 30 minutes at room temperature. Counterstaining was obtained with 25 $\mu$ l of Gill hematoxylin. Twice each slide was placed in 100% ethanol solution followed by CitriSolv for 1 minute at room temperature. A 1.5 glass cover slip (Thermo Scientific) was mounted to each slide and staining was observed.

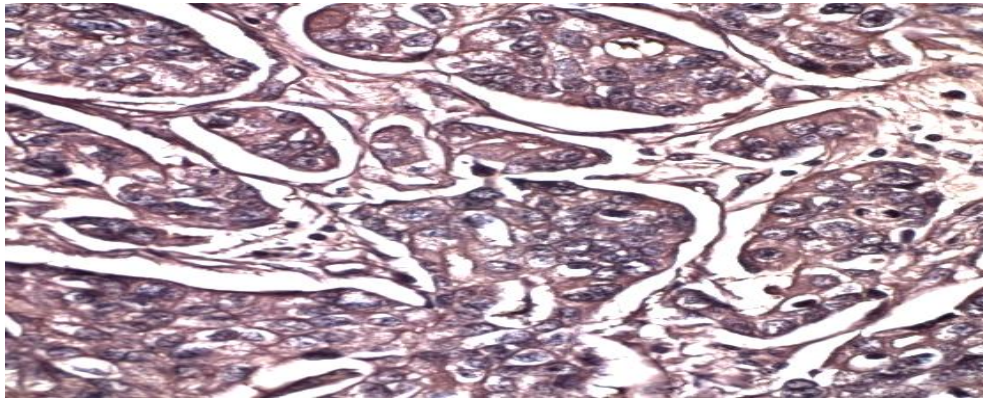


## Results

### Detection of ER $\alpha$ and ER $\beta$ in Breast Cancer Tissues

Evaluation of human breast cancer tissues designated as ER negative demonstrates intense staining of ER- $\alpha$  and ER- $\beta$  outside of the nucleus (Fig. 11 A & B), and it is predominantly located in the nucleus of estrogen receptor positive tumor samples (Fig 12 A & B). ER localization is determined based on the intensity of brown staining seen in the sample compared to slides with non-specific staining.

A.



B.

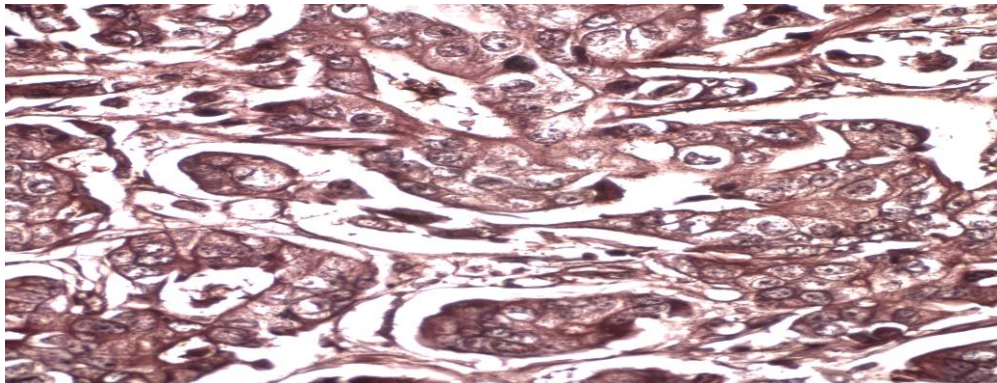
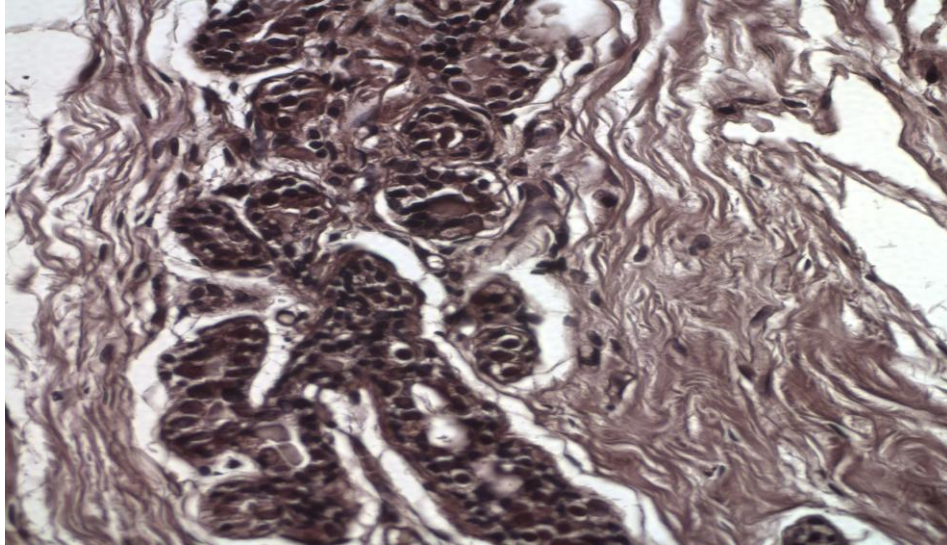


Figure 11. IHC of ER $\alpha$  (A) and ER $\beta$  (B) in African-American ER Negative Breast Cancer Tissues. The ER $\alpha$  and ER $\beta$  staining is outside of the nucleus in these ER Negative breast cancer tissues.

A.



B.

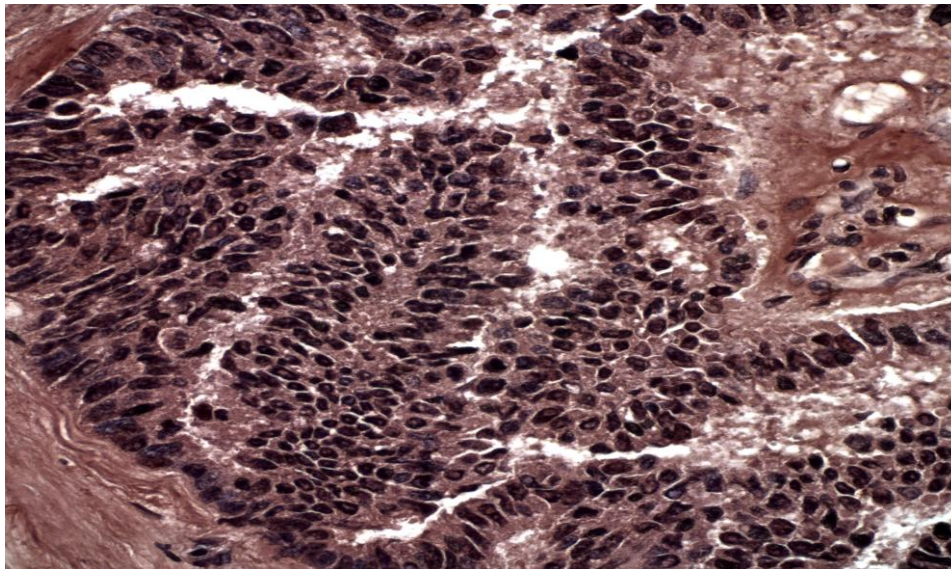


Figure 12. IHC of ER $\alpha$  (A) and ER $\beta$  (B) in African-American ER Positive Breast Cancer Tissues. There is strong nuclear staining of ER $\alpha$  and ER $\beta$  in these ER positive breast cancer tissues.

### Discussion

Breast tumors are given ER designation in order to determine available treatment options. ER positive tumors are treated with hormonal therapies directed towards

estrogen and the estrogen receptor (Jordan, 2007). There is a lack of targeted therapies for ER negative tumors (Reis-Filho et al., 2008). Tumor designation is based on the staining pattern of ER- $\alpha$  in the nuclei of the breast cancer cells using IHC (Nadji et al. 2005; Swain, 2001). This was the method used to determine the designation of the breast cancer tissues used in this study as ER negative (Figure 11 A and B) and ER positive (Figure 12 A and B). Our IHC results confirmed that the ER negative breast cancer tissues (Fig 11) lack significant ER $\alpha$  nuclear staining and that there is strong nuclear staining of ER $\alpha$  in the ER positive breast cancer tissues (Fig 12). However, the current method used to assign ER designation does not consider the extranuclear expression of ER $\alpha$  and ER $\beta$  in ER negative breast cancer.

Importantly, studies in this dissertation demonstrate the expression and significance of extranuclear ER $\alpha$  and ER $\beta$  in ER negative breast cancer. Our studies show that when stimulated by IGF2 extranuclear ERs translocate to the mitochondria and plasma membrane. In the mitochondria, ERs bind to estrogen response like elements in the mitochondrial DNA and prevent apoptosis, and ERs translocated to the plasma membrane promote ER and growth factor receptor cross-talk which activates the cell survival MAP kinase pathway (Singh, 2007; Bjornstrom and Sjoberg 2005; Pietras and Marquez-Garban 2007; Klotz et al. 2002). In light of these studies the current IHC method guidelines used in the clinic to determine ER designation based on the nuclear staining of ER $\alpha$  needs to be revised to include the evaluation of extranuclear ER- $\alpha$  and ER- $\beta$  because they play a major role in breast cancer growth and survival.

## CHAPTER FOUR

### DISCUSSION

#### **Conclusions and Future Directions**

Breast cancer is the most common form of cancer in women (Tian et al., 2010; Greenlee et al., 2001). The risk of a women developing breast cancer in her lifetime has nearly tripled over the last sixty years (Tian et al., 2010; Feuer et al., 1993). The estrogen receptor (ER) is the primary target for both chemoprevention and endocrine therapy for breast cancer treatment (Griekspoor et al., 2007; Jordan, 2007; Reis-Filho et al., 2008; Robson and Offit 2007). The majority of breast cancer tissues have an increased expression of both the estrogen receptor alpha and estrogen receptor beta (ER- $\alpha$  and ER- $\beta$ ) (Speirs, 2002; Herynk et al., 2004; Mueller et al., 2002; Song et al., 2004). However, most breast cancers are estrogen dependent at the time of diagnoses, but eventually develop a mechanism that allows estrogen independent tumor growth as the disease progresses (Szepeshazi, 1992). This estrogen independent breast tumor growth affects different ethnic groups disproportionately (Chlebowski et al. 2005; Ihemelandu et al. 2007; Reis-Filho et al., 2008). When compared to Caucasian women, African-American (AA) women are more likely to have aggressive tumors that grow independent of estrogen (Kobayashi 2008; Ihemelandu et al., 2007; Reis-Filho et al., 2008). These tumors are resistant to the endocrine therapy currently used and they are therefore referred to as being chemoresistant (Turner et al., 2004; Brenton, 2005; Jumppanen et al., 2007; Reis-Filho et al., 2008). This dissertation addressed the need for a comprehensive

approach to understanding the biological factors associated with chemoresistance and increased mortality among AA patients diagnosed with breast cancer.

This dissertation investigated how insulin-like growth factor-II (IGF-II) contributes to the survival disparity among AA women. IGF-II is an important hormone in the development of normal breast tissue and breast cancer (Singh et al., 2008; Singh et al., 2007; Vyas et al., 2005). It is alternatively spliced into two forms, the precursor IGF-II (proIGF-II) and the mature IGF-II (mIGF-II). ProIGF-II is the predominant form expressed in cancer (Singh et al., 2008; Singh et al., 2007).

The mitogenic effects of IGF-II are mediated through the Insulin-like growth factor I receptor (IGF-IR) and the Insulin Receptor (IR) (Abbas et al., 2007; Ellis et al. 1998; LeRoith et al. 1995). The importance of growth factor activation of IGF-IR in Estrogen Receptor activation has not been fully deciphered but, it has been shown that *IGF-I* binding to the IGF-IR leads to the phosphorylation of ER- $\alpha$  (Schiff, 2004; Gee, 2005; Shou, 2004; Fagan and Yee, 2008). However, IGF-II and not IGF-I is overexpressed in breast cancer cells. Moreover, most breast cancer cells express both ER- $\alpha$  and ER- $\beta$ .

This dissertation has provided evidence demonstrating that *IGF-II* abrogates the requirement for estrogen in breast cancer cells by acting through the IGF-IR and IR to phosphorylate/activate ER- $\alpha$  and ER- $\beta$ , thus promoting estrogen independent growth.

We used two BLBC cell lines, CRL-2335 and Hs578T, for the studies in this dissertation. These ATCC breast cancer cell lines are designated as ER negative. However, in our studies we determined that these cell lines not only expressed ER- $\alpha$  and

ER- $\beta$  but these receptors also had the ability to become activated and translocate to the mitochondria and plasma membrane to regulate cell survival.

The studies in this dissertation show that the activation of ER in Hs578T and CRL-2335 basal like breast cancer (BLBC) cell lines is independent of estrogen. IR and IGF-1R was successfully knocked down in Hs578T and CRL-2335 cell lines and ER- $\alpha$  and ER- $\beta$  phosphorylation in response to IGF2 treatment was evaluated and IGF2 was able to activate both the IGF-1R and IR in BLBC cells leading to ER activation.

The BLBC cell lines used in this dissertation were designated, by ATCC, as being ER negative based on the cells nuclear staining pattern using IHC (Nadji et al. 2005; Swain 2001). This dissertation not only demonstrates the extranuclear expression of ER- $\alpha$  and ER- $\beta$  in breast cancers cell lines and breast cancer tissues designated as ER negative, but it also shows that these ERs are activated by IGF2 and promote breast cancer cell survival. Notably, the ER- $\alpha$  and ER- $\beta$  antibodies used in these western blot and IHC studies are standard antibodies used for breast cancer ER designation in the clinic. We suggest that the ER designation of breast tumors must take into consideration extranuclear ER expression.

There are many future studies that can be done from the groundwork of this dissertation. There is a big gap in knowledge concerning the treatment of women with BLBC. The main reason for this is because the cell survival pathways used by these cells are poorly understood. This dissertation outlines a novel mechanism used by BLBC to promote cell survival. Studies in our laboratory show that MAP kinase pathway is one of the cell survival pathways activated by IGF2 in BLBC cells. However, in order to develop targeted treatment for BLBCs there is a need to further elucidate the role of these

pathways in promoting breast cancer cell survival.

Hormonal therapies, such as Tamoxifen (Tamox), are used as the standard of care for women with estrogen-dependent breast cancer (Jaiyesimi, et al. 1995) (Massarweh, et al. 2008). While Tamoxifen use in estrogen-dependent breast cancer has been shown to be beneficial, there is increasing evidence that the treatment of estrogen-independent tumors, such as BLBC, with Tamoxifen is detrimental and leads to a decrease in patient survival (Merglen, et al. 2009). The underlying mechanism for this detrimental effect is unknown, but evidence suggests that increased growth factor receptor signaling plays a significant role (Shou, 2004; Gee et al., 2004; Britton et al., 2006). Studies in our laboratory shed some light on the subject by demonstrating that Tamox leads to the activation of ER phosphorylation and the upregulation of ER-regulated survival genes (IGF-II, Survivin and BCL-X) in BLBC cell lines. This increase in IGF-II activates IGF-1R and IR potentiating ER activation via cross-talk mechanisms. This suggests a novel mechanism in which anti-estrogen therapy, Tamox, plays a role in promoting breast cancer cell survival in BLBC. However, there is a need to future elucidate the connection between Tamox treatment and its effects on BLBC growth and survival.

Lens epithelium-derived growth factor (LEDGF) was used in this dissertation as a marker for the integrity of the nuclear compartment extractions. LEDGF is alternatively spliced into LEDGF/p75 and LEDGF/p52. Evidence suggests that LEDGF p75 and p52 behave as antagonist in the cellular stress response in cancer cells (Brown-Bryan, 2008). In our studies we observed a differential expression of LEDGF in the CRL-2335, Hs578T and MCF-7 breast cancer cell lines. LEDGF/p52 was expressed in the CRL-2335 and Hs578T cell lines, and LEDGF/p75 was detected in MCF-7 cells. There is a need to

further elucidate how the differential expression of LEDGF in breast cancer cell lines mediates cell survival.

In conclusion, the studies demonstrated in this dissertation are significant and will have a great impact in the breast cancer field once implemented in the clinical setting. Our studies provide evidence for a need to change the way breast cancer is designated. Currently, breast cancer tissues are designated as ER negative if there is less than 10 percent nuclear staining of ER $\alpha$  (Nadji et al. 2005; Swain 2001). Breast cancer designated as ER negative is considered to lack ER mediated signaling and thus it is not treated with ER targeted therapies.

In addition, ER negative breast cancer cells are able to grow independent of estrogen and therefore thought to have no need for ER signaling. However, our studies show that breast cancer cells and tissues designated as ER negative actually do express ER but it is outside of the nucleus (extranuclear). Moreover, these breast cancer cells not only express extranuclear ER $\alpha$  but they also express extranuclear ER $\beta$ .

In this dissertation we have demonstrated the importance of this extranuclear expression of ER $\alpha$  and ER $\beta$ . Our studies show that IGF2 binds to the IR and IGF-1R leading to the activation and translocation of ER $\alpha$  and ER $\beta$  to the plasma membrane and mitochondria where ERs are then able to promote breast cancer cell survival independent of estrogen. This represents a novel mechanism in which these breast cancer cells are able to promote ER activation without the need for estrogen. In the light of these studies which show that extranuclear ERs promote breast cancer cell survival there is a need to reconsider breast cancer designation based on nuclear staining for ER $\alpha$  to include extranuclear expression of both ER $\alpha$  and ER $\beta$ . Also, the recognition of the importance



and role of extranuclear ER signaling in breast cancer cells will allow for the investigation of targeted therapies for these cancers.

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