THE ROLE OF ESTROGEN RECEPTOR-ALPHA 36 IN THE MEMBRANE EFFECT OF 17BETA-ESTRADIOL ON TUMOR PROGRESSION

A Dissertation
Presented to
The Academic Faculty

By

Reyhaan Ali Chaudhri

In Partial Fulfillment
Of the Requirements for the Degree
Doctorate of Philosophy in Molecular and Cell Biology
In the School of Biology

Georgia Institute of Technology

August 2013

THE ROLE OF ESTROGEN RECEPTOR-ALPHA 36 IN THE MEMBRANE EFFECT OF 17BETA-ESTRADIOL ON TUMOR PROGRESSION

Approved by:

Dr. Barbara D. Boyan, Advisor
School of Biology
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Chong Shin
School of Biology
Georgia Institute of Technology

Dr. Zvi Schwartz
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Nael McCarty
School of Biology
Emory University

Dr. Kirill Lobachev
School of Biology
Georgia Institute of Technology

Date Approved: [March 28, 2013]

ACKNOWLEDGEMENTS

First and foremost, I have to acknowledge my advisors, Dr. Barbara Boyan and Dr. Zvi Schwartz. They have continued to support me for many years, not only as a graduate student, but first as an undergraduate student and a lab manager. I first met Dr. Boyan as an undergraduate student taking an intro class to biomedical engineering. After being fortunate enough to witness two lectures from Dr. Boyan, I was immediately intrigued by her research. She was very approachable, and for some reason, the fact that I was born in Canada and my educational background seemed to pique her interest. She told me she spent much of her childhood in Toronto and she liked biologists. Check and check. Within a few days, I entered the lab as an undergraduate research assistant and the first person I met was Dr. Schwartz, who has been extremely influential on my approach to research. As a mentor, Dr. Schwartz was extremely influential in my choice to pursue a PhD, and he showed me how to approach science with such an admirable passion, that I hope I can continue in my professional career with a similar joyous passion.

As an undergraduate student, I was placed under the direct mentorship of Dr. Liping Wang. In retrospect, I have seen undergraduate students come and go, and I have witnessed different mentorship techniques from numerous graduate students and post-doctoral fellows, and I can honestly say that I had a very good mentor. There are many different types of undergraduates: those who want to do research because they are trying to get a recommendation or add research experience to their resume and those who are truly interested in answering questions through research. I truly appreciate that Dr. Wang did not treat me like an extra pair of hands to help him perform

experiments; instead, he let me feel like I had ownership of the experiments I was performing, which I believe is the best way to instill interest in an undergraduate student.

The next person I would like to acknowledge helped me greatly in my development as a graduate student. After my undergraduate years, I became lab manager for 2 years, and during this time, I worked with Dr. Rene Olivares-Navarrete, who at the time was a post-doctoral fellow. I first met Dr. Olivares-Navarrete as an undergraduate. When I first joined the lab, he had also joined from Universidad Nacional Autonoma de Mexico in order to spend 6 months of his PhD research with Dr. Boyan and Dr. Schwartz. We reconnected during my tenure as lab manager when he joined the lab as a post-doc and worked together on the role of integrins in osteoblast responses to microrough titanium surfaces, which resulted in a co-authored publication. I performed some of my own research as lab manager, but we also continued to work together on other projects. In fact, one of the projects we worked on together was the precursor to my PhD work. I must credit Dr. Olivares-Navarrete in helping me to begin this work. Without his friendship and me observing his example as a passionate scientist, I would probably not be where I am now.

I would also like to thank my committee members Dr. Kirill Lobachev, Dr. Nael McCarty, and Dr. Chong Shin for their interest and insight into my work. I understand their time is valuable and I greatly appreciate the time they have devoted to guiding me through the end of my PhD career, especially the laborious hours they devoted to my committee meetings. I have actually known Dr. Lobachev and Dr. McCarty since my years as an undergraduate student, and taking their classes during my undergraduate years played a major role in me refocusing myself from an undergraduate to a graduate student. I would also like to thank Dr. Lobachev for his help as a collaborator with the production of mutant plasmids for ERα36, the focus of this thesis.

I would like to briefly mention Dr. Henry Blumberg and Dr. Thomas Ziegler for mentoring me through the MSCR program at Emory University, not to mention Cheryl Sroka who truly made my life easier while completing the master's degree. In addition, I would like to thank Dr. Ruth O'Regan for her advice in tissue microarray analysis. I would like to acknowledge Dr. Boyan again for advising me to enter the MSCR program, which has given me exciting opportunities as to where my career may take me.

As for those who have come and gone from the Boyan lab who were especially helpful during my time here, I would like to thank Dr. Christopher Hermann, Dr. Yun Wang, Dr. Tracy Denison, Dr. Jung Hwa Park, Dr. Ge Zhao, Dr. Ming Zhong, Crystal Branan, Maya Fisher, Jamie Lazin, Erin Plute, Leang Chhun, and Kevin Wong. All of these individuals offered help and friendship during my time in the lab and I will forever reflect on our time together with fondness. I would especially like to thank Sharon Hyzy for everything she has helped me with through the years. As the lab manager during my time as a graduate student, she truly helped everything in the lab function smoothly to the point where the only limiting factor in any of my experiments was my own success. Special thanks to Maribel Baker for all her help the last few years and for putting up with me during grant writing times. Also, thanks to Ms. Brentis Henderson for her administrative help with ordering materials and planning conference trips. To my fellow biologists, Dr. Jennifer Hurst-Kennedy, Dr. Khairat El-Baradie, and Maryam Doroudi, we were a special group in a tissue engineering lab, and I enjoyed working together, and together offering a biological perspective to the engineers in the lab. Dr. Hurst-Kennedy was especially instrumental in helping me to better approach my research from the start and I greatly appreciate her for that. To the current members of the lab: Dr. Joshua Cohen, Alice Cheng, Erin Hewett, Kelsey Lawrence, Shirae Leslie, Ethan Lotz, Qingfen Pan, James Wade, and Marcus Walker, who have helped me most recently with

preparing for my defense. I would like to especially recognize Dr. Cohen for our current work together on *in vivo* tumor models. He has really taken the reigns with these studies, allowing me to focus on finishing experiments and writing. I cannot forget to mention the numerous undergraduate students James Camba and Monica Liou who I had the pleasure of mentoring during my first few years of graduate school, and especially the most recent students Agreen Hadadi, Natalia Cuenca, and Anastasia Lobacheva, who worked with me and gave me a fresh, unbiased perspective on my own research.

I would like to acknowledge my former colleagues Dr. Jiaxuan Chen, Dr. Rolando Gittens, Dr. Christopher Lee, and Dr. Andrew Raines not only as insightful and essential in offering their valuable perspective into my research, but they were also truly incredible friends and I am very proud and happy to call them friends. I would also like to acknowledge a few friends who have always been there for me when I needed to take my mind out of the lab and Georgia Tech. Sherman Ku, Deale Carruth, Eddie Rhodes, and John Rhodes (all former Georgia Tech students, but now spread across the US from Atlanta to Marietta to Dallas to San Jose to Seattle): you are my brothers from other mothers and I know the time we've interacted during these last 5+ years has been sporadic, but I truly appreciate your friendship.

Finally, I would like to thank everyone in my family for always being there for me. To my parents, Haroon and Fawziyeh Chaudhri, I love you dearly, and I would not have been able to get here without your support. To my sisters Maheen and Mehreen, and my brother Adnaan: thank you for dealing with my mood swings and moments of frustration; and thank you for coming to visit knowing that it was very difficult for me to leave Atlanta for extended periods of time. To my grandfather, Mirza Mehdi, I am finally out of school and I will commence my search for a suitable wife. To my brother-in-law Obaid and my nephew Jaasim-I'm sorry but I have to mention that I am writing these

acknowledgements while Syracuse just lost the Big East tournament. I just wanted to mention it. To all my extended family in Toronto, Ottawa, Montreal, Brattleboro, Chicago, Las Vegas, Houston, Baltimore, Denver, Pakistan, Iran, and I think we have distant relatives somewhere in Japan: I love you all and thank you all for not being too hard on me for being in school for the last 25 years. Also, Dr. Boyan, if you are reading this deep into the short story that is my acknowledgements section, you can truly see that it was difficult to avoid family whenever we went to a conference.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-BP	2-bromopalmitate
AR	androgen receptor
Bax	bcl2-associated x protein
Bcl2	b-cell lymphoma 2 protein
Cav1	caveolin-1
CDH1	e-cadherin
CTX	cholera toxin
CXCR4	C-X-C chemokine receptor 4
DAG	diacylglycerol
DNABD	DNA binding domain
E_2	17β-estradiol
E_2-BSA	17β-estradiol conjugated to bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial mesenchymal transition
ER	estrogen receptor
ERK1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
FGF2	fibroblast growth factor 2 (also basic fibroblast growth factor, bFGF)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPR30	G-protein coupled receptor (GPER, G-protein coupled estrogen receptor)
HER2	human epidermal growth factor 2 receptor (also HER2/neu)
HMA	2-hydroxymyristic acid
IHC	immunohistochemistry
IP3	inositol trisphosphate
JNK	c-jun N-terminal kinase
LBD	ligand binding domain

LPA	lysophosphatidic acid
LPAR	lysophosphatidic acid receptor
MAPK	mitogen activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAT	palmitoylacetyltransferase
PC-PLC	phosphatidylcholine specific PLC
PC-PLD	phosphatidylcholine specific PLD
PI-PLC	phosphatidylinositol specific PLC
PI3K	phosphoinositide 3 kinase
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PR	progesterone receptor
PTX	pertussis toxin
RANKL	receptor activator of nuclear factor kappa B ligand
RT-PCR	reverse transcription polymerase chain reaction
Tm	tunicamycin
TMA	tissue microarray
TNBC	triple negative breast cancer
TNM	tissue, Node, Metastasis classification
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor

SUMMARY

Breast cancer is a heterogeneous disease that afflicts all patients differently, and therefore requires individualized treatment depending on a large variety of factors. Several methods of classification exist to divide patients into meaningful groups in order to better personalize their treatment regimens. Healthcare is evolving into more use of personalized treatments that can more effectively treat patients on an individual level, rather than by using more generalized treatments that may not prove effective in all patients. In addition, personalized treatment also aims to reduce adverse effects, while increasing effectiveness.

Estrogen receptor (ER) status is one such method of grouping breast cancer patients into different treatment groups. Based on stage diagnosis and determination of receptor status, initial treatments such as surgery or radiotherapy may be used. Standard chemotherapy is another method, however, side effects may vary among patients and may be quite adverse. Other treatments include hormone or receptor blocking. This thesis has identified an alternatively spliced variant of classical ERα that resides in the plasma membrane of breast cancer cells and plays a major role in rapid signaling by estrogen. The overall aim of this thesis was to examine the role of the membrane receptor for 17β-estradiol (E_2) in breast cancer that enhances breast tumor aggressiveness and to evaluate the mechanisms by which it functions. The general hypothesis was that nonclassical estrogen signaling through the proposed membrane-associated ER, ERα36, can promote breast tumor aggressiveness by enhancing cell survivability while altering expression of angiogenic and metastatic factors. This work examined the mechanisms of ERα36-dependent signaling in breast cancer cells, and the correlation of ERα36 to clinical outcome in human breast cancer tissue through

histological evaluation. These data provide significant research as they provide a greater understanding of estrogen signaling in breast cancer through ERα36 and its role in tumorigenicity and metastasis. This study also proposes further clinical examination of ERα36, and suggests drug design to target ERα36 followed by preclinical studies to determine if drugs targeting ERα36 would benefit breast cancer patients by reducing tumorigenicity and increasing survival.

CHAPTER 1: INTRODUCTION

1.1 Cancer: Causes, risks, and approaches to treatment

Cancer comprises a large group of diseases that arise from abnormal cell growth. This cell growth can then lead to the formation of a neoplasm that may become malignant, leading to what is commonly known as a cancer. Cancerous tumors can arise in any tissue in the body, and local causes may vary from one tissue to another. Several factors have been identified as playing a role in cancer occurrence, but to identify a single cause of cancer is virtually impossible. Mutations occur normally during cell division, and several checkpoint proteins, one of the most recognizable being the tumor suppressor p53, are susceptible to mutations themselves. These proteins can regulate cell growth, but mutations in DNA can cause many of these proteins, either oncogenic or tumor suppressor proteins, to function abnormally, allowing further accumulation of mutations and eventually leading to unregulated cell proliferation.

Mutations can occur spontaneously from the imperfect replication fidelity of DNA polymerase or may even be caused by external mutagens, either physical or chemical(1). Carcinogens, mutagens that cause cancer, have been identified from experimental evidence, and as individuals, we take it upon ourselves to limit exposure to carcinogens in everyday life. Unfortunately, although more than 90% of cancers arise specifically from external forces(2), several cancers are genetically inherited, and although all cancer patients should be treated with the ultimate goal of curing the disease, the unfortunate cases of genetic heredity of cancer pose more difficult approaches to prevention, diagnosis, and treatment.

As much as some would like to believe a cure for cancer is imminent, the majority of people making these statements are not scientists, and do not understand that cancer itself is not a single disease but rather a class of diseases. In addition, direct prevention of cancer is not as simple as that of many infectious diseases such as some virally-induced diseases that can be prevented and eradicated through effective vaccination(3). Because cancer is heterogeneous in development, and several causes exist, many of which are unknown, the most effective approach to alleviate the deleterious effects of cancer is by continually improving current treatments, and discovering novel approaches to personalized treatment and prevention. Early diagnosis of any cancer, but specifically breast cancer, is one of the most important ways to successfully combat the disease by allowing either complete removal of the neoplasias causing the disease, or by preventing further growth and eventual spread of the disease throughout the body. Cancer treatment is not performed with one specific approach. Rather, several approaches may be necessary for a single patient, beginning with surgery or radiotherapy and followed by adjunct chemotherapeutic approaches and lifestyle changes(2). Research into surgical techniques and radiotherapy are limited to the scope of the method, but possible additions to chemotherapeutic approaches are practically endless, due to the vast possibilities for drug targets, and the fact that new discoveries are constantly being made into identifying novel targets. Therefore, the identification of new targets for treatment is crucial to the development of effective and successful treatments that can specifically target and kill cancer cells, or prevent their growth.

Breast cancer is one specific type of cancer in which environmental factors play a much smaller role in development of the disease than other factors. The primary risk factors for developing breast cancer are sex and age(4). Although less than 5% of breast

cancer cases are attributed to genetic heredity(2), those citing such a statistic ignore the inherent fact that sex is specifically genetic and aging occurs in all individuals. Although this is a more philosophical argument for genetics being more responsible for breast cancer than other factors, it is undeniable that women are almost helpless in preventing breast cancer occurrence.

As the most common non-cutaneous cancer among women in the United States, breast cancer is expected to afflict over 200,000 women in the United States in 2013, and according to the National Cancer Institute, almost 40,000 deaths are predicted to occur due to breast cancer in this year. Although the 5-year survival for breast cancer patients diagnosed in the early stages exceeds 90%, survival in patients with distant metastases drops below 25% indicating very poor prognosis for these patients(5). The complexities of breast cancer growth and metastasis present several problems in development of treatments for patients. Just as any form of cancer, breast cancer can present itself differently among patients. Although an actual cure for breast cancer is elusive, novel approaches to diagnosis and treatment can help to reduce mortality and allow patients, specifically those with more advanced stage cancer, to live normal lives.

1.2 Estrogen biosynthesis

Estrogen is a general name for the group of hormones that are named for their role in the estrous cycles of females and are the primary female sex hormones, although they are still produced and function in males. The three naturally occurring steroidal estrogens are estrone (E_1), estradiol (E_2), and estriol (E_3). Estradiol, the most commonly chemically substituted form being 17b-estradiol, is the most predominant estrogen in women during their reproductive years(6). After menopause, estrone is the predominant

estrogen, and during pregnancy, estriol is the predominant circulating estrogen. E_2, however, is the most potent of the three steroidal estrogens(7)

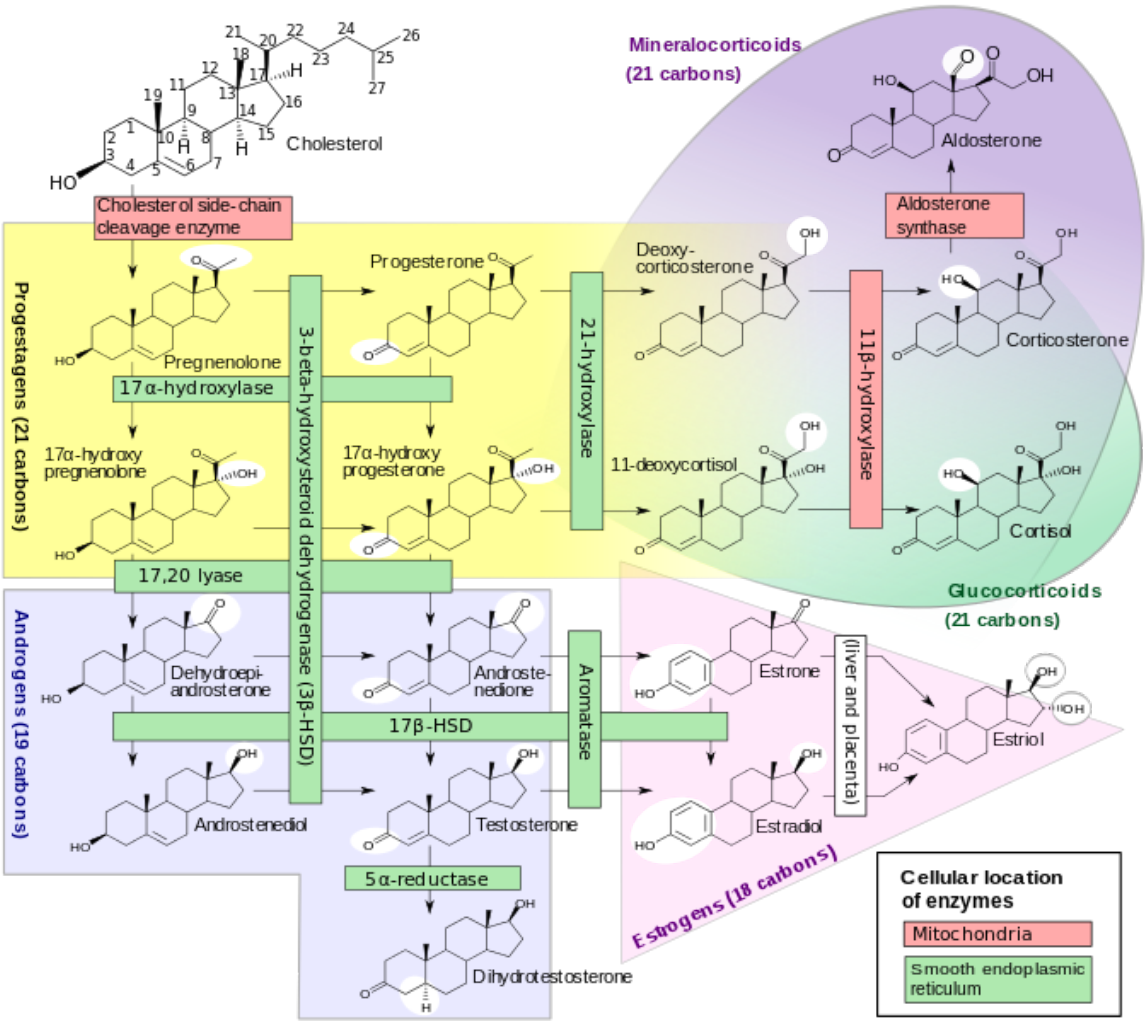

Figure 1.1: Steroid hormone biosynthetic pathways (Häggström et al, 2009)(8)

As steroid hormones, estrogens arise from the conversion of cholesterol through several intermediary compounds. In the ovaries, estrogen is produced by conversion of the androgen, androstenedione, which serves as a precursor to other androgens such as testosterone and dihydrotestosterone, as well as the estrogenic compounds, estrone and estradiol. Several enzymes catalyze these conversions. 17b-hydroxysteroid dehydrogenase catalyzes the conversion of androstenedione to testosterone, while aromatase catalyzes the conversion of androstenedione or testosterone to estrone or estradiol, respectively. The biosynthesis of the androgens and estrogens with the enzymes that catalyze the key steps of the biosynthetic pathways is clearly illustrated in figure 2.1(8). Many of these enzymes can be produced by cells outside of the ovaries, especially aromatase(9), and this allows for conversion of androgens to estrogens locally, which is particularly important after menopause, when estrogen production from the ovaries dramatically declines(10, 11).

1.3 Physiological roles of estrogen

As previously stated, estrogens are the group of primary female sex hormones that play major roles in body development and reproductive development. Estradiol is responsible for the formation of primary and secondary female sex characteristics, development of the uterus, and regulation of the menstrual cycle. In males, estrogen regulates male reproductive development, especially maturation of sperm. Numerous other developmental and structural changes are mediated by estrogen function, including regulation of skeletal development and breast development(12). These two functions are of particular interest as they are unfortunately affected by deficiency or abnormalities in estrogen signaling resulting in debilitating conditions, such as osteopenia or breast cancer progression(13, 14). Moreover, while estrogen normally

regulates osteoblast and osteoclast function, abnormal estrogen levels following menopause may result in dysregulation of bone remodeling, leading to decreases in bone mineral retention, and ultimately causing osteopenia, a precursor to osteoporosis(15). Causes of breast cancer are not as easily identified, but estrogen is well accepted as a mediator of breast cancer progression and abnormal estrogen can promote progression of hormone-responsive cancers, such as uterine, ovarian, cervical, and especially, breast cancer(16). Therefore, the functioning of estrogen and the role of estrogen receptors in normal and abnormal physiological conditions is extremely important to understand.

1.4 Estrogen receptors and the classical nuclear receptor pathway

Estrogens belong to the class of biomolecules known as the steroid hormones, which traditionally confer their effects through direct interaction with hormone-specific receptors. These include vitamin D3 metabolites such as 1α,25-dihydroxyvitamin-D3 (1,25(OH)2D3) and 24R,25(OH)2D3 with the vitamin D3 receptor (VDR or nVDR), androgens such as testosterone and dihydrotestosterone with androgen receptors (AR), and most relevant to this study, estrogens, particularly estradiol, with the estrogen receptors, ERα and ERb(17). The classical mechanism by which steroid hormones such as 17b-estradiol elicit cellular effects begins with hormone binding to steroid hormone receptors in the cytosol, dimerization of receptors, either by homo- or heterodimerization, and translocation to the nucleus. In the nucleus, these hormone-receptor dimers traditionally function as transcription factors by binding to specific response elements on the DNA and either activating or repressing transcription(18).

1.5 Estrogen receptors and non-classical pathways

Recently, there is increasing evidence of non-nuclear cytosolic or plasma membrane-associated receptors that mediate non-genomic effects of several steroid hormones(19-21). For some of these hormones, the traditional nuclear receptors have been found to function outside of the nucleus to direct non-genomic effects(22, 23), and in some cases, orphan receptors for many of these hormones have been identified that actually may be alternative receptors to the traditional steroid hormone receptors(21, 24). Although some contention exists on the function of these non-traditional receptors, there is ample evidence to support the existence and ability of these receptors to bind their corresponding steroid hormones, one such example being the membrane receptor for 1,25(OH)2D3. Although several groups have identified nVDR in the plasma membrane(23, 25), protein disulfide isomerase family A, member 3 (PDIA3), traditionally recognized as a chaperone protein, has been the main focus as the potential membrane receptor for 1,25(OH)2D3. Also known as Erp60, Erp57, GRP58, and 1,25D3-MARRS, PDIA3 has been identified by numerous groups as a putative membrane receptor for 1,25(OH)2D3(26-29).

Classical steroid hormone signaling pathways that typically occur through hormone-dependent activation of receptors in the cytosol leading to transcriptional effects can take hours to days. However, steroid hormones can exert effects on cells rapidly within seconds to minutes through non-classical mechanisms outside of the nucleus. These can either be cytosolic or membrane-associated and their effects can be non-genomic, but crosstalk with genomic pathways can also occur(15, 19, 20, 30). The activation of signaling by steroid hormone receptor interaction with their corresponding hormones in the cytosol can activate signaling cascades that result in rapid activation of enzymes such as kinases or phospholipases that can lead to rapid physiological

changes in cells(19, 31). Typically these changes lead to activation of pathways that can affect cell proliferation or apoptosis. These pathways can also lead to downstream activation of transcription factors causing genomic regulation, which again takes hours to days. Figure 2.2 illustrates several mechanisms by which steroid hormones can activate rapid membrane-associated signaling(32).

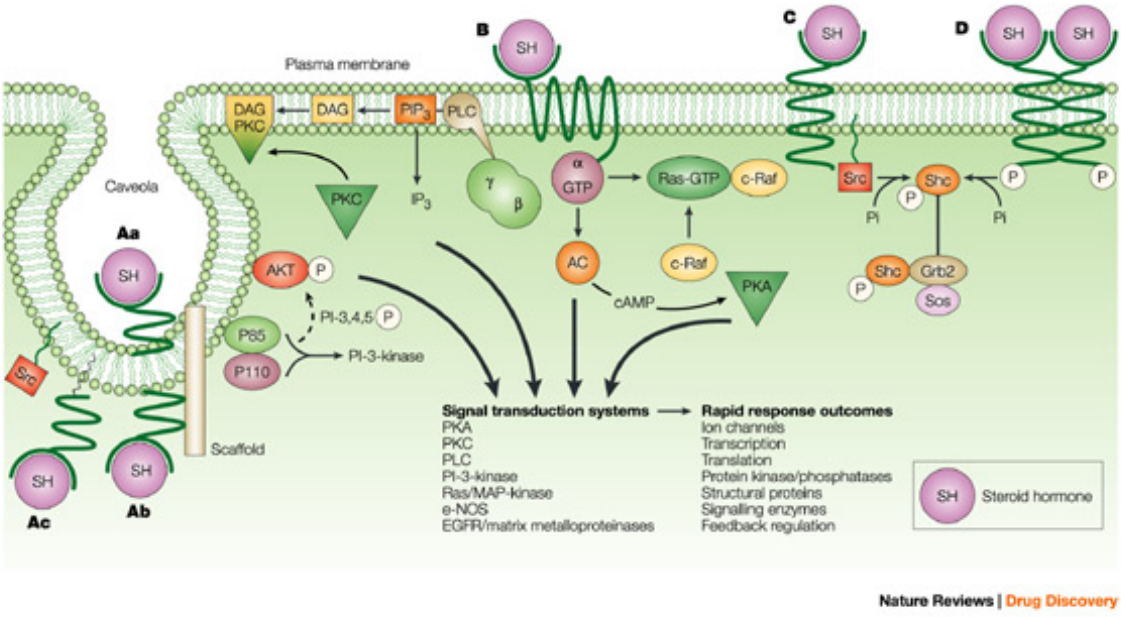

Figure 1.2: Steroid hormone signaling through membrane receptors (Norman et al, 2004)(32)

These rapid signaling events cannot only occur in the cytosol, but can in fact be mediated through receptors residing in the plasma membrane. Lipid rafts are regions of the plasma membrane that are highly concentrated in cholesterol and proteins,

especially steroid hormone receptors. Caveolae are specialized lipid rafts that are characterized as membrane invaginations, which contain the specific caveolin proteins caveolin-1, caveolin-2, and caveolin-3(33). Several steroid hormone receptors have been found in caveolae, including the nVDR, PDIA3, and the traditional ERs(26, 28, 34-41). It is possible that signaling through membrane-associated receptors can promote internalization of the receptors, but activation of these receptors at the membrane can in fact result in activation of signaling pathways directly without receptor translocation(41). The mechanism of how these receptors signal from the membranes is not completely understood, but in many cases, these responses are hypothesized to occur through what are known as membrane-delimited pathways(42, 43). In membrane-delimited signaling, G-proteins are activated and this activation leads to downstream activation of associated signaling cascades. If translocation of receptors occurs and the signaling does not in fact require membrane-associated proteins in order to mediate pathway activation, these responses might be considered membrane-associated or membrane-mediated, rather than membrane-delimited, which actually require membrane activation of the receptors(44). Previous work in breast cancer cells showing the ability of E_2 to rapidly activate PKC through membrane-associated signaling, actually shows that these effects are mediated through G-protein activation, suggesting that the membrane-associated effect of E_2 as far as PKC activation is concerned, is in fact through a membrane-delimited pathway.

As far as estrogen signaling is concerned, the main candidates for membrane-associated estrogen receptors include the traditional receptors ERα and ERb, but most recently, an orphan GPCR, GPR30 (also known as GPER), has been studied as a possible membrane-associated receptor for estrogen(45). However, the function and presence of GPR30 as a membrane-associated estrogen receptor, remains

controversial, as some groups contend that it is strictly a cytosolic or endoplasmic reticulum-associated receptor(5, 46), while other groups have maintained that it is a membrane-associated receptor(47-49). While GPR30 remains a controversial candidate, several splice variants have been identified for the traditional receptors, both ERα and ERb. Until recently, two known splice variants of ERα had been identified: the traditional ERα, which has been alternatively named ERα66 to distinguish it from the other variants has a molecular weight of 66 kDa, and a lower molecular weight variant, ERα46, which not surprisingly has a molecular weight of 46kDa(39). Both ERα66 and ERα46 can translocate to the nucleus upon ligand binding, but they have also been identified in plasma membranes(39, 50-52). More recently, a novel ERα splice variant was discovered with a molecular mass of approximately 36 kDa and has been named ERα36 (Figure 2.3). It differs from ERα66 by lacking both transcriptional activation domains (AF1 and AF2) (exons 7 and 8) but retains the DNA-binding domain and partial dimerization and ligand-binding domains(53). This suggests that although it may still bind to DNA, it may not directly activate transcription. It is possible that it may affect transcriptional function as a co-factor due to its ability to dimerize with other members and bind to DNA, but ERα36 also contains a novel exon 9, which encodes 27 amino acids of unknown function(53). There are limited data on the function of ERα36, but it has been found in the membranes of several cells and has been shown to mediate some rapid effects of estrogen(54-56).

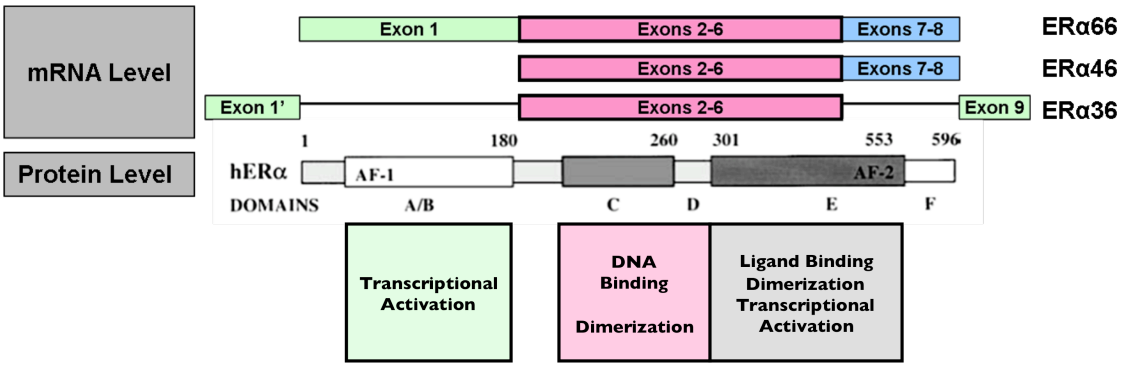

Figure 1.3: mRNA and protein domain maps of ERα splice variants

Several studies have reported the presence of both ERα and ERβ in plasma membranes, including truncated forms of the receptors, suggesting that they may be involved in the membrane-mediated effects of E_2. This is supported by studies using E_2 conjugated to bovine serum albumin (E_2-BSA), which cannot pass through the plasma membrane and reach the nuclear receptor, yet it elicits many of the same effects as E_2. E_2 and E_2-BSA rapidly increase protein kinase C (PKC)-specific activity without new gene expression or protein synthesis, and antibodies to nuclear and cytosolic ERα and ERβ as well as activators and inhibitors of classical ERs do not block this effect. However, which one of the ER isoforms is responsible is not known.

1.6 Estrogen and Cancer

Estrogen receptor (ER) status is a major point of concern in diagnosis and treatment of breast cancer. Based on stage diagnosis and determination of receptor status, treatments such as surgery, chemotherapy, or radiotherapy may be used. Other treatments include hormone or receptor blocking. Growth of ER-positive breast cancers

is typically enhanced by estrogen, but ER interactions with DNA are not necessary for this growth to occur(57), suggesting that non-nuclear actions of ERs may play a role. ER-negative and triple negative breast cancers, which are ER-negative, progesterone receptor-negative, and human epidermal growth factor receptor 2-negative, are typically characterized as more aggressive and less responsive to hormone treatments(58). These tumors are also less responsive to treatments such as tamoxifen, a commonly used estrogen antagonist, which reduces tumor aggressiveness in ER-positive breast cancer and prevents recurrence of cancer after chemotherapy or radiotherapy. However, estrogen may have some effects in ER-negative breast cancers that are not fully understood, and with the discovery of novel splice variants of traditional ERs(53), patients with ER-negative breast cancer should not be assumed as nonresponsive to treatments typical for ER-positive breast cancer patients. Moreover, rapid responses to E_2 and E_2-BSA are seen in ERα-negative HCC38 breast cancer cells as well as in ERα-positive MCF7 cells(57), suggesting that either ERβ is responsible or that another ERα isoform mediates the effects of E_2.

ERα36 was shown to be involved in estrogen-stimulated MAPK (ERK) activation in HEK293 cells in which the receptor was overexpressed(55), as well as in testosterone-stimulated ERK and Akt activation in endometrial cancer cells(59), raising the possibility that this ERα isoform is involved. The G-protein-coupled receptor GPR30 has also been shown to mediate non-nuclear responses of estrogen and has been reported as an alternative membrane receptor for E_2(60). However, Kang et al reported that GPR30 is not responsible for nongenomic signaling of estrogen in the context of rapid enzyme activation such as ERK1 and ERK2(61). While GPR30 has been hypothesized as a receptor for estrogen, ERα36 is known to retain a ligand-binding domain for estrogen; however, it does not retain transcriptional activation function,

suggesting that it may have functions unrelated to traditional nuclear receptor transcriptional function in breast cancer cells, particularly ERα-negative breast cancer cell that respond to estrogen.

Epidemiological studies have implied that sex hormones are involved in the tumorigenesis of not only breast cancer but also several other cancers, especially cancers that are thought of as sex-specific cancers(62-64). The larynx is a secondary sex organ, which undergoes trophic changes in response to hormonal changes during puberty, and morphostructural changes during adulthood. Nonetheless, while other tumors of secondary sex organs have been accepted as hormone dependent cancers(65-67), laryngeal carcinoma is still a subject of controversy, and as such, its treatment is not oriented to counteract the hormonal effect.

1.7 Thesis objective

This thesis aims to gain a better understanding of the role of E_2 in breast cancer tumorigenicity and progression. The research presented used *in vitro* approaches to identify the receptor responsible for the membrane-mediated effects of E_2 in breast cancer cells, and to elucidate the mechanism by which it functions. Similar effects were examined in other cancers to determine if these effects and the role of the membrane receptor are present in a more generalized context of cancer. Finally, human cancer tissues were examined histologically to determine if the membrane receptor may provide diagnostic value *in vivo* or if it could potentially be a suitable target for drug design for novel treatments.

CHAPTER 2: SPECIFIC AIMS

The complexities of breast cancer growth and metastasis present several problems in development of treatments for patients. Just as any form of cancer, breast cancer can present itself differently among patients. Estrogen receptor (ER) status is a major point of concern in diagnosis and treatment. Based on stage diagnosis and determination of receptor status, treatments such as surgery, chemotherapy, or radiotherapy may be used. Other treatments include hormone or receptor blocking. We have previously shown that E_2 can induce rapid, non-genomic activation of PKC in breast cancer cells leading to enhanced proliferation *in vitro*. At the time, we did not identify a receptor that mediates this effect, but we did determine that the effect was membrane-mediated. Recently, a novel alternatively spliced variant of classical ERα known as ERα36 has been cloned and identified in breast cancer cells. This study provides a better insight into mechanisms by which estrogen enhances breast cancer tumorigenicity and metastasis. The **overall objective** was to identify the membrane receptor for 17β-estradiol (E_2) in breast cancer that enhances breast tumor aggressiveness and to evaluate the mechanisms by which it functions. The **general hypothesis** was that nonclassical estrogen signaling through the membrane-associated ER, ERα36, can promote breast tumor aggressiveness by enhancing cell survivability while altering expression of angiogenic and metastatic factors. The **alternative to this hypothesis** was that rapid effects of estrogen on breast cancer cells occur through other mechanisms including ERβ or other nonclassical ERs, such as GPR30. The **overall approach** used antibody blocking, protein inhibition, gene silencing, and mutagenesis to assess the role of ERα36 in rapid membrane signaling leading to tumorigenicity and metastasis of breast cancer cells. Three specific aims were to be evaluated in order to address the hypothesis:

2.1 Specific Aim 1: To determine if the estrogen receptor, ERα36, plays a role in rapid membrane signaling of E_2 in breast cancer cells and if this signaling leads to enhanced breast cancer cell survivability *in vitro*

Rapid protein kinase C (PKC) activation occurs in response to estrogen treatment in both ERα-positive MCF7 and ERα-negative HCC38 breast cancer cells. PKC is associated with enhanced tumorigenicity(1). The **objective** was to examine the mechanism by which E_2 can cause rapid signaling events in breast cancer cells that do not contain classical forms of ERα and to determine the physiological effects of these signaling events. The **hypothesis** was that E_2-induced rapid PKC activation in breast cancer cells is mediated by ERα36 from the membrane. The alternative hypothesis is that ERβ, the non-classical ER, GPR30, or another unidentified receptor of estrogen that resides on the membrane mediates the rapid activation of PKC in breast cancer cells.

ER blocking by specific antibodies and RNA interference was used to determine the specific receptor responsible for the membrane response of E_2. The antibodies and RNA interference targeted known splice variants of ERα, ERβ, and the nonclassical ER, GPR30. The mechanism of action of E_2 through this receptor was elucidated further by analysis of localization and determination of associated signaling events followed by examination of the pro-proliferative and anti-apoptotic effects of E_2. Following initial studies, the effects of E_2 on enhanced cell survivability were examined by analysis of DNA synthesis, cell viability, and apoptosis. In order to examine E_2's antiapoptotic effects in these breast cancer cells, taxol, a common chemotherapeutic agent used in breast cancer treatment, will be used to induce apoptosis followed by treatment with E_2 to determine if E_2 can promote anti-apoptosis.

2.2 Specific Aim 2: To determine the mechanism by which ERα36 mediates the membrane-associated effect of E_2 in breast cancer cells and if this pathway exists on other cancers

ERα has three known alternatively-spliced variants: the traditional ERα66, as well as the more recently identified ERα46 and ERα36(53). Interestingly, our preliminary results showed that HCC38 breast cancer cells do not express ERα66 and ERα46, but do express ERα36. The mRNA for ERα66 is coded for by 8 exons containing characteristic features of traditional steroid hormone receptors such as transcriptional activation domains (AF1 and AF2), a DNA-binding domain (DNABD), a ligand-binding domain (LBD), and regions that confer dimerization. ERα36 retains the DNABD, LBD, and partial dimerization, but does not retain AF1 or AF2. Intriguingly, ERα36 also contains a novel exon (exon 9) not found in the mRNA of ERα66 that codes for 27 amino acids of unknown function.

Previous work has shown that E_2 can rapidly activate PKC in MCF7 and HCC38 cells via phosphatidyl inositol specific phospholipase C (PI-PLC) and G-protein(57). Non-genomic signaling of the vitamin D3 metabolite, 24R,25-dihydoxyvitamin-D3 has been shown to mediate anti-apoptosis through activation of PLD, LPA, and PI3K in chondrocytes(68), while estrogen is known to be anti-apoptotic in ER-positive breast cancer cells(69). The ***objectives*** were to elucidate the mechanistic pathway of E_2's effects on breast cancer cells via membrane signaling and to determine if exon 9 of ERα36 is required in mediating the membrane-initiated effect of E_2 in breast cancer. The ***hypotheses*** were as follows: (1) E_2 interaction with ERα36 in breast cancer cells leads to activation of a pathway involving G-protein, PI-PLC, and PLD, and this pathway results in activation of PKC, leading to anti-apoptotic effects of E_2 in breast cancer cells, and (2) exon 9 is necessary for mediating rapid signaling of E_2 from the plasma

membrane. The ***alternative hypotheses*** were that E_2 functioning through ERα36 activates a separate pathway from that including PLC and PLD activation such as activation of phospholipase A2 (PLA2), and exon 9 plays no role in the membrane-mediated functioning of ERα36 in breast cancer cells. Complete deletion of exon 9 was performed in order to evaluate its function. To examine the PKC activation pathway in HCC38 cells, functional analyses using antibodies and inhibitors to specific mediators in the hypothesized pathway were performed to determine involvement. In addition, we wanted to determine if ERα36 can mediate similar effects in other cancers, or if these effects are limited only to breast cancer. Therefore, we also performed similar studies in thyroid and laryngeal cancer cells.

2.3 Specific Aim 3: To examine the role of ERα36 in enhancing breast cancer aggressiveness and metastasis, and to determine its role *in vivo*

Several factors associated with tumor metastasis are known to be up-regulated in breast carcinomas. The molecular mechanisms of breast cancer metastases, which commonly occur in bone, are not well understood. The general events that lead to metastatic cancer include initial tumor formation, growth and vascularization, detachment and migration, and eventual re-localization of tumorigenic cells to secondary sites of the body such as bone. The ***objectives*** were to examine presence of ERα36 in human breast cancer tissue and determine if ERα36 presence can predict clinical outcome. The ***hypothesis*** was that ERα36 presence is associated with VEGF presence and clinical variables that are related to poor clinical outcome.

In evaluating aim 1, we examined the indirect genomic effect of this membrane-mediated signaling of E_2 through ERα36 on the production of angiogenic growth factors,

migratory factors, and osteoclastogenic factors, thereby allowing us to further understand the mechanism by which breast cancer progresses *in vitro*. Following up with this study, we determined if there is a correlation between expression and presence of ERα36 and clinicopathological variables of the cancer *in vivo* in blinded analysis of tissue samples from breast tumors of patients diagnosed with varying stages of breast cancer.

The outcomes of this work were expected to show that signaling via ERα36 could enhance cell proliferation, protection against apoptosis, and production of growth factors, and other proteins that promote tumor aggressiveness and metastasis. This research is significant because it provides a greater understanding of estrogen signaling in breast cancer and its role in tumorigenicity and metastasis. The study provides scientific support for the targeting of ERα36 for drug design or gene therapy in the diagnosis and treatment of metastatic breast cancer.

CHAPTER 3: Membrane Estrogen Signaling Enhances Tumorigenesis and Metastatic Potential of Breast Cancer Cells via Estrogen Receptor-α36 (ERα36)

[Chapter 3 is published as: Chaudhri RA, Olivares-Navarrete R, Cuenca N, Hadadi A, Boyan BD, Schwartz Z. Membrane estrogen signaling enhances tumorigenesis and metastatic potential of breast cancer cells via estrogen receptor-alpha36 (ERalpha36). J Biol Chem. 2012;287(10):7169-81. PMCID: 3293594.]

Protein kinase C (PKC) signaling can be activated rapidly by 17β-estradiol (E_2) via nontraditional signaling in ERα-positive MCF7 and ERα-negative HCC38 breast cancer cells and is associated with tumorigenicity. Additionally, E_2 has been shown to elicit anti-apoptotic effects in cancer cells counteracting pro-apoptotic effects of chemotherapeutics. Supporting evidence suggests the existence of a membrane-associated ER that differs from the traditional receptors, ERα and ERβ. Our aim was to identify the ER responsible for rapid PKC activation and to evaluate downstream effects, such as proliferation, apoptosis, and metastasis. RT-PCR, Western blot, and immunofluorescence were used to determine the presence of ER splice variants in multiple cell lines. E_2 effects on PKC activity were measured with and without ER-blocking antibodies. Cell proliferation was determined by [^{3}H]-thymidine incorporation, and cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (MTT) whereas apoptosis was determined by DNA fragmentation and TUNEL. Quantitative RT-PCR and sandwich ELISA were used to determine the effects on metastatic factors. The role of membrane-dependent signaling in cancer cell invasiveness was examined using an *in vitro* assay. The results indicate the presence of an ERα splice variant, ERα36, in ERα-positive MCF7 and ERα-negative HCC38 breast cancer cells, which localized to plasma membranes and rapidly activated PKC in response to E_2, leading to deleterious effects such as enhancement of proliferation,

protection against apoptosis, and enhancement of metastatic factors. These findings propose ERα36 as a novel target for the development of therapies that can prevent progression of breast cancer in the primary tumor as well as during metastasis.

3.1 INTRODUCTION

The complexities of breast cancer growth and metastasis present several problems in development of treatments for patients. The main screening process in determining the treatment and prognosis of breast cancer patients is receptor status. Growth of estrogen receptor (ER)-positive breast cancers is typically enhanced by estrogen, but ER interactions with DNA are not necessary for this growth to occur (70, 71), suggesting that non-nuclear actions of ERs may play a role. Triple negative breast cancers, which are ER-negative, progesterone receptor (PR) negative, and human epidermal growth factor receptor 2 (HER2) negative, are typically characterized as more aggressive and less responsive to hormone treatments (72). These tumors are also less responsive to treatments such as tamoxifen, a commonly used estrogen antagonist, which reduces tumor aggressiveness in ER-positive breast cancer and prevents recurrence of cancer after chemotherapy or radiotherapy (73). However, estrogen may have some effects in ER-negative breast cancers that are not fully understood, and with the discovery of novel splice variants of traditional ERs (74, 75), patients with ER-negative breast cancer should not be assumed as non-responsive to treatments typical for ER-positive breast cancer patients.

Steroid hormone receptors traditionally function as transcription factors upon ligand binding, but many studies have identified them in plasma membranes and have shown that they can rapidly activate signal transduction pathways, leading to events such as increased proliferation or attenuated apoptosis (21). Estrogens, in particular,

elicit many of their effects on cells through classical steroid hormone receptor mechanisms involving two primary receptor classes: ERα and ERβ.

It is now understood that 17β-estradiol (E_2) exerts some of its effects via signaling mechanisms other than traditional nuclear receptor-mediated pathways (70, 76-78). Several studies have reported the presence of both ERα and ERβ in plasma membranes (21, 70), including truncated forms of the receptors, suggesting that they may be involved in membrane-mediated effects of the hormone. This is supported by studies using E_2 conjugated to bovine serum albumin (E_2-BSA), which cannot pass through the plasma membrane and reach the nuclear receptor, yet elicits many of the same effects as E_2 (57, 79, 80). E_2 and E_2-BSA rapidly increase protein kinase C (PKC) specific activity without new gene expression or protein synthesis, and antibodies to nuclear and cytosolic ERα and ERβ, as well as activators and inhibitors of classical ERs do not block this effect (57). However, which one of the ER isoforms is responsible is not known. Moreover, rapid responses to E_2 and E_2-BSA are seen in ERα-negative HCC38 breast cancer cells as well as in ERα-positive MCF7 cells (57), suggesting that either ERb is responsible or that another ERα isoform mediates E_2's effects.

There are multiple isoforms of ERα: the traditional ERα66 (MW=66 kDa) and a lower molecular weight variant ERα46. Both ERα66 and ERα46 are localized to the nucleus upon ligand binding (53). Recently, a novel ERα variant was discovered with a molecular mass of approximately 36 kDa (ERα36) (53, 55, 59). It differs from ERα66 by lacking both transcriptional activation domains (AF1 and AF2) (exons 7 and 8), but retains the DNA-binding domain and partial dimerization and ligand-binding domains (53). It also contains a novel exon 9, which encodes 27 amino acids of unknown function. ERα36 was shown to be involved in estrogen-stimulated mitogen activated protein kinase (ERK) activation in HEK293 cells in which the receptor was

overexpressed as well as in testosterone-stimulated ERK and Akt activation in endometrial cancer cells (55), raising the possibility that this ERα isoform is involved.

The G-protein coupled receptor GPR30 has also been shown to mediate non-nuclear responses of estrogen and has been reported as an alternative membrane receptor for E_2 (81). Kang et al reported that GPR30 is not responsible for non-genomic signaling of estrogen in the context of rapid enzyme activation such as ERK1 and ERK2 (82). However, we show in this study that GPR30 does not play a role in membrane-associated E_2-dependent cell proliferation, but it is not known if GPR30 mediates other responses related to apoptosis or metastasis and further examination is needed.

The purpose of the present study was to evaluate the role of ERα36 in membrane-associated estrogen signaling in breast cancer. We hypothesized that ERα36-associated E_2 membrane signaling in breast cancer cells leads to enhanced cancer cell survival by promoting proliferation, protecting against apoptosis, and stimulating downstream gene expression associated with enhanced tumorigenicity and metastasis. The main goal of this investigation was to help us gain a greater understanding of the underlying mechanisms of breast cancer tumor aggression and invasion, providing us with new knowledge vital in the development of novel treatments to control breast cancer growth and metastasis.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

ERα-positive MCF7 and ERα-negative HCC38 human breast cancer cells as well as SkBr3, COS7, and HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The PKC assay kit was purchased from GE

LifeSciences (Piscataway, NJ). Minimal essential media (MEM) and Roswell Park Memorial Institute 1640 media (RPMI 1640) were purchased from Gibco/Invitrogen (Carlsbad, CA). Charcoal-dextran filtered fetal bovine serum (CD-FBS) was purchased from Hyclone (Logan, UT). E_2, E_2-BSA, and taxol (paclitaxel) were purchased from Sigma-Aldrich (St. Louis, MO). Chelerythrine, a PKC inhibitor, was purchased from EMD Chemicals (Gibbstown, NJ). Protein content of samples was measured using the Macro BCA reagent kit from Pierce Chemicals/Thermo Scientific (Rockford, IL). Primers were purchased from Eurofins (Des Moines, IA). Reverse transcription and polymerase chain reaction (PCR) reagents were purchased from BioRad, Inc. (Hercules, CA). Quantitative reverse transcription PCR (qRT-PCR) reagents were purchased from Applied Biosystems (Carlsbad, CA). [^{32}P]-ATP and [^{3}H]-thymidine were obtained from Perkin-Elmer (Boston, MA). Polyclonal ERα66 and ERα36 antibodies were purchased from Chi Scientific (Maynard, MA). Polyclonal ERβ and monoclonal glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) antibodies were obtained from Millipore (Billerica, MA). Polyclonal antibodies to caveolin-1 and GPR30 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to cytochrome C oxidase (COX) IV were from Abcam (Cambridge, MA). Goat anti-rabbit-horse radish peroxidase (HRP) and goat anti-mouse-HRP conjugated secondary antibodies were obtained from BioRad. Goat anti-rabbit Alexa 488, goat anti-rabbit Alexa 594, Hoechst 4322 (nuclear stain), and Select FX 488 endoplasmic reticulum stain were purchased from Molecular Probes (Carlsbad, CA).

3.2.2 Cell Culture

All cells (SkBr3, MDA-MB-231, HEK293, and COS7) were cultured in appropriate media as specified by the ATCC containing 10% charcoal/dextran-filtered FBS and

lacking phenol red, which can mimic the effects of E_2 at low levels. MCF7 cells were maintained in minimum Eagle's-based media, and HCC38 cells were maintained in RPMI 1640-based media.

3.2.3 Presence of ER Isoforms

In order to determine if ERα36 is expressed in both ERα-positive MCF7 and ERα-negative HCC38 breast cancer cells, we designed sequence-specific primers that would selectively identify the three known alternative splicing variants of ERα: ERα66, ERα46, and ERα36. Primers used are shown in Table 3.1. Due to the sequence homology of ERα66 and ERα46, we could not identify ERα46 mRNA independent of ERα66. However, due to the existence of exon 9, which is not expressed in ERα66 or ERα46 (53), we successfully designed primers that spanned this exon in order to analyze expression of ERα36.

Table 3.1: Primer sequence for ERα splice variants.

Vector NTI software was used to determine alignment of the three alternatively spliced variants for ERα: ERα66, ERα46, and ERα36. We determined that it is not possible to distinguish ERα46 from ERα66, however it is possible to recognize ERα66 from both smaller isoforms. Primers were designed for ERα66 that spanned exon 1 in its mRNA. Primers for ERα46 and ERα66, designated for ERα46/66, spanned exons 7 and 8, which are both found in ERα46 and ERα66. Finally, primers for ERα36 were designed to span exon 9.

Target	Primer direction	Sequence (5' to 3')
ERα66	Sense	TGCCTGGAGTGATGTTTAAGC
	Antisense	ACGGGAGCAAGTGCAGTC
ERα66/46	Sense	CCACACGGTTCAGATAATCC
	Antisense	ATCCCTTTGGCTGTTCCC
ERα36	Sense	GTGGTTTCCTCGTGTCTAAAGC
	Antisense	GGTGTTGAGTGTTGGTTGCC

RNA was extracted from cells using the TRIzol method. Reverse transcription was performed to produce cDNA for various ERs from both MCF7 and HCC38 cells as well as several other cell lines reported as lacking ERα including SkBr3, MDA-MB-231, HEK293 and COS7 (83-85). PCR was then performed in order to determine if mRNAs for the various alternative splice variants of ERα were expressed in these cells.

Western blots of isolated membrane fractions were used to determine the subcellular location of ERα36. For western blots of whole cell lysates, cells were lysed with RIPA buffer containing 5mM NP-40 (Sigma-Aldrich). We first performed western blots using unfiltered lysates from MCF7, HCC38, and COS7 cells, which are derived from a non-human primate embryonic kidney cell line, and reported to not contain ERs (85). Whole cell lysates were filtered with a molecular weight cut-off of 100kDa prior to separation on 4-20% SDS-polyacrylamide gels (SDS-PAGE). In addition, cell lysates were fractionated according to the method previously described by Smart et al. (86) in order to obtain crude nuclear, plasma membrane, and pure caveolae fractions.

Following SDS-PAGE of the isolated membrane fractions, protein was transferred onto nitrocellulose. Membranes were blocked with 2% bovine serum albumin in 1X phosphate buffered saline (PBS) containing 0.05% Tween-20 (Sigma-Aldrich) and probed with primary antibodies. Secondary HRP-conjugated antibodies were used for detection by chemiluminescence using the West Pico Chemiluminescence Substrate kit (Pierce Biotechnologies, Rockford, IL).

Immunofluorescence microscopy was used to identify the subcellular localization of ERa66 and ERα36 in MCF7 cells. Cells were cultured on 4-well chamber slides. At the time of harvest, cells were fixed with 4% paraformaldehyde. Some samples were then permeabilized with 0.01% Triton-X 100 for 10 minutes and subsequently stained for

ERs using specific antibodies. These samples were imaged with a Leica DMLB fluorescent microscope using a Hamamatsu Orca camera. To determine if ERα36 colocalizes to lipid rafts and caveolae, cells fixed in chamber slides were either pre-stained for lipid rafts (Vybrant Lipid Raft Labeling kit, Invitrogen, Carlsbad, CA) and/or probed with antibodies against ERα36 and protein disulfide isomerase (PDI) (endoplasmic reticulum) followed by incubation with fluorescent-tagged secondary antibodies (488 and 594nm). All samples were treated with Hoechst dye for nuclear staining (333nm). These samples were then imaged using a Nikon LSM510 confocal laser-scanning microscope and multiple Z-slices were obtained in order to obtain representative images.

3.2.4 PKC Activity

To determine if ERa36 mediated the stimulatory effect of E2 on PKC activity, MCF7 and HCC38 cells were cultured to confluence in 24-well tissue culture-treated plates. Cells were pre-treated with antibodies against ERα36 followed by treatment with E2 for 9 minutes. Cells were washed two times in cold 1XPBS and lysed using RIPA buffer at time of harvest. Samples were then aliquoted and assayed for protein content by Macro BCA protein assay (Pierce Biotechnologies) and measurement of PKC activation using the Biotrak Protein Kinase C Assay kit (GE LifeSciences).

HCC38 cultures were also treated with 1mM methyl β-cyclodextrin (MebCD) for 30 minutes to deplete the membranes of cholesterol, thereby disrupting the caveolae and lipid rafts (34). The cells were then treated with E2 for 9 minutes and protein content and PKC activity were determined as described.

3.2.5 Cell Proliferation, Viability, and Apoptosis

In order to determine the consequences of rapid activation of PKC in the context of breast cancer cell survival we performed assays to determine the effect of inhibiting PKC on apoptosis and proliferation. PKC activity was inhibited using chelerythrine as described previously (57). Confluent cultures of HCC38 cells were cultured in the presence of 0.1μM, 1.0μM, and 10μM chelerythrine for 24 hours and MTT was measured. In addition, HCC38 cells were treated with 5mM and 7.5mM phosphate to induce apoptosis, as we have previously shown that phosphate induces apoptosis in other cell types (87). We previously showed that tamoxifen, an ER agonist that inhibits PKC, blocks the stimulatory effects of E2 on proliferation. Finally to determine if ERα36 mediates the anti-apoptotic effect of E_2, we took advantage of the observation that E_2 inhibits taxol-induced apoptosis via membrane-associated signaling (69). Sub-confluent cultures of HCC38 cells were pretreated with E_2 for 90 minutes followed by taxol treatment for 4 hours after which cells were assays for caspase-3 activity and TUNEL.

Cell proliferation was assessed as a function of [3H]-thymidine incorporation, as described previously (57). Subconfluent cultures of HCC38 cells were made quiescent by starvation for 48 hours prior to treatment in starvation media containing 0.1% charcoal-dextran filtered FBS. Cells were then treated with varying concentrations of E_2 or E_2-BSA for 24 hours. In addition, cells were treated with antibodies to ERa36 and GPR30. At 20 hours after the start of treatment time, 1.0mCi/mL of [3H]-thymidine was added to all samples for the remainder of the treatment. At 24 hours, media were removed and cells were washed two times with 1X PBS. Cells were then fixed by washing three times with cold 5% trichloroacetic acid (TCA). During the third wash, the TCA was left on the cells for 30 minutes at 4°C. After fixation, the TCA was removed and the cell layers were allowed to dry at which point, 100mL of 1% sodium dodecyl sulfate

was added. The cell layers were then scraped and transferred to glass scintillation vials for measurement of radioactive decay (DPM) to determine relative incorporation of [3H]-thymidine.

The number of viable cells was determined by the MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (Sigma-Aldrich) to purple formazan by mitochondrial reductase of living cells. In order to determine the role of PKC signaling in breast cancer cell viability, confluent cultures of HCC38 cells were treated with the PKC inhibitor chelerythrine (10-7, 10-6, and 10-5 M) for 24 hours and MTT activity and DNA fragmentation were measured. In order to determine if signaling via ERα36 could protect breast cancer cells against apoptosis, HCC38 cells were treated with E_2 and E_2-BSA in conjunction with taxol, which induces cell death in breast cancer cells (88). Apoptosis was assessed using DNA fragmentation as described previously (89). TUNEL assay was used to determine the protective effect of E_2 against taxol-induced cell death. In addition, caspase-3 activity was measured using the CaspAce assay kit from Promega (Madison, WI).

3.2.6 E_2 Effects on Factors that Promote Bone Metastasis

Breast cancer has a particular affinity for metastasizing to bone (90, 91). Therefore, we measured the effects of E_2 and E_2-BSA on expression of factors associated with bone metastasis at 12 hours. qRT-PCR was used to determine the quantitative effect of E_2 on expression of receptor activator of nuclear factor kappa-B ligand (RANKL), Snail1, and e-cadherin (CDH1) in HCC38 cells. In addition, we assessed expression of C-X-C chemokine receptor type 4 (CXCR4), which is a receptor for the chemotactic ligand, stromal cell-derived factor-1 (SDF1) that is present in high

levels in bone (92), syndecan-4, which is involved cell interaction with matrix (93), and matrix metalloproteinase-9 (MMP9), which has been shown to be regulated through signaling of ERα (93). We also examined the effects of E_2-BSA on secretion of factors related to osteoclast activation and inhibition. Sandwich ELISAs, were used to measure levels of osteoprotegerin (OPG) and interleukin-6 (IL6) (R&D Systems, Minneapolis, MN) in the conditioned media 24 hours after treatment with E_2 or E_2-BSA.

3.2.7 Scratch-wound assay to measure in vitro breast cancer cell invasion

A 10μL micropipette tip was used to create scratch-wounds in confluent cultures of HCC38 cells. After washing with media to remove detached cells and debris, the cultures were treated with E_2-BSA, anti-ERα36 antibody, or both. Phase-contrast images were obtained at the beginning of the assay as well as at 6 and 12-hour increments until all wounds were healed. MatLab was used to quantify mean diameter of the wounds over time and data are presented as percent wound closure.

3.2.8 Statistical Analyses

For all experiments, statistical analyses were performed by analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons at a significance level of 0.05. Identification of symbols to signify statistical significance is found in the respective figure legends.

3.3 RESULTS

3.3.1 ERα36 Is Expressed in Both ERα-positive and ERα-negative Cells

Fig. 3.1A shows the different exons expressed in the mRNA of the three known splice variants of ERα. RT-PCR analysis showed expression of all ERα variants in MCF7 cells. HCC38 cells expressed only ERα36; neither ERα46 nor ERα66 was detected by RT-PCR. We performed a screen of more than 20 human cell lines for the expression of ERα36 and did not find a suitable negative control for ERα36 expression (data not shown). MDA-MB-231, SkBr3, and HEK293 cells exhibited expression of only ERα36 but not the other variants of ERα (Figure 3.1, A and B). COS7 African green monkey embryonic kidney cells, although they do not express ERα36, are not a relevant negative control for our studies as they are not human cells, and they are of embryonic origin. All cells expressed GPR30.

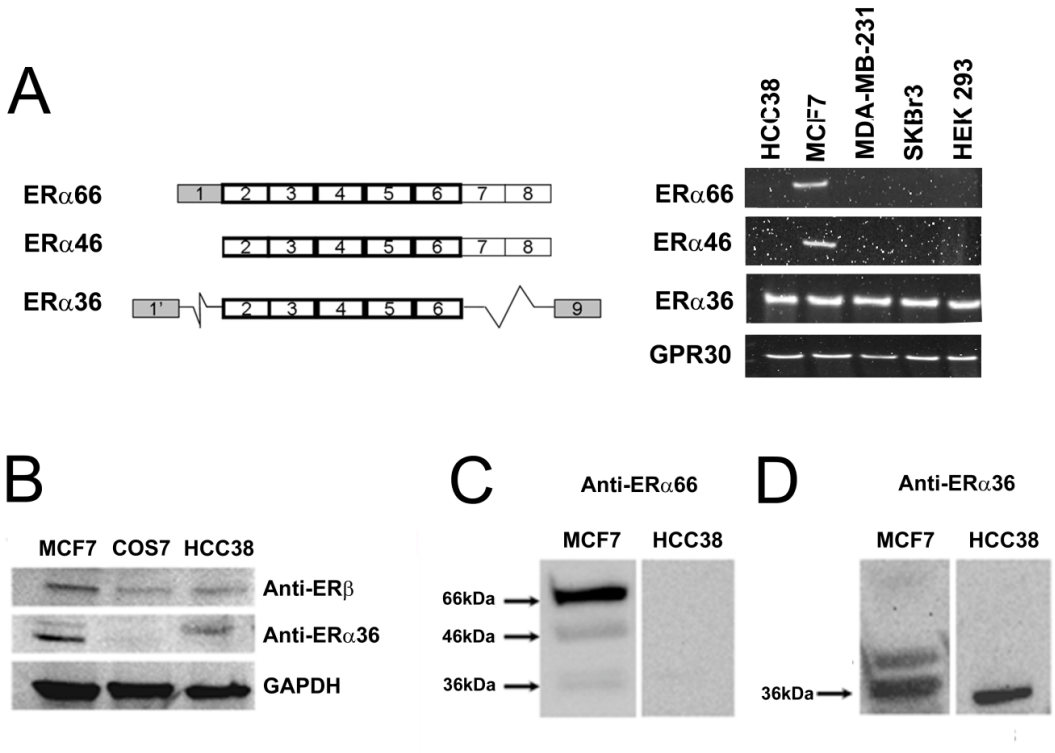

Figure 3.1: mRNA expression and protein presence of ERα splice variants in MCF7 and HCC38 cells.

(A) Different cell types show variable expression of ERα splicing variants. MCF7 express all variants, while ERα-negative HCC38, MDA-MB-231, SkBr3, and HEK293 cells only express ERα36. (B) Western blots of unfiltered cell lysates verify protein presence of ERβ and ERα36 in MCF7 and HCC38 cells but no ERα36 in COS7 cells. Filtered lysates of MCF7 and HCC38 cells clearly show presence of (C) ERα66 and (D) ERα36 by western blot, while HCC38 only show presence of ERα36.

Western blot analysis confirmed the presence of ERα36 in both MCF7 and HCC38 cell lines but not in COS7 cells, and ERβ was detected in lysates from MCF7, COS7, and HCC38 cells (Figure 3.1B). Because we are limited to using polyclonal antibodies to identify ERα66 separately from ERα36 in this study, and we noticed nonspecific bands above 100 kDa in blots of our unfiltered lysates, we performed Western analysis on filtered lysates to verify the specificity of the ERα36 antibody. This allowed us to look more closely at the recognition bands from 36 to 66 kDa. The

antibodies to ERα66 recognized three bands of varying intensity on blots of MCF7 cells but did not recognize a single band in HCC38 cells (Figure 3.1C). Polyclonal antibodies raised against the unique C-terminal end of ERα36 recognized two bands in MCF7 cells (Fig. 1C), but they only recognized a single band at ~37 kDa in HCC38 cells (Figure 3.1D). The presence of a second band in the MCF7 cell lysates does not appear to be ERα46 but more likely may be representative of a post-translationally modified form of ERα36.

3.3.2 Rapid E_2-induced PKC Activation in MCF7 and HCC38 Cells Occurs via Membrane Signaling through ERα36

E_2 caused a dose-dependent increase in PKC activity in MCF7 cells (Figure 3.2A) and in HCC38 cells (Figure 3.2B) at 9 min. Antibodies to ERα36 completely abolished this effect in both cell types, and a nonspecific IgG had no effect (Figure 3.2, C and D).

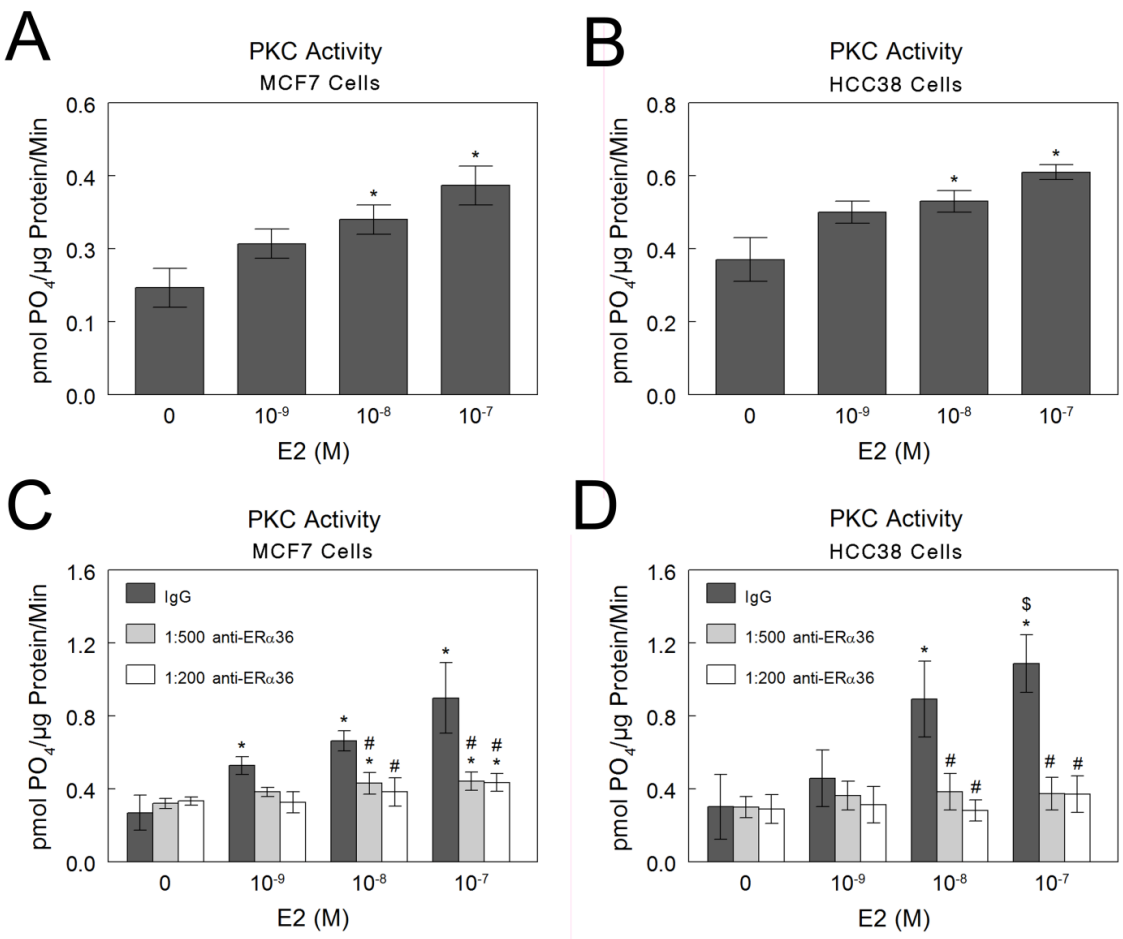

Figure 3.2: E_2 rapidly activates PKC in breast cancer cells through ERα36.

E_2-induces activation of PKC in MCF7 and HCC38 cells rapidly within 9 minutes of treatment (A,B). E_2-induced rapid activation of PKC in MCF7 and HCC38 cells occurs through ERα36. E_2 rapidly induces PKC activity within 9 minutes in both MCF7 and HCC38 cells and antibody blocking of ERα36 on the cells' membranes abolishes this effect (C,D). * $p<0.05$ compared to 0M E_2, $ $p<0.05$ compared to 10^{-9}M E_2, # $p<0.05$ compared to IgG.

3.3.3. PKC Maintains Cell Survival in HCC38 Cells

Chelerythrine caused a dose-dependent decrease in MTT activity in HCC38 cells (Figure 3.3A), indicating decreased cell viability. In addition, chelerythrine caused a

dose-dependent increase in DNA fragmentation (Figure 3.3B), indicating that inhibition of PKC promotes apoptosis of HCC38 cells.

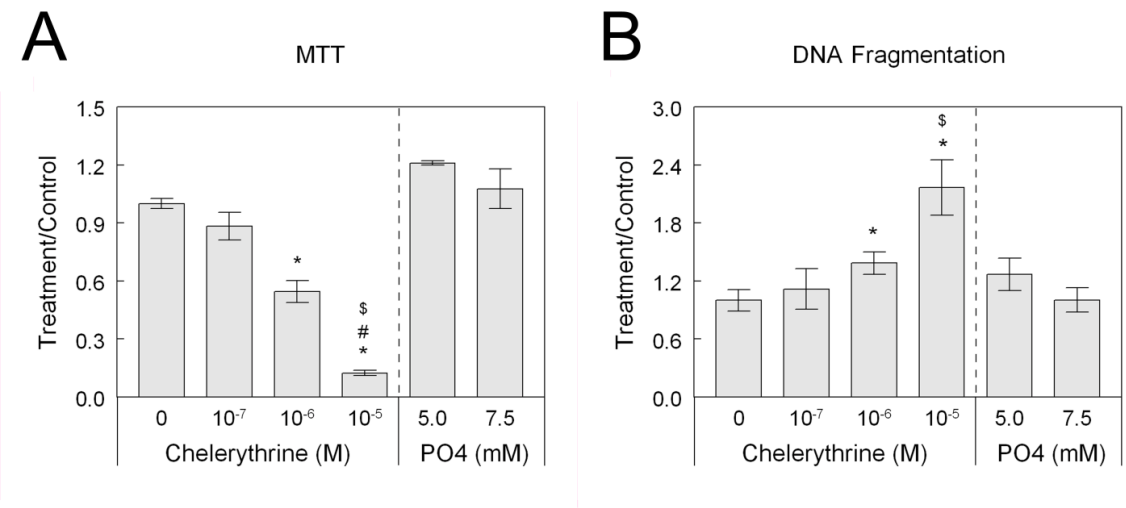

Figure 3.3: PKC activity is required to maintain cell survival.

Inhibition of PKC with chelerythrine causes dose-dependent decreases in MTT (A) and dose dependent increases in DNA fragmentation (B). Phosphate, which induces apoptosis in other cell types such as chondrocytes, does not induce apoptosis in breast cancer cells. * $p<0.05$ compared to 0M chelerythrine, \$ $p<0.05$ compared to 10^{-7}M chelerythrine, # $p<0.05$ compared to 10^{-6}M chelerythrine.

3.3.4 E_2-dependent Activation of PKC Requires Intact Caveolae

Immunofluorescence of ERα-positive MCF7 cells showed that ERα66 and ERα36 were differentially distributed. In MCF7 cells that were not permeabilized by detergent, ERα66 and ERα36 were both present on the plasma membrane (Figure 3.4A). When cells were permeabilized by Triton X-100, ERα66 was mainly localized peri-nuclearly.

ERα36 was primarily non-nuclear, but it was also distributed throughout the cells, although not to the same extent peri-nuclearly as ERα66.

Confocal microscopy indicated that ERα36 co-localized with lipid rafts and caveolin-1 protein in both MCF7 and HCC38 cells (Figure 3.4B). Staining with antibodies to protein-disulfide isomerase, which is primarily found in the endoplasmic reticulum, showed that ERα36 did not co-localize to endoplasmic reticulum. Western blots detected ERα36 and caveolin-1 in pure caveolae fractions (F3) from both cell lines (Figure 3.4C). COX-IV was not found, demonstrating that the plasma membrane fractions were not contaminated with mitochondrial membranes. E_2 did not stimulate PKC activity in HCC38 cells after treatment with methyl β-cyclodextrin, which destroys caveolae (Figure 3.4D), indicating the importance of the specialized membrane compartment for E_2-dependent PKC signaling.

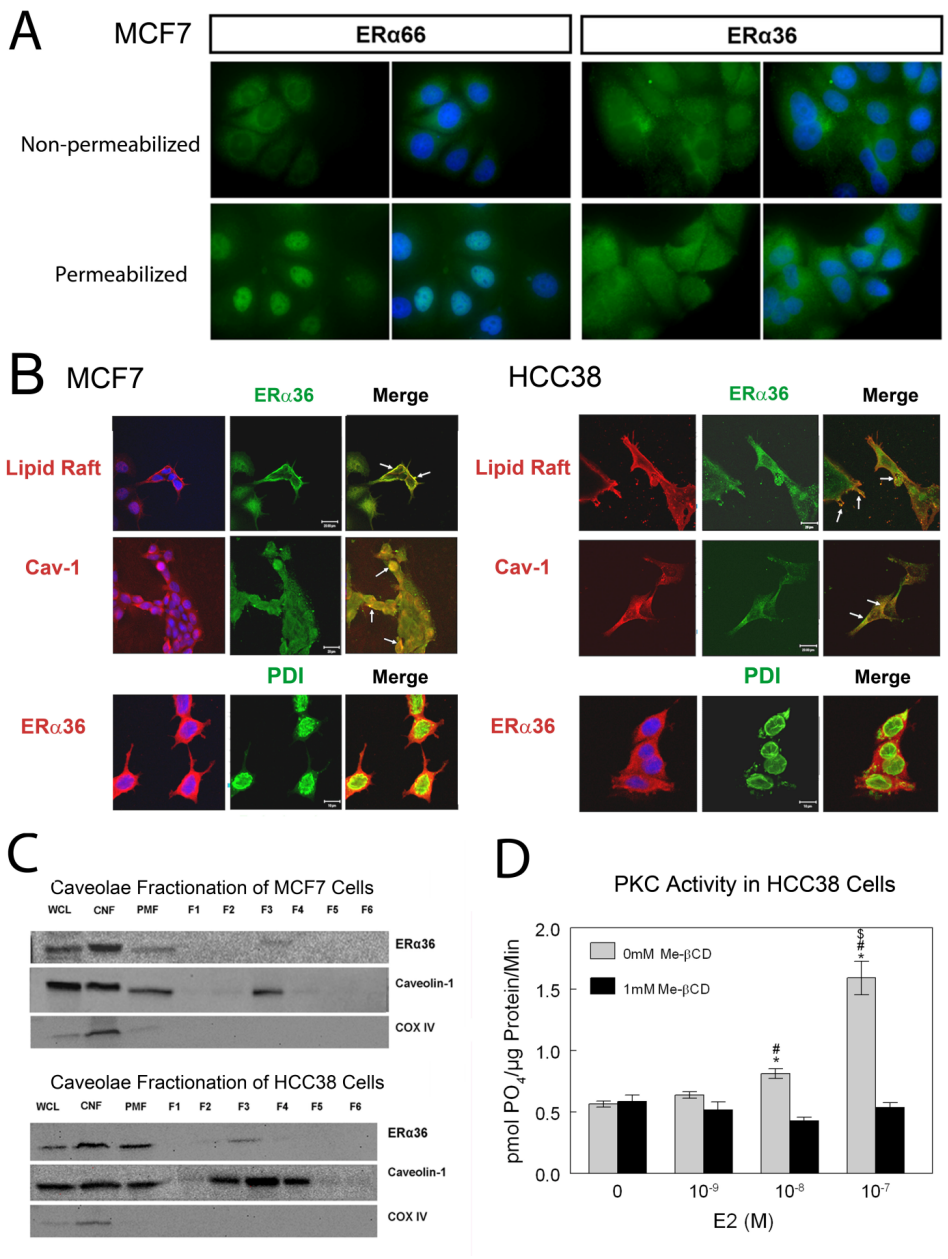

Figure 3.4: Immunofluorescence shows that ERα66 primarily exhibits peri-nuclear localization in MCF7 cells, while ERα36 exhibits non-specific localization throughout the cells.

Qualitatively, it appears that ERα36 exhibits greater cell surface expression than ERα66 (A). ERα36 colocalizes with lipid rafts and caveolin-1 in both MCF7 and HCC38 cells and does not appear to localize to endoplasmic reticulum in either cell (B). Caveolae fraction of both MCF7 and HCC38 cells show the existence of ERα36 in caveolae (F3) (C) and digestion of cholesterol and removal of lipid raft and caveolae from the cell membranes of HCC38 cells by β-cyclodextrin (MeβCD) signify the requirement of caveolae for rapid signaling of E_2 from the membrane (D). * $p<0.05$ compared to 0M E_2, # $p<0.05$ compared to 10^{-9}M E_2, $ $p<0.05$ compared to 10^{-8}M E_2.

3.3.5 Rapid E_2 Signaling in HCC38 Breast Cancer Cells Protects Cells from Taxol-induced Apoptosis through ERα36 while Enhancing Proliferation

Treatment of HCC38 cells with taxol increased apoptosis (Figure 3.5, A–D). When cells were pretreated with E_2, the apoptotic effects of taxol were reduced, based on decreases in TUNEL (Figure 3.5A) and caspase-3 activity (Figure 3.5B). The fact that E_2-BSA also exhibited this effect (Figure 3.5, C and D) indicates that reduction of taxol-induced apoptosis by E_2 was through a membrane-dependent mechanism.

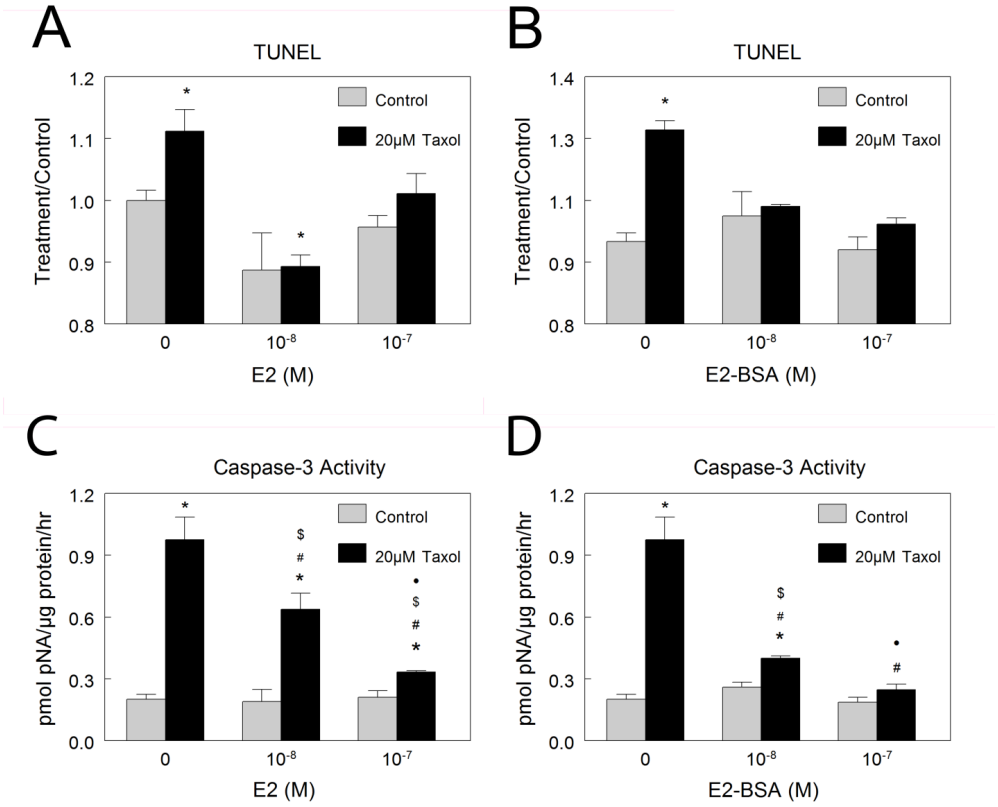

Figure 3.5: Effect of estrogen on taxol-induced apoptosis.

E_2 and E_2-BSA protected HCC38 cells against taxol-induced apoptosis as measured by TUNEL **(A,B)** and Caspase-3 activity **(C,D)**. * $p<0.05$ compared to control + 0M E_2, # $p<0.05$ compared to 20uM taxol + 0M E_2, $ $p<0.05$ compared to control + 10^{-8}M E_2, • $p<0.05$ compared to control + 10^{-7}M E_2.

The proliferative effect of E_2 also involved a membrane-associated mechanism. Both E_2 and E_2-BSA enhanced [^{3}H]-thymidine incorporation in HCC38 cells (Figure 3.6, A and B). Treatment of the cultures with antibodies to ERα36 blocked this effect. In contrast, neither nonspecific IgG nor antibody against GPR30 reduced the effect of E_2 or E_2-BSA.

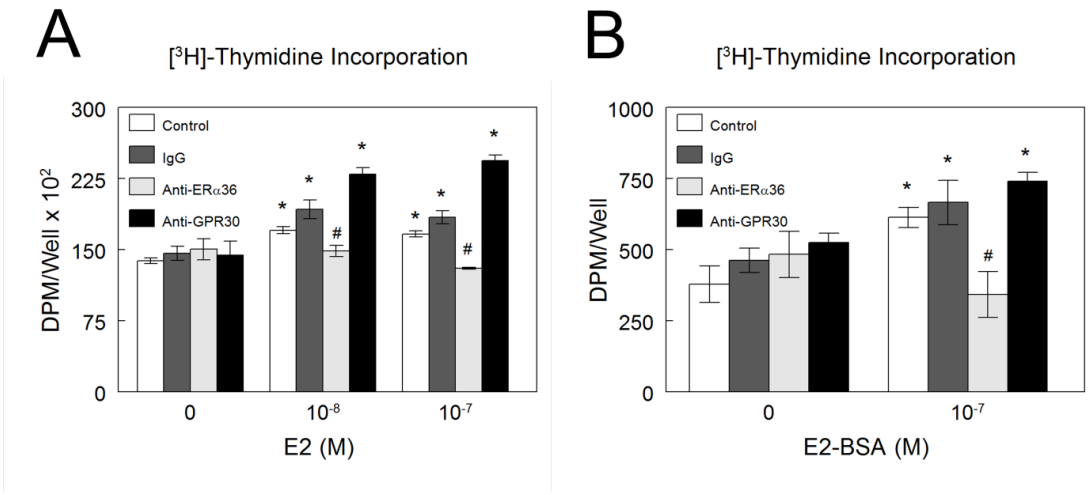

Figure 3.6: Effect of estrogen through ERα36 on cell proliferation.

E_2 (A) and E_2-BSA (B) caused an increase in DNA synthesis as measured by [3H]-thymidine incorporation, and when cells were pre-treated with antibody against ERα36, E_2-BSA induced cell proliferation was inhibited. * $p<0.05$ compared to control + 0M E_2, # $p<0.05$ compared to control + 10^{-8}M E_2 or 10^{-7}M E_2 or 10^{-7}M E_2-BSA.

3.3.6 Estrogen Signaling through ERα36 Increases Expression of Factors That Can Enhance Cancer Cell Metastasis and Bone Resorption

E_2-BSA treatment of HCC38 cells caused increased expression of the metastatic factor Snail1, although pretreatment with antibody against ERα36 blocked this effect

(Figure 3.7A). At the same time, E_2-BSA down-regulated e-cadherin (CDH1) (Figure 3.7C) indicating that expression of factors associated with epithelial to mesenchymal transition may also be mediated by ERα36-dependent signaling. E_2-BSA also had a stimulatory effect on CXCR4 expression (Figure 3.7E). We also saw an inhibitory effect of E_2 and E_2-BSA on syndecan-4 expression when normalized to GAPDH, but this was not blocked by antibodies to ERα36 (supplemental Figure A3.1). We did not see any effect of estrogen on MMP9 expression under these experimental conditions (data not shown). E_2-BSA increased expression of the osteoclast activator RANKL by an ERα36-dependent mechanism, based on inhibition of the effect by anti-ERα36 antibodies (Figure 3.7B). E_2-BSA had no effect on osteoprotegerin production (Figure 3.7D). Levels of this RANKL decoy receptor remained unchanged. Interestingly, E_2 treatment led to a slight decrease in osteoprotegerin levels (data not shown), potentially via a mechanism not mediated by ERα36. Finally, E_2-BSA increased production of interleukin-6 (Figure 3.7F).

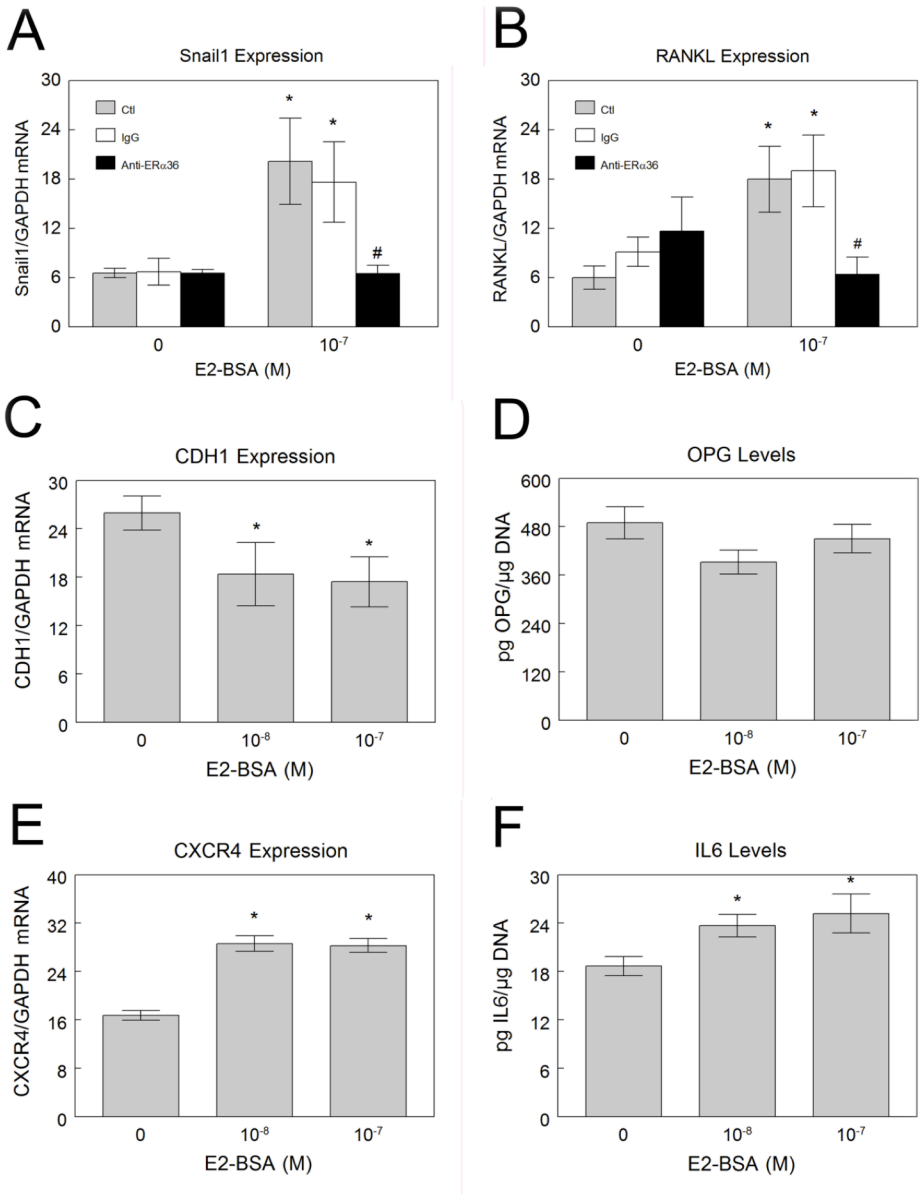

Figure 3.7: Membrane estrogen signaling via ERα36 enhances expression of factors that can enhance metastasis of breast cancer cells.

qRT-PCR analysis indicates that E_2 signaling in HCC38 cells increases expression of Snail1 (A) and a correlating downregulation of E-Cadherin expression is also observed (C). E_2-BSA also upregulates expression of CXCR4, the chemokine receptor for SDF-1 (E). E_2-BSA signaling via ERα36 enhances expression of osteoclastogenic factors. qRT-PCR analysis indicates that E_2 signaling in HCC38 cells increases expression of RANKL through ERα36 at the membrane (B) with no observed change in OPG production (D), while IL-6 production is enhanced (F). * $p<0.05$ compared to control + 0M E_2-BSA, # $p<0.05$ compared to control + 10^{-7}M E_2-BSA.

3.3.7 Estrogen Signaling through Membrane-associated ERα36 Promotes in vitro Invasiveness of HCC38 Breast Cancer Cells

Using the scratch-wound method, we observed that E_2-BSA treatment of HCC38 cells caused more rapid closure of the wound created in a two-dimensional culture (Figure 3.8A). Furthermore, we found that treatment with antibody against ERα36 inhibited this effect (Figure 3.8B).

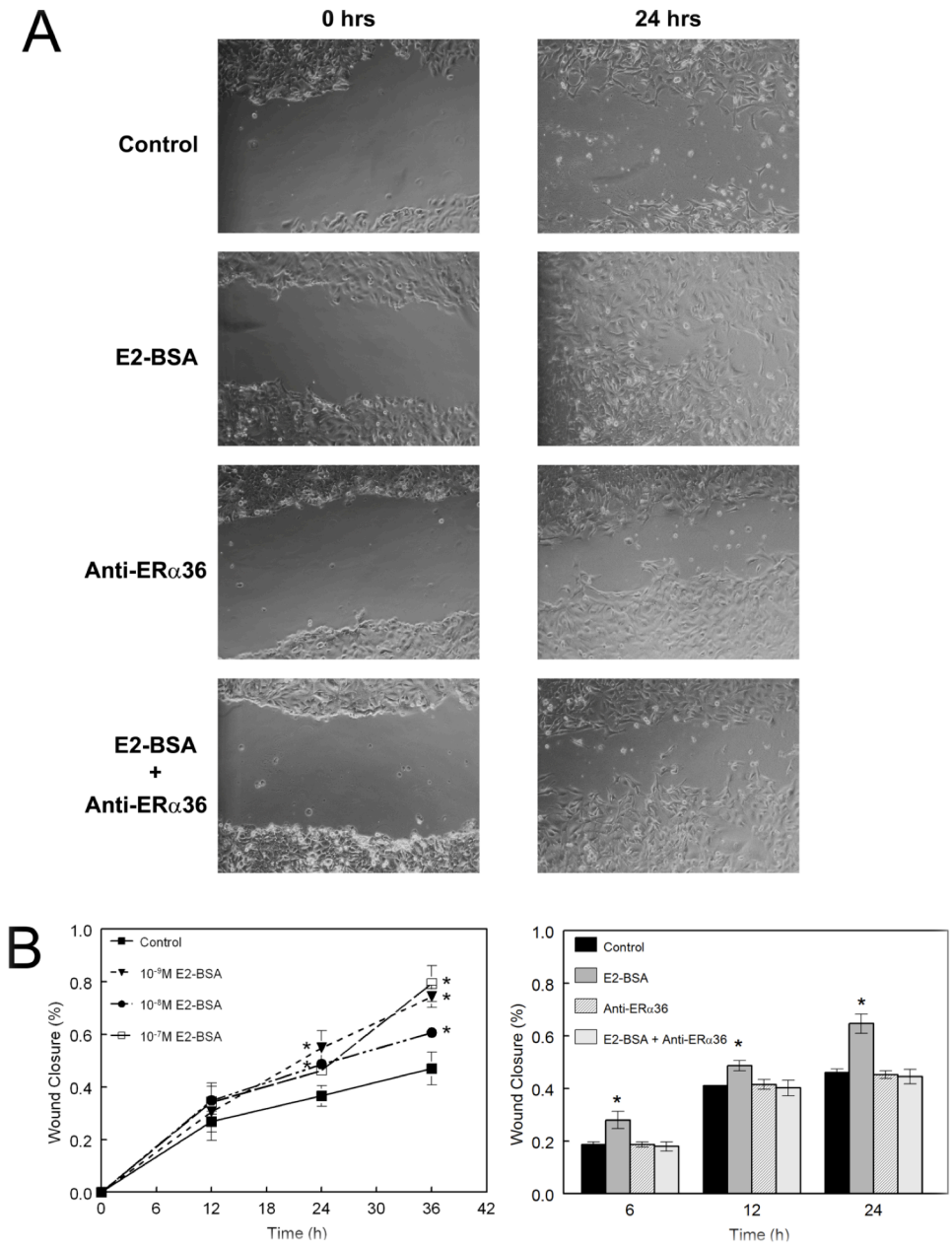

Figure 3.8: Membrane estrogen signaling via ERα36 promotes invasiveness of breast cancer cells *in vitro*.

Results of the scratch wound assay indicate that E_2 signaling in HCC38 cells through membrane-associated ERα36 can lead to more rapid closure of the wound created in the culture. (A) shows select phase-contrast images of representative samples from control samples, E_2-BSA-treated samples, anti- ERα36-treated samples, and samples treated with both. (B) shows time course data of samples treated with increasing concentrations of E_2-BSA and samples treated as described in (A). *$p<0.05$ compared to control.

3.4 DISCUSSION

This study exhibits that ERα-negative HCC38 breast cancer cells do in fact express ERα36 and that rapid activation of PKC in these cells in response to E_2 occurs through membrane-associated ERα36. It is unknown if ERα36 is expressed in all cancers; however, the findings presented in this article open the possibility that many ERα-negative breast cancers may actually express ERα36. In fact, the HCC38 cell line used in this study is triple-negative (57, 94), and therefore does not express PR or HER-2, suggesting that triple-negative breast cancers may also express this specific splice variant of ER. Although MDA-MB-231 and SkBr3 breast cancer cells have been cited in the literature as ERα-negative (95), our results show that even these cells express ERα36, which further supports the claim that ERα36 has a function in ER-negative breast cancers. We did however see that the COS7 cell line did not contain protein for ERα36, but this may be due to the fact that this cell line is derived from a non-human primate and we can account for this based on inter-species differences as well as the fact that the cell line is of embryonic origin.

GPR30 is a very well studied alternative receptor for estrogen that remains controversial as far as its subcellular localization is concerned(49, 82, 96, 97). We saw that all ER-negative cells, including HCC38 expressed GPR30 mRNA. This may suggest a role of GPR30 in ER-negative breast cancer, but our results here do not indicate a role for GPR30 in breast cancer cell proliferation. The fact that the antibody against GPR30 did not block the effect of E_2 nor E_2-BSA on [^{3}H]-thymidine incorporation is evidence that GPR30 does not mediate the membrane-associated response of ERα66-negative cells to E_2, at least where cell proliferation is concerned. However, ERα36 is responsible for mediating breast cancer cell proliferation. We did observe that when cells were treated with estrogen and pre-treated with antibodies against GPR30, [^{3}H]-thymidine

incorporation increased above baseline estrogen treatment. We can speculate that by blocking GPR30, we may be altering the kinetics of E_2 signaling by allowing more E_2 to be available for signaling through ERα36, but this requires more in depth analysis to come to this conclusion.

The present study focused on non-traditional mechanisms of ERα36, as we were highly interested in the role that this protein plays in rapid membrane-initiated signaling. Our findings suggest that ERα66 plays a greater role in nuclear-receptor signaling than it does in the cytosol or membrane, while ERα36 may have functions more prevalent outside of the nucleus. Localization of ERα36 to caveolae within the cell membrane suggests that ERα36 plays a major role in rapid membrane-initiated signaling. Caveolae typically house several proteins that are involved in many rapidly activated membrane-associated pathways (34, 86), such as those associated with vitamin D_3 metabolites (37, 98), testosterone, and especially estrogen (37). The loss of caveolae by β-cyclodextrin treatment indicates the requirement of caveolae for these membrane-specific responses of estrogen. Due to the complex nature of caveolae and the many proteins that may be found within caveolae, it is quite possible that ERα36 does not work alone in these pathways.

As previously observed, we found that E_2 rapidly activated PKC within minutes in both MCF7 and HCC38 cells. When we pretreated cells with antibodies that specifically target ERα36, the effects of E_2 were completely abolished. When we account for the fact that in our previous study(57), we did not see inhibition of this PKC effect when we used antibodies to block ERα66, we can conclude that ERα36 is responsible for this effect, and this response is initiated from the membrane due to the fact that the antibodies cannot cross the membrane and are therefore targeting membrane associated receptors.

We have found that these rapid membrane-mediated effects are associated with enhanced breast cancer cell survival. The PKC pathway appears to be crucial to the anti-apoptotic effects of estrogen in these cells as evidenced by the effect of chelerythrine on MTT and DNA fragmentation. Because we did not observe cell death in response to phosphate treatment, yet we did when we inhibited PKC with chelerythrine, we can conclude that PKC plays a major role in breast cancer cell survival and tumorigenesis.

One mechanism by which E_2-dependent activation of PKC may function in cell survival is by promoting cell proliferation. We previously demonstrated that the addition of tamoxifen, an antagonist of the estrogen receptor, which actually inhibits PKC in breast cancer, caused inhibition of DNA synthesis in MCF7 and HCC38 breast cancer cells(57). Together with data from Marino et al. showing that estradiol increases DNA synthesis through PKC activation (99), our observations support this hypothesis.

In addition, numerous studies(100-104), including a previous study by our group(57), have implicated PKC signaling as a major contributor to tumor progression. We found a strong correlation among PKC activity and tumor size and recurrence, which contained well over 100 subjects. This observation indicated that patients diagnosed as ER-negative according to the current screening method may in fact respond to tamoxifen.

Because we observed that tamoxifen inhibits PKC through this specific pathway of E_2, we decided to observe E_2's effects on different pathways of other chemotherapeutics, such as taxol. Our results indicate that not only does E_2 enhance cell survival by increasing cell proliferation through this membrane-mediated mechanism, as evidenced by effects of E_2-BSA, but E_2 also elicits anti-apoptotic effects

on these cells that can oppose the effects of very commonly used chemotherapeutic agents, such as taxol. Enhancement of cell proliferation in conjunction with the anti-apoptotic effects of E_2 can be implicated in primary aggressive tumor growth. The ability of estrogen to counteract the effects of taxol in these breast cancer cells poses a great problem in the treatment of the disease.

The progression of a tumor and development of metastasis is a very complex process. As the tumor continues to grow, the intracellular signaling of local factors associated with angiogenesis alleviates the requirement of a neo-vasculature. Expression of several factors such as Snail1 and Snail2 leads to a down-regulation of cell-cell interaction proteins such as cadherins, leading to what is known as an epithelial to mesenchymal transition(105-107). This alteration in cancer cell phenotype imparts the ability of the cells to detach from the primary tumor and migrate to distant sites of the body. Breast, as well as prostate cancer, appear to have a particular affinity for metastasizing to osseous tissue (90), which indicates poor prognosis for the patient. The effect of estrogen on RANK ligand in our system indicates the possibility that estrogen signaling within the primary breast tumor can enhance secretion of factors by the tumor inducing osteoclastogenesis, thereby promoting bone remodeling. This can create a “fertile” soil for the attachment of migrating cancer cells and because bone is so highly vascularized (90), the probability of migrating cancer cells to find a location in bone on which to attach and form metastases is extremely high. Increases in chemotactic receptors such as CXCR4, as we observed; also promote migration to sites where ligands for this receptor, such as SDF-1, reside in high levels. In addition, effects of estrogen on extracellular matrix interacting components, such as membrane syndecan-4, can also promote detachment of cancer cells from the primary tumor.

Our results suggest that many breast cancer cell responses to estrogen include upregulation of many of these factors, and the fact that ERα36 directly mediates these responses, as well as promoting *in vitro* invasiveness, suggests that this receptor may play a major role in breast cancer metastasis. ERα36 has even been shown to have a high association with lymph node metastasis in subjects with gastric cancer (108).

Because ERα36 does retain the DNA-binding domain found in traditional ERα, it is still possible that this receptor may play some role in directly regulating gene expression; however, the fact that it does not retain either transcriptional activation domains (AF1/AF2) suggests that it may only function as a co-factor for transcription or even as a transcriptional repressor. This suggests that ERα36 may play multiple roles in regulating cell differentiation and survivability. Taking this into account with data showing different effects of estrogen signaling through ERα36 in rat costochondral resting zone chondrocytes(109), the possibility remains that ERα36 also plays different roles depending on the cell type, especially whether the cells are cancerous or not.

From our previous data on estrogen signaling in MCF7 and HCC38 breast cancer cells and with our current findings, we hypothesize that E_2 interacts with ERα36 at the cell membrane to rapidly activate PKC through a PI-PLC-dependent mechanism that cleaves PIP_2 to IP_3 and DAG. IP_3 promotes calcium influx from the smooth endoplasmic reticulum while DAG helps to anchor PKC to the cell membrane, thereby promoting activation of PKC. Because PKC can activate ERK1/2 (80), which can rapidly enhance phosphorylation of proteins that promote proliferation, as well as factors that may enhance downstream gene expression, this pathway may lead to indirect effects on gene expression. This can be inhibited by chelerythrine, which directly inhibits PKC. Genes that may be affected, such as RANKL and Snail1, can promote metastatic activity of the breast cancer cells. These responses that can rapidly activate ERK1/2 can lead to

diverging pathways by which PIP_2 cleavage can also enhance anti-apoptotic effects of E_2 possibly through activation of Akt. Taxol has been shown to activate apoptosis of breast cancer cells by enhancing activity of c-Jun kinase (JNK), which alters the ratio of the Bcl-2 family of proteins such as Bax (pro-apoptotic) and BAD (anti-apoptotic), which in turn leads to enhanced permeability of the mitochondrial membrane and release of cytochrome C(69). This eventually leads to activation of the apoptotic caspase cascade, eventually leading to activation of caspase-3 and apoptosis. This study has specifically shown that membrane-associated estrogen signaling can inhibit taxol-induced caspase-3 activity. A schematic representation of this proposed pathway is seen in Figure 3.9.

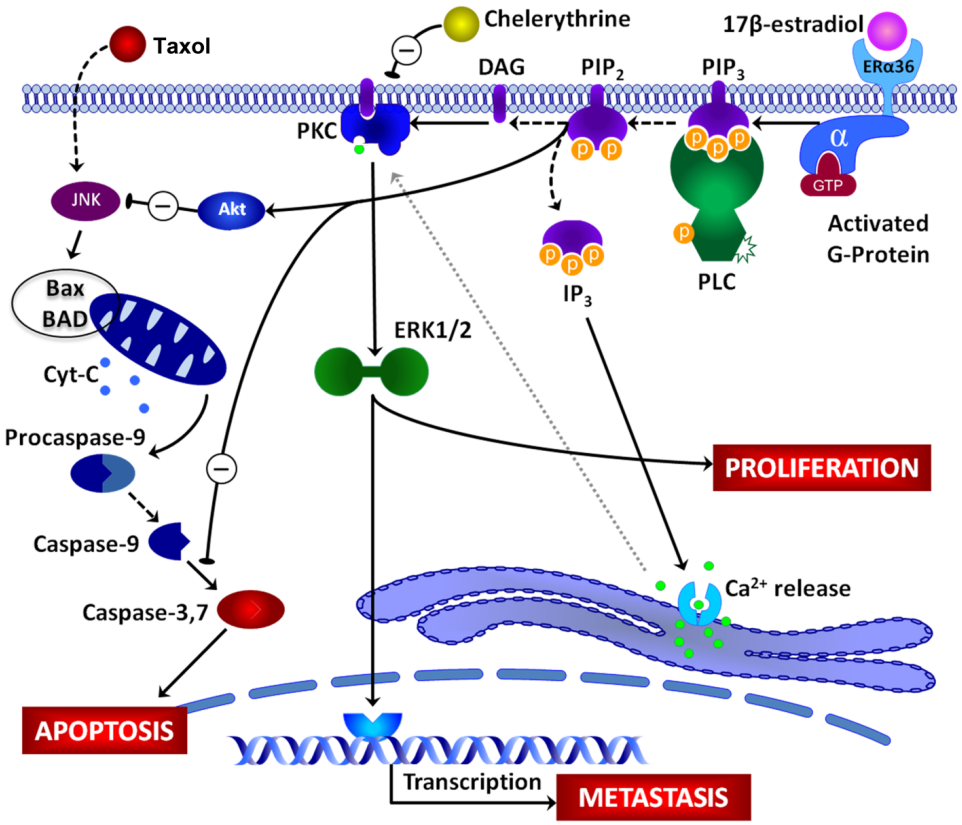

Figure 3.9: Proposed model of rapid membrane-initiated signaling of estrogen via ERα36.

E_2 interacts with ERα36 at the cell membrane initiating a signaling cascade consisting of phosphatidylinositol-specific phospholipase C (PI-PLC) dependent cleavage of phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 signals Ca^{2+} release from the smooth endoplasmic reticulum, while DAG anchors protein kinase C (PKC) to the membrane. The membrane localization along with Ca^{2+} association with PKC leads to its activation, which can be inhibited by chelerythrine, with downstream activation of ERK1/2 mitogen activated protein kinase (MAPK). Activation of ERK1/2 can affect cell proliferation as well as phosphorylation of transcription factors that can activate or repress gene transcription, possibly leading to expression of factors associated with metastasis and tumor aggressiveness. Taxol induces apoptosis through a mechanism involving JNK MAPK and the Bcl2-associated proteins Bax and BAD, leading to release of cytochrome C from the mitochondria. This initiates the caspase cascade involving caspases-9 and -7, eventually leading to activation of caspase-3, which causes apoptosis. E_2 signaling through membrane-associated receptor prevents taxol-induced apoptosis through a mechanism that may activate Akt and cause inhibition of caspase-3.

The findings of this article present a novel target for development of new therapies such as pharmaceutical intervention against progression of breast cancer primary tumor growth and prevention of metastasis. The mechanism of the membrane receptor for estrogen in breast cancer tumorigenesis and metastasis has not been previously reported. This study identifies a membrane receptor for estrogen in breast cancer cells that activates an array of pathways. We show that estrogen signaling via ERα36 initiated at the cell membrane activates cross-talk among multiple pathways important for breast cancer aggressiveness. Figure 3.9 proposes a model for the mechanism by which membrane estrogen signaling through ERα36 enhances cell proliferation, anti-apoptotic effects, and metastatic effects. A drug that specifically targets the membrane-associated estrogen receptor, ERα36, may not pose harmful effects on normal estrogen processes that occur within the cells, as ERα36 cannot activate expression of genes containing traditional estrogen response elements (EREs). A multi-faceted approach to treatment remains necessary as cancer, especially breast cancer, exemplifies a class of diseases rather than a single disease. Every patient is unique, but further investigation and understanding of membrane-associated signaling of estrogen in breast cancer can possibly lead to new routes of treatment for this devastating disease.

CHAPTER 4: ERα36 Mediates the Anti-apoptotic Effect of Estradiol in Triple Negative Breast Cancer Cells via a Membrane-Associated Mechanism

[Chapter 4 has been prepared for manuscript submission as: Chaudhri RA, Hadadi A, Lobachev KS, Boyan BD, Schwartz Z. The Mechanism by which ERα36 Mediates the Anti-apoptotic Effect of Estradiol in Triple Negative Breast Cancer Cells. 2013.]

Estrogen receptor (ER)-positive breast cancer typically responds to anti-estrogen treatment as estrogen enhances cell proliferation and tumor growth. However, previous work has shown that some ER-negative breast cancers respond to 17b-estradiol and anti-estrogens such as tamoxifen. Not only do these cancers respond to 17b-estradiol, but also the effect is rapid and non-genomic, suggesting that these effects can be mediated by the presence of a membrane-associated ER. ERα36 has been shown to mediate rapid, non-genomic, membrane-associated effects of 17b-estradiol in several cancer cell lines, including ER-negative HCC38 breast cancer cells. Moreover, the effect of 17b-estradiol in these cells is anti-apoptotic. The aim of this study was to determine if ERα36 mediates this anti-apoptotic effect, and to elucidate the mechanism involved. Taxol was used to induce apoptosis in HCC38 cells, as indicated by increased caspase-3 activity, and the effect of 17b-estradiol pre-treatment was determined. Antibodies to ERα36, inhibitors to specific cell signaling molecules, ERα36 deletion mutants, and ERα36-silencing were used prior to these treatments to determine the role of ERα36 in these effects and to determine which signaling molecules were involved. We found that the anti-apoptotic effect of 17b-estradiol in HCC38 breast cancer cells is in fact mediated by membrane-associated ERα36. We also showed that this signaling occurs through a pathway that requires PLD, LPA, and PI3K; Gαs and calcium signaling may also be involved. In addition, dynamic palmitoylation is required for the membrane-associated

effect of 17b-estradiol. Exon 9 of ERα36, a unique exon to ERα36 not found in other identified splice variants of ERα with previously unknown function, is necessary for these effects. This study provides a working model for the mechanism by which estradiol promotes anti-apoptosis through membrane-associated ERα36, suggesting that ERα36 may be a potential membrane target for drug design against breast cancer, particularly triple negative breast cancer.

4.1 INTRODUCTION

Breast cancer is the most common non-cutaneous cancer among women in the United States. The National Cancer Institute estimates that there will be 232,340 new cases among women in the U.S. in 2013, which correlates to about 1 in 8 women receiving diagnosis. The NCI also predicts 39,620 deaths due to breast cancer in the same year. Although the 5-year survival for patients diagnosed in the early stages of breast cancer exceeds 90%, survival in patients with distant metastasis drops below 25% indicating very poor prognosis for these individuals (5). While an actual cure for breast cancer is elusive, novel approaches to diagnosis and treatment can help to reduce mortality and allow patients, specifically those with more advanced stage cancer, to live normal lives.

The progression of cancer is a dynamic process that begins with primary tumor growth, depending on cancer cell proliferation simultaneously with the ability of cancer cells to evade apoptosis (110). In some cases, aggressive cancer cells can evade apoptosis even in the presence of radiotherapy and chemotherapeutic drugs that are used to target these cells and specifically induce apoptosis (69, 88). Current approaches to treatment have evolved to combination therapy, usually beginning with surgery and/or targeted radiotherapy, followed by adjunctive chemotherapy (111-113).

Taxol, a very commonly used chemotherapeutic drug, is the first of a group of drugs known as taxanes, which induce apoptosis in cells by inhibiting mitosis (114, 115). Currently, taxol is synthetically prepared and various formulations and carriers are being developed to increase the effectiveness of the drug (116). Moreover, the main problem with drugs such as taxol is that they do not only target cancer cells, but can also induce apoptosis in normal cells (117, 118). This necessitates current approaches of targeted therapy, by which newer targets are being discovered that are either unique or highly upregulated in cancer cells.

Estrogen receptors (ERs) play a major role in classification, diagnosis, and treatment of breast cancer (119-121). Patients that contain hormone responsive or ER-positive tumors are expected to take the ER-agonist tamoxifen continuously for 5 years following initial treatment (122). Tamoxifen functions as an anti-estrogen, and we previously showed that it could block the stimulatory effect of 17b-estradiol (E_2) on ER-negative breast cancer cells as well as in ER-positive cells (57). Traditional inhibitors of ERs, such as ICI 182,780 and diethylsilbesterol, did not block E_2-induced cell proliferation or protein kinase C (PKC) activity in the ER-negative cells, nor did traditional antibodies to ERα and ERβ (57).

Membrane-associated E_2 signaling also elicits anti-apoptotic effects against taxol, leading to an aggressive cancer phenotype (54, 69). Taxol induces apoptosis through a c-Jun N-terminal kinase (JNK)-dependent mechanism that promotes mitochondrial efflux of cytochrome C, leading to activation of an apoptotic caspase cascade (69). This suggests that receptor-mediated signaling is involved, but traditional ERα is not responsible.

We recently showed that ERα36, an alternatively spliced variant to traditional ERα, is responsible for the membrane-mediated effect of E_2 in breast cancer cells that promotes cell survivability (54). The mRNA of ERα36 lacks the first exon found in traditional ERα, ERα66, as well as exons 7 and 8 (53). This results in a truncated form of ERα66 that does not contain the transcriptional activation domains AF1 and AF2 and a truncated ligand-binding domain, however, ERα36 still exhibits ligand-dependent effects of E_2 (53, 55, 123). In addition, ERα36 contains a novel exon at the C-terminus known as exon 9. This exon contains 27 amino acids of unknown function, but it is hypothesized to contain myristolation or palmitoylation-specific sequences (53, 124), which would help to explain any membrane-associated effects mediated by ERα36. This study investigated the mechanism by which ERα36 prevents taxol-induced apoptosis. We hypothesized that ligand-dependent activation of ERα36 induces receptor-dependent inhibition of signaling cascades associated with activation of apoptosis, as well as activation of anti-apoptotic signaling cascades.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

ERα-negative HCC38 human breast cancer cells and human embryonic kidney HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Roswell Park Memorial Institute 1640 medium (RPMI 1640) was purchased from Invitrogen (Grand Island, NY). Charcoal/dextran-filtered fetal bovine serum (FBS) was purchased from Gemini Bioproducts (Sacramento, CA). E_2 enantiomer (Ent-E_2) was kindly provided as a gift from Dr. Douglas Covey (Washington University, St. Louis, MO) (125). E_2, E_2-BSA, taxol, 2-hydroxymyristic acid (HMA), 2-bromohexadecanoic acid (2-bromopalmitate, 2-BP), and tunicamycin (Tm) were

purchased from Sigma (St. Louis, MO). Cycloheximide (CHM), Wortmannin, D609, U73122, LY294002, thapsigargin, pertussis toxin (PTX), and cholera toxin (CTX) were purchased from EMD Chemicals (Gibbstown, NJ). VPC32183S and lysophosphatidic acid (LPA) were purchased from Avanti Polar Lipids (Alabaster, AL). Protein content of samples was measured using the Macro BCA reagent kit from Pierce/Thermo Scientific (Rockford, IL). Polyclonal ERα36 antibodies against the unique C-terminal 27 amino acids were generated by Cell Applications Inc. (San Diego, CA). 740 Y-P and the Titertacs TUNEL assay were purchased from R&D Systems (Minneapolis, MN). Goat anti-rabbit horseradish peroxidase (HRP) and goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). Bax and Bcl2 primers were purchased from Eurofins MWG Operon (Huntsville, AL). The cytochrome C apoptosis assay kit was purchased from MBL International (Woburn, MA). The Amplex Red Phospholipase D (PLD) Assay kit was purchased from Life Technologies (Grand Island, NY). Caspase-3 activity was measured using the CaspAce Assay system from Promega (Madison, WI). ERα36 overexpression plasmids were purchased from Chi Scientific (Maynard, MA). Polyfect transfection reagent was obtained from Qiagen (Germantown, MD).

4.2.2 Cell Culture

HCC38 cells and HEK293 cells were cultured in RPMI 1640-based media or DMEM, respectively, as specified by the ATCC containing 10% charcoal/dextran-filtered FBS and lacking phenol red, which can mimic the effects of E_2 at low levels (126, 127). Specific modifications for each experimental question are described below.

4.2.3 Requirement for ERα36 in E_2's Anti-apoptotic Effect

4.2.3.1 Apoptotic Effect of Taxol in HCC38 Cells

The experimental design for this study was based on the ability of E2 to block the apoptotic effects of taxol. Initial experiments were performed to establish the effect of taxol on HCC38 cells. 24 hours after plating, HCC38 cells were treated with increasing concentrations of taxol (5, 10, 20μM) for 4 hours, after which TUNEL and caspase-3 activity were measured using assays kits according to the manufacturers' directions. To confirm that the effects of taxol were apoptotic, as caspase-3 activity is implicated in the terminal differentiation of some cell types (128-131), HCC38 cells were treated with 20μM taxol for 12 hours and *BAX/BCL2* mRNA levels were determined and cytochrome C translocation from the mitochondria to the cytosol was examined by the cytochrome C apoptosis assay kit from MBL International according to the manufacturer's instructions.

4.2.3.2 Requirement for a Receptor-mediated Membrane-associated Mechanism

E_2 conjugated to bovine serum albumin (E_2-BSA), which cannot cross the plasma membrane (21, 49, 132) was used to verify that the anti-apoptotic effect of E_2 was via a membrane-mediated mechanism. E_2-BSA has previously been shown to have similar effects to E_2 and can interact with ERs. BSA conjugation prevents E_2 from crossing the plasma membrane, and therefore, E_2-BSA effects can be attributed to either membrane receptor effects or alterations in membrane fluidity due to it's the hydrophobic nature of E_2-BSA (49, 57, 132, 133). To address the possibility that E_2's effect is due to a non-specific interaction with the plasma membrane, cells were also treated with the E_2 enantiomer Ent-E_2 (125). While Ent-E_2 is an enantiomer of E_2, it cannot directly interact with ERs, and therefore, any effects caused by Ent-E_2 could be attributed to its direct effect on membrane fluidity as it possesses the same hydrophobic properties of E_2.

PLD activity was determined as an outcome measure, based on our previous observation that the anti-apoptotic effect of the vitamin D3 metabolite 24R,25-dihydroxyvitamin-D3 (24,25(OH)2D3) occurs through activation of PLD (68). PLD activity was measured using the Amplex Red PLD assay from Life Technologies. Subconfluent cultures of HCC38 cells in 24-well TCPS plates were treated with E_2 or Ent-E2. Also, prior to E_2 treatment, a 15 minute pretreatment of cells with ERα36 specific antibodies (1:500 dilution) was performed to block the membrane receptor in order to determine if the effect of E_2 was through membrane-associated ERα36. While antibodies cannot enter the cells, any inhibition of E_2's effect in the presence of antibody could be attributed with E_2's direct interaction with membrane-associated ERα36.

4.2.3.3 ERα36 Silencing, Overexpression, and Mutation

In order to confirm the role of ERα36 in the anti-apoptotic effect of E_2, HCC38 cells were transiently transfected with an ERα36 shRNA expression plasmid in order to transiently knockdown ERα36. The shRNA expression plasmid was produced by cloning a microRNA specific anti-sense target sequence for the 3'UTR of ERα36 cDNA using the DNA oligonucleotides, 5'-GGATCCCATGCCAATAGGTACTGAATTGATATCCGTTCAG--TACCTATTGGCATTTTTTTCCAAAAGCTT-3', and was prepared by Sigma-Aldrich using their Mission shRNA purified plasmid expression system. HCC38 cells were seeded at a density of 1.25 x 10^5 cells/cm^2 in tissue culture treated polystyrene (TCPS) and cultured in media containing no antibiotics. One day after plating, when cells were approximately 75% confluent, transient transfection was performed using Polyfect transfection reagent from Qiagen according to the manufacturer's instructions. Media were changed after 24 hours to full growth media, and 48 hours after transfection, cells were treated according to experimental procedures. The PLD and caspase-3 activity assays were performed with HCC38 silenced for ERα36 (denoted as shERα36), wildtype

HCC38 cells (denoted as wt), and mock transfected HCC38 cells. Whole cell lysates were harvested in RIPA and western blot was performed on wt and shERα36 HCC38 cells using ERα36 antibodies (1:500 dilution). Densitometry analysis using Quantity One software was performed to determine percentage of knockdown in shERα36 cells.

In order to determine the importance of ERα36 on a more generalized platform, the human embryonic kidney cell line, HEK293, which has been previously demonstrated to not express functional levels of endogenous ERα36 (55), was used as a model for analysis of wildtype exogenous ERα36 overexpression (denoted as 293ovrx36). Use of HEK293 cells thus allowed us to examine the role of wildtype exogenous ERα36 and mutated ERα36 independent of the presence of functional endogenous ERα36.

Because exon 9 is unique to ERα36, and ERα66 has not been shown to mediate the membrane-associated effect of E_2 in ER-positive MCF7 cells (57), we hypothesized that exon 9 was required for these responses. An exon 9 deletion mutant (denoted as 293ex9d36) was created using overlap extension PCR cloning as previously described by Bryksin and Matsumura (134). Exon 9 deleted HEK293 cells were then created according to the methods described below, allowing us to examine the requirement of this exon in the anti-apoptotic pathway of E_2.

HEK293 cells were transiently transfected with ERα36 wt overexpression and exon 9-deletion plasmids using Polyfect transfection reagent according to manufacturer's instructions. HEK293 cells were seeded at a density of 1.25×10^5 cells/cm^2 in TCPS plates and cultured in medium containing no antibiotics. One day after plating, when cells were approximately 75% confluent, transient transfection was performed using Polyfect transfection reagent according to the manufacturer's protocol.

Media were changed after 24 hours to full growth media, and 48 hours after transfection, cells were treated as previously described for measurement of PLD and caspase-3 activity. PLD and caspase-3 activity assays were performed in HEK293 overexpressed with wildtype ERα36 (293ovrx36) and exon 9-deleted ERα36 (293ex9d26). Wildtype HEK293 cells mock transfected with a non-targeting vector and plated at the same time as the mutant cells were used as positive controls. Whole cell lysates were harvested in RIPA and western blot was performed on 293wt, 293ovrx36, and 293ex9d36 HEK293 cells using ERα antibodies (1:500 dilution) that can also detect ERα36. These antibodies were used rather than ERα36 antibodies because the ERα36 antibodies detect the C-terminal domain of ERα36, and the exon 9 deletion mutants (293ex9d36) do not contain this region. Bands shown in western blots were detected at ~36kDa. Densitometry analysis using Quantity One software was performed to determine percentage of knockdown in shERα36 cells.

4.2.3.4 Examination of E_2's Anti-apoptotic Pathway

In order to determine the signaling pathway involved in the anti-apoptotic effect of E_2, we took advantage of the observation that E_2 inhibits taxol-induced apoptosis via membrane-associated signaling by attenuating the effect of taxol on caspase-3 activity (54, 69). For the experiments described below, subconfluent cultures of HCC38 cells were pretreated with 10^{-8}M E_2 for 90 minutes followed by 20μM taxol treatment, after which time, E_2 was removed from the cultures. After 24 hours of taxol treatment, he number of viable cells was determined by the MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple formazan by mitochondrial reductase in living cells (135). After 4 hours of taxol treatment, caspase-3 activity was determined as described above. Requirement for ERα36 was determined by pretreating the HCC38 cells with antibodies generated against the unique C-terminus of

ERα36. Phosphatidylcholine-specific PLD (PC-PLD) was inhibited using 10^{-5}M wortmannin (136, 137). Phosphatidylcholine-specific phospholipase C (PC-PLC) was inhibited using $5x10^{-5}$M D609 (76, 137). Phosphatidylinositol-specific phospholipase C (PI-PLC) was inhibited using 10^{-5}M U73122 (136, 137). 10^{-6}M VPC32183S was used to block lysophosphatidic acid (LPA) signaling through LPA1 and LPA3 receptors, while LPA was used to activate LPA signaling (68). 10^{-5}M LY294002 was used to block phosphoinositide-3-kinase (PI3K) (68). 10^{-6}M 740 Y-P was used to activate PI3K signaling (138). 3μM thapsigargin was used to inhibit calcium translocation from the rough endoplasmic reticulum to the cytosol. Pertussis toxin (25ng/mL) was used to inhibit Gαi signaling, while cholera toxin (100ng/mL) was used to inhibit Gαs signaling (68). E_2-BSA was also used in several of these experiments to further determine if these effects were membrane-associated. As previously described, E_2-BSA cannot cross the plasma membrane, but still has many similar effects to E_2. For all caspase-3 experiments using E_2-BSA, subconfluent cultures of HCC38 cells were pretreated as with the inhibitors stated above followed with 10^{-8}M E_2-BSA for 90 minutes. 20μM taxol was then added, and after 4 hours of taxol treatment, caspase-3 activity was measured as described above.

4.2.3.5 Requirement for Dynamic Palmitoylation

Previous work has shown that ER's, particularly the ERα variants ERα66 and ERα46, can be targeted to the plasma membrane by palmitoylation (39, 139). ERα66 and ERα46 have been identified as translocating to the plasma membrane due to palmitoylation. In order to examine this, tunicamycin and 2-bromohexadecanoic acid (2-bromopalmitate, 2-BP) were used to inhibit palmitoylacetyltransferase and thus prevent palmitoylation of ERα36 (39). Cycloheximide (8μM) was used to block N-glycosylation

and HMA (0.5mM) was used to block myristolation (39). HCC38 cells were pre-treated with 30μM tunicamycin (Tm) and 10μM 2-BP to inhibit the activity of palmitoylacetyltransferase, which is responsible for post-translational palmitoylation of proteins that are targeted to the plasma membrane (39, 140). Tunicamycin, however, also inhibits N-linked glycosylation (39), and because of this, we also pre-treated cells with 8μM cycloheximide (CHM) as a control for N-glycosylation inhibition (39). In order to invalidate the possibility that the membrane effects of E_2 through ERα36 do not occur due to myristolation, we also pre-treated cells with 0.5mM 2-hydroxymyristic acid (HMA), which blocks post-translational myristolation (39). In order to determine if palmitoylation of ERα36 is necessary for the anti-apoptotic effect of E_2, HCC38 cells were pre-treated with 30μM Tm and 10μM 2-BP for 2 hours prior to E_2 and taxol treatment for 4 hours, after which, caspase-3 activity was measured.

4.2.4 Statistical Analyses

For all experiments, statistical analyses were performed by analysis of variance with Bonferroni's correction for multiple comparisons at a significance level of 0.05. All experiments were performed with n=6 individual cultures per variable. Experiments were performed multiple times to ensure validity of the data. Results of individual experiments are presented. Identification of symbols to signify statistical significance is found in the respective figure legends.

4.3 RESULTS

4.3.1 Effect of Taxol on Apoptosis

Taxol induced apoptosis in the HCC38 cells. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and caspase-3 activity both exhibited a dose-dependent increase (Figure 4.1A,B). *BAX/BCL2* also increased, confirming that the cells were apoptotic (Figure 4.1C). Results were further confirmed by an increase in cytochrome C translocation from the mitochondria to the cytosol in the presence of 20μM taxol (Figure 4.1D).

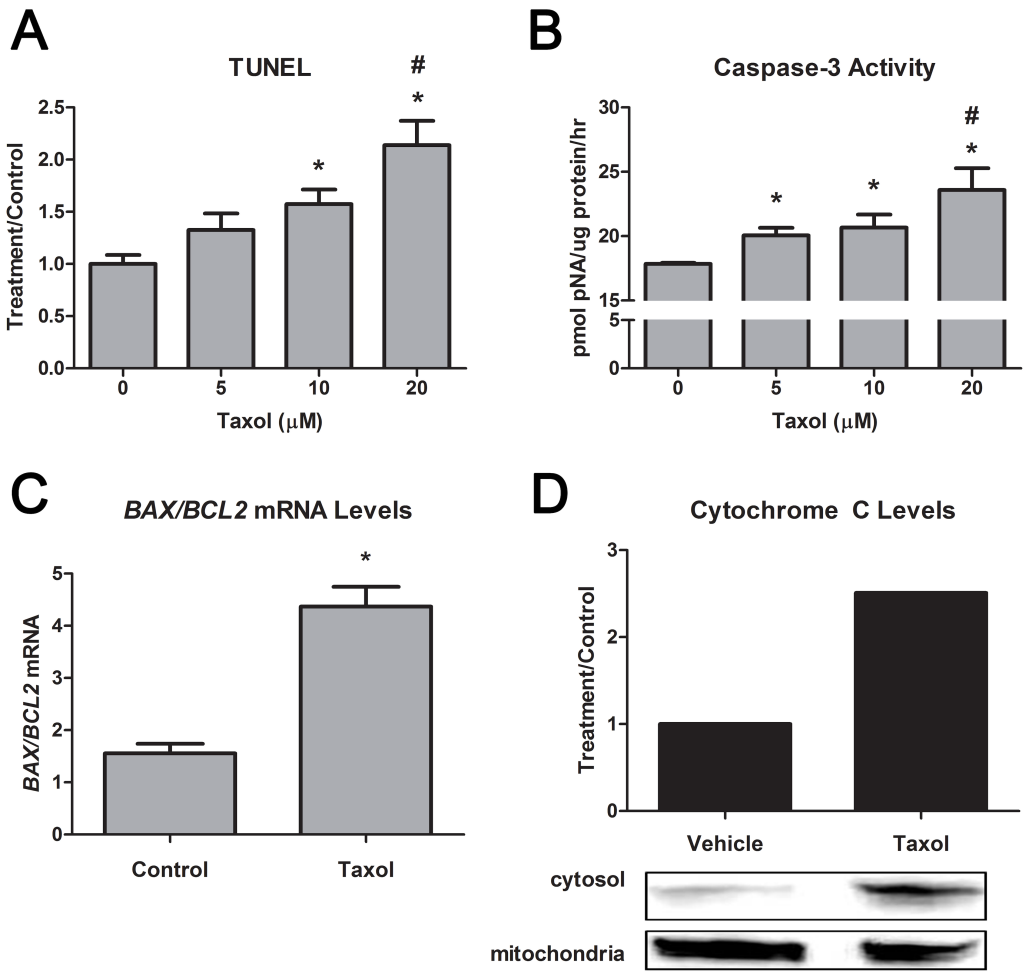

Figure 4.1: Effect of taxol on apoptosis of HCC38 cells

(A) Taxol's (0, 5, 10, 20μM) effect on TUNEL and (B) caspase-3 activity is dose-dependent. (C) Taxol also increased bax/bcl2 mRNA levels and (D) cytochrome C levels in the cytosol versus the mitochondria. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 5μM taxol.

4.3.2 Role of ERα36 in Activation of PLD by E_2

We found that 10^{-8}M E_2 activated phospholipase D (PLD) in HCC38 cells at 30 and 60 minutes (Figure 4.2A). The effect was receptor-mediated. Unlike E_2, E_2

enantiomer (Ent-E_2) concentrations used in this study did not have the same effect at 30 minutes (Figure 4.2B). Antibodies to ERα36 blocked the effect of E_2 on PLD activity (Figure 4.2C). Additionally, E_2 was unable to increase PLD activity in HCC38 cells transiently transfected with ERα36 shRNA expression plasmids (shERα36) (Figure 4.2D).

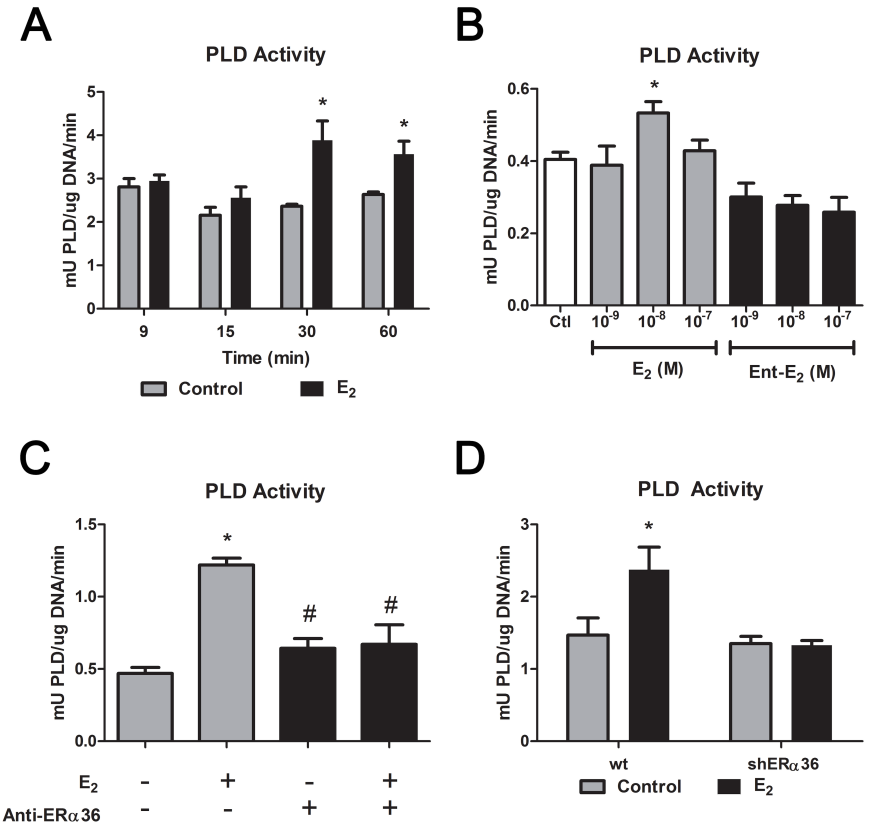

Figure 4.2: Role of ERα36 in the Effect of E_2 on Phospholipase D Activity

(A) Time course study of the effect of E_2 on PLD activity in HCC38 cells. (B) Dose dependent effect of E_2 on PLD activity after 30 minutes in HCC38 cells. Ent-E_2 showed no ability to enhance PLD at any concentration. (C) ERα36 inhibits the effect of 10^{-8}M E_2 on PLD activity after 30 minutes. (D) Transient transfection of HCC38 cells with ERα36 shRNA expression plasmid blocks the effect of E_2 on PLD activity after 30 minutes. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the corresponding untreated control group while # represents $p<0.05$ compared to E_2-treatment.

4.3.3 Role of ERα36 in the Anti-apoptotic Effect of E_2

Membrane activation of ERα36 signaling by E_2 caused the anti-apoptotic effect of E_2 against taxol. E_2 blocked taxol-induced effects on MTT and caspase-3 activation while the antibody to ERα36 prevented the effect of E_2 (Figure 4.3A,B) and E_2 conjugated to bovine serum albumin (E_2-BSA) (Figure A4.1). Additionally, HCC38 cells transiently transfected with ERα36 shRNA expression plasmids (shERα36), with greater than 70% knockdown (Figure A4.2A), exhibited a reduced ability of E_2 to block taxol-induced caspase-3 activity (Figure 4.3C).

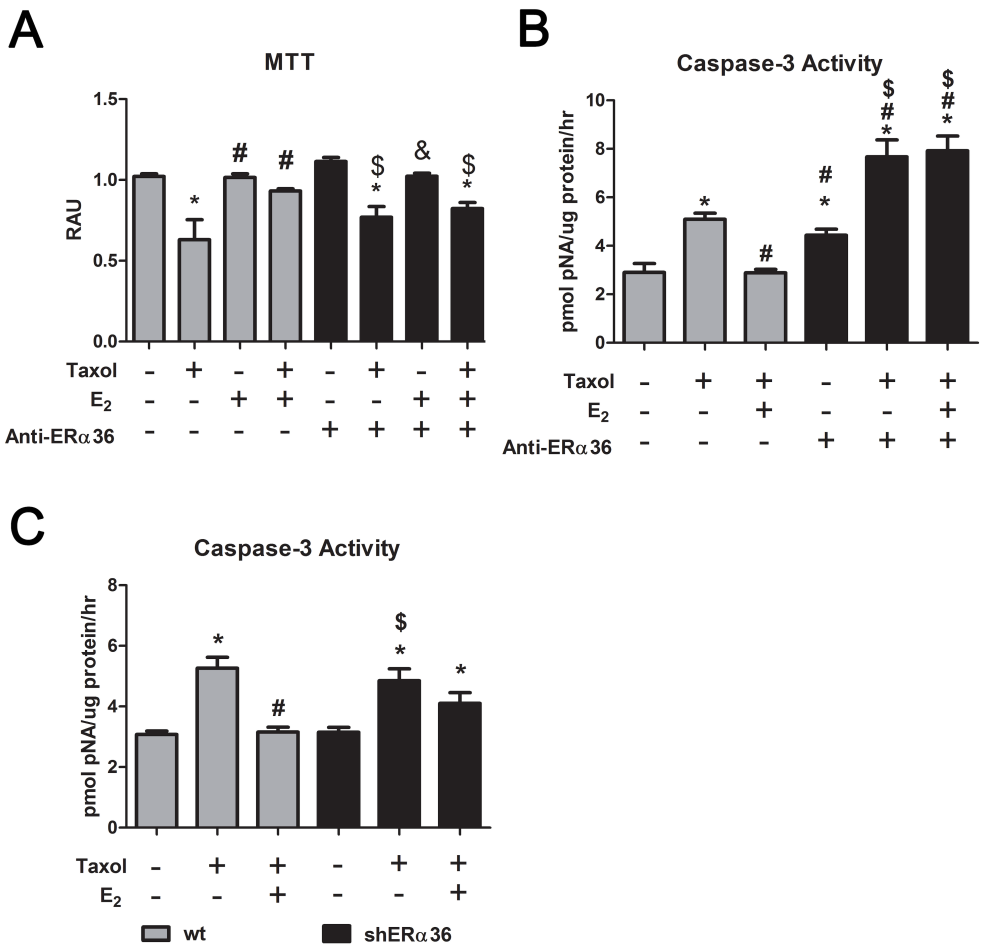

Figure 4.3: Role of ERα36 in the Anti-apoptotic Effect of E_2

(A) MTT is reduced by 20μM taxol, while this effect is prevented by 10^{-8}M E_2. ERα36 antibodies block this effect of E_2. (B) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while ERα36 antibodies block this effect. (C) While E_2 inhibits taxol-induced caspase-3 activity in wildtype HCC38 cells, HCC38 cells transiently transfected with shERα36 expression plasmids did not show the same effect. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to anti-ERα36 or shERα36 alone.

4.3.4 Role of Exon 9

HEK293 cells transiently transfected with wildtype ERα36 cDNA expression vectors and mutant vectors designed to express exon 9-deleted ERα36 cDNA were shown to have greater detectable levels of ERα36 protein compared to wildtype HEK293 cells, which exhibited very low levels of ERα36 shown by western blot using ERα antibodies that detect ERα36 (Figure A4.2B). Although wildtype HEK293 cells exhibited decreased PLD activity after 30 minutes of treatment with 10^{-8}M E_2, HEK293 cells transiently overexpressing exogenous wildtype ERα36 (293ovrx36) exhibited increased PLD activity after 30 minutes of E_2 treatment. Exon 9-deleted exogenous ERα36 expression mutants (293ex9d36) were unable to promote this effect of E_2 on PLD activity (Figure 4.4A). Additionally, while overexpression of wildtype ERα36 allowed E_2 to block taxol-induced caspase-3 activity in HEK293 cells, exon 9 deletion did not mediate E_2's effect against taxol induced caspase-3 activity. Interestingly, exon 9 deletion increased caspase-3 activity alone (Figure 4.4B).

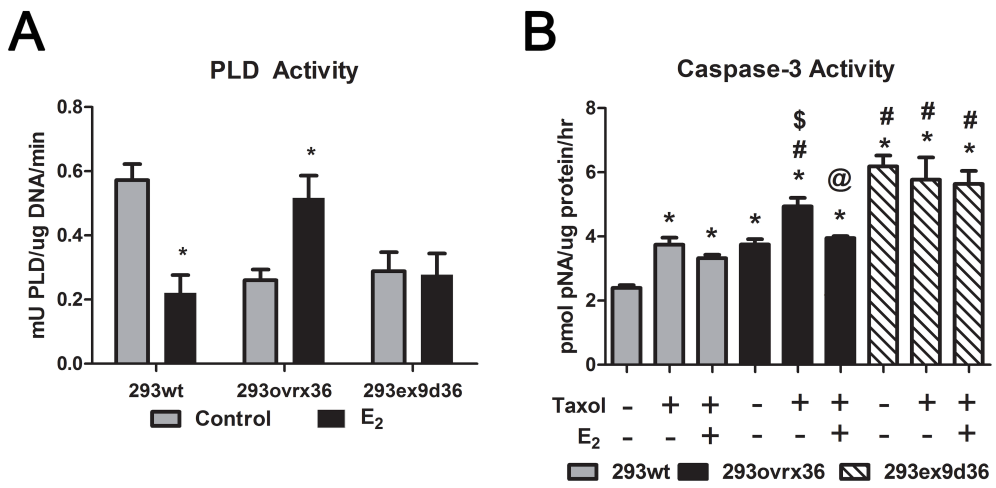

Figure 4.4: Requirement of Exon 9 in the Anti-apoptotic Effect of E_2

(A) PLD activity is reduced with 10^{-8}M E_2 wildtype HEK293 cells, while E_2 increases PLD activity in ERα36 overexpressed HEK293 cells. In cells overexpressing exon 9-deleted ERα36, E_2 did not exhibit this effect. * represents $p<0.05$ compared to the corresponding untreated control group. (B) Similarly, ERα36 overexpression mediated the anti-apoptotic effect of E_2 against taxol-induced caspase-3 activity, but in cells with exon 9-deleted ERα36, this effect was not evident. * represents $p<0.05$ compared to the corresponding untreated 293wt, # represents $p<0.05$ compared to taxol only in 293wt, $ represents $p<0.05$ compared to untreated 293ovrx36, and @ represents $p<0.05$ compared to taxol only in 293ovrx36.

4.3.5 Role of PLD

We also found that inhibition of phosphatidylcholine specific PLD (PC-PLD) with wortmannin blocked the anti-apoptotic effect of E_2 on taxol-induced caspase-3 activity (Figure 4.5A), indicating that the effect of E_2 on PLD leads to its anti-apoptotic effect. We did not see a similar effect when we inhibited phosphatidylcholine specific phospholipase C (PC-PLC) with D609 (Figure 4.5B) or phosphatidylinositol specific PLC (PI-PLC) with U73122 (Figure 4.5C), nor in the case of E_2-BSA's effect on taxol-induced caspase-3 activity (Figure A4.3A,B).

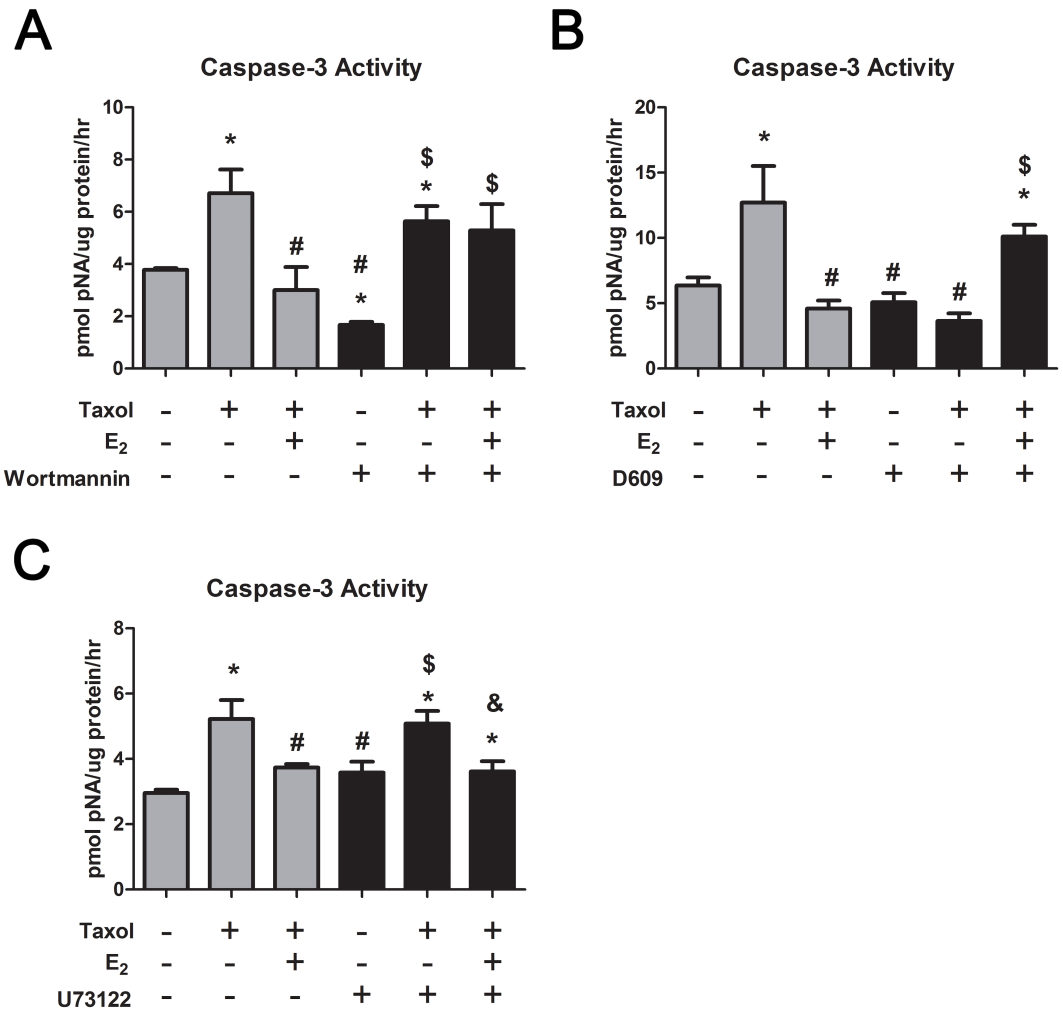

Figure 4.5: Role of Phospholipases in the Anti-apoptotic Effect of E_2

(A) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while wortmannin blocks this effect. (B) D609 and (C) U73122 do not exhibit the same effect as wortmannin. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone.

4.3.6 Role of Lysophosphatidic Acid

The anti-apoptotic pathway of E_2 was mediated by lysophosphatidic acid (LPA) signaling. When LPA signaling through the LPA1/3 receptors was inhibited with VPC32183S, the anti-apoptotic effect of E_2 and E_2-BSA on taxol-induced caspase-3

activity was prevented (Figure 4.6A, Figure A4.4A). VPC32183S also inhibited the effect of taxol (Figure 4.6A), suggesting some crosstalk in the taxol and E_2 pathways. LPA blocked the effect of taxol on caspase-3 activity in a similar manner as E_2 (Figure 4.6B).

4.3.7 Role of Phosphoinositide-3-kinase

Phosphoinositide-3-kinase (PI3K) plays a role in the anti-apoptotic pathway of E_2. Inhibition of PI3K with LY294002 prevented the effect of E_2 and E_2-BSA on taxol-induced caspase-3 activity (Figure 4.6C, Figure A4.4B). Conversely, the PI3K activator 740 Y-P prevented taxol-induced caspase-3 activity (Figure 4.6D).

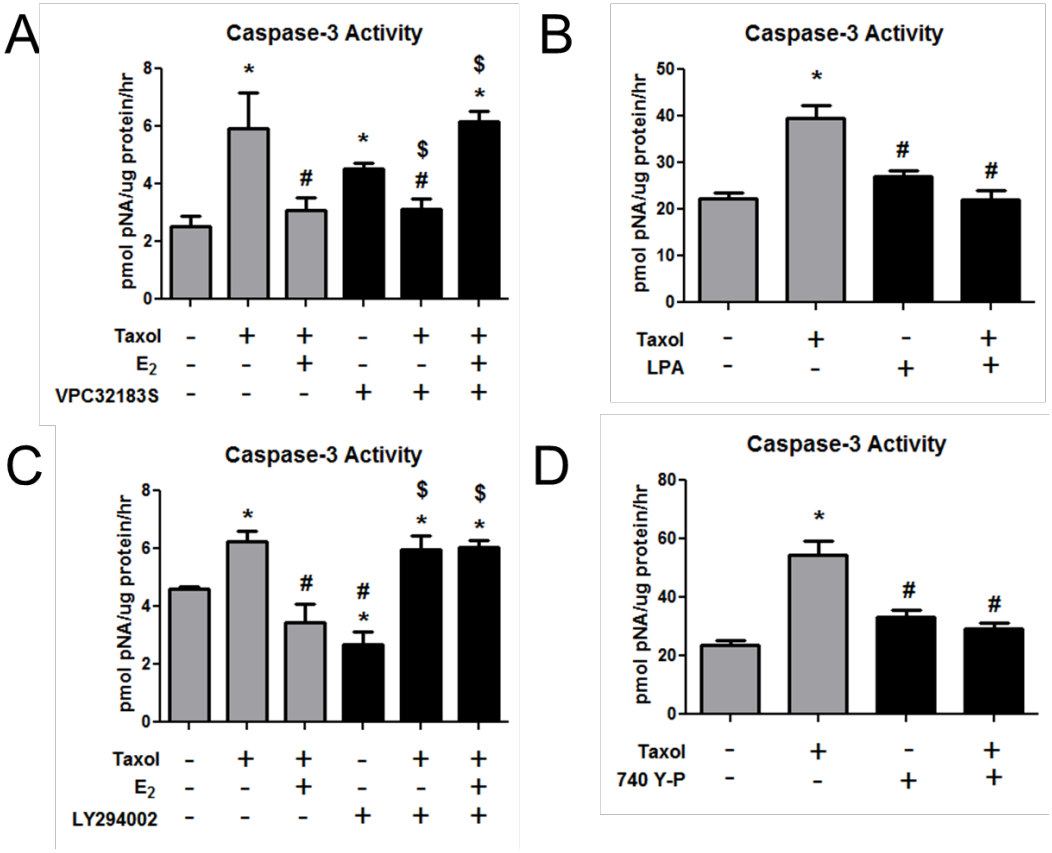

Figure 4.6: Role of LPA and PI3K in the Anti-apoptotic Effect of E_2

(A) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while the LPAR1/3 antagonist, VPC32183S, does not allow E_2 to block the effect of taxol. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone. (B) Taxol-induced (20μM) caspase-3 activity is reduced by LPA. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol. (C) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while the LY294002 does not allow E_2 to block the effect of taxol. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone. (D) Taxol-induced (20μM) caspase-3 activity is reduced by the PI3K activator 740 Y-P. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol.

4.3.8 Effect of Calcium and G-protein Signaling

Pre-treatment of cells with thapsigargin, which inhibits cytosolic calcium influx from the endoplasmic reticulum, increased caspase-3 activity to a comparable extent as taxol, indicating that blocking of calcium signaling can induce apoptosis (Figure 4.7A,B). E_2 or E_2-BSA (Figure A4.5A) reversed this effect. Pertussis toxin (PTX), which inhibits Gαi signaling, also increased caspase-3 activity, as did cholera toxin (CTX), which inhibits Gαs signaling (Figure 4.7B). Neither E_2 nor E_2-BSA had an effect on PTX-induced caspase-3 activity; however, both E_2 and E_2-BSA (Figure A4.5B) reduced the effect of CTX and taxol on caspase-3 activity, indicating that the anti-apoptotic effect of E_2 may require membrane activation of Gαs.

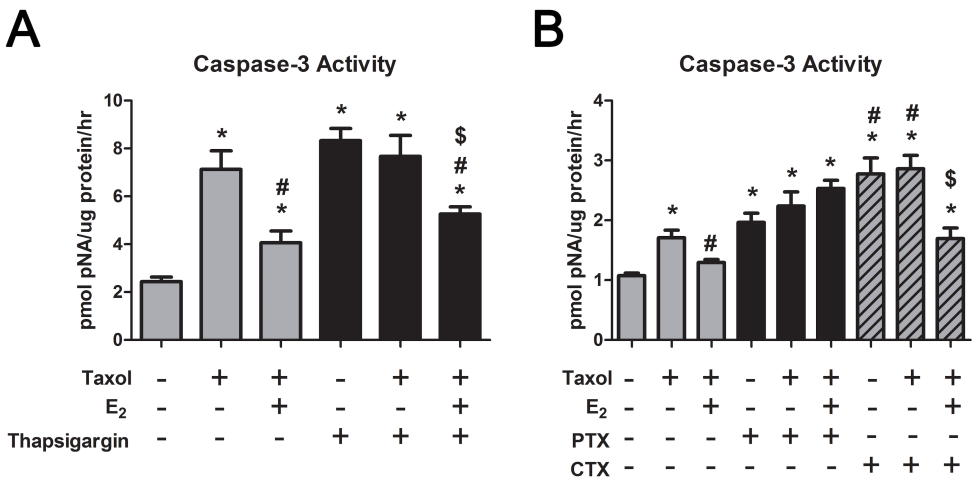

Figure 4.7: Role of Ca^{++} and G-protein Signaling in Apoptotic Signaling of E_2

(A) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while thapsigargin enhances caspase-3 activity alone and this is also reduced by E_2. (B) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while pertussis toxin and cholera toxin both enhance caspase-3 activity alone. The effect of CTX is reduced by E_2. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone.

4.3.9 Role of Palmitoylation

HCC38 cells treated with 10^{-8}M E_2 for 9 minutes exhibited increased plasma membrane localization of ERα36, as determined by densitometry analysis of western blots (Figure A4.6A), while cells pre-treated for 2 hours with tunicamycin (Tm) or 2-bromopalmitate (2-BP) did not (Figure 4.8A). Cycloheximide (CHM) had no effect on translocation of ERα36 to the membrane in response to E_2 (data not shown), nor did it alter E_2-dependent increases in PKC (Figure 4.8B) or PLD (Figure 4.8C). 2-hydroxymyristic acid (HMA) reduced translocation of ERα36 (Figure 4.8A), but did not alter E_2-dependent PKC or PLD. In contrast, Tm and 2-BP blocked ERα36 translocation and the stimulatory effects of E_2 on PKC and PLD indicating that palmitoylation played a role in these effects. In addition, palmitoylation was required for the anti-apoptotic effect of E_2. Tm blocked the effect of E_2 on taxol-induced caspase-3 activity (Figure 4.8D) as did 2-BP (Figure 4.8E). Similar effects were observed in cells treated with E_2-BSA (Figure A4.6B) indicating that a plasma membrane receptor was involved.

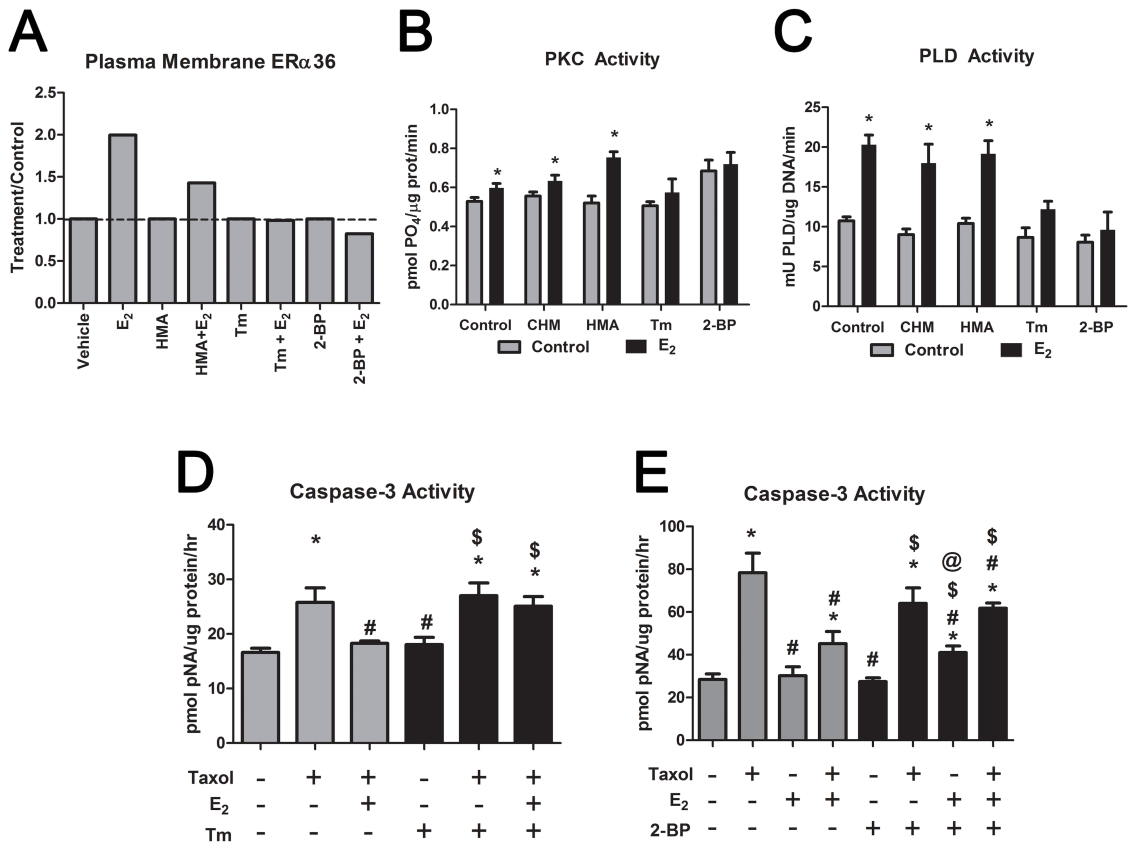

Figure 4.8: Role of Palmitoylation in the Anti-apoptotic Effect of E_2

(A) Western blot of plasma membrane fractions of HCC38 cells treated with 10^{-8}M E_2 for 9 minutes, pre-treated with 0.5mM HMA, 30μM Tm, or 10μM 2-BP for 2 hours indicates that ERα36 membrane-association occurs rapidly and is blocked by Tm and 2-BP. (B,C) Tm and 2-BP prevent the effect of 10^{-8}M E_2 on (B) PKC activation after 9 minutes and (C) PLD activation after 30 minutes. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to corresponding untreated control. (D,E) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while (D) Tm (30μM) and (E) 2-BP (10μM) block this effect. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor (Tm or 2-BP) alone. @ represents $p<0.05$ compared to taxol and 2-BP alone.

4.3.10 Proposed Mechanism by which E_2 Blocks Taxol-induced Apoptosis in ER-negative Breast Cancer

Based on the results of this study, along with results from our previous work regarding E_2's non-genomic, membrane-mediated effects in HCC38 cells, we have developed a working model for the mechanism by which E_2 promotes triple negative breast cancer cell survival, specifically through proliferative and anti-apoptotic effects. Interaction of E_2 with ERα36 on the plasma membrane leads to a signaling cascade that begins with G-protein activation, specifically Gαs, leading to activation of phosphatidylcholine-specific PLD (Figure 4.9). PLD then converts phosphatidylcholine (PC) to phosphatidic acid (PA), which after conversion to lysophosphatidic acid (LPA) activates LPA receptors, which promote activation of PI3K. PI3K then promotes anti-apoptotic activity, possibly through an Akt-dependent mechanism, which can prevent activation of a caspase signaling cascade that ultimately leads to caspase-3 activation and apoptosis. As taxol promotes caspase-3 activation through a mechanism detailed by Levin et al. (69), which includes JNK phosphorylation and cytochrome C release from the mitochondria, the mechanism of E_2 inhibitory effect on caspase-3 activity counteracts the pro-apoptotic effect of taxol. At the same time, the activation of signaling through ERα36 can also promote PKC activation through PLC, diacylglycerol (DAG), and inositol trisphosphate (IP3). PLD can also further activate PKC, indicating cross talk between diverging pathways that are simultaneously activated by E_2 at the plasma membrane. Overall, in the context of cancer cell physiology, we conclude that E_2-membrane activation of ERα36 promotes proliferation, while simultaneously activating an anti-apoptotic pathway.

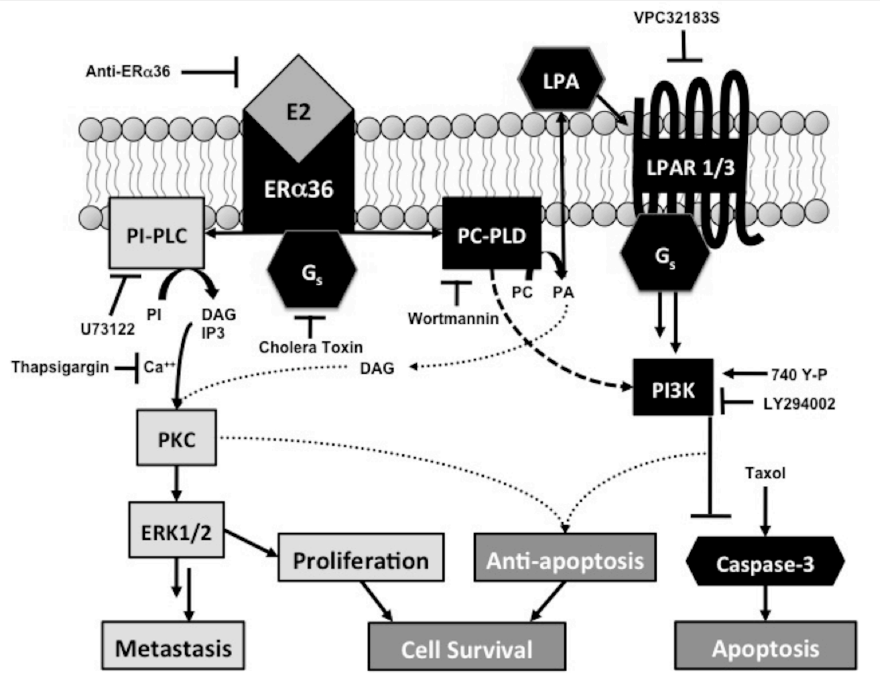

Figure 4.9: Proposed Mechanism by which E_2 Promotes Cancer Cell Survival

E_2 signaling through membrane-associated ERα36 activates two pathways, mediating the proliferative effect through a G-protein-PLC-PKC specific pathway and the anti-apoptotic effect through PLD-LPA-PI3K specific pathway. Cross-talk can occur between these pathways as PLD can activate PKC via DAG and calcium-dependent PKC can also mediate anti-apoptosis.

4.4 DISCUSSION

Estrogen receptor (ER) status typically dictates classification, diagnosis, and treatment of breast cancer. ER-negative breast cancer, and particularly triple negative breast cancer (TNBC), must be treated with more rigorous means than ER-positive cancers, which traditionally respond more readily to anti-estrogens such as tamoxifen. Taxol is commonly used as an adjuvant chemotherapeutic drug in the treatment of several cancers including breast cancer (141-144). However, E_2 is known to abrogate the effect of taxol (69), thereby promoting cancer cell survival through an anti-apoptotic mechanism. Therefore, a better understanding of the mechanism by which E_2 promotes anti-apoptosis in breast cancer is necessary.

This study examined the mechanism by which E_2 functions to promote breast cancer cell survivability. Our approach used antibodies, inhibitors, and activators of hypothesized proteins involved in the pathway. We used caspase-3 activity as our endpoint measurement, as we showed that caspase-3 is a downstream regulator of taxol-induced apoptosis and we previously showed that membrane-associated E_2 signaling abrogates the effect of taxol on caspase-3 activity (54). Taxol caused apoptosis in HCC38 cells in a similar manner to that seen in ER-positive MCF7 and ZR-75-1 breast cancer cells through activation of Bcl2 associated proteins, cytochrome-C translocation, and caspase-3 activation (69). As we previously showed that membrane-associated E_2 signaling abrogated the effect of taxol in HCC38 cells (54), we wanted to elucidate the mechanism by which this occurs.

HCC38 cells served as an in vitro model for TNBC. This cell line does not contain progesterone receptor (PR), Her2 receptor, or traditional ER. However, we have previously shown that HCC38 cells do express ERα36, an alternatively spliced variant of the traditional ERα (54). ERα36 was responsible for the membrane-mediated effect of E_2 that promotes rapid activation of PKC and proliferation. Membrane-associated E_2 signaling promoted anti-apoptosis against taxol-induced caspase-3 activity. However, at the time, we did not implicate ERα36 as the receptor responsible for this, although the results did suggest that ERα36 could be responsible for the activation of multiple membrane-associated pathways that promote cancer cell aggressiveness. The ability of antibodies against ERα36 and ERα36 silencing to prevent the anti-apoptotic effect of E_2 against taxol in this study proves that the anti-apoptotic effect is mediated specifically through membrane-associated ERα36.

In this study, we have shown that ERα36 does in fact mediate the membrane-associated anti-apoptotic effect of E_2. Not only did our studies using antibodies against

ERα36 implicated it as the membrane receptor responsible for these effects, but membrane association is required for these effects, as is seen with the results using inhibitors to palmitoylation. Because we have determined that the anti-apoptotic effect of E_2 is through a non-genomic, membrane-mediated mechanism that begins with E_2/ERα36 interaction at the plasma membrane, we hypothesized that dynamic palmitoylation by palmitoylacetyltransferase (PAT), mediates the membrane effect of E_2 through ERα36 against taxol-induced caspase-3 activity. As we expected, due to several previous studies indicating membrane association of ERs, particularly ERα66 and ERα46 occurs due to palmitoylation of these receptors (39, 139), the membrane effect of E_2 through ERα36 is mediated via palmitoylation and not myristolation. The use of 2-hydroxymyristic acid, which prevents myristolation of proteins, did not abolish membrane association of ERα36. Our work using cycloheximide, HMA, tunicamycin, and 2-bromohexadecanoic acid, indicates that membrane association of ERα36 can occur rapidly within ten minutes of treatment with E_2, suggesting that E_2 itself promotes rapid association of ERα36 with the plasma membrane via palmitoylation. Tunicamycin, a known inhibitor of palmitoylacetyltransferase, completely blocked the E_2-induced membrane association of ERα36. However, tunicamycin is also known to be a potent inhibitor of N-glycosylation (39), indicating that it is not the most ideal inhibitory candidate for studying palmitoylation. The inability of cycloheximide to block the E_2-induced plasma membrane association of ERα36, cycloheximide a suitable control for this undesired effect of tunicamycin, as cycloheximide also inhibits N-glycosylation of proteins.

To further support the role of palmitoylation of ERα36 in these anti-apoptotic membrane-associated effects of E_2, we used 2-bromohexadecanoic acid, or 2-bromopalmitate (2-BP) to specifically inhibit palmitoylacetyltransferase activity (140),

thereby preventing dynamic palmitoylation of ERα36. The effects of 2-BP were similar to those seen with tunicamycin, and with both inhibitors, we observed a reduction in rapid membrane association of ERα36, an inhibitory effect on E_2-induced PKC and PLD activation, and complete abolishment of the anti-apoptotic effect of E_2. Because 2-BP is a more specific inhibitor of PATs than tunicamycin, we concluded that the anti-apoptotic effect of E_2 occurs with rapid and dynamic palmitoylation of ERα36 leading to membrane association of the receptor within minutes.

In addition, ERα36 contains a unique C-terminal exon, exon 9, which codes for 27 amino acids of unknown function. Wang et al. hypothesized that this exon contains a potential myristolation sequence (55). Therefore, we created ERα36 deletion mutants to determine if the anti-apoptotic membrane effect of E_2 is dependent on the presence of exon 9. HEK293 cells were used a model for overexpression of wildtype exogenous ERα36 and exon 9-deleted ERα36 expression plasmids. Overexpression of ERα36 lacking exon 9 suggested that this exon is required for the anti-apoptotic membrane effect of E_2. We observed the requirement for exon 9 in the effect of E_2 on PLD activity, but the role of exon 9 in the effect of E_2 against taxol-induced caspase-3 was less clear. Because we observed an increase in caspase-3 activity when we removed exon 9 without any other treatment, it is possible that the removal of exon 9 alters the overall effect of ligand-independent ERα36. Because our results indicate palmitoylation is the mechanism by which ERα36 translocates dynamically to the plasma membrane, and HMA did not prevent the effect of E_2, further studies are necessary to determine if exon 9 is a target for palmitoylation of ERα36.

Previous results were not clear as to how E_2 promotes anti-apoptosis (69). The vitamin-D3 metabolite, 24R,25-dihydroxyvitamin-D3, another steroid hormone which activates PKC through a similar mechanism as E_2, has anti-apoptotic effects by

activating phospholipase D in chondrocytes (68). Based on this, we investigated whether E_2 can rapidly activate PLD in breast cancer cells and found that E_2 activated PC-PLD within 30 minutes, which occurred specifically through membrane-associated ERα36. Moreover, the ability of wortmannin to block the effect of E_2 on taxol-induced caspase-3 activity indicates that E_2-specific PLD activity mediates the anti-apoptotic effect of E_2 in breast cancer, and in this case, particularly in triple negative breast cancer cells.

While PC-PLD was shown to play a role in the anti-apoptotic effect of E_2, neither PI-PLC, nor PC-PLC appeared to be involved. Neither U73122 nor D609 abrogated the effect of E_2; however, they both appeared to block the effect of taxol on caspase-3 activity, suggesting a possible role of PLC in the mechanism by which taxol induces apoptosis. As Levin et al. showed that taxol induces apoptosis through a JNK-dependent mechanism (145), it is quite possible that JNK is activated through a pathway that requires PLC. Other studies show JNK activation can occur through a PKC-associated pathway. This may explain the effects seen on taxol-induced caspase-3 activity in the presence of D609 and U73122, which inhibit PC-PLC and PI-PLC respectively.

The PI3K/Akt signaling pathway has been shown in several studies to be intimately associated with apoptotic and anti-apoptotic signaling in several cell types (146-149). Due to its well-known role in anti-apoptotic pathways, we hypothesized that PI3K is part of the pathway by which E_2 exerts its anti-apoptotic effect. Although wortmannin is also known to inhibit PI3K, the concentration used in our study (10μM), can potently inhibit PLD, without measurable effects on PI3K activity (137). Not only did LY294002, a specific inhibitor of PI3K, block E_2's anti-apoptotic effect against taxol, but also the PI3K activator, 740 Y-P, alone prevented taxol's apoptotic effect in a similar manner to E_2. Similarly, the addition of LPA, which is also known to regulate anti-apoptosis through a PI3K-Akt-dependent mechanism (68), prevented the effect of taxol

on cancer cell apoptosis. However, the use of the LPA signaling inhibitor, VPC32183S, which inhibits signaling of LPA via the LPA1 and LPA3 receptors (150), appeared to not only block the E_2 anti-apoptotic effect, but also appeared to block taxol's effect as well. Because this inhibitor is not specific to one isoform of the LPA receptor, it may have multiple competing effects on LPA signaling. These effects on taxol-induced apoptosis suggests that LPA signaling is a promiscuous process that may have differential effects on apoptotic signaling pathways, and although further investigation into the role of these receptors is necessary to achieve a definitive conclusion on which specific receptor is involved in the anti-apoptotic effect of E_2, it may be that LPA signaling, while it appears to be a component of the anti-apoptotic effect of E_2, may also function in the pathway by which taxol induces apoptosis.

We originally hypothesized that G-protein and intracellular calcium signaling may also play a role in the anti-apoptotic effect of E_2 against taxol-induced apoptosis. We used thapsigargin, an inhibitor of endoplasmic reticulum-associated calcium channels to block the influx of calcium to the cytosol. Interestingly, we observed a marked increase in caspase-3 activity when HCC38 cells were treated with thapsigargin alone. Although this effect was reduced by the use of E_2, it was not clear whether calcium involvement in this anti-apoptotic effect of E_2 was specifically through the pathway that includes PLD. Interestingly, as we have shown that PKC activity is important for the maintenance of cancer cell survival (54), these results suggest that PLC-specific PKC activation, which depends on calcium efflux from the endoplasmic reticulum, may not only promote cancer cell proliferation, but may also crosstalk with the anti-apoptotic pathway to block caspase-3 activation. Similarly, we also observed that pertussis toxin and cholera toxin, inhibitors of G-protein signaling through inhibition of Gαi and Gαs respectively, also showed marked increases in caspase-3 activity when used alone. Although we cannot

come to solid conclusions on the role of these G-protein subunits due to these results alone, it is interesting to note that the rapid activation of PKC in HCC38 breast cancer cells occurs via Gαs activation. We can also suggest that these effects of pertussis toxin and cholera toxin may be due to attenuation of LPA receptor signaling, which depends on G-protein function. It is important to note that the effect of pertussis toxin on caspase-3 activity was not abrogated by E_2. However, the effect of cholera toxin, which specifically inhibits Gαs signaling, was reduced with the addition of E_2, and this result is consistent with the idea that PKC activation by E_2 occurs by Gαs activation (57) and is anti-apoptotic. Although further investigation into the role of Gαs signaling in this pathway is necessary, the results suggest a membrane-delimited role of ERα36 in the anti-apoptotic pathway of E_2. Either ERα36 directly activates Gαs, or another player may be involved.

This study provides in-depth knowledge into the mechanism of membrane-associated E_2 signaling in triple negative HCC38 breast cancer cells producing a working model for E_2 signaling through ERα36. The use of antibodies against ERα36, ERα36 silencing, analysis of palmitoylation, and results with E_2-BSA, which cannot cross the plasma membrane, prove that these effects of E_2 are initiated at the plasma membrane. We previously showed that rapid activation of PKC is mediated by ERα36 at the plasma membrane and this effect leads to cancer cell proliferation. This study showed that the activation of ERα36 by E_2 could also lead to activation of another pathway of anti-apoptosis, which involves PC-PLD, LPA, and PI3K. Crosstalk can occur between these pathways due to the ability of PLD to activate PKC through a DAG-dependent mechanism, as well as PKC's ability to promote anti-apoptosis as well. The elucidation of this signaling pathway permits a greater understanding of how E_2 functions in breast cancer cells, particularly triple negative breast cancer cells, to block the effects of

chemotherapeutic drugs that target cancer cells for apoptosis. The role of ERα36 in this pathway, and the fact that we have shown that ERα36 mediates this effect from the plasma membrane, suggests that ERα36 may be a suitable target for diagnosis and treatment of breast cancer. Perhaps production of ERα36 specific monoclonal antibodies would provide another avenue to more robustly target and kill breast cancer cells overexpressing the receptor. In theory, targeting of ERα36 would prevent the anti-apoptotic effect of E_2 and thus provide a novel approach to personalized, adjuvant therapy against breast cancer.

FINANCIAL DISCLOSURE

This work was supported in part by the Price Gilbert, Jr. Chair. It was also supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number UL1TR000454 and TL1TR000456. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CHAPTER 5: 17beta-estradiol Promotes Aggressive Laryngeal Cancer through Membrane-Associated Estrogen Receptor-Alpha 36

[Chapter 5 has been prepared for manuscript submission as: Chaudhri RA, Schwartz N, Hadadi A, Schwartz Z, Boyan BD. Estradiol Enhances Proliferation and Anti-apoptosis Through Membrane-associated ERα36 in Laryngeal Cancer Cells. 2013.]

17β-estradiol (E_2) plays a key role in tumorigenesis by enhancing cell survivability and metastasis through its cytoplasmic receptors. Recently, a variant of estrogen receptor alpha, ERα36 has been implicated as a substantial mediator of E_2's proliferative and anti-apoptotic effects through rapid membrane-associated signaling, and cancers previously regarded as hormone-independent due to the absence of traditional receptors, may in fact be susceptible to E_2. Despite rising from a secondary sex organ and having a clear gender disposition, laryngeal cancer is not uniformly accepted as hormone-dependent, even in the face of compelling evidence of E_2 responsiveness. The aim of this study was to further elucidate the role of E_2 in the tumorigenesis of laryngeal cancer, both in vitro and in vivo. ERα36 presence was evaluated in membranes of the laryngeal carcinoma cell line, Hep2, as well as in laryngeal tumor samples. In vitro, ERα36 was found to mediate rapid activation of protein kinase C and phospholipase D by E_2, leading to increased proliferation and protection against chemotherapy-induced apoptosis. Furthermore, in response to E_2 activation of ERα36, an upregulation of angiogenic and metastatic factors was observed. Clinical analysis of laryngeal tumors revealed a similar association between the amount of ERα36 and VEGF, and indicated a role in lymph node metastasis. These findings present compelling evidence of ERα36-dependent E_2 signaling in laryngeal cancer. Thus, targeting ERα36 may reduce the deleterious effects of E_2 in laryngeal cancer,

ultimately suggesting the importance of anti-estrogen therapy or the production of novel drugs that specifically target ERα36.

5.1 INTRODUCTION

The mechanisms responsible for tumor growth and metastasis are complex and are only partially understood. One of the factors contributing to this process that has major clinical implications for both prognosis and treatment is the presence of hormone and growth factor receptors in cancer cells. 17β-estradiol (E_2) traditionally acts as a sex hormone, involved in sexual development and the reproductive systems of males and females, but it has also many other effects both in non-reproductive tissues such as bone and in cancerous tissue. The importance of E_2 in the pathogenesis of breast and prostate cancer is well established (70, 151). E_2 is known to enhance cell proliferation, survivability, and metastasis (152-154). This deleterious effect of E_2 is in fact the target of one of the main treatments in breast cancer, the anti-estrogen tamoxifen.

Classically, the mechanism of E_2 action is mediated through interaction with its cytoplasmic receptors, ERα and ERβ, which in turn act in the nucleus as transcription factors (155). There is increasing evidence that E_2 acts via plasma membrane receptor(s), which rapidly activate signal transduction pathways, thus enhancing proliferation and attenuating apoptosis (31, 81). This membrane-mediated mechanism does not necessitate new gene expression or protein synthesis and is thought to be responsible for the E_2 activation of protein kinase C (PKC) (57).

One of the key candidates for the membrane mediated action of E_2 is a novel ERα variant, with a molecular mass of ~36kDa (ERα36). It differs from the classical ERα66 by lacking both transcriptional activation domains (AF1/AF2), but retains the

DNA-binding domain as well as partial dimerization and an intact ligand-binding domain (53, 55). It also contains a novel C-terminal exon that encodes 27 amino acids of unknown function. We have previously shown that this alternative ER resides in the plasma membranes of breast cancer cells and mediates E_2's proliferative and anti-apoptotic effects (54).

Epidemiological studies have implied that sex hormones may be involved in the tumorigenesis of laryngeal carcinomas, due to the vast differences in gender susceptibility, with a male: female ratio of 11:1 (156). The larynx is a secondary sex organ, which undergoes trophic changes in response to hormonal changes during puberty, and morphostructural changes during adulthood. Nonetheless, while other tumors of secondary sex organs have been accepted as hormone dependent cancers, laryngeal carcinoma is still a subject of controversy, and as such, its treatment is not oriented to counteract the hormonal effect.

The presence of classical cytosolic ERs is controversial in laryngeal cancer (157, 158). Despite this, laryngeal cancer cells display E_2 responsiveness, as E_2 was found to increase proliferation of laryngeal cancer cells (159), and a non-genomic mechanism was implicated (160). Furthermore, the effects of E_2 were inhibited by tamoxifen (161). These findings advocate a role for E_2 in the tumorigenesis of laryngeal cancer and warrants further exploration of its effects.

In order to further improve the understanding of the role of E_2 in the tumorigenesis and metastasis of laryngeal cancer, we examined the underlying mechanism of E_2's effect in vitro using laryngeal cancer cell lines as the model. We hypothesized that the effect exerted by E_2 on laryngeal cancer is similar to that found in breast cancer and that ERα36-associated E_2 membrane signaling mediated by PKC is

involved in promoting proliferation, protecting against apoptosis and stimulating gene expression resulting in enhanced aggressiveness and metastasis of laryngeal cancer. In addition, we used immunohistochemistry to assess expression of ERa36 and vascular endothelial growth factor (VEGF) in histological samples from human laryngeal tumors in order to help clarify the role of E_2 *in vivo*.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

The Hep2 laryngeal carcinoma, TT thyroid carcinoma, HeLa cervical carcinoma, and HCC38 breast cancer cell lines were validated and obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Minimum essential medium (MEM) and Roswell Park Memorial Institute (RPMI) 1640, both lacking phenol red were purchased from Invitrogen (Grand Island, NY, USA) and F12K nutrient mixture was purchased from Cellgro (Manassas, VA, USA). Charcoal/dextran-filtered fetal bovine serum (FBS) was purchased from Gemini Bioproducts (Sacramento, CA, USA). The estradiol enantiomer ent-17β-estradiol (Ent-E_2) was kindly provided as a gift from Douglas Covey (Washington University, St. Louis, MO, USA) (125). E_2, E_2 conjugated to bovine serum albumin (E_2-BSA), and taxol were purchased from Sigma (St. Louis, MO, USA). The PLD inhibitor wortmannin was purchased from EMD chemicals (Billerica, MA, USA). Protein content of samples was measured using the Macro BCA reagent kit from Pierce/Thermo Scientific (Rockford, IL, USA). Polyclonal ERα66 antibodies were purchased from Chi Scientific (Maynard, MA, USA), ERα36 antibodies were purchased from Cell Applications Inc. (San Diego, CA, USA), GAPDH antibodies were purchased from EMD-Millipore (Billerica, MA, USA), and caveolin-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). VEGF antibodies were purchased

from Abcam (Cambridge, MA, USA). The PKC assay kit was purchased from GE Lifesciences (Pittsburgh, PA, USA). The Amplex Red Phospholipase D Assay kit was purchased from Life Technologies (Grand Island, NY, USA). The Titertacs TUNEL assay kit was purchased from R&D Systems (Minneapolis, MN, USA). The CaspAce assay kit was purchased from Promega (Madison, WI, USA). All primers were purchased from Eurofins (Huntsville, AL, USA). The IRB-approved larynx tissue microarray was purchased from Imgenex (San Diego, CA, USA).

5.2.2 Cell Culture

Hep2 and HeLa cells were cultured in MEM-based media, as specified by the ATCC, containing 10% charcoal/dextran-filtered FBS and lacking phenol red, which can mimic the effects of E_2 at low levels. HCC38 cells were maintained in RPMI 1640-based media, while TT cells were cultured in F12K media.

5.2.3 Presence of ER isoforms

In order to determine whether laryngeal cancer cells express ERα36, sequence-specific primers, designed in our lab previously (54), were used to selectively identify the traditional ERα66 and the alternatively spliced variant ERα36, as well as the E_2 receptor, GPR30. RNA was extracted from Hep2, HeLa, HCC38, and TT thyroid medullary carcinoma cell lines cells using the TRIzol method. Reverse transcription was performed to produce cDNA for ERs from the laryngeal cell line. PCR was then performed to determine whether mRNAs for the receptors were expressed. Western blots of isolated membrane fractions were used to identify the subcellular localization of ERα66 and ERα36 in the cell lines.

5.2.4 PKC and PLD activity

Subconfluent cultures of Hep2 cells were treated with E_2, Ent-E_2, and E_2-BSA at the indicated time points and at a concentration of 10^{-8}M unless otherwise specified. Ent-E_2 was used in order to determine if E_2 response relies on a stereospecific receptor response. The use of E_2-BSA, which cannot pass through the plasma membrane and reach the nuclear receptor (49, 132, 133), enables confirmation that the effects elicited by E_2 are membrane mediated. The PLD inhibitor, wortmannin, was used at a concentration of 10^{-5}M. ERα36 antibodies were used to block the effect of E_2, in order to determine the role of ERα36 in the E_2 activation of PKC and PLD. Wortmannin and ERα36 antibodies were used 15 minutes prior to E_2 treatment. Cells were washed twice with phosphate buffer saline (PBS) and lysed in RIPA buffer at time of harvest. PKC activity was measured using the BioTrak PKC assay kit from GE Lifesciences. PLD activity was measured using the Amplex Red PLD assay from Life Technologies and normalized using the PicoGreen DNA quantification assay. ERα36 antibodies were used to block the effect of E_2, in order to determine the role of ERα36 in the E_2 activation of the PLD signal cascade.

5.2.5 Cell proliferation, viability and apoptosis

In order to establish whether signaling via ERα36 can protect against apoptosis, the Hep2 cell line was treated with taxol, a known chemotherapeutic agent that induces cell death, as previously demonstrated in breast cell lines (54). Subconfluent cultures were pretreated with E_2 for 90 min followed by taxol treatment for 4 h, after which cells were assayed for caspase-3 activity, using the CaspAce assay kit from Promega (Madison, WI) and DNA fragmentation via TUNEL. Furthermore, cell proliferation was assessed as a function of DNA synthesis, using the Click-IT Edu assay from Invitrogen

(Grand Island, NY). ERα36 antibodies were used to block the effect of E_2, thus verifying the role of ERα36 in the E_2 signal cascade.

5.2.6 Expression of angiogenic and metastatic factors

Quantitative RT-PCR was used to determine the effect of E_2 on expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2), which have been implicated in pathological angiogenesis associated with tumors (162-164). Snail1 and e-cadherin (CDH1), which are metastatic factors leading to epithelial to mesenchymal transitions (110) and the metastatic factor CXC chemokine receptor type 4 (CXCR4) (165), were also measured in the Hep2 cell line. RT-PCR was performed with Applied Biosystems Fast SYBR mastermix in the Applied Biosystems StepOne Realtime system.

5.2.7 Immunohistochemistry

A laryngeal cancer tissue microarray containing 39 histological slices of laryngeal cancer tissue was obtained from Imgenex Corp. Clinical information for the 39 patients with laryngeal carcinoma is presented in Table 5.1. Prior to immunohistochemistry, slides were placed in a 60°C oven for 30 minutes, deparaffinized, and rehydrated, and antigen presentation was achieved by heating in a pressure cooker for 3 minutes in 0.1M sodium citrate buffer. ERα36 and VEGF presence was examined by immunohistochemistry using the LSAB immunohistological staining kit from Santa Cruz Biotechnology. Slides were counterstained with haematoxylin.

Table 5.1: Larynx TMA characteristics.

A total of 39 laryngeal cancer subject tissues were examined by immunohistochemistry and blindly scored by 3 independent observers. The characteristics in the table above describe the age, sex, tissue type, TNM classification, and staging characteristics for these 39 subjects.

Table 5.1: Laryngeal Cancer Patient Characteristics (N=39)					
Age					
Mean(SD)	60(8.68)				
> 50	36				
≤ 50	3				
Sex					
Male	38				
Female	1				
Tissue Type					
Supraglottic	16				
Glottic	7				
Transglottic	6				
Pyriform Sinus	5				
Subglottic	5				
TNM Classification	**0**	**1**	**2**	**3**	**4**
Tumor Size	0	1	1	16	21
Lymph Node Metastasis	16	4	17	1	N/A
Metastasis	38	1	N/A	N/A	N/A
Stage	**I**	**II**	**III**	**IV(A, B, C)**	
	1	1	12	25 (23, 1, 1)	

5.2.8 Imaging and scoring

Slides were imaged using a Leica DMLB microscope system with a 63X objective oil immersion lens. Images were taken with ImagePro for all samples and blindly scored

by three independent observers according to the scoring method listed in Table 5.2. ERα36 presence in all tissues was scored according to two classifications: ERα36 number describes the number of cells in the tissue that are positive for ERα36; ERα36 intensity describes the intensity of the staining, which describes cells that express either low amounts of ERα36 or high amounts of ERα36. VEGF was scored in a similar manner to ERα36 for VEGF number and VEGF intensity.

Table 5.2: Histology Scoring Method

All samples were scored blindly by three independent observers. Scores were then compared and for samples that were not scored the same by all three observers, the two most common scores were used. If no scores matched, a fourth observer blindly determined the score.

Table 5.2: Immunohistological Scoring Method

	Score	Representation
ERα36 Number	0	<1% cells positive
	1	1<10% cells positive
	2	10<50% cells positive
	3	≥50% cells positive
ERα36 Location	0	Nuclear
	1	Non-nuclear
ERα36 Intensity	0	Low
	1	High
VEGF Number	0	<1% cells positive
	1	1<10% cells positive
	2	10<50% cells positive
	3	≥50% cells positive
VEGF Intensity	0	Low
	1	High

5.2.9 Statistical analyses

For all in vitro experiments, data is represented as the mean and standard error among 6 individual samples. Statistical analyses were performed by analysis of variance with Bonferroni's correction for Student's t-test at a significance level of $\alpha \leq 0.05$. Histological samples were scored blindly by three independent observers, and correlations of ERα36 number and intensity to clinicopathological variables were performed using Fisher's exact test.

5.3 RESULTS

RT-PCR results showed that Hep2 laryngeal carcinoma epithelial cells expressed the alternative splicing variant to the traditional ERα, ERα36 (Figure 1A). The cells also expressed mRNA for the classical ERα66, as well as the non-classical G-protein coupled estrogen receptor GPR30 (Figure 5.1A). The presence of ERα36 protein was confirmed by western blot analysis, which demonstrated that Hep2 cells contained protein for ERα36. While ERα36 was also identified in the TT thyroid cancer cell line, it was more pronounced in the Hep2 laryngeal cells (Figure 5.1B). Plasma membrane isolation of Hep2 cells and western blot analysis showed that ERα36 was present in Hep2 plasma membranes along with caveolin-1, a protein found in the caveolae, which are specifically invaginated compartments of the plasma membrane that are known to house many integral membrane receptors (Figure 5.1C); however, TT cells did not appear to have detectable levels of plasma membrane-associated ERα36, although caveolin-1 was found in this fraction (Figure 5.1D). HCC38 breast cancer cells and HeLa cervical cancer cells were used as positive controls for ERα36 presence in the plasma membrane (Figure 5.1C,D). This is in agreement with our previous results, which placed this receptor in the plasma membrane of different cell lines (54).

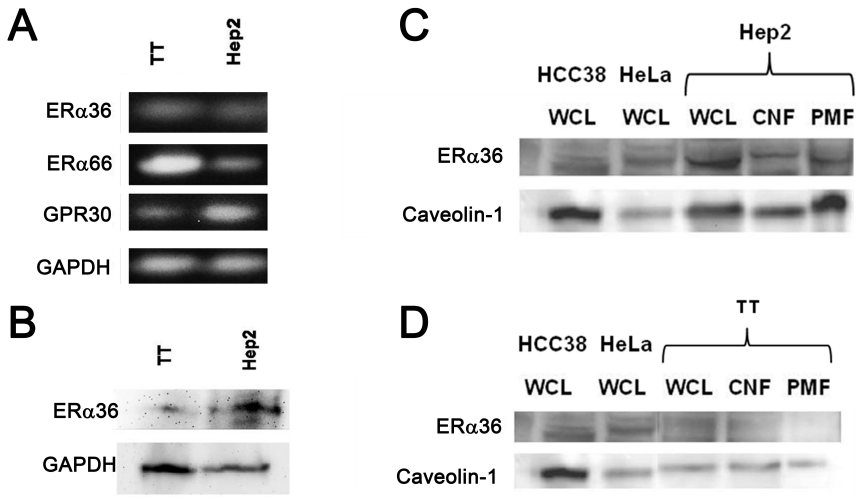

Figure 5.1: Presence of ERs in laryngeal and thyroid cell lines

RT-PCR shows that both the TT thyroid medullary and Hep2 laryngeal carcinoma cell lines express both the traditional ER, ERα66, and the novel variant, ERα36, as well as the non-traditional G-protein coupled ER, GPR30 (A). Western blots of whole cell lysates from both the TT and Hep2 cell lines show that they both contain protein for the novel receptor, ERα36, but it appears at least qualitatively, that the Hep2 cell line contains more (B). Western blots on plasma membrane fractions show the presence of ERα36 in the plasma membranes of Hep2 cells, but not in TT cells(C, D).

E_2 caused a significant increase in PKC activity after 30 minutes of treatment (Figure 5.2A). The effect of E_2 was biphasic, with a significant increase in PKC at 10^{-9}M and 10^{-8}M but with no effect at 10^{-7}M E_2 (Figure 5.2B). E_2's effect was abolished with the specific PLD inhibitor wortmannin (Figure 5.2C) or by antibodies specifically against ERα36 (Figure 5.2D). Furthermore, E_2 stimulated PLD activity. This effect appeared after 60 minutes (Figure 5.3A), between the concentrations $5X10^{-9}$-10^{-8}M E_2 (Figure 5.3B). The enantiomer of E_2 (Ent-E_2) had no effect on PLD activity (Figure 5.3C). In addition, E_2-BSA had a stimulatory effect at a concentration of 10^{-9}M on PLD activity in the Hep2 cells (Figure 5.3D). Using antibodies specifically against ERα36 to block the receptor prior to E_2 treatment abolished the effect of E_2 on PLD activity (Figure 5.3E).

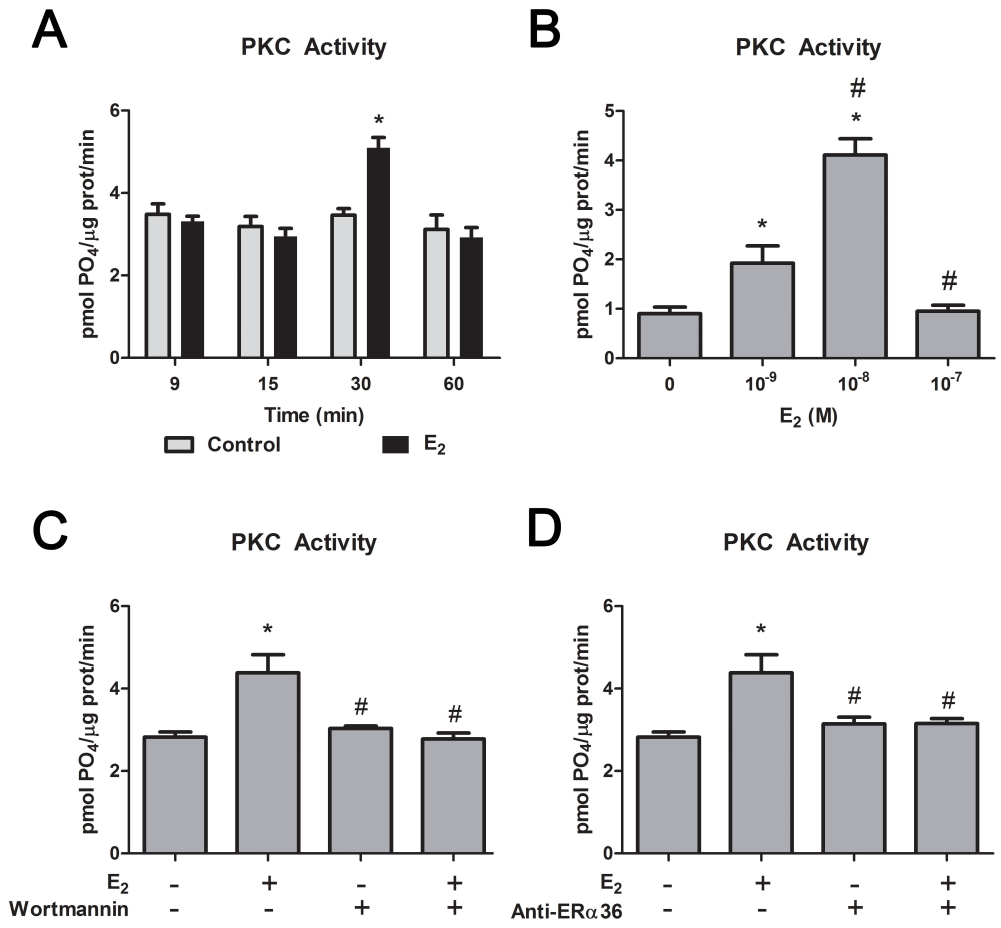

Figure 5.2: Estradiol effects on PKC activity in Hep2 laryngeal carcinoma cells

A time course evaluation of PKC activity in response to 10^{-8}M E_2 treatment of Hep2 cells shows that PKC activity is enhanced 30 minutes after treatment (A), while in response to different doses of E_2, a biphasic effect is observed (B). Pre-treatment for 15 minutes with 10^{-5}M wortmannin and 1:500 ERα36 antibody, prevent the effect of E_2 on PKC (Figures 2C and 2D, respectively). *p<0.05 vs. corresponding control; #p<0.05 compared to 10^{-9}M E_2.

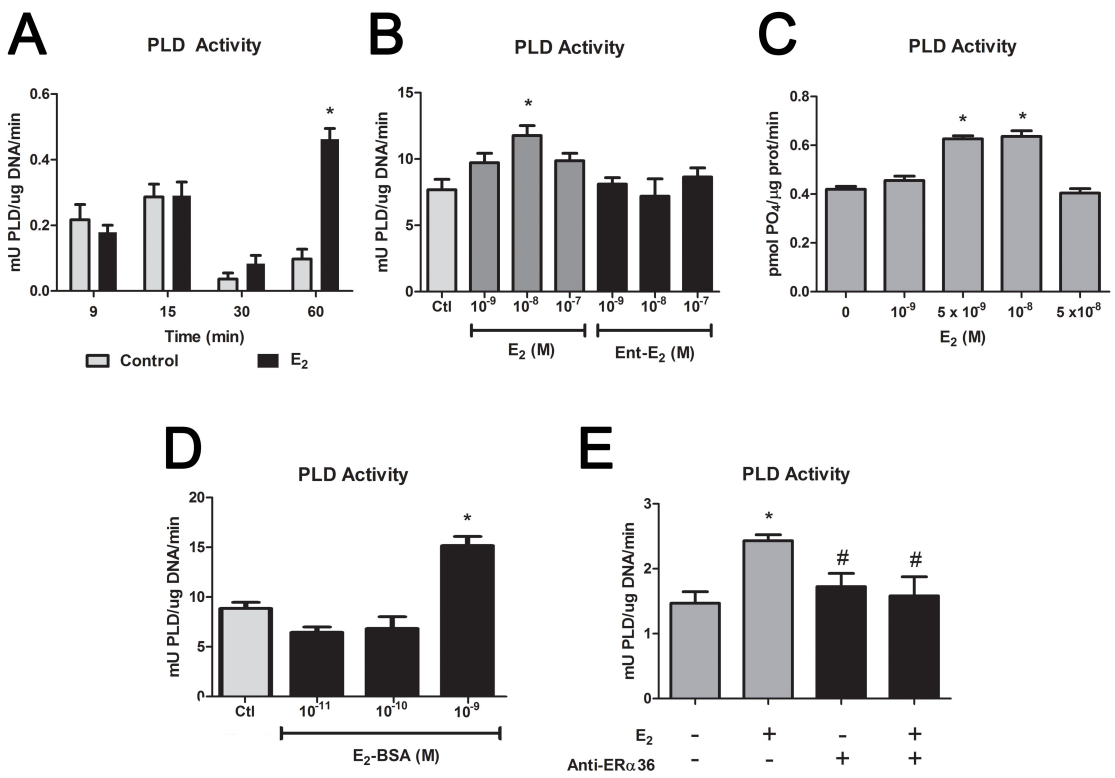

Figure 5.3: Estradiol effect on PLD activity in Hep2 laryngeal carcinoma cells

A time course evaluation of PLD activity in response to 10^{-8}M E_2 treatment of Hep2 cells shows that PLD activity is enhanced 60 minutes after treatment(A), while a biphasic effect is observed in response to different doses(B). When the enantiomer for E_2, Ent-E_2, is used to treat Hep2 cells, no increase in PLD activity is observed(C). When E_2-BSA is used to treat Hep2 cells, an increase in PLD activity is only observed with 10^{-9}M E_2-BSA(D). When Hep2 cells were pre-treated with antibody against ERα36, the effect of E_2 on PLD activity was abolished(E). *p<0.05 vs. corresponding control; #p<0.05 compared to E_2 only.

In order to determine the physiological effects of E_2 signaling mediated by ERα36, we further examined cell survivability and downstream gene expression related to angiogenesis and metastasis. Initially, taxol was used to induce apoptosis, in order to assess the anti-apoptotic effects of E_2 in cancer cells. The TUNEL assay, which measures DNA nicks associated with apoptosis, showed that taxol enhanced TUNEL, while pre-treatment with 10^{-8} and 10^{-7}M E_2 prevented this effect (Figure 5.4A). While

taxol additionally enhanced caspase-3 activity, an enzyme that plays a major role in apoptosis, 10^{-8}M E_2 also blocked this effect (Figure 5.4B). . E_2 also exhibited a proliferative effect in Hep2 cells, as evident from the enhanced DNA synthesis (Figure 5.4C). Pre-treatment of Hep2 cells with ERα36 antibodies (1:500 dilution) blocked these anti-apoptotic and proliferative effects of E_2 (Figure 5.4B,C).

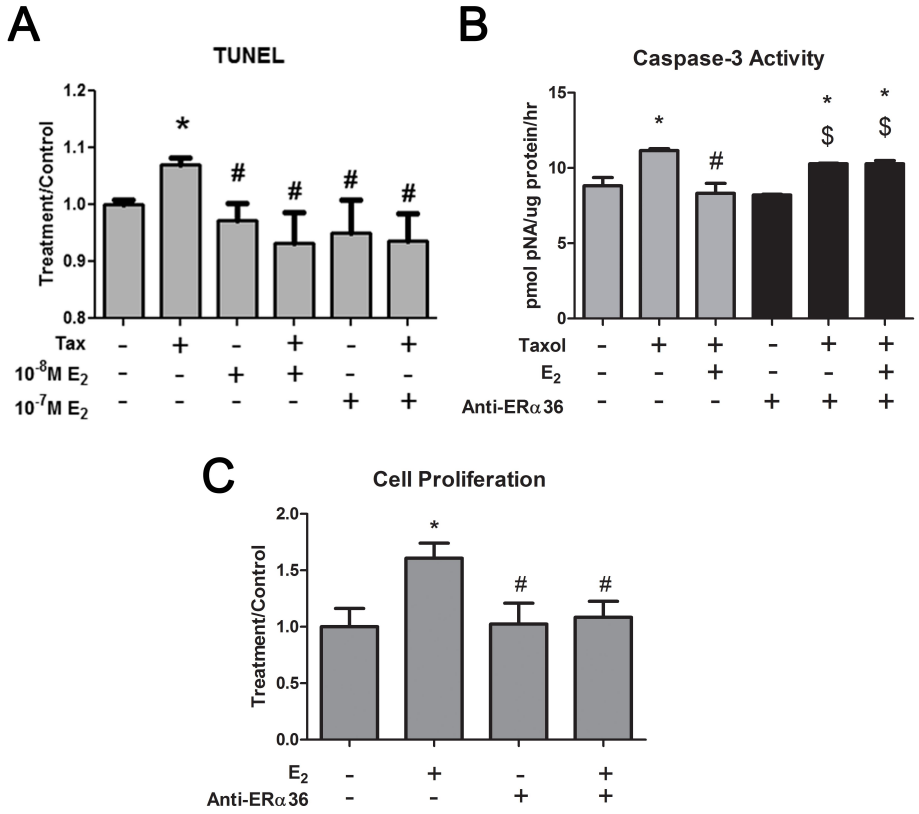

Figure 5.4: Role of E_2 in Hep2 laryngeal carcinoma cell survival

Taxol increased TUNEL and this was blocked by 10^{-8}-10^{-7}M E_2(A). 10^{-8}M E_2 also inhibited the ability of taxol to increase caspase-3 activity, while antibodies against ERα36 reduced this effect of E_2(B). 10^{-8}-10^{-7}M E_2 increased cell proliferation, but in the presence of ERα36 antibodies, this effect was not evident(C). *p<0.05 vs. corresponding control; #p<0.05 compared to taxol only; $p<0.05 compared to anti-ERα36 only.

Quantitative RT-PCR analysis showed that 10^{-8}M E_2 increased mRNA levels of the angiogenic factors *VEGF* (Figure 5.5A) and *FGF2* (Figure 5.5B), while ERα36 antibodies (1:500 dilution) blocked this effect. The migratory factor *CXCR4* (Figure 5.5C) showed a similar enhancement with E_2 treatment and this was again blocked with ERα36 antibodies. Furthermore, E_2 enhanced the metastatic factor *Snail1* (Figure 5.5D), while down-regulating the expression of e-cadherin (*CDH1*) (Figure 5.5E), and both of these effects by E_2 were blocked by ERα36 antibodies. mRNA levels were all normalized to *GAPDH* (Figure 5.5) or *RPS18* (Figure A5.1).

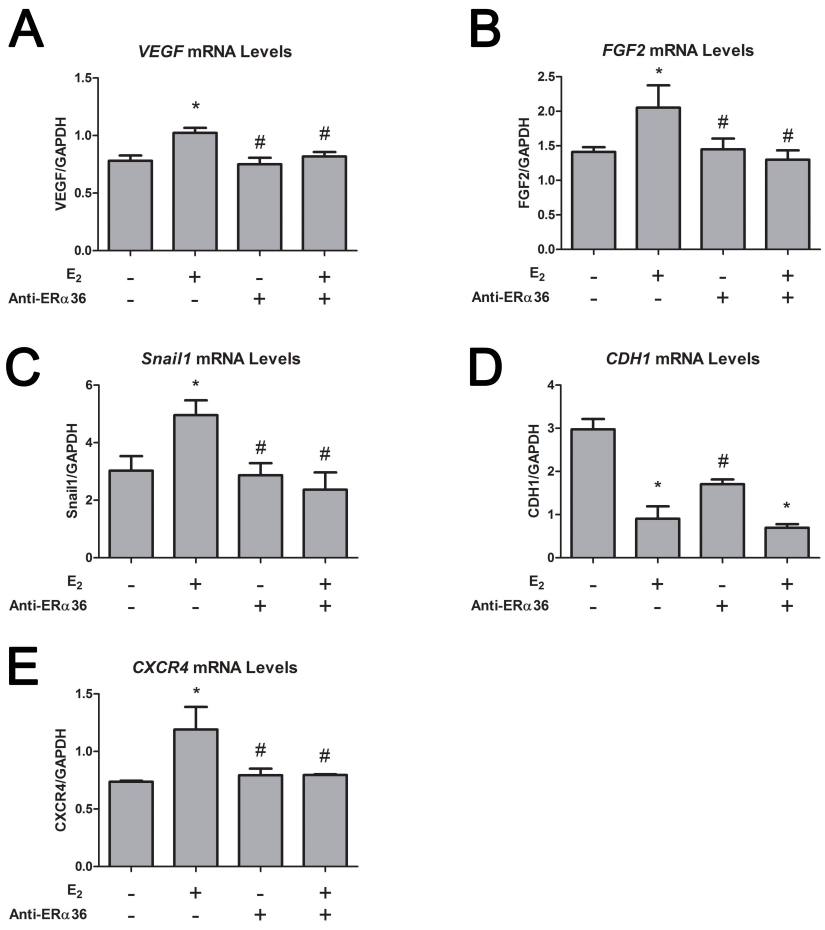

Figure 5.5: Role of ERα36 in E_2's effect angiogenic and metastatic factor expression

10^{-8}M E_2 increased mRNA levels of pro-angiogenic factors *VEGF*(A) and *FGF2*(B), and the metastatic factors *CXCR4*(C) and *Snail1*(D), while antibodies to ERα36 blocked this effect. The effects on *Snail1* were accompanied by a corresponding decrease in E-Cadherin (*CDH1*) mRNA levels that was also inhibited by ERα36 antibodies(E). All values were normalized to *GAPDH* mRNA levels. *p<0.05 vs. corresponding control; #p<0.05 vs. E_2 only.

The blinded analysis of laryngeal cancer immunohistochemical staining for ERα36 demonstrated that all laryngeal cancer patient tissues were positive for the presence of the membrane receptor. There was a variance in the distribution of the receptor and in the degree of positive staining for the receptor. Tissues containing less

ERα36 also appeared to contain less VEGF, and vice versa (Figure 5.6). Samples with relatively low ERα36 appeared to also contain less VEGF (Figure 5.6A). Conversely, samples that exhibited strong punctate staining of ERα36 also had punctate staining of VEGF, which appeared to occur around blood vessels (Figure 5.6B). While some samples with relatively high ERα36 exhibited moderate amounts of VEGF (Figure 5.6C), others appeared to stain strongly for VEGF (Figure 5.6D). Amongst the study sample, all samples exhibited positive staining for ERα36 and VEGF, and a correlation was found between the number of ERα36 receptors and the number of VEGF (p=0.0178) (Table 5.3). ERα36 number and intensity was further found to correlate with metastasis to regional lymph nodes (p=0.0263 and p=0.0119, respectively). However, we did not observe a correlation between ERα36 number and intensity with other variables such as age, tumor size, or VEGF intensity (Table 5.3).

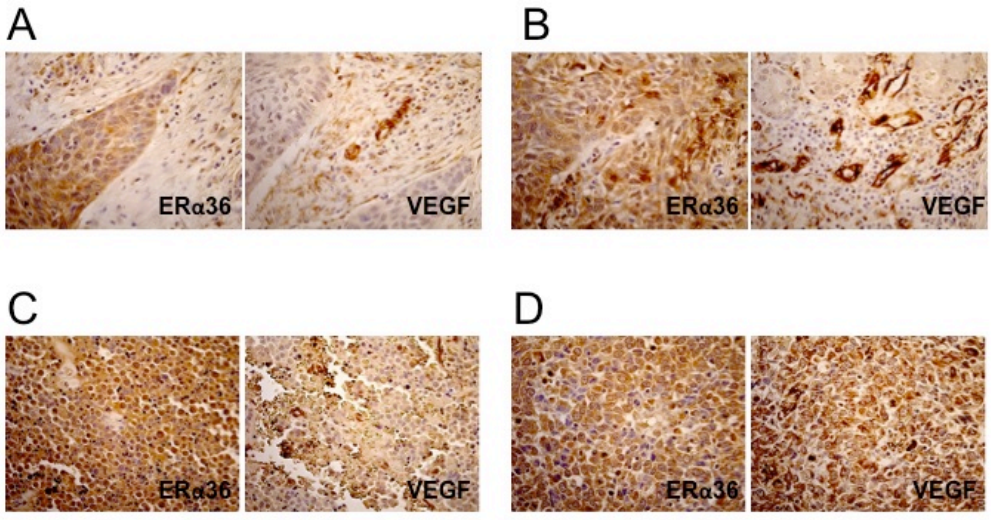

Figure 5.6: Larynx TMA ERα36 and VEGF Immunohistochemistry

Sample with low ERα36 and low VEGF levels(A). Samples with strong punctate staining of ERα36 accompanied by strong punctate staining of VEGF in blood vessels(B). Sample with high levels of ERα36, and moderate amounts of VEGF(C). Sample with strong ERα36 and VEGF staining(D).

Table 5.3: Larynx TMA statistics

Table 5.3: Larynx TMA Statistics			
Association of:	**With:**	**Significant?**	**P-value**
ERα36 Number	Age (≤50 vs. >50)	no	0.6057
	Tumor size by TNM staging	no	0.6057
	Metastasis to regional lymph nodes	**yes**	**0.0263**
	VEGF intensity	no	0.2156
	VEGF number	**yes**	**0.0178**
ERα36 Intensity	Age (<50 vs. >50)	no	0.4520
	Tumor size by TNM staging	no	0.3698
	Metastasis to regional lymph nodes	**yes**	**0.0119**
	VEGF number	no	0.7250
	VEGF intensity	no	0.2973

5.4 DISCUSSION

E_2 has been studied in multiple ER expressing cells as a potential factor that influences tumorigenesis. In breast cancer, the prototype of ER expressing cancer cells, ER-negative tumors have been found to respond to E_2 with increases in PKC activity, which correlates with enhanced tumorigenicity (54). The discovery of a novel ER variant, ERα36, opened the possibility that cancers previously labeled as non-hormone dependent and ER negative might in fact be susceptible to the effects of E_2 via this membrane receptor, as was demonstrated in ER negative breast cancers (54, 57). In breast cancer, ERα36 was found to be a key cellular and transcriptional regulator of proliferation and enhanced aggressiveness (54, 55), thus emphasizing the importance of characterizing further its presence and role in other cancers that are subject to the influences of sex hormones.

Previous work has suggested that laryngeal squamous cell carcinomas exhibit sex-hormone dependent behavior (158). Here we show that laryngeal carcinoma cell lines possess functional ERα36 and it is present in the plasma membranes. The existence of ERα36 in the plasma membrane was not uniform in all cancer cells, however, as demonstrated by the lack of ERa36 in TT thyroid cancer cells.

Previous studies in breast cancer demonstrated that ERα36 is localized specifically in the caveolae and is responsible for the E_2 activation of the PKC signaling cascade (54). This was also the case for Hep2 cells; ERa36 was localized with caveolin-1, indicating its present in caveolae. Moreover, E_2 stimulated PKC activity in Hep2 cells via a mechanism comparable to that seen in the breast cancer cells. E_2 caused an increase in PKC activity that was d36ependent on PLD, based on its inhibition by wortmannin. E_2 activated PLD via a receptor mediated mechanism; the enantiomer of E_2 had no effect and antibodies to ERa36 blocked E_2-dependent increases in PLD activity. It was also membrane-dependent as E_2-BSA was able to elicit the response. Furthermore, blocking ERα36 signaling with antibodies abolished the protective and proliferative effects of E_2, indicating that ERα36 has a role in tumorigenesis of laryngeal cancer and in the anti-apoptotic effect of E_2 against chemotherapeutics.

PKC has a key role in promoting tumorigenesis (166). Higher PKC activity correlates *in vivo* to enhanced tumor aggressiveness and progression (54, 103, 166), while *in vitro*, activation of the PKC signaling pathway enhances cell proliferation and survival via an anti-apoptotic mechanism (54, 57). Activation of PKC initiates a signaling cascade that results in activation of the ERK1/2 family of mitogen activated protein kinases (MAPK), providing an alternative method for steroid hormones to modulate gene expression rather than by traditional nuclear receptor mediated pathways (54).

PKC expression in laryngeal cancer was reported to be significantly higher than that found in normal laryngeal epithelium and adjacent non-cancerous laryngeal epithelium and was correlated with clinical stage and cervical lymph node metastasis (167). In our study, in response to E_2, Hep2 cells demonstrated an increase in PKC activity followed by an upregulation of angiogenic and metastatic factors. Clinical analysis of patients with laryngeal cancer revealed an association between the number and concentration of ERα36 and metastasis to regional lymph nodes. A similar relationship was observed in other cancer cells, such as breast (54) and gastric carcinoma (108). Previous studies found that E_2 activation of PKC mediates the expression of several metastatic factors such as *Snail1* and *Snail2*, which down-regulate the cell-cell interaction proteins such as cadherins, leading to what is known as an epithelial to mesenchymal transition (105), thus promoting tumor metastasis (54). Tamoxifen was found to exert its effect, among others, by inhibiting the PKC signal pathway (57, 168), thus opening the possibility that tamoxifen may be considered in the treatment of laryngeal cancer.

There are a number of alternative mechanisms by which PKC is activated and regulated via a membrane receptor, of which phospholipase A2, phospholipase C and phospholipase D have a prominent role. The mechanism by which this family of phospholipases cleaves different membrane phospholipids, such as phosphatidylcholine or phosphatidylinositol, to form diacylglycerol (DAG), thus activating PKC is well studied (68). However, there is increasing evidence that this mechanism isn't one directional, as in the case of PLD, in which PKC was found to be an upstream regulator of PLD (167). Moreover, PLD in itself has anti-apoptotic effects, through the production of lysophosphatidic acid, which in turn leads to decreased caspase-3 activity (68). Our findings support that in laryngeal cancer, E_2 exerts its effects through the PLD-PKC

pathway. Further research is needed in order to elucidate the mechanism by which this pathway renders laryngeal cancer cells sensitive to the effects of E_2.

These effects can explain the ability of E_2 to enhance tumor aggressiveness directly by allowing cells to proliferate and evade the effects of common chemotherapeutics. Not only can these cells proliferate and evade apoptosis in the presence of E_2, but E_2 may also lead to a more aggressive cancer by leading the primary tumor and metastasizing to other secondary sites in the patient. As tumors grow, cells within the tumor secrete VEGF, leading to vascularization of the tumor and providing a network through which these tumor cells can enter the vasculature and travel throughout the body. Our previous results showed that E_2 enhanced VEGF and FGF expression *in vitro* and the *in vivo* correlation between ERα36 and VEGF suggest that patients with high levels of ERα36 may have a more aggressive tumor phenotype as evidenced by the correlation to lymph node metastasis. These patients may benefit from treatments such as tamoxifen, which can block signaling of E_2 through ERα36, regardless of whether they contain traditional ERs.

As opposed to breast cancer in which the role of E_2 signaling has been studied mainly in female patients, in this study we observed that E_2 plays also a major role in development of cancer in male patients. Our findings suggest that male patients with laryngeal cancer, and possibly other cancers that are hormone-dependent, may benefit from ER-targeting treatments. Originally we sought to determine if the role of ERα36 in laryngeal cancer is sex-dependent, but were unable to do so due to the low frequency of laryngeal cancer in women and thus our sample of tissues contained samples from mostly male patients.

The findings of this study present compelling evidence that laryngeal cancer is hormone responsive, specifically to the effects of E_2, via ERα36. This membrane receptor activates several pathways, which were found, both *in vitro* and *in vivo*, to correlate with laryngeal cancer progression and aggressiveness. E_2 also elicits anti-apoptotic effects that can oppose the effects of chemotherapeutic agents, such as taxol, and might thus confer resistance to treatment. Further investigation is warranted in order to elucidate the role of E_2 and this membrane-mediated mechanism and to shed light on its impact on the treatment and management of laryngeal cancer clinically.

Funding

Supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number UL1TR000454 and TL1TR000456. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Partially funded by the Price Gilbert, Jr. Foundation.

CHAPTER 6: Association of Estrogen Receptor-Alpha 36 and Vascular Endothelial Growth Factor with Clinical Variables in Breast Cancer

[Chapter 6 has been prepared for manuscript submission as: Chaudhri RA, Hadadi A, Lobacheva A, O'Regan RM, Schwartz Z, Boyan BD. ERα36 in Human Breast Cancer Tissue Microarray and Association with Clinicopathological Variables. 2013.]

Estrogen receptor alpha 36 (ERα36), an alternatively spliced variant of traditional ERα, has been shown to play a major role in non-traditional estrogen signaling, especially in ER-negative and triple negative breast cancer (TNBC) cells. ERα36 mediates effects of 17b-estradiol (E_2) by promoting cancer cell proliferation, anti-apoptosis, and production of local growth factors. The aim of this study was to investigate the association of ERα36 with clinical variables such as patient age, tumor size, metastasis, and stage, levels of vascular endothelial growth factor (VEGF), and patient survival. Immunohistochemistry for ERα36 and VEGF was performed on a breast cancer tissue microarray (TMA) consisting of a 40-subject cohort. Slides were imaged and blindly scored by three independent observers assessing the number of immunopositive cells, location, and intensity of the stain. Univariate and multivariate statistical analyses were performed to determine the relation of ERα36 and VEGF with clinical variables. Survival analyses were also performed. Our results show an association of age and VEGF intensity with ERα36 intensity. These results, in combination with *in vitro* results showing that ERα36 can mediate the effect of E_2 on mRNA levels of angiogenic and metastatic factors, suggest that ERα36 signaling in breast tumors can promote tumor vascularization leading to tumor progression and poor clinical outcome. In addition, survival analysis showed that even though only human epidermal growth factor receptor 2 (HER2), stage, and node metastasis showed a

predictive value of survival in the overall cohort, a subset of patients with high amounts of ERα36 additionally showed that progesterone receptor (PR) status, VEGF intensity, and tumor size also showed significant effect on survival. This study shows that ERα36 presence is significantly correlated to several clinicopathological factors in breast cancer tissue and may have an effect on survival. These data suggest that therapeutic intervention targeting ERα36 can potentially reduce tumor growth, angiogenesis, and metastasis, and may provide a survival benefit for patients overexpressing ERα36.

6.1 INTRODUCTION

Breast cancer is a heterogeneous disease of which several risk factors may predict outcome depending on specific patient characteristics (5, 169). The presence of estrogen receptors (ERs) is one major method of classifying patients and may determine treatment regimens (170). Typically, ER-positive tumors are hormone-dependent, growing in the presence of estrogen, and therefore, many adjuvant treatments aim to counteract the effects of estrogen. Tamoxifen, the most common anti-estrogen used in the treatment of breast cancer, is typically prescribed as an adjuvant treatment in conjunction with either surgery or radiotherapy, and in patients post-operatively, it is recommended to be prescribed for 5 years following tumor removal (171). ER-negative tumors, however, are typically thought of as non-responsive to anti-estrogens, and therefore, treatment is much more difficult in these patients (58). Furthermore, triple negative breast cancer (TNBC) patients tend to be the most difficult patients to treat as they not only lack ERs, but also progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (58). Both PR and HER2 play roles in tumor development and may be targeted for treatment, but in patients lacking all three receptors treatment options after surgery and radiotherapy are limited to standard

chemotherapy rather than targeted chemotherapy, leading to adverse side effects in patients as standard chemotherapeutics exhibit cytotoxic effects that do not tend to differentiate normal cells from cancer cells.

The identification of novel targets for treatment is crucial in the fight against breast cancer, especially in breast cancer patients who have limited options for treatment. ERα36, an alternatively spliced variant of traditional ERα, or ERα66, has been shown by several *in vitro* studies to play a major role in non-traditional estrogen signaling in breast cancer cells, especially in ER-negative or TNBC cells (54, 59, 82, 108, 172). The consequences of this non-traditional signaling are widespread, but most importantly, ERα36 can mediate effects of 17b-estradiol (E_2) that promote cancer cell proliferation, anti-apoptosis, and production of local factors (54). These local factors include growth factors that promote angiogenesis, epithelial to mesenchymal transition (EMT), and cell migration. Therefore, we wanted to investigate the association of ERα36 with variables such as patient age, tumor size, metastasis, stage, and levels of vascular endothelial growth factor (VEGF). We used a commercially available breast cancer tissue microarray (TMA), which allowed us to perform consistent immunohistochemistry for ERα36 and VEGF in all subject samples of the cohort. The TMA permits direct comparison of every sample in the cohort without experimental bias that normally could be found in immunohistochemistry (IHC) experiments using a large amount of samples on individual slides. We also determined the role of ERα36 and VEGF, as well as several clinical variables, with 5-year survival.

6.2 MATERIALS AND METHODS

6.2.1 Breast Tissue Microarray

The Institutional Review Board-approved breast tissue microarray (TMA) used in this study was purchased from Imgenex (San Diego, CA). Slides provided contained fifty-nine samples that were represented by forty different patient cases. The TMA also included samples from 10 of the subjects in the original cohort of 40 that were from metastatic tissue from lymph nodes. Nine additional samples from matched adjacent normal tissue were also included.

Characteristics of the patient cohort used in this study can be seen in Table 6.1. The mean age was 47(SD=8), but for this study, age was divided into patients under the age of 50 and patients 50 and older. As we were unable to obtain information on menopausal status, and although this is not directly representative of patient menopausal status, we made the general assumption based on published statistics that the vast majority of women over the age of 50 are post-menopausal, whereas women under the age of 50 are typically pre-menopausal (173). The other variables we analyzed in this study included TNM variables, p53 status, receptor status, and histological stage. TNM, which describes tumor growth and spread, is broken down into three variables: T describes the tumor size or extent and is scored from T0 to T4, with T0 being no observable tumor and T1-4 representing increasing growth and spread of the tumor; N describes spread of the cancer from the primary site to regional lymph nodes, with N0 indicating no observable metastasis to regional lymph nodes, and N1-3 describing extent of spread from proximal lymph nodes to more distant lymph nodes; M indicates the presence of distant metastases, with M0 indicating no distant metastasis and M1 indicating metastasis has occurred beyond regional lymph nodes. In this cohort, we only observed patients whom were classified as either T2 or T3, and therefore, all

analyses concerning this characteristic excluded T0, T1, or T4. Lymph node metastasis was observed in 65% of the subjects, while 14 subjects were classified as having no evident lymph node metastases (N0). ER status and PR status were both observed as being divided 70% to 30%, negative and positive, respectively, while HER2 status was observed 77.5% to 22.5%. Histological stage was observed as 30% of the subjects classified as IIA, 30% as IIB, 22.5% IIIA, and 17.5% IIIB. In this study, we analyzed the difference in associations between stage II and stage III subjects as survival drastically reduce from stage II to stage III (174, 175).

Table 6.1: Breast cancer patient characteristics

Breast Cancer Patient Characteristics			
Variable		**No. of Patients (N=40)**	**%**
Age	≤50	23	57.5
	>50	17	42.5
TNM			
Tumor Size	T2	32	80
	T3	8	20
Lymph Node Metastasis	N0	14	35
	N1	12	30
	N2	6	15
	N3	8	20
Metastasis*	M0	40	100
p53 status	Negative	22	55
	Positive	18	45
Receptor status			
ER	Negative	28	70
	Positive	12	30
PR	Negative	28	70
	Positive	12	30
HER2/neu	Negative	31	77.5
	Positive	9	22.5
Histological Stage	IIA	12	30
	IIB	12	30
	IIIA	9	22.5
	IIIB	0	0
	IIIC	7	17.5

6.2.2 Immunohistochemistry

The sections were paraffin-embedded onto a single slide containing all fifty-nine samples. This allowed for consistent immunohistochemical staining among all samples, thereby permitting direct comparisons among samples. Prior to immunohistochemistry, slides were deparaffinized by heating in a 60°C oven for 30 minutes, followed by washing three times in xylene for 1 minute each wash, two times in 100% ethanol for 1 minute each, two times in 95% ethanol for 1 minute each, and tap water for 5 minutes. In order to unmask antigens, slides were heated in a pressure cooker for 3 minutes immersed in 10mM sodium citrate buffer (pH 6.0). After removal from the pressure cooker, slides were placed in room temperature sodium citrate buffer to cool for 20 minutes, after which time they were washed in tap water three times for 2 minutes each wash.

After antigen retrieval, immunohistochemistry was performed using the using the Immunocruz™ rabbit LSAB staining system from Santa Cruz Biotechnology (Santa Cruz, CA). In order to quench endogenous peroxidase activity, slides were incubated for 5 minutes in hydrogen peroxide solution, rinsed with 1X phosphate-buffered saline (PBS), and washed for 2 minutes in 1X PBS at room temperature. Slides were then blocked with 5% normal goat serum for 20 minutes. Anti-ERα36 specific antibodies were purchased from Cell Applications, Inc. (San Diego, CA). Anti-VEGF antibodies were purchased from Abcam (Cambridge, MA). Anti-ERα36 and anti-VEGF antibodies were then applied to the slides at a 1:50 dilution. After 2 hours, the slides were washed two times with 1X PBS for 2 minutes each. Slides were then incubated for 30 minutes with biotinylated secondary antibody and washed twice again with 1X PBS for 2 minutes each wash. After secondary antibody incubation, slides were incubated for 30 minutes with horseradish peroxidase (HRP)-streptavidin solution followed by two washes with 1X

PBS for 2 minutes each. After the HRP-streptavidin incubation, slides were then incubated for 10 minutes with diaminobenzoate (DAB) chromogen and peroxide, which was prepared following the manufacturer's instructions. Following this incubation, slides were washed two times with deionized H_2O for 2 minutes each and then counterstained with Gill's haematoxylin for 10 seconds. Slides were immediately washed with tap water until all excess haematoxylin cleared and then were destained with acid alcohol and bluing reagent, followed by another wash with tap water. Slides were finally dehydrated twice with 95% ethanol for 10 seconds each, twice with 100% ethanol for 10 seconds each, and three times in xylene for 10 seconds each. Excess xylene was removed from the slides and slides were finished with Cytoseal mounting medium (ThermoFisher Scientific, Waltham, MA) and coverslipped.

6.2.3 Imaging and scoring

Slides were imaged using a Leica DMLB microscope system with a 63X objective oil immersion lens. Images were taken with ImagePro for all samples and blindly scored by three independent observers according to the scoring method listed in Table 6.2. ERα36 presence in all tissues was scored according to three classifications: ERα36 number describes the number of cells in the tissue that are positive for ERα36. ERα36 location describes whether the majority of positive cells express ERα36 primarily in the nucleus or if ERα36 is present throughout the cells, indicating cytosolic and membrane presence. ERα36 intensity describes the intensity of the staining, which describes cells that express either low amounts of ERα36 or high amounts of ERα36. VEGF was scored in a similar manner to ERα36; however, no score for VEGF location was included as all VEGF is a secreted growth factor.

Originally, we wanted to determine the role of ERα36 by comparing patient tissues positive for ERα36 to patient tissues negative for ERα36. The fact that all samples contained ERα36 prevented us from answering our original question; however, we did perform the analysis comparing patient tissues showing more than 50% of cells positive for ERα36 (ERα36 number score of 3) to patient tissues showing less than 50% of cells positive for ERα36 (ERα36 number score of 2).

Table 6.2: Histology Scoring Method

All samples were scored blindly by three independent observers. Scores were then compared and for samples that were not scored the same by all three observers, the two most common scores were used. If no scores matched, a fourth observer blindly determined the score.

	Score	Representation
ERα36 Number	0	<1% cells positive
	1	1-10% cells positive
	2	<10<50% cells positive
	3	>50% cells positive
ERα36 Location	0	Nuclear
	1	Non-nuclear
ERα36 Intensity	0	Low
	1	High
VEGF Number	0	<1% cells positive
	1	1-10% cells positive
	2	<10<50% cells positive
	3	>50% cells positive
VEGF Intensity	0	Low
	1	High

6.2.4 Statistical analysis

All statistical analyses were performed in SAS. The PROC FREQ procedure was used to analyze comparisons of variables using Fisher's exact test. Fisher's exact test is a more accurate measurement of comparison than the Chi-square test because it gives

an exact p-value rather than an approximation, and it is especially useful in analyses where sample size is a concern. Survival analyses were performed using the PROC LIFETEST procedure, which was also used to produce survival curves. Patients with final follow up times prior to 5 years who were not deceased were censored at the time that they last attended follow-up. All predictors of survival were determined by the Log-Rank test for differences in survival.

6.3 RESULTS

The blinded analysis of immunohistochemical staining for ERα36 demonstrated that all breast cancer patient tissues were positive for the presence of the membrane receptor. Figure 6.1 shows 3 representative samples stained for the presence of ERα36 and VEGF by immunohistochemistry. Figure 6.1A shows the majority of cells in the tissue are positive for ERα36 and the location is primarily nuclear. The corresponding VEGF staining was scored as 1 for VEGF number, indicating greater than 1% and less than 10% of cells positive for VEGF, and 0 for VEGF intensity, indicating low intensity. Figure 6.1B shows a sample in which less than 50% of the cells are positive for ERα36 and the presence in the positive cells is dispersed throughout the cells. This sample was also scored as low for ERα36 intensity, according to all observers. The corresponding VEGF staining was scored as 3 for VEGF number, indicating greater than 50% of cells positive for VEGF, and 0 for VEGF intensity, indicating low intensity. Figure 6.1C shows a sample with an ERα36 number score of 3, indicating greater than 50% positive cells, an ERα36 location score of 1, indicating presence throughout the cells, and an ERα36 intensity score of 1, indicating high intensity. The corresponding VEGF staining was scored as 3 for VEGF number, indicating greater than 50% of cells positive for VEGF, and 1 for VEGF intensity, indicating high intensity.

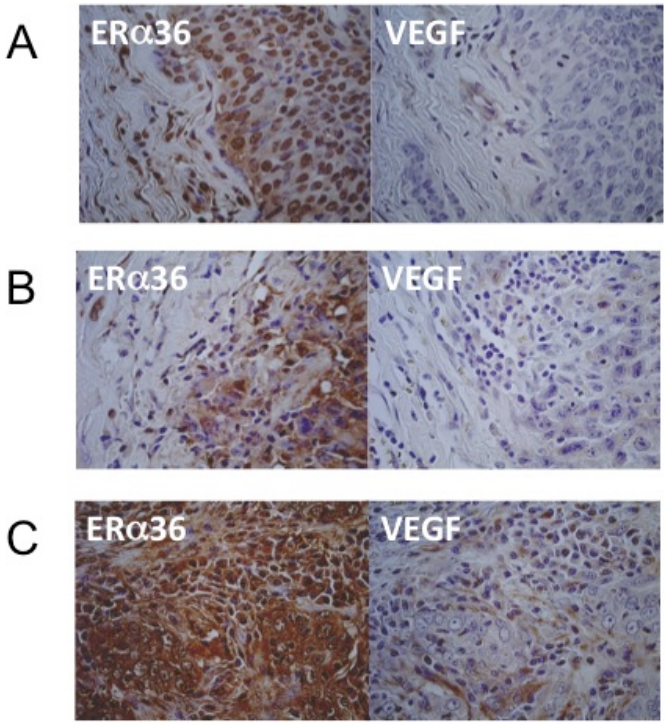

Figure 6.1: ERα36 and VEGF Immunohistochemistry in breast TMA samples.

(A) Sample with high ERα36 number (score=3), nuclear ERα36 location (score=0), low ERα36 intensity (score=0), low VEGF number (score=1), and low VEGF intensity (score=0). (B) Sample with moderate ERα36 number (score=2), cytosolic ERα36 location (score=1), low ERα36 intensity (score=0), moderate VEGF number (score=2), and low VEGF intensity (score=0). (B) Sample with high ERα36 number (score=3), cytosolic ERα36 location (score=1), high ERα36 intensity (score=1), high VEGF number (score=3), and high VEGF intensity (score=1).

Scoring results for breast cancer samples can be seen in Table 6.3. No differences were found in scoring of the 10 samples of tissue from tumors that metastasized to lymph nodes (data not shown). As stated previously, all tumor tissues were positive for ERα36 and 82.5% of the samples exhibited ERα36 positivity in more than 50% of the cells. The majority of samples exhibited ERα36 predominantly non-nuclearly (80%), and 85% of the samples exhibited high levels of ERα36. VEGF scoring was more evenly distributed across all scores for VEGF number, with the exception of a single sample exhibiting a VEGF number score of 3, indicating more than 50% of the

cells in the sample were positive for VEGF. VEGF intensity scoring was somewhat even with 57.5% of subjects scored with low VEGF intensity and 42.5% of subjects scored with high VEGF intensity.

Table 6.3: Scoring of breast tumor samples

Scoring Characteristics for Breast Tumor Samples				
	Score	Representation	No. of Patients (N=40)	%
ERα36 Scoring				
Number	0	<1% positive cells	0	0
	1	1%-10% positive cells	0	0
	2	10%-50% positive cells	7	17.5
	3	>50% positive cells	33	82.5
Location	0	>50% nuclear only	8	20
	1	>50% cytosolic	32	80
Intensity	0	Low	6	15
	1	High	34	85
VEGF Scoring				
Number	0	<1% positive cells	15	37.5
	1	1%-10% positive cells	16	40
	2	10%-50% positive cells	8	20
	3	>50% positive cells	1	2.5
Intensity	0	Low	23	57.5
	1	High	17	42.5

Included in the TMA were 9 controls from matched adjacent normal tissue of which scoring characteristics are seen in Table 6.4. Fisher's exact test determined no association of tumor status, comparing the 9 tumor samples to their matched non-tumor controls, with ERα36 number, ERα36 location, ERα36 intensity, VEGF number, or VEGF intensity. No other analyses were performed comparing these two groups.

Table 6.4: Scoring of normal adjacent tissue

Scoring Characteristics for Normal Tissue Samples				
	Score	Representation	No. of Patients (N=40)	%
ERα36 Scoring				
Number	0	<1% positive cells	0	0
	1	1%-10% positive cells	0	0
	2	10%-50% positive cells	1	11.1
	3	>50% positive cells	8	88.9
Location	0	>50% nuclear only	1	11.1
	1	>50% cytosolic	8	88.9
Intensity	0	Low	3	33.3
	1	High	6	66.7
VEGF Scoring				
Number	0	<1% positive cells	2	22.2
	1	1%-10% positive cells	4	44.4
	2	10%-50% positive cells	3	33.3
	3	>50% positive cells	0	0
Intensity	0	Low	5	55.6
	1	High	4	44.4

Table 6.5 shows the results of Fisher's exact test comparing ERα36 number to patient variables, Table 6.6 shows results for comparison of ERα36 location with patient variables, and Table 6.7 shows results for comparison of ERα36 intensity and patient variables. In this study, neither ERα36 number nor ERα36 location in all subjects surveyed was significantly associated with any variable presented. However, ERα36 intensity was found to be directly associated with VEGF intensity, as well as age. After performing the same analysis in only ER-negative subjects (Tables A6.1, A6.2, and A6.3), no variables were determined to be significantly associated with ERα36 number, location, or intensity. However, a Fisher's exact p-value of 0.0691 approached significance when analyzing the association of ERα36 number and p53 status in ER-negative patients (Table 6.8). The same analyses were performed in a subset of TNBC subjects (Tables A6.4, A6.5, and A6.6). In TNBC samples, the association of ERα36

number and lymph node metastasis (N0 vs. N1-3) also approached a significant correlation (p=0.0525).

Table 6.5: ERα36 number in all patients

ERα36 Number in All Patients (ERα36 Positive in <50% vs >50%)			
Characteristic	Comparison	P-value	Significant?
Age	<50 vs >50	0.6770	No
TNM			
Tumor Size	T2 vs T3	0.1282	No
Node	N0 vs N1-3	0.6789	No
	N0-1 vs N2-3	1.0000	No
	N0-2 vs N3	1.0000	No
Stage	II vs III	1.0000	No
p53 status	Negative vs Positive	0.2110	No
Receptor status			
ER	Negative vs Positive	0.6521	No
PR	Negative vs Positive	0.0810	No
HER2/neu	Negative vs Positive	0.6446	No
VEGF Scoring			
Number	0 vs 1-3	N/A	N/A
	0-1 vs 2-3	0.2097	No
	0-2 vs 3	N/A	N/A
Intensity	Low vs High	0.6770	No

Table 6.6: ERα36 location in all patients

ERα36 Location in All Patients (Nuclear vs Cytosolic)			
Characteristic	**Comparison**	P-value	Significant?
Age	<50 vs >50	0.4284	No
TNM			
Tumor Size	T2 vs T3	1.0000	No
Node	N0 vs N1-3	0.2216	No
	N0-1 vs N2-3	1.0000	No
	N0-2 vs N3	0.6499	No
Stage	II vs III	0.6905	No
p53 status	Negative vs Positive	1.0000	No
Receptor status			
ER	Negative vs Positive	1.0000	N/A
PR	Negative vs Positive	1.0000	N/A
HER2/neu	Negative vs Positive	1.0000	N/A
VEGF Scoring			
Number	0 vs 1-3	N/A	N/A
	0-1 vs 2-3	0.6905	No
	0-2 vs 3	N/A	N/A
Intensity	Low vs High	1.0000	No

Table 6.7: ERα36 intensity in all patients

ERα36 Intensity in All Patients (ERα36 Low vs High)			
Characteristic	Comparison	P-value	Significant?
Age	<50 vs >50	**0.0295**	**Yes**
TNM			
Tumor Size	T2 vs T3	1.0000	No
Node	N0 vs N1-3	1.0000	No
	N0-1 vs N2-3	0.6456	No
	N0-2 vs N3	0.5803	No
Stage	II vs III	0.6678	No
p53 status	Negative vs Positive	1.0000	No
Receptor status			
ER	Negative vs Positive	0.6479	No
PR	Negative vs Positive	0.6479	No
HER2/neu	Negative vs Positive	0.6016	No
VEGF Scoring			
Number	0 vs 1-3	0.3725	No
	0-1 vs 2-3	N/A	N/A
	0-2 vs 3	N/A	N/A
Intensity	Low vs High	**0.0295**	**Yes**

Multivariate analysis confirmed the null hypothesis in all patients in logistic regression models separately for ERα36 number, ERα36 location, and ERα36 intensity. No variables were found to significantly predict outcome, whether outcome was number, location, or intensity, nor was any variable found to significantly predict outcome when patients were stratified based on ER status or if they were TNBC subjects.

Figure 6.2 is an example of a typical Kaplan-Meier curve showing differences in mortality from subjects of the entire cohort with HER2 negative vs. HER2 positive status, which was found to be a significant predictor of 5-year survival in this patient cohort. Survival analysis using the PROC LIFETEST procedure in SAS did not reveal any predictive value of ERα36 where 5-year mortality was concerned; however, we do see that HER2 status (p=0.0147), stage (p=0.0058), and lymph node status (p=0.0382, 0.0014, and 0.0023 for N0 vs. N1-3, N0-1 vs. N2-3, and N0-2 vs. N3, respectively), predicted mortality in this group of patients as shown in Table 6.8. Survival analysis in ER-negative (Table A6.7) and TNBC (Table A6.8) subjects revealed that only node status was predictive of mortality in these patients.

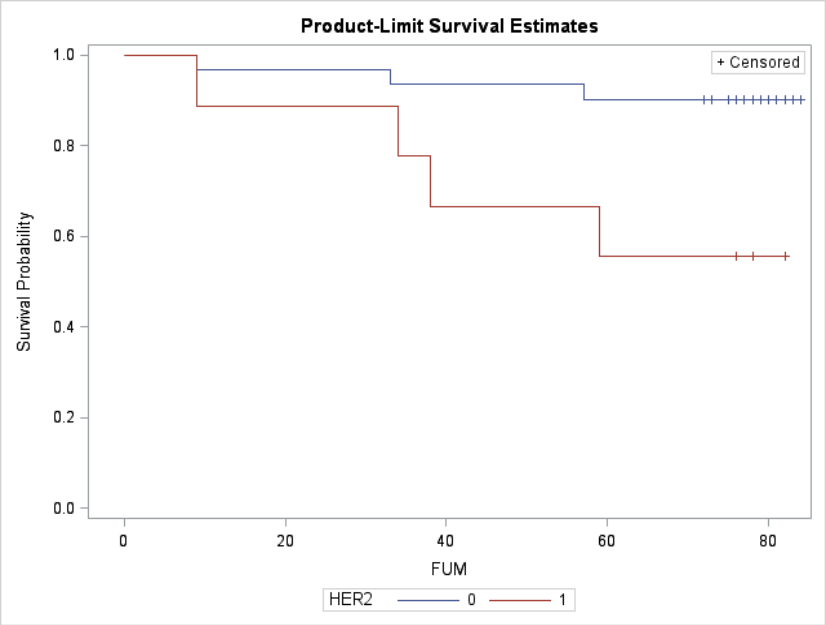

Figure 6.2: Kaplan-Meier Curves for all patients.

Kaplan-Meier Curve comparing survival HER2-negative (HER2=0) vs. HER2-positive (HER2=1) patients.

Table 6.8: Survival in Breast TMA patients.

Log-rank p-values in all patients.

Survival in All Subjects		
Variable	**Significant?**	**Log-Rank P-value**
ERα36 Number	no	0.8518
ERα36 Localization	no	0.5062
ERα36 Intensity	no	0.2349
ER status	no	0.8992
PR status	no	0.0657
HER2 status	**yes**	**0.0147**
VEGF Number	no	0.7411
VEGF Intensity	no	0.1124
P53 status	no	0.1102
Stage	**yes**	**0.0058**
Tumor size (T2 vs T3)	no	0.1192
Node status (0 vs 1-3)	**yes**	**0.0382**
Node status (0-1 vs, 2-3)	**yes**	**0.0014**
Node status (0-2 vs 3)	**yes**	**0.0023**
Age (<50 vs >50)	no	0.4233

Patients with high ERα36 number (score=3), which is the group of patients in which greater than 50% of the cells in their tumor samples were positive for ERα36, exhibited a correlation with mortality. Log-rank p-values for all variables can be seen in Table 6.9. After stratifying this group based on their ERα36 number, the log-rank test showed that PR status (p=0.0477), VEGF intensity (p=0.0155), stage (0.0139), tumor size (p=0.0092) and node status greater than 1 (p=0071) affected survival.

Table 6.9: Survival in subjects with high ERα36 number

Log-rank p-values in patients with high ERα36 number (score=3), which corresponds to samples showing greater than 50% ERα36 positive cells.

Survival in Subjects with High ERα36 Number		
Variable	**Significant?**	**Log-Rank P-value**
ER status	no	0.9783
PR status	**yes**	**0.0477**
HER2 status	no	0.0677
VEGF Number	no	0.3142
VEGF Intensity	**yes**	**0.0155**
P53 status	no	0.1164
Stage	**yes**	**0.0139**
Tumor size (T2 vs T3)	**yes**	**0.0092**
Node status (0 vs 1-3)	no	0.0649
Node status (0-1 vs, 2-3)	**yes**	**0.0071**
Node status (0-2 vs 3)	N/A	N/A
Age (<50 vs >50)	no	0.1157

A similar relationship was observed when analyzing survival of patients with high ERα36 intensity (score=1), which is the group of patients with qualitatively high levels of ERα36 present in positive cells. Log-rank p-values for all variables can be seen in Table

6.10. After stratifying this group based on their ERα36 intensity, the log-rank test showed that HER2 status (p=0.0162), stage (p=0.0386), node status greater than 1 (p=0.0131), and node status greater than 2 (p=0.0029) affected survival (Table 6.10).

Table 6.10: Survival in subjects with high ERα36 intensity

Log-rank p-values in patients with high ERα36 intensity (score=1).

Survival in Subjects with High ERα36 intensity		
Variable	**Significant?**	**Log-Rank P-value**
ER status	no	0.5176
PR status	no	0.1043
HER2 status	**yes**	**0.0162**
VEGF Number	no	0.4520
VEGF Intensity	no	0.1630
P53 status	no	0.0726
Stage	**yes**	**0.0386**
Tumor size (T2 vs T3)	no	0.2982
Node status (0 vs 1-3)	**yes**	**0.0821**
Node status (0-1 vs, 2-3)	**yes**	**0.0131**
Node status (0-2 vs 3)	**yes**	**0.0029**
Age (<50 vs >50)	no	0.6423

We finally performed survival analysis on only patients with predominantly cytosolic ERα36 location (score=1), which is the group of patients with predominantly cytosolic and membrane presence of ERα36 in cells rather than predominant presence in the nucleus. Log-rank p-values for all variables can be seen in Table 6.11. After stratifying this group based on their ERα36 location, the Log-rank test showed that HER2 status (p=0.0208), stage (p=0.0015), tumor size (p=0.0292), node status greater

than 0 (p=0.0499), node status greater than 1 (p=0.0006), and node status greater than 2 (p=0.0038) could predict survival.

Table 6.11. Survival in subjects with cytosolic ERα36 location

Log-rank p-values in patients with predominantly cytosolic ERα36 location (score=1).

Survival in Subjects with Cytosolic ERα36 Location		
Variable	**Significant?**	**Log-Rank P-value**
ER status	no	0.6743
PR status	no	0.1130
HER2 status	**yes**	**0.0208**
VEGF Number	no	0.8751
VEGF Intensity	no	0.2784
P53 status	no	0.3947
Stage	**yes**	**0.0015**
Tumor size (T2 vs T3)	**yes**	**0.0292**
Node status (0 vs 1-3)	**yes**	**0.0499**
Node status (0-1 vs, 2-3)	**yes**	**0.0006**
Node status (0-2 vs 3)	**yes**	**0.0038**
Age (<50 vs >50)	no	0.7645

6.4 DISCUSSION

In this study, we were able to independently identify several variables that were associated with ERα36 in a 40-subject cohort of breast cancer collected on a single slide in a TMA. ERα36 has been shown to mediate non-nuclear responses of estrogen *in vitro* leading to more aggressive cancer phenotype such as enhanced cell proliferation, anti-apoptosis, and production of factors that promote angiogenesis and metastasis (54, 55). VEGF has been shown to be elevated in tumor tissue undergoing vascularization and is

associated with increased risk of metastasis (176-179). We therefore performed immunohistochemistry for ERα36 and VEGF in all samples in order to determine if ERα36 was associated with any physiologically relevant variable such as patient age, tumor size, metastasis, tumor stage, or VEGF presence.

As this study only provides a snapshot of ERα36 presence in these subjects and not an idea of ERα36 functionality, we could not determine the actual functional role of ERα36 in these patients. However, the direct association of ERα36 and age, as well as the association of ERα36 and VEGF, provides intriguing insight into the possible role of ERα36 in this cohort of breast cancer patients. In addition, *in vitro* data suggests a tumorigenic role of ERα36 in a variety of cancers (59, 108, 180-182). The positive association of ERα36 intensity and VEGF intensity suggests that breast cancer tissue with higher levels of ERα36 at the cellular level, may lead to enhanced vascularization. Enhanced tumor vascularization would understandably aid in tumor growth and spread and provide a path by which cells from the primary tumor can enter the circulation either via the circulatory system or the lymphatic system and travel to distant sites (162, 164, 183). Qualitatively, it appeared that ERα36 exhibited punctate expression in several samples that contained blood vessels and VEGF levels in these blood vessels was highly evident. We could not determine if these blood vessels were immature or mature blood vessels; however, it was notable to have seen this qualitative correlation of expression between ERα36 and VEGF.

Interestingly, in this limited cohort, we did see an association between age and ERα36 intensity, suggesting that in older patients, ERα36 levels are higher. Although menopausal status of the subjects in this cohort was not obtained, we made the assumption that the average woman over the age of 50 is post-menopausal, whereas women under the age of 50 are generally pre-menopausal (173). This may not be

completely accurate, but as an educated assumption, if this were true for this cohort, it may give an idea as to the relation of ERα36 presence in breast cancer tissue in these patients and menopausal status. As circulating estrogen levels decline following menopause, breast tissue can actually maintain estrogen levels, indicating the functional presence of estrogen in the breast after menopause. Not only are estrogen levels maintained, but also in many cases, estrogen levels can actually be elevated in breast tumor tissue, and this can be attributed to local production of estrogen through multiple mechanisms including the conversion of pre-estrogens such as androstenedione and testosterone via the enzyme aromatase (184, 185).

Although univariate analysis only showed an association of ERα36 with age and VEGF intensity, we were able to perform survival analysis and assess the individual prognostic value of all variables and 5-year survival. HER2 status, stage, and lymph node metastasis were associated with decreased mortality in the entire cohort, indicating that these variables could possibly predict survival. In ER-negative patients and TNBC patients, node status also affected survival, which was not surprising as lymph node metastasis is a logical precursor to distant metastasis, which makes cancer more difficult to treat leading to poor prognosis.

Survival analysis of patients in which their tumors showed greater than 50% of cells positive for ERα36 showed the same variables predictive of survival than the entire cohort, as did the subset of patients with high ERα36 intensity or predominantly non-nuclear localization of ERα36. In fact, in the subset of patients with high amounts of ERα36 (ERα36 number=3), PR status also showed significant predictive value for survival. The entire undivided cohort showed a p-value for PR prediction for survival of 0.0657 and although this is not statistically significant at a significance level of 0.05, it is approaching significance. When we performed the analysis on the patient subset that

expressed high amounts of ERα36, we did see a significant predictive value of PR status, suggesting some unknown association of ERα36 and PR where survival is concerned. Interestingly, VEGF intensity and node status were associated with decreased survival in this subset of patients, and the fact that ERα36 and VEGF were directly associated by univariate analysis, and ERα36 promotes VEGF expression in breast cancer cells, the poor outcome may be explained by the ability of ERα36 to promote tumor vascularization, leading to metastasis, and thus, poor survival.

Limitations of this study include missing data such as treatment, race, status of genetic mutations such as BRCA1/2, non-cancerous abnormal breast conditions, weight, circulating E_2 levels, history of alcohol use, history of pregnancy, number of children, history of birth control use, family history and the low sample size. Most if not all of these limitations could be alleviated by performing this study on a prospective cohort in which all of these factors could be included with patient information with all patients enrolled in the study. A retrospective cohort from whom more patient information is obtainable would also provide a more accurate idea of some of the associations of these variables with outcome. Unfortunately, due to the constraints of this study as far as sample size and subject information is concerned, the inability to find significant associations between variables and outcome does not provide a generalizable idea of whether these variables do in fact play a role in outcome. However, the data presented in this study, especially in showing significant association of several variables and ERα36, as well as many of these variables and effects on survival in this cohort, suggest the robustness of these variables' predictive values. If these variables could be found significantly predictive of the outcomes examined here considering the low sample size, they will most likely prove to be significant in a study with a larger sample size.

This study, as limited as it may be due to small sample size, is one of the first studies showing the association of ERα36 and VEGF in human breast cancer patients using a tissue microarray that allows for consistent immunohistochemical evaluation among all samples in the TMA. The results of this preliminary study show independent association of ERα36 and VEGF, as well as ERα36 and age. Furthermore, it shows that survival in patients with high amounts of ERα36 can be associated with several variables including PR status, HER2 status, stage, tumor size, and node status. Although ERα36 itself was not able to show a predictive value for survival in this patient cohort, the significant predictive value of the other variables in patients with high levels of ERα36 suggest that ERα36 may be an important factor with which to perform a further, more extensive analysis. A larger cohort may give a clearer idea of the role of ERα36 on patient outcome. The fact that several of the variables analyzed in this study did in fact show a significant association with the outcomes measured suggests that these factors in particular may be very robust in predicting outcome.

CHAPTER 7: Conclusions and Future Perspectives

Due to the heterogeneity of causes and characteristics of cancer, the idea of finding a single, defined cure is impractical. However, new discoveries and greater insight into the mechanisms of cancer development and progression can provide novel methods for treatment. In some cases, treatments can be so effective, they can extend life expectancy and patient quality of life in such a way as to essentially cure the patient of the physical manifestations of cancer. Because every patient presents a unique case, discovery of novel drug targets allows for several routes of treatment in single subjects. Instead of just simple physical removal of cancerous tumors by surgery or prevention of tumor growth by radiotherapy, several adjunct chemotherapeutic treatments have been developed that allow for more personalized treatment, depending on the specific characteristics of the patient and the tumor. Standard chemotherapy, which aims to fight cancer through cytotoxicity, does not differentiate among cells and not only kills cancer cells, but may also affect normal cells, leading to adverse side effects. Especially due to these generalized effects of standard chemotherapy in patients, the need for more tailored and effective treatments with reduced side effects is more prevalent than ever.

Personalized medicine is rapidly become the ideal route of treatment for several diseases. In cancer, personalization of treatment is especially beneficial due to the heterogeneity of cancer. Several drugs have either recently been approved or are in the process of achieving approval by the FDA for use as adjuvant treatments for cancer. One of the most common approaches to personalized medicine in cancer treatment is immunotherapy or targeting of biomarkers that are overexpressed in cancer tissue. The discovery of novel biological targets that are more prevalent in cancer tissue than normal tissue provides a more practical route of treatment than standard chemotherapeutics,

which tend to not differentiate between normal and cancerous cells. Several companies currently produce drugs that exploit the overexpression of biological targets in cancer tissue compared to normal tissue. Trastuzumab, a monoclonal antibody that targets HER2 receptor in breast cancer, is especially effective in treating breast cancer tumors that highly overexpress HER2. HER2/neu receptors, which can be found in the plasma membrane of some breast cancer cells, promote cell proliferation, and trastuzumab binding to HER2/neu receptors causes cell cycle arrest and decreased proliferation of cancer cells overexpressing the receptor. This drug can only be beneficial to a subset of subjects that have HER2/neu overexpression in the cancerous tissue, and therefore, not all patients can benefit from its use. Moreover, like most chemotherapeutics, trastuzumab is meant for use as an adjunct treatment along with more conventional methods such as surgery or radiotherapy. However, trastuzumab provides an excellent example of a novel chemotherapeutic that can lead to beneficial, personalized treatment in a select subset of breast cancer patients.

The identification and investigation of the role of novel biological targets in cancer progression is necessary to predict and evaluate the possibility of drugs that may specifically bind and alter the function of these targets. This thesis identified ERα36 as a potential target in the treatment of a select group of breast cancer subjects exhibiting high expression of the membrane receptor. The fact that this receptor functions specifically from the membrane in ER-negative and TNBC patients creates hopeful optimism that targeting this receptor at the membrane with a drug that cannot enter the cells, would provide an alternative and effective method of treatment in patients who are difficult to treat with conventional methods.

In this thesis, ERα36 was shown to mediate the rapid membrane-delimited effects of E_2 in triple negative breast cancer cells as well as other cells. The activation of

this pathway occurred in ER-positive breast cancer cells (MCF7), TNBC cells (HCC38), and laryngeal cancer cells (Hep2), as well as an embryonic kidney cell line that was transfected to overexpress ERα36 (Hek293). Several other cell lines were screened for the presence of ERα36, and in all cells, ERα36 expression was apparent, however, the extent of expression to the level of functionality was not evaluated in all of these cell lines. This work proposes a mechanism, based on experimental evidence, of the rapid effects of E_2 through membrane-associated ERα36 on cell proliferation and anti-apoptosis. This mechanism was found to include divergent pathways from the plasma membrane that reconverge downstream to affect cancer cell survivability. The rapid activation of PKC by E_2 through membrane-associated ERα36 was found to require activation of G-proteins that lead to activation of PLC, DAG, IP3, calcium signaling, and PKC. Activation of PKC leads to downstream activation of MAPkinase, which can effect proliferation, as well as gene transcription via MAP kinase phosphorylation of an array of transcription factors. At the same time, ERα36 can mediate an effect of E_2 on PLD at the membrane leading to activation of LPA signaling and PI3K, which in turn leads to attenuation of the caspase cascade that promotes apoptosis, and therefore, E_2's effect on this pathway can promote anti-apoptosis, especially in the presence of chemotherapeutics that aim to induce apoptosis. In this study, we found that E_2 reduced the effect of taxol, a common chemotherapeutic drug that causes cancer cell death, and that this reduction occurred through the specific pathway mediated by ERα36.

Through examination of the presence of ERα36 in a breast cancer tissue microarray and a laryngeal cancer tissue microarray, ERα36 was found to be associated with specific factors of poor outcome such as tumor vascularization and metastasis. It was also found that in breast tissues with high levels of ERα36, patient survival was reduced when previously identified predictors of survival were examined, suggesting that

ERα36 may have a role in the effect of these predictors on outcome. Although in many of our analyses, ERα36 was not directly correlated to outcome, the results presented suggest further examination of the role of ERα36 in breast cancer and laryngeal cancer in a larger cohort of subjects with more complete information, especially including patient treatment, is necessary. The results of a larger study would more definitely describe a role of ERα36 in breast cancer and would support the diagnostic value of ERα36 or the importance of developing drugs that may target ERα36.

We have currently commenced *in vivo* studies modeling breast tumor growth and breast tumor osteolysis in mice. The goal of these studies is to first establish models of tumor growth and osteolysis in order to determine the role of ERα36 *in vivo*. In our current models, we have included treatment groups with E_2 and tamoxifen to evaluate the importance of E_2 in the growth and osteolytic effects of the tumors. The next step is to create tumors with reduced ERα36 expression by mRNA knockdown in the cancer cells prior to injection into the mice. We hypothesize that ERα36-silenced tumors exhibit reduced growth and reduced osteolysis *in vivo* compared to wildtype tumors. Further studies include production of monoclonal antibodies against ERα36, which can then be used in several studies to first evaluate the effectiveness of the antibodies, followed by preclinical studies to determine if use of monoclonal antibodies *in vivo* can reduce tumor progression. If these preclinical studies prove efficacy in the use of monoclonal antibodies to target ERα36 *in vivo*, we would suggest clinical trials of the monoclonal antibodies in breast cancer patients exhibiting tumor overexpression of ERα36. Although these future approaches provide optimism in a novel approach to personalized breast cancer therapy by targeting ERα36, it would be tragic to not pursue these studies, which would give a definitive conclusion into the role of ERα36 in breast cancer and the possibility of ERα36 as a potential drug target.

APPENDICES

APPENDIX I: Chapter 3 Supplemental Figure

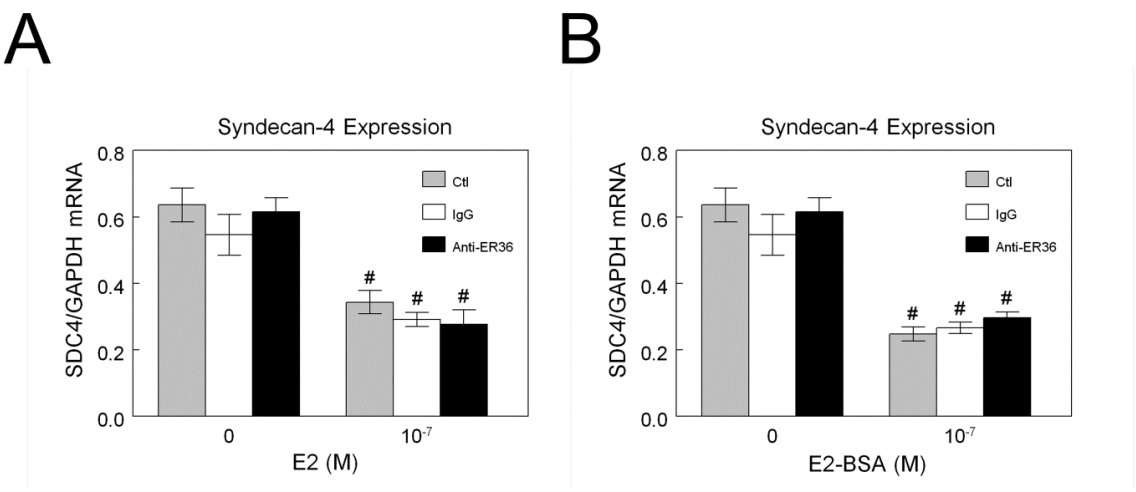

Figure A3.1: Membrane estrogen signaling increases expression of membrane syndecan-4, but not through ERα36.

qRT-PCR analysis indicates that E_2 signaling in HCC38 cells increases expression of syndecan-4 **(A),** and E_2-BSA has a similar effect **(B)**. * $p<0.05$ compared to control + vehicle (0M E_2-BSA), # $p<0.05$ compared to control + 10^{-7}M E_2-BSA.

APPENDIX II: Chapter 4 Supplemental Figures

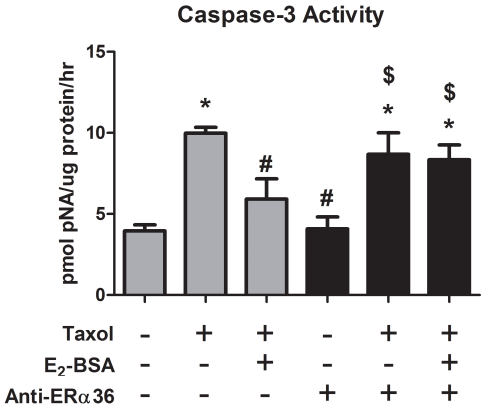

Figure A4.1: ERα36 and the Anti-apoptotic Effect of E_2-BSA

Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2-BSA, while ERα36 antibodies block this effect. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to anti-ERα36 alone.

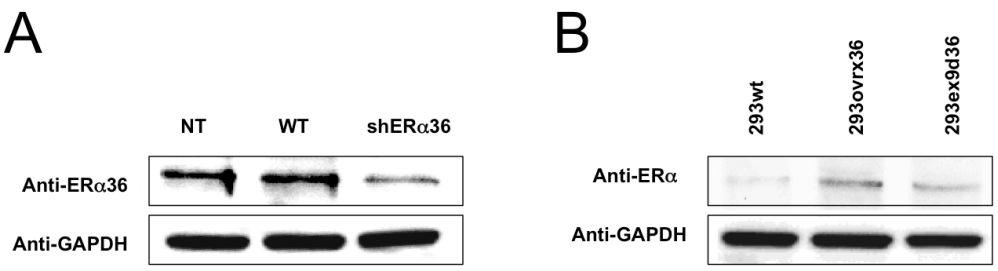

Figure A4.2: ERα36 Silencing and Overexpression

(A) Using anti-ERα36 antibodies, western blots were performed on whole cell lysates from HCC38 cells transiently transfected with a non-target control vector (NT), wildtype HCC38 cells (WT), and HCC38 cells transiently transfected with ERα36 shRNA vector (shERα36). Densitometry analysis showed greater than 70% knockdown of ERα36 protein levels in the shERα36 cells compared to wildtype controls. All samples were normalized to GAPDH. (B) Using ant-ERα antibodies that also recognize ERα36, western blots were performed on whole cell lysates from wildtype HEK293 cells (293wt), and HEK293 transiently transfected to overexpress wildtype ERα36 cDNA (293ovrx36) and exon 9-deleted ERα36 cDNA (293ex9d36).

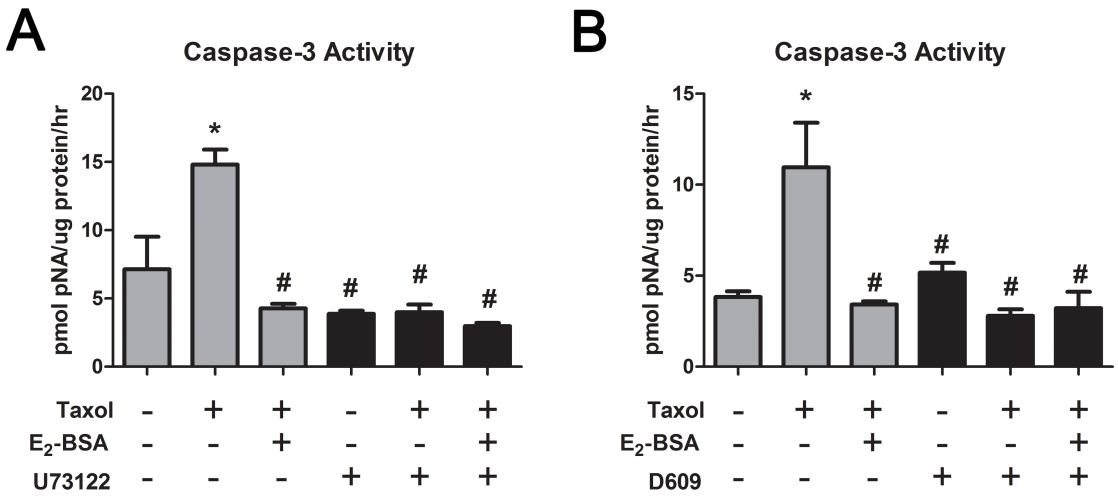

Figure A4.3: Phospholipases and the Anti-apoptotic Effect of E_2-BSA

(A) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2, while U73122 and (B) D609 block taxol's effect. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone.

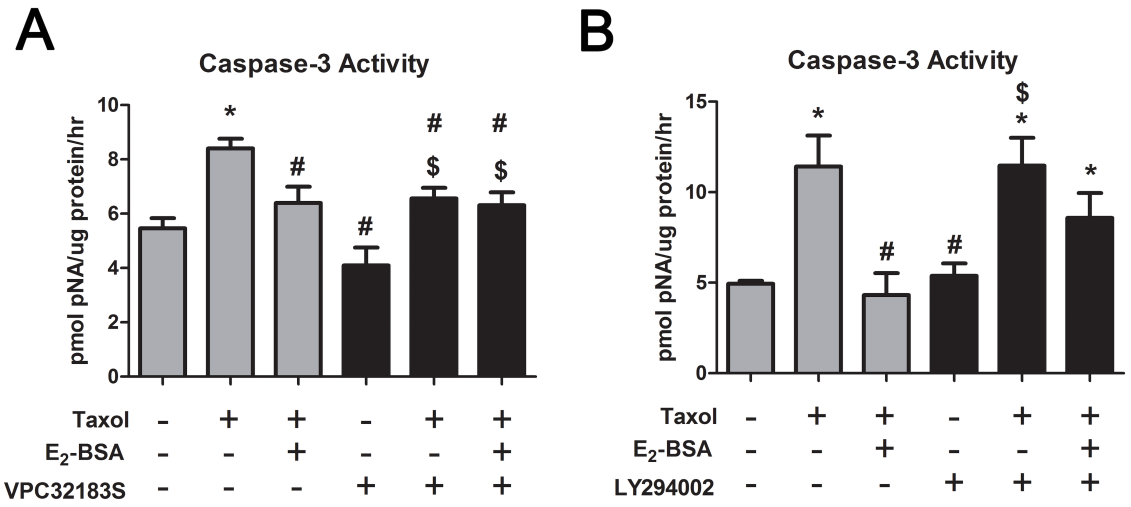

Figure A4.4: LPA Signaling, PI3K and the Anti-apoptotic Effect of E_2-BSA

(A) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2-BSA, while the LPAR1/3 antagonist, VPC32183S, does not allow E_2-BSA to block the effect of taxol. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone. (B) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2-BSA, while the LY294002 does not allow E_2-BSA to block the effect of taxol. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone.

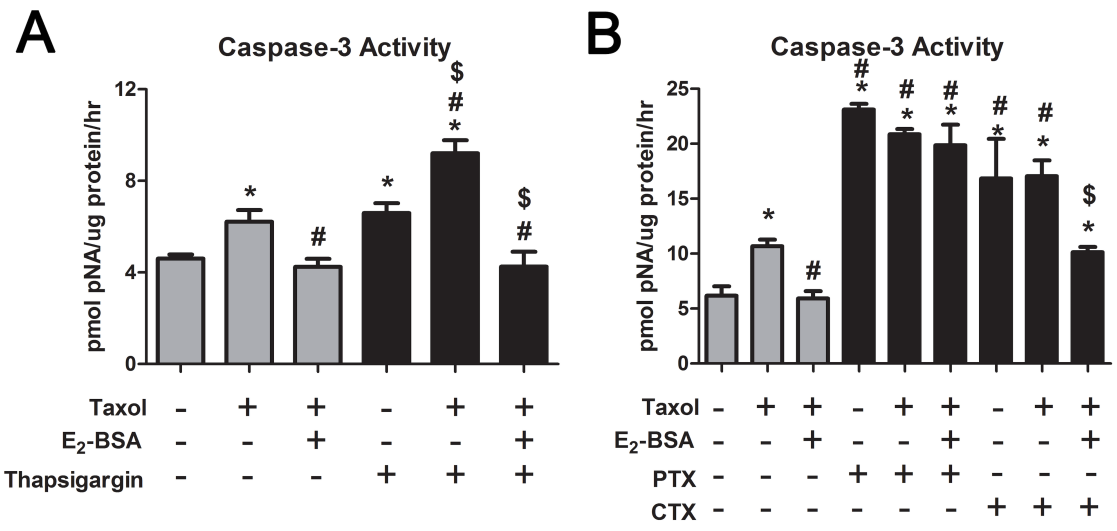

Figure A4.5: Role of Ca^{++} and G-protein Signaling in Apoptotic Signaling of E_2-BSA

(A) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2-BSA , while thapsigargin enhances caspase-3 activity alone and this is also reduced by E_2-BSA . (B) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2-BSA , while pertussis toxin and cholera toxin both enhance caspase-3 activity alone. The effect of CTX is reduced by E_2-BSA . Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone.

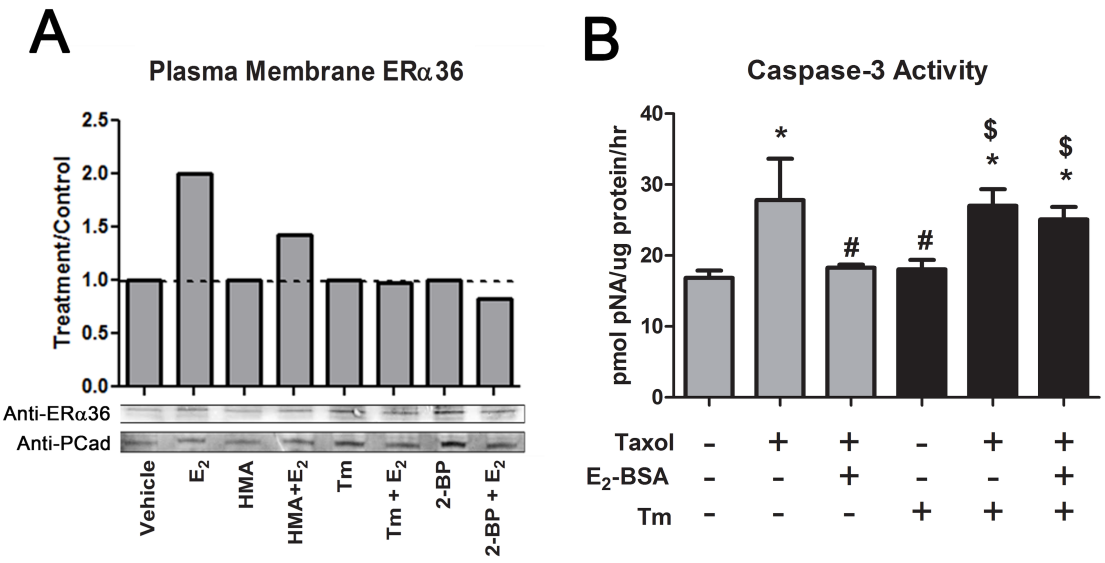

Figure A4.6: Palmitoylation and the Anti-apoptotic Effect of E_2-BSA

(A) Western blot of plasma membrane fractions of HCC38 cells treated with 10^{-8}M E_2 for 9 minutes, pre-treated with 0.5mM HMA, 30μM Tm, or 10μM 2-BP for 2 hours indicates that ERα36 membrane-association occurs rapidly and is blocked by Tm and 2-BP. (B,C) Tm and 2-BP prevent the effect of 10^{-8}M E_2 on (B) Taxol-induced (20μM) caspase-3 activity is reduced by 10^{-8}M E_2-BSA, while Tm (30μM) blocked this effect. Data is represented by the mean of 6 individual samples and error bars represent standard error of the mean. * represents $p<0.05$ compared to the untreated control group while # represents $p<0.05$ compared to 20μM taxol and $ represents $p<0.05$ compared to inhibitor alone. @ represents $p<0.05$ compared to taxol and 2-BP alone.

APPENDIX III: Chapter 5 Supplemental Figures

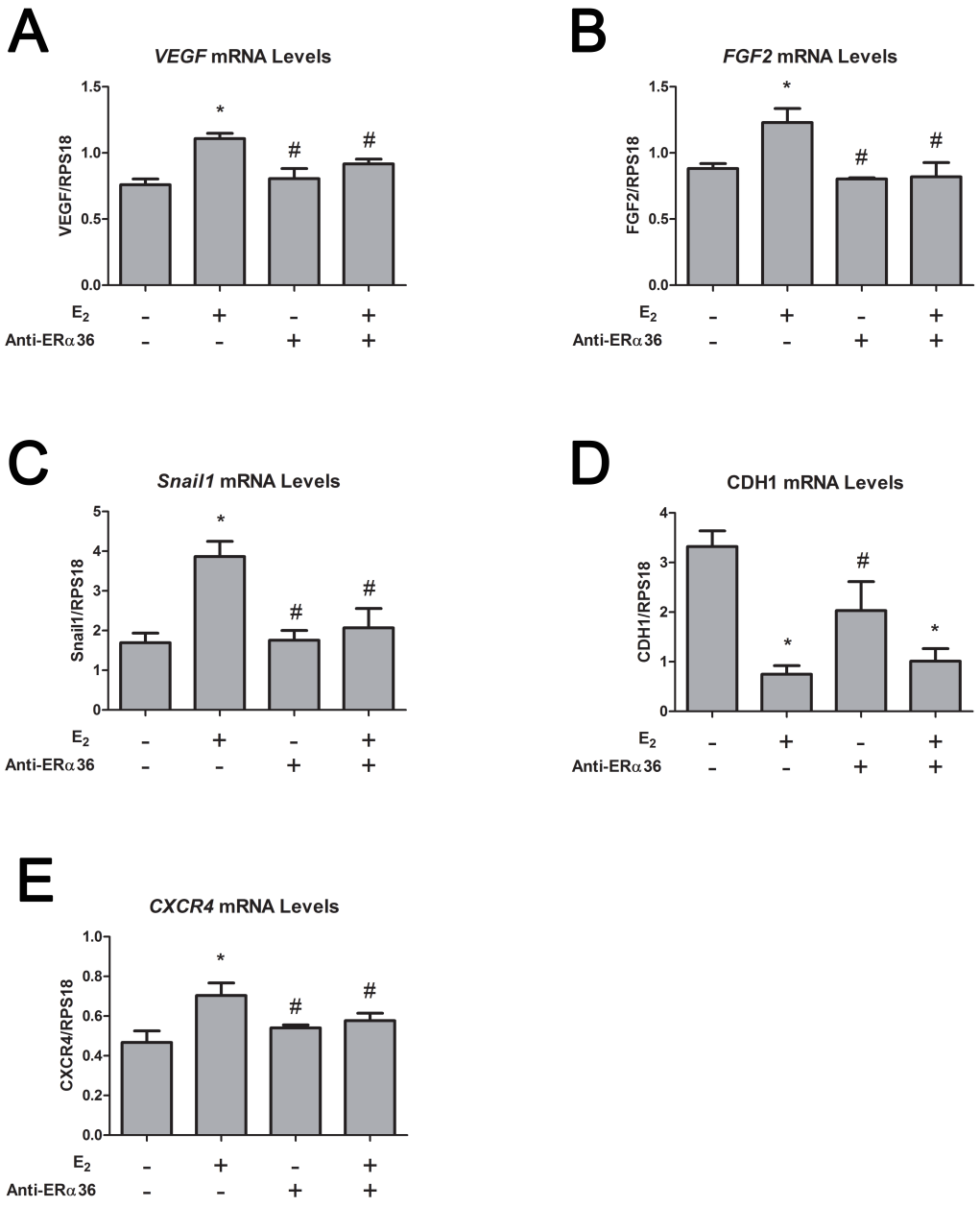

Figure A5.1: mRNA levels normalized to RPS18

10^{-8}M E_2 increased mRNA levels of pro-angiogenic factors VEGF(A) and FGF2(B), and the metastatic factors CXCR4(C) and Snail1(D), while antibodies to ERα36 blocked this effect. The effects on Snail1 were accompanied by a corresponding decrease in E-Cadherin mRNA levels that was also inhibited by ERα36 antibodies(E). All values were normalized to *RPS18* mRNA levels. *p<0.05 vs. corresponding control; #p<0.05 vs. E_2 only.

APPENDIX IV: Chapter 6 Supplemental Tables

Table A6.1: ERα36 number in ER-negative subjects

ERα36 Number in ER Negative Patients (ERα36 Positive in <50% vs >50%)			
Characteristic	Comparison	P-value	Significant?
Age	<50 vs >50	1.0000	No
TNM			
Tumor Size	T0-2 vs T3-4	0.5806	No
Node	N0 vs N1-4	0.3107	No
	N0-1 vs N2-4	1.0000	No
	N0-2 vs N3-4	1.0000	No
Stage	II vs III	0.6730	No
p53 status	Negative vs Positive	0.0691	No
Receptor status			
ER	Negative vs Positive	N/A	N/A
PR	Negative vs Positive	0.5534	No
HER2/neu	Negative vs Positive	0.6291	No
VEGF Scoring			
Number	0 vs 1-3	0.3518	No
	0-1 vs 2-3	0.6219	No
	0-2 vs 3	1.0000	No
Intensity	Low vs High	0.3547	No

Table A6.2: ERα36 location in ER-negative subjects

ERα36 Location in ER- Patients (Nuclear vs Cytosolic)			
Characteristic	**Comparison**	P-value	Significant?
Age	<50 vs >50	0.3746	No
TNM			
Tumor Size	T2 vs T3	0.2894	No
Node	N0 vs N1-3	0.1412	No
	N0-1 vs N2-3	0.6525	No
	N0-2 vs N3	0.5806	No
Stage	II vs III	1.0000	No
p53 status	Negative vs Positive	1.0000	No
Receptor status			
ER	Negative vs Positive	N/A	N/A
PR	Negative vs Positive	1.0000	No
HER2/neu	Negative vs Positive	0.6219	No
VEGF Scoring			
Number	0 vs 1-3	1.0000	No
	0-1 vs 2-3	1.0000	No
	0-2 vs 3	0.2143	No
Intensity	Low vs High	1.0000	No

Table A6.3: ERα36 intensity in ER-negative subjects

ERα36 Intensity in ER Negative Patients (ERα36 Low vs High)			
Characteristic	Comparison	P-value	Significant?
Age	<50 vs >50	0.1282	No
TNM			
Tumor Size	T2 vs T3	1.0000	No
Node	N0 vs N1-3	0.6056	No
	N0-1 vs N2-3	1.0000	No
	N0-2 vs N3	0.2855	No
Stage	II vs III	1.0000	No
p53 status	Negative vs Positive	0.6389	No
Receptor status			
ER	Negative vs Positive	N/A	N/A
PR	Negative vs Positive	1.0000	No
HER2/neu	Negative vs Positive	1.0000	No
VEGF Scoring			
Number	0 vs 1-3	1.0000	No
	0-1 vs 2-3	0.5737	No
	0-2 vs 3	1.0000	No
Intensity	Low vs High	0.1247	No

Table A6.4: ERα36 number in triple negative subjects

ERα36 Number in TN Patients (ERα36 Positive in <50% vs >50%)			
Characteristic	Comparison	P-value	Significant?
Age	<50 vs >50	1.0000	No
TNM			
Tumor Size	T0-2 vs T3-4	0.5378	No
Node	N0 vs N1-4	0.0525	No
	N0-1 vs N2-4	0.6029	No
	N0-2 vs N3-4	0.5193	No
Stage	II vs III	0.5675	No
p53 status	Negative vs Positive	0.2941	No
Receptor status			
ER	Negative vs Positive	N/A	N/A
PR	Negative vs Positive	N/A	N/A
HER2/neu	Negative vs Positive	N/A	N/A
VEGF Scoring		0.2374	No
Number	0 vs 1-3	0.5378	No
	0-1 vs 2-3	0.5378	No
	0-2 vs 3	1.0000	No
Intensity	Low vs High	0.2374	No

Table A6.5: ERα36 location in triple negative subjects

ERα36 Location in TN Patients (Nuclear vs Cytosolic)			
Characteristic	**Comparison**	P-value	Significant?
Age	<50 vs >=50	1.0000	No
TNM			
Tumor Size	T2 vs T3	0.5147	No
Node	N0 vs N1-3	0.5147	No
	N0-1 vs N2-3	0.5368	No
	N0-2 vs N3	0.1206	No
Stage	II vs III	0.5765	No
p53 status	Negative vs Positive	1.0000	No
Receptor status			
ER	Negative vs Positive	N/A	N/A
PR	Negative vs Positive	N/A	N/A
HER2/neu	Negative vs Positive	N/A	N/A
VEGF Scoring			
Number	0 vs 1-3	0.5147	No
	0-1 vs 2-3	1.0000	No
	0-2 vs 3	0.1765	No
Intensity	Low vs High	1.0000	No

Table A6.6: ERα36 intensity in triple negative subjects

ERα36 Intensity in TN Patients (ERα36 Low vs High)			
Characteristic	**Comparison**	P-value	Significant?
Age	<50 vs >50	0.2279	No
TNM			
Tumor Size	T2 vs T3	1.0000	No
Node	N0 vs N1-3	1.0000	No
	N0-1 vs N2-3	0.5368	No
	N0-2 vs N3	0.1206	No
Stage	II vs III	0.5765	No
p53 status	Negative vs Positive	0.5765	No
Receptor status			
ER	Negative vs Positive	N/A	N/A
PR	Negative vs Positive	N/A	N/A
HER2/neu	Negative vs Positive	N/A	N/A
VEGF Scoring			
Number	0 vs 1-3	1.0000	No
	0-1 vs 2-3	1.0000	No
	0-2 vs 3	1.0000	No
Intensity	Low vs High	0.5147	No

Table A6.7. Survival in subjects with ER-negative

Survival in ER-negative Subjects		
Variable	**Significant?**	**Log-Rank P-value**
ERα36 Number	no	0.9740
ERα36 Localization	no	0.2512
ERα36 Intensity	no	0.8832
ER status	N/A	N/A
PR status	no	0.2728
HER2 status	no	0.0530
VEGF Number	no	0.6995
VEGF Intensity	no	0.3634
P53 status	no	0.0869
Stage	no	0.0618
Tumor size (T2 vs T3)	no	0.3052
Node status (0 vs 1-3)	no	0.1340
Node status (0-1 vs, 2-3)	**yes**	**0.0368**
Node status (0-2 vs 3)	**yes**	**0.0079**
Age (<50 vs >50)	no	0.4549

Table A6.8. Survival in subjects with TNBC

Survival in TNBC Subjects		
Variable	**Significant?**	**Log-Rank P-value**
ERα36 Number	no	0.9740
ERα36 Localization	no	0.1663
ERα36 Intensity	no	0.2437
ER status	N/A	N/A
PR status	N/A	N/A
HER2 status	N/A	N/A
VEGF Number	no	0.5450
VEGF Intensity	no	0.2846
P53 status	no	0.1208
Stage	no	0.1208
Tumor size (T2 vs T3)	no	0.5450
Node status (0 vs 1-3)	no	0.3507
Node status (0-1 vs, 2-3)	no	0.0788
Node status (0-2 vs 3)	**yes**	**0.0079**
Age (<50 vs >50)	no	0.2242

REFERENCES

1. Stuart GR, Oda Y, de Boer JG, Glickman BW. Mutation frequency and specificity with age in liver, bladder and brain of lacI transgenic mice. Genetics. 2000;154(3):1291-300. PMCID: 1460990.

2. Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, et al. Cancer is a preventable disease that requires major lifestyle changes. Pharm Res. 2008;25(9):2097-116. PMCID: 2515569.

3. Lombard M, Pastoret PP, Moulin AM. A brief history of vaccines and vaccination. Rev Sci Tech. 2007;26(1):29-48.

4. Reeder JG, Vogel VG. Breast cancer prevention. Cancer Treat Res. 2008;141:149-64.

5. SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations) [database on the Internet]. National Cancer Institute. Bethesda, MD, . 2012. Available from: http://seer.cancer.gov/csr/1975_2009_pops09/, .

6. Barlow JJ, Logan CM. Estrogen secretion, biosynthesis and metabolism: their relationship to the menstrual cycle. Steroids. 1966;7(4):309-20.

7. Guiochon-Mantel A, Milgrom E, Schaison G. [Estrogen biosynthesis and receptors]. Ann Endocrinol (Paris). 1999;60(5):381-91.

8. Häggström M RD. Steroidogenesis. In: steroidogenesis.svg, editor. Wikipedia: Wikipedia; 2009.

9. Kamat A, Hinshelwood MM, Murry BA, Mendelson CR. Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans. Trends Endocrinol Metab. 2002;13(3):122-8.

10. Nagatomi K. [Estrogen biosynthesis in the human ovary]. Nihon Sanka Fujinka Gakkai Zasshi. 1968;20(9):1098-104.

11. Kinoshita Y, Honjo H. [Estrogen structure and biosynthesis]. Nihon Rinsho. 2006;64 Suppl 4:339-45.

12. Frank GR. Role of estrogen and androgen in pubertal skeletal physiology. Med Pediatr Oncol. 2003;41(3):217-21.

13. Wronski TJ, Cintron M, Doherty AL, Dann LM. Estrogen treatment prevents osteopenia and depresses bone turnover in ovariectomized rats. Endocrinology. 1988;123(2):681-6.

14. Kato S. [Hormones and osteoporosis update. Estrogen and bone remodeling]. Clin Calcium. 2009;19(7):951-6.

15. Almeida M, Iyer S, Martin-Millan M, Bartell SM, Han L, Ambrogini E, et al. Estrogen receptor-alpha signaling in osteoblast progenitors stimulates cortical bone accrual. J Clin Invest. 2013;123(1):394-404. PMCID: 3533305.

16. Gulyaeva LF, Mikhailova ON, PustyInyak VO, Kim IVt, Gerasimov AV, Krasilnikov SE, et al. Comparative analysis of SNP in estrogen-metabolizing enzymes for ovarian, endometrial, and breast cancers in Novosibirsk, Russia. Adv Exp Med Biol. 2008;617:359-66.

17. Bellido T, Girasole G, Passeri G, Yu XP, Mocharla H, Jilka RL, et al. Demonstration of estrogen and vitamin D receptors in bone marrow-derived stromal cells: up-regulation of the estrogen receptor by 1,25-dihydroxyvitamin-D3. Endocrinology. 1993;133(2):553-62.

18. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83(6):835-9.

19. Almeida M, Han L, O'Brien C A, Kousteni S, Manolagas SC. Classical genotropic versus kinase-initiated regulation of gene transcription by the estrogen receptor alpha. Endocrinology. 2006;147(4):1986-96.

20. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, et al. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell. 2001;104(5):719-30.

21. Levin ER. Cellular Functions of the Plasma Membrane Estrogen Receptor. Trends Endocrinol Metab. 1999;10(9):374-7.

22. Evinger AJ, 3rd, Levin ER. Requirements for estrogen receptor alpha membrane localization and function. Steroids. 2005;70(5-7):361-3.

23. Huhtakangas JA, Olivera CJ, Bishop JE, Zanello LP, Norman AW. The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25(OH)2-vitamin D3 in vivo and in vitro. Mol Endocrinol. 2004;18(11):2660-71.

24. Norman AW, Olivera CJ, Barreto Silva FR, Bishop JE. A specific binding protein/receptor for 1alpha,25-dihydroxyvitamin D(3) is present in an intestinal caveolae membrane fraction. Biochem Biophys Res Commun. 2002;298(3):414-9.

25. Capiati D, Benassati S, Boland RL. 1,25(OH)2-vitamin D3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. J Cell Biochem. 2002;86(1):128-35.

26. Boyan BD, Chen J, Schwartz Z. Mechanism of Pdia3-dependent 1alpha,25-dihydroxy vitamin D3 signaling in musculoskeletal cells. Steroids. 2012;77(10):892-6.

27. Chen CJ, Nguyen T, Shively JE. Role of calpain-9 and PKC-delta in the apoptotic mechanism of lumen formation in CEACAM1 transfected breast epithelial cells. Exp Cell Res. 2010;316(4):638-48. PMCID: 2868383.

28. Chen J, Olivares-Navarrete R, Wang Y, Herman TR, Boyan BD, Schwartz Z. Protein-disulfide isomerase-associated 3 (Pdia3) mediates the membrane response to 1,25-dihydroxyvitamin D3 in osteoblasts. J Biol Chem. 2010;285(47):37041-50. PMCID: 2978632.

29. Nemere I, Farach-Carson MC, Rohe B, Sterling TM, Norman AW, Boyan BD, et al. Ribozyme knockdown functionally links a 1,25(OH)2D3 membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells. Proc Natl Acad Sci U S A. 2004;101(19):7392-7. PMCID: 409929.

30. Norman AW, Nemere I, Zhou LX, Bishop JE, Lowe KE, Maiyar AC, et al. 1,25(OH)2-vitamin D3, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. J Steroid Biochem Mol Biol. 1992;41(3-8):231-40.

31. Levin ER. Cell localization, physiology, and nongenomic actions of estrogen receptors. J Appl Physiol. 2001;91(4):1860-7.

32. Norman AW, Mizwicki MT, Norman DP. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat Rev Drug Discov. 2004;3(1):27-41.

33. Anderson RG. The caveolae membrane system. Annu Rev Biochem. 1998;67:199-225.

34. Boyan BD, Wang L, Wong KL, Jo H, Schwartz Z. Plasma membrane requirements for 1alpha,25(OH)2D3 dependent PKC signaling in chondrocytes and osteoblasts. Steroids. 2006;71(4):286-90.

35. Chambliss KL, Shaul PW. Rapid activation of endothelial NO synthase by estrogen: evidence for a steroid receptor fast-action complex (SRFC) in caveolae. Steroids. 2002;67(6):413-9.

36. Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, et al. Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. Circ Res. 2000;87(11):E44-52.

37. Kim HP, Lee JY, Jeong JK, Bae SW, Lee HK, Jo I. Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. Biochem Biophys Res Commun. 1999;263(1):257-62.

38. Gilad LA, Schwartz B. Association of estrogen receptor beta with plasma-membrane caveola components: implication in control of vitamin D receptor. J Mol Endocrinol. 2007;38(6):603-18.

39. Li L, Haynes MP, Bender JR. Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proc Natl Acad Sci U S A. 2003;100(8):4807-12. PMCID: 153637.

40. Doroudi M, Schwartz Z, Boyan BD. Phospholipase A2 activating protein is required for 1alpha,25-dihydroxyvitamin D3 dependent rapid activation of protein kinase C via Pdia3. J Steroid Biochem Mol Biol. 2012;132(1-2):48-56.

41. Bondar G, Kuo J, Hamid N, Micevych P. Estradiol-induced estrogen receptor-alpha trafficking. J Neurosci. 2009;29(48):15323-30. PMCID: 2836237.

42. Straub SG, Sharp GW, Meglasson MD, De Souza CJ. Progesterone inhibits insulin secretion by a membrane delimited, non-genomic action. Biosci Rep. 2001;21(5):653-66.

43. Gamberucci A, Giunti R, Benedetti A. Progesterone inhibits capacitative Ca2+ entry in Jurkat T lymphocytes by a membrane delimited mechanism, independently of plasma membrane depolarization. Cell Calcium. 2004;36(2):175-80.

44. Brown AM. Membrane-delimited cell signaling complexes: direct ion channel regulation by G proteins. J Membr Biol. 1993;131(2):93-104.

45. Mizukami Y. In vivo functions of GPR30/GPER-1, a membrane receptor for estrogen: from discovery to functions in vivo. Endocr J. 2010;57(2):101-7.

46. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annu Rev Physiol. 2008;70:165-90.

47. Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Ando S, et al. 17 beta-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ER alpha. Mol Cell Endocrinol. 2010;320(1-2):136-44.

48. Filardo EJ, Quinn JA, Sabo E. Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. Steroids. 2008;73(9-10):870-3.

49. Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, et al. Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology. 2007;148(7):3236-45.

50. Nadal A, Ropero AB, Fuentes E, Soria B. The plasma membrane estrogen receptor: nuclear or unclear? Trends Pharmacol Sci. 2001;22(12):597-9.

51. Arvanitis DN, Wang H, Bagshaw RD, Callahan JW, Boggs JM. Membrane-associated estrogen receptor and caveolin-1 are present in central nervous system myelin and oligodendrocyte plasma membranes. J Neurosci Res. 2004;75(5):603-13.

52. Azuma K, Horie K, Inoue S, Ouchi Y, Sakai R. Analysis of estrogen receptor alpha signaling complex at the plasma membrane. FEBS Lett. 2004;577(3):339-44.

53. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochem Biophys Res Commun. 2005;336(4):1023-7.

54. Chaudhri RA, Olivares-Navarrete R, Cuenca N, Hadadi A, Boyan BD, Schwartz Z. Membrane estrogen signaling enhances tumorigenesis and metastatic potential of breast cancer cells via estrogen receptor-alpha36 (ERalpha36). J Biol Chem. 2012;287(10):7169-81. PMCID: 3293594.

55. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. A variant of estrogen receptor-{alpha}, hER-{alpha}36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. Proc Natl Acad Sci U S A. 2006;103(24):9063-8. PMCID: 1482566.

56. Lau MY, Han H, Hu J, Ji C. Association of cyclin D and estrogen receptor alpha36 with hepatocellular adenomas of female mice under chronic endoplasmic reticulum stress. J Gastroenterol Hepatol. 2013;28(3):576-83. PMCID: 3584191.

57. Boyan BD, Sylvia VL, Frambach T, Lohmann CH, Dietl J, Dean DD, et al. Estrogen-dependent rapid activation of protein kinase C in estrogen receptor-positive MCF-7 breast cancer cells and estrogen receptor-negative HCC38 cells is membrane-mediated and inhibited by tamoxifen. Endocrinology. 2003;144(5):1812-24.

58. Cicin I, Karagol H, Usta U, Sezer A, Uzunoglu S, Alas-Cosar R, et al. Triple negative breast cancer compared to hormone receptor negative/HER2 positive breast cancer. Med Oncol. 2009;26(3):335-43.

59. Lin SL, Yan LY, Liang XW, Wang ZB, Wang ZY, Qiao J, et al. A novel variant of ER-alpha, ER-alpha36 mediates testosterone-stimulated ERK and Akt activation in endometrial cancer Hec1A cells. Reprod Biol Endocrinol. 2009;7:102. PMCID: 2761922.

60. Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, et al. G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. Cancer Res. 2007;67(4):1859-66.

61. Kang L, Zhang X, Xie Y, Tu Y, Wang D, Liu Z, et al. Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. Mol Endocrinol.24(4):709-21.

62. Ito K. Hormone replacement therapy and cancers: the biological roles of estrogen and progestin in tumorigenesis are different between the endometrium and breast. Tohoku J Exp Med. 2007;212(1):1-12.

63. Larsson SC, Bergkvist L, Wolk A. Folate intake and risk of breast cancer by estrogen and progesterone receptor status in a Swedish cohort. Cancer Epidemiol Biomarkers Prev. 2008;17(12):3444-9.

64. Brinton LA, Schwartz L, Spitz MR, Park Y, Hollenbeck AR, Gierach GL. Unopposed estrogen and estrogen plus progestin menopausal hormone therapy and lung cancer risk in the NIH-AARP Diet and Health Study Cohort. Cancer Causes Control. 2012;23(3):487-96. PMCID: 3328187.

65. Bakken K, Alsaker E, Eggen AE, Lund E. Hormone replacement therapy and incidence of hormone-dependent cancers in the Norwegian Women and Cancer study. Int J Cancer. 2004;112(1):130-4.

66. Calle EE, Miracle-McMahill HL, Thun MJ, Heath CW, Jr. Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. J Natl Cancer Inst. 1995;87(7):517-23.

67. Persson I, Yuen J, Bergkvist L, Schairer C. Cancer incidence and mortality in women receiving estrogen and estrogen-progestin replacement therapy--long-term follow-up of a Swedish cohort. Int J Cancer. 1996;67(3):327-32.

68. Hurst-Kennedy J, Zhong M, Gupta V, Boyan BD, Schwartz Z. 24R,25-Dihydroxyvitamin D3, lysophosphatidic acid, and p53: A signaling axis in the inhibition of phosphate-induced chondrocyte apoptosis. J Steroid Biochem Mol Biol. 2010.

69. Razandi M, Pedram A, Levin ER. Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. Mol Endocrinol. 2000;14(9):1434-47.

70. Kampa M, Pelekanou V, Castanas E. Membrane-initiated steroid action in breast and prostate cancer. Steroids. 2008;73(9-10):953-60.

71. DeNardo DG, Cuba VL, Kim H, Wu K, Lee AV, Brown PH. Estrogen receptor DNA binding is not required for estrogen-induced breast cell growth. Mol Cell Endocrinol. 2007;277(1-2):13-25.

72. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13(15 Pt 1):4429-34.

73. Ferno M, Baldetorp B, Bendahl PO, Borg A, Ewers SB, Olsson H, et al. Recurrence-free survival in breast cancer improved by adjuvant tamoxifen--especially for progesterone receptor positive tumors with a high proliferation. Breast Cancer Res Treat. 1995;36(1):23-34.

74. Madsen MW, Reiter BE, Lykkesfeldt AE. Differential expression of estrogen receptor mRNA splice variants in the tamoxifen resistant human breast cancer cell line, MCF-7/TAMR-1 compared to the parental MCF-7 cell line. Mol Cell Endocrinol. 1995;109(2):197-207.

75. Poola I, Koduri S, Chatra S, Clarke R. Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach. J Steroid Biochem Mol Biol. 2000;72(5):249-58.

76. Sylvia VL, Boyan BD, Dean DD, Schwartz Z. The membrane effects of 17beta-estradiol on chondrocyte phenotypic expression are mediated by activation of protein kinase C through phospholipase C and G-proteins. J Steroid Biochem Mol Biol. 2000;73(5):211-24.

77. Song RX, Zhang Z, Santen RJ. Estrogen rapid action via protein complex formation involving ERalpha and Src. Trends Endocrinol Metab. 2005;16(8):347-53.

78. Kim KH, Bender JR. Membrane-initiated actions of estrogen on the endothelium. Mol Cell Endocrinol. 2009;308(1-2):3-8. PMCID: 2701909.

79. Schwartz Z, Sylvia VL, Guinee T, Dean DD, Boyan BD. Tamoxifen elicits its anti-estrogen effects in growth plate chondrocytes by inhibiting protein kinase C. J Steroid Biochem Mol Biol. 2002;80(4-5):401-10.

80. McMillan J, Fatehi-Sedeh S, Sylvia VL, Bingham V, Zhong M, Boyan BD, et al. Sex-specific regulation of growth plate chondrocytes by estrogen is via multiple MAP kinase signaling pathways. Biochim Biophys Acta. 2006;1763(4):381-92.

81. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 2005;307(5715):1625-30.

82. Kang L, Zhang X, Xie Y, Tu Y, Wang D, Liu Z, et al. Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. Mol Endocrinol. 2010;24(4):709-21. PMCID: 2852353.

83. Hu P, Kinyamu HK, Wang L, Martin J, Archer TK, Teng C. Estrogen induces estrogen-related receptor alpha gene expression and chromatin structural changes in estrogen receptor (ER)-positive and ER-negative breast cancer cells. J Biol Chem. 2008;283(11):6752-63.

84. Touitou I, Mathieu M, Rochefort H. Stable transfection of the estrogen receptor cDNA into Hela cells induces estrogen responsiveness of endogenous cathepsin D gene but not of cell growth. Biochem Biophys Res Commun. 1990;169(1):109-15.

85. Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, Grohe C. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem. 2000;275(24):18447-53.

86. Smart EJ, Ying YS, Mineo C, Anderson RG. A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci U S A. 1995;92(22):10104-8.

87. Denison TA, Koch CF, Shapiro IM, Schwartz Z, Boyan BD. Inorganic phosphate modulates responsiveness to 24,25(OH)2D3 in chondrogenic ATDC5 cells. J Cell Biochem. 2009;107(1):155-62.

88. Sui M, Huang Y, Park BH, Davidson NE, Fan W. Estrogen receptor alpha mediates breast cancer cell resistance to paclitaxel through inhibition of apoptotic cell death. Cancer Res. 2007;67(11):5337-44.

89. Zhong M, Carney DH, Ryaby JT, Schwartz Z, Boyan BD. Inhibition of phosphate-induced apoptosis in resting zone chondrocytes by thrombin peptide 508. Cells Tissues Organs. 2009;189(1-4):56-9.

90. Akhtari M, Mansuri J, Newman KA, Guise TM, Seth P. Biology of breast cancer bone metastasis. Cancer Biol Ther. 2008;7(1):3-9.

91. Rose AA, Siegel PM. Breast cancer-derived factors facilitate osteolytic bone metastasis. Bull Cancer. 2006;93(9):931-43.

92. Nakayama T, Mutsuga N, Tosato G. FGF2 posttranscriptionally down-regulates expression of SDF1 in bone marrow stromal cells through FGFR1 IIIc. Blood. 2007;109(4):1363-72. PMCID: 1794071.

93. Kousidou O, Berdiaki A, Kletsas D, Zafiropoulos A, Theocharis AD, Tzanakakis GN, et al. Estradiol-estrogen receptor: a key interplay of the expression of syndecan-2 and metalloproteinase-9 in breast cancer cells. Mol Oncol. 2008;2(3):223-32.

94. Merrell M, Suarez-Cuervo C, Harris KW, Vaananen HK, Selander KS. Bisphosphonate induced growth inhibition of breast cancer cells is augmented by p38 inhibition. Breast Cancer Res Treat. 2003;81(3):231-41.

95. Peng J, Jordan VC. Expression of estrogen receptor alpha with a Tet-off adenoviral system induces G0/G1 cell cycle arrest in SKBr3 breast cancer cells. Int J Oncol. 2010;36(2):451-8. PMCID: 2842990.

96. Revankar CM, Mitchell HD, Field AS, Burai R, Corona C, Ramesh C, et al. Synthetic estrogen derivatives demonstrate the functionality of intracellular GPR30. ACS Chem Biol. 2007;2(8):536-44.

97. Olde B, Leeb-Lundberg LM. GPR30/GPER1: searching for a role in estrogen physiology. Trends Endocrinol Metab. 2009;20(8):409-16.

98. Buitrago C, Boland R. Caveolae and caveolin-1 are implicated in 1alpha,25(OH)2-vitamin D3-dependent modulation of Src, MAPK cascades and VDR localization in skeletal muscle cells. J Steroid Biochem Mol Biol.121(1-2):169-75.

99. Marino M, Distefano E, Caporali S, Ceracchi G, Pallottini V, Trentalance A. beta-estradiol stimulation of DNA synthesis requires different PKC isoforms in HepG2 and MCF7 cells. J Cell Physiol. 2001;188(2):170-7.

100. Carey I, Noti JD. Isolation of protein kinase C-alpha-regulated cDNAs associated with breast tumor aggressiveness by differential mRNA display. Int J Oncol. 1999;14(5):951-6.

101. Weldon CB, McKee A, Collins-Burow BM, Melnik LI, Scandurro AB, McLachlan JA, et al. PKC-mediated survival signaling in breast carcinoma cells: a role for MEK1-AP1 signaling. Int J Oncol. 2005;26(3):763-8.

102. Jain S, Chakraborty G, Kundu GC. The crucial role of cyclooxygenase-2 in osteopontin-induced protein kinase C alpha/c-Src/IkappaB kinase alpha/beta-dependent prostate tumor progression and angiogenesis. Cancer Res. 2006;66(13):6638-48.

103. Ways DK, Kukoly CA, deVente J, Hooker JL, Bryant WO, Posekany KJ, et al. MCF-7 breast cancer cells transfected with protein kinase C-alpha exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. J Clin Invest. 1995;95(4):1906-15. PMCID: 295735.

104. Connolly J, Rose D. Expression of the invasive phenotype by MCF-7 human breast cancer cells transfected to overexpress protein kinase C-alpha or the erbB2 proto-oncogene. Int J Oncol. 1997;10(1):71-6.

105. Tomaskovic-Crook E, Thompson EW, Thiery JP. Epithelial to mesenchymal transition and breast cancer. Breast Cancer Res. 2009;11(6):213.

106. Blick T, Widodo E, Hugo H, Waltham M, Lenburg ME, Neve RM, et al. Epithelial mesenchymal transition traits in human breast cancer cell lines. Clin Exp Metastasis. 2008;25(6):629-42.

107. Vincent-Salomon A, Thiery JP. Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. Breast Cancer Res. 2003;5(2):101-6.

108. Deng H, Huang X, Fan J, Wang L, Xia Q, Yang X, et al. A variant of estrogen receptor-alpha, ER-alpha36 is expressed in human gastric cancer and is highly correlated with lymph node metastasis. Oncol Rep. 2010;24(1):171-6.

109. Zhong M, Carney DH, Boyan BD, Schwartz Z. 17beta-Estradiol regulates rat growth plate chondrocyte apoptosis through a mitochondrial pathway not involving nitric oxide or MAPKs. Endocrinology. 2011;152(1):82-92.

110. Herranz N, Pasini D, Diaz VM, Franci C, Gutierrez A, Dave N, et al. Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. Mol Cell Biol. 2008;28(15):4772-81. PMCID: 2493371.

111. Kiyotani K, Mushiroda T, Sasa M, Bando Y, Sumitomo I, Hosono N, et al. Impact of CYP2D6*10 on recurrence-free survival in breast cancer patients receiving adjuvant tamoxifen therapy. Cancer Sci. 2008;99(5):995-9.

112. DiMartino L, Demontis B, Mitchell IP, Hayward SW, Deshpande N. A randomized clinical trial to investigate the usefulness of the addition of prednisolone to tamoxifen as adjuvants to mastectomy in primary breast cancer patients with a high risk of recurrence: a preliminary report. Anticancer Res. 1991;11(2):869-72.

113. Harris EE, Correa C, Hwang WT, Liao J, Litt HI, Ferrari VA, et al. Late cardiac mortality and morbidity in early-stage breast cancer patients after breast-conservation treatment. J Clin Oncol. 2006;24(25):4100-6.

114. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc. 1971;93(9):2325-7.

115. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci U S A. 1980;77(3):1561-5. PMCID: 348536.

116. Zhang YL, Zhang ZH, Jiang TY, Ayman W, Jing L, Lv HX, et al. Cell uptake of paclitaxel solid lipid nanoparticles modified by cell-penetrating peptides in A549 cells. Pharmazie. 2013;68(1):47-53.

117. Hartmann JT, Fels LM, Knop S, Stolt H, Kanz L, Bokemeyer C. A randomized trial comparing the nephrotoxicity of cisplatin/ifosfamide-based combination chemotherapy with or without amifostine in patients with solid tumors. Invest New Drugs. 2000;18(3):281-9.

118. Schmid P, Schippinger W, Nitsch T, Huebner G, Heilmann V, Schultze W, et al. Up-front tandem high-dose chemotherapy compared with standard chemotherapy with

doxorubicin and paclitaxel in metastatic breast cancer: results of a randomized trial. J Clin Oncol. 2005;23(3):432-40.

119. Thorpe SM. Immunological quantitation of nuclear receptors in human breast cancer: relation to cytosolic estrogen and progesterone receptors. Cancer Res. 1987;47(7):1830-5.

120. Jatoi I, Chen BE, Anderson WF, Rosenberg PS. Breast cancer mortality trends in the United States according to estrogen receptor status and age at diagnosis. J Clin Oncol. 2007;25(13):1683-90.

121. Schneider J, Ruschhaupt M, Buness A, Asslaber M, Regitnig P, Zatloukal K, et al. Identification and meta-analysis of a small gene expression signature for the diagnosis of estrogen receptor status in invasive ductal breast cancer. Int J Cancer. 2006;119(12):2974-9.

122. Fisher B, Redmond C, Brown A, Fisher ER, Wolmark N, Bowman D, et al. Adjuvant chemotherapy with and without tamoxifen in the treatment of primary breast cancer: 5-year results from the National Surgical Adjuvant Breast and Bowel Project Trial. J Clin Oncol. 1986;4(4):459-71.

123. Xie H, Sun M, Liao XB, Yuan LQ, Sheng ZF, Meng JC, et al. Estrogen receptor alpha36 mediates a bone-sparing effect of 17beta-estrodiol in postmenopausal women. J Bone Miner Res. 2011;26(1):156-68.

124. Rao J, Jiang X, Wang Y, Chen B. Advances in the understanding of the structure and function of ER-alpha36,a novel variant of human estrogen receptor-alpha. J Steroid Biochem Mol Biol. 2011;127(3-5):231-7.

125. Green PS, Yang SH, Nilsson KR, Kumar AS, Covey DF, Simpkins JW. The nonfeminizing enantiomer of 17beta-estradiol exerts protective effects in neuronal cultures and a rat model of cerebral ischemia. Endocrinology. 2001;142(1):400-6.

126. Rajendran KG, Lopez T, Parikh I. Estrogenic effect of phenol red in MCF-7 cells is achieved through activation of estrogen receptor by interacting with a site distinct from the steroid binding site. Biochem Biophys Res Commun. 1987;142(3):724-31.

127. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A. 1986;83(8):2496-500. PMCID: 323325.

128. Finckbone V, Oomman SK, Strahlendorf HK, Strahlendorf JC. Regional differences in the temporal expression of non-apoptotic caspase-3-positive bergmann glial cells in the developing rat cerebellum. Front Neuroanat. 2009;3:3. PMCID: 2691149.

129. Gulyaeva NV. Non-apoptotic functions of caspase-3 in nervous tissue. Biochemistry (Mosc). 2003;68(11):1171-80.

130. Sztiller-Sikorska M, Jakubowska J, Wozniak M, Stasiak M, Czyz M. A non-apoptotic function of caspase-3 in pharmacologically-induced differentiation of K562 cells. Br J Pharmacol. 2009;157(8):1451-62. PMCID: 2765302.

131. Zhao X, Wang D, Zhao Z, Xiao Y, Sengupta S, Zhang R, et al. Caspase-3-dependent activation of calcium-independent phospholipase A2 enhances cell migration in non-apoptotic ovarian cancer cells. J Biol Chem. 2006;281(39):29357-68.

132. Sylvia VL, Walton J, Lopez D, Dean DD, Boyan BD, Schwartz Z. 17 beta-estradiol-BSA conjugates and 17 beta-estradiol regulate growth plate chondrocytes by common membrane associated mechanisms involving PKC dependent and independent signal transduction. J Cell Biochem. 2001;81(3):413-29.

133. Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE. Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology. 1999;140(11):5455-8.

134. Bryksin AV, Matsumura I. Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. Biotechniques. 2010;48(6):463-5. PMCID: 3121328.

135. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63.

136. Sylvia VL, Schwartz Z, Del Toro F, DeVeau P, Whetstone R, Hardin RR, et al. Regulation of phospholipase D (PLD) in growth plate chondrocytes by 24R,25-(OH)2D3 is dependent on cell maturation state (resting zone cells) and is specific to the PLD2 isoform. Biochim Biophys Acta. 2001;1499(3):209-21.

137. Schwartz Z, Sylvia VL, Luna MH, DeVeau P, Whetstone R, Dean DD, et al. The effect of 24R,25-(OH)(2)D(3) on protein kinase C activity in chondrocytes is mediated by phospholipase D whereas the effect of 1alpha,25-(OH)(2)D(3) is mediated by phospholipase C. Steroids. 2001;66(9):683-94.

138. Derossi D, Williams EJ, Green PJ, Dunican DJ, Doherty P. Stimulation of mitogenesis by a cell-permeable PI 3-kinase binding peptide. Biochem Biophys Res Commun. 1998;251(1):148-52.

139. Marino M, Ascenzi P. Membrane association of estrogen receptor alpha and beta influences 17beta-estradiol-mediated cancer cell proliferation. Steroids. 2008;73(9-10):853-8.

140. Anania VG, Coscoy L. Palmitoylation of MIR2 is required for its function. J Virol. 2011;85(5):2288-95. PMCID: 3067770.

141. Meden H, Rath W, Kuhn W. [Taxol--a new cytostatic drug for therapy of ovarian and breast cancer]. Geburtshilfe Frauenheilkd. 1994;54(4):187-93.

142. Gunby P. National Cancer Institute making more taxol available for refractory ovarian, breast cancers. JAMA. 1992;268(14):1824-5.

143. Taxol improves outlook for lung, breast, and ovarian cancer. Oncology (Williston Park). 1992;6(8):16.

144. Borovik R, Steiner M, Atad J, Sneiderman B, Rosenberg T, Palti S. [Taxol (paclitaxel) as second-line therapy in breast and ovarian cancer]. Harefuah. 1998;134(8):605-8, 71.

145. Razandi M, Pedram A, Merchenthaler I, Greene GL, Levin ER. Plasma membrane estrogen receptors exist and functions as dimers. Mol Endocrinol. 2004;18(12):2854-65.

146. Morgensztern D, McLeod HL. PI3K/Akt/mTOR pathway as a target for cancer therapy. Anticancer Drugs. 2005;16(8):797-803.

147. Cortot A, Armand JP, Soria JC. [PI3K-AKT-mTOR pathway inhibitors]. Bull Cancer. 2006;93(1):19-26.

148. Yap TA, Garrett MD, Walton MI, Raynaud F, de Bono JS, Workman P. Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. Curr Opin Pharmacol. 2008;8(4):393-412.

149. LoPiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. Drug Resist Updat. 2008;11(1-2):32-50. PMCID: 2442829.

150. Hurst-Kennedy J, Boyan BD, Schwartz Z. Lysophosphatidic acid signaling promotes proliferation, differentiation, and cell survival in rat growth plate chondrocytes. Biochim Biophys Acta. 2009;1793(5):836-46.

151. Yager JD, Liehr JG. Molecular mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol. 1996;36:203-32.

152. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res. 1987;47(16):4355-60.

153. van den Brule FA, Engel J, Stetler-Stevenson WG, Liu FT, Sobel ME, Castronovo V. Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progestin in human breast-cancer cells. Int J Cancer. 1992;52(4):653-7.

154. Carruba G. Estrogen and prostate cancer: an eclipsed truth in an androgen-dominated scenario. J Cell Biochem. 2007;102(4):899-911.

155. Enmark E, Gustafsson JA. Oestrogen receptors - an overview. J Intern Med. 1999;246(2):133-8.

156. Cook MB, Dawsey SM, Freedman ND, Inskip PD, Wichner SM, Quraishi SM, et al. Sex disparities in cancer incidence by period and age. Cancer Epidemiol Biomarkers Prev. 2009;18(4):1174-82. PMCID: 2793271.

157. Reiner Z, Cvrtila D, Petric V. Cytoplasmic steroid receptors in cancer of the larynx. Arch Otorhinolaryngol. 1988;245(1):47-9.

158. Hagedorn HG, Nerlich AG. Analysis of sex-hormone-receptor expression in laryngeal carcinoma. Eur Arch Otorhinolaryngol. 2002;259(4):205-10.

159. Dorsey M, Benghuzzi H, Tucci M, Cason Z. Growth and cell viability of estradiol and IP-6 treated Hep-2 laryngeal carcinoma cells. Biomed Sci Instrum. 2005;41:205-10.

160. Robbins KT, Vu TP, Diaz A, Varki NM. Growth effects of tamoxifen and estradiol on laryngeal carcinoma cell lines. Arch Otolaryngol Head Neck Surg. 1994;120(11):1261-6.

161. Ferrandina G, Almadori G, Maggiano N, Lanza P, Ferlini C, Cattani P, et al. Growth-inhibitory effect of tamoxifen and quercetin and presence of type II estrogen binding sites in human laryngeal cancer cell lines and primary laryngeal tumors. Int J Cancer. 1998;77(5):747-54.

162. Pinto MP, Badtke MM, Dudevoir ML, Harrell JC, Jacobsen BM, Horwitz KB. Vascular endothelial growth factor secreted by activated stroma enhances angiogenesis and hormone-independent growth of estrogen receptor-positive breast cancer. Cancer Res. 2010;70(7):2655-64. PMCID: 2848872.

163. Yamasaki T, Tsuda H, Imazeki N, Matsubara O. Vascular endothelial growth factor mRNA levels quantified by reverse transcription-polymerase chain reaction in microdissected breast carcinoma tissues are correlated with histological type and grade of both invasive and intraductal components. Pathol Int. 2005;55(5):255-63.

164. Van der Auwera I, Van Laere SJ, Van den Eynden GG, Benoy I, van Dam P, Colpaert CG, et al. Increased angiogenesis and lymphangiogenesis in inflammatory versus noninflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. Clin Cancer Res. 2004;10(23):7965-71.

165. Mukherjee D, Zhao J. The Role of chemokine receptor CXCR4 in breast cancer metastasis. Am J Cancer Res. 2013;3(1):46-57. PMCID: 3555200.

166. Blobe GC, Obeid LM, Hannun YA. Regulation of protein kinase C and role in cancer biology. Cancer Metastasis Rev. 1994;13(3-4):411-31.

167. Liu J, Pan S, Zhou Y, Yu Z, Liu L. [Expression and significance of IGF-IR and PKC in laryngeal squamous cell carcinoma]. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi. 2010;24(4):152-5.

168. Ahn SJ, Yoon MS, Hyuk S, Han W, Yoon YD, Han JS, et al. Phospholipase C-protein kinase C mediated phospholipase D activation pathway is involved in tamoxifen induced apoptosis. J Cell Biochem. 2003;89(3):520-8.

169. Polyak K. Heterogeneity in breast cancer. J Clin Invest. 2011;121(10):3786-8. PMCID: 3195489.

170. Zhu K, Bernard LJ, Levine RS, Williams SM. Estrogen receptor status of breast cancer: a marker of different stages of tumor or different entities of the disease? Med Hypotheses. 1997;49(1):69-75.

171. Bardon S, Vignon F, Derocq D, Rochefort H. The antiproliferative effect of tamoxifen in breast cancer cells: mediation by the estrogen receptor. Mol Cell Endocrinol. 1984;35(2-3):89-96.

172. Feng S, Wang Y, Wang X, Wang Z, Cui Y, Liu J, et al. Caveolin-1 gene silencing promotes the activation of PI3K/AKT dependent on Eralpha36 and the transformation of MCF10ACE. Sci China Life Sci. 2010;53(5):598-605.

173. Kato I, Toniolo P, Akhmedkhanov A, Koenig KL, Shore R, Zeleniuch-Jacquotte A. Prospective study of factors influencing the onset of natural menopause. J Clin Epidemiol. 1998;51(12):1271-6.

174. Chu KC, Smart CR, Tarone RE. Analysis of breast cancer mortality and stage distribution by age for the Health Insurance Plan clinical trial. J Natl Cancer Inst. 1988;80(14):1125-32.

175. Coburn NG, Chung MA, Fulton J, Cady B. Decreased breast cancer tumor size, stage, and mortality in Rhode Island: an example of a well-screened population. Cancer Control. 2004;11(4):222-30.

176. Duan L, Ye L, Zhao G, Wu Z, Jin C, Cai X, et al. Serum spleen tyrosine kinase and vascular endothelial growth factor-C levels predict lymph node metastasis of oesophageal squamous cell carcinoma. Eur J Cardiothorac Surg. 2013;43(3):e58-63.

177. Li EX, Shi F, Wu YY, Wu Y, Guo JJ, Dong DF. [The relationship between lymphatic metastasis and serum vascular endothelial growth factor C and cyclooxygenase 2 expression in breast cancer]. Zhonghua Yi Xue Za Zhi. 2008;88(2):88-91.

178. Nakamura Y, Yasuoka H, Tsujimoto M, Yoshidome K, Nakahara M, Nakao K, et al. Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. Clin Cancer Res. 2006;12(4):1201-7.

179. Koyama Y, Kaneko K, Akazawa K, Kanbayashi C, Kanda T, Hatakeyama K. Vascular endothelial growth factor-C and vascular endothelial growth factor-d messenger RNA expression in breast cancer: association with lymph node metastasis. Clin Breast Cancer. 2003;4(5):354-60.

180. Lin SL, Yan LY, Zhang XT, Yuan J, Li M, Qiao J, et al. ER-alpha36, a variant of ER-alpha, promotes tamoxifen agonist action in endometrial cancer cells via the MAPK/ERK and PI3K/Akt pathways. PLoS One. 2010;5(2):e9013. PMCID: 2814868.

181. Zhang XT, Kang LG, Ding L, Vranic S, Gatalica Z, Wang ZY. A positive feedback loop of ER-alpha36/EGFR promotes malignant growth of ER-negative breast cancer cells. Oncogene. 2011;30(7):770-80. PMCID: 3020987.

182. Wang J, Li J, Fang R, Xie S, Wang L, Xu C. Expression of ERalpha36 in gastric cancer samples and their matched normal tissues. Oncol Lett. 2012;3(1):172-5. PMCID: 3362625.

183. Zhao YC, Ni XJ, Li Y, Dai M, Yuan ZX, Zhu YY, et al. Peritumoral lymphangiogenesis induced by vascular endothelial growth factor C and D promotes lymph node metastasis in breast cancer patients. World J Surg Oncol. 2012;10:165. PMCID: 3499230.

184. Tekmal RR, Durgam VR. A novel in vitro and in vivo breast cancer model for testing inhibitors of estrogen biosynthesis and its action using mammary tumor cells with an activated int-5/aromatase gene. Cancer Lett. 1997;118(1):21-8.

185. Bulun SE, Price TM, Aitken J, Mahendroo MS, Simpson ER. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. J Clin Endocrinol Metab. 1993;77(6):1622-8.

VITA

REYHAAN ALI CHAUDHRI

Reyhaan Ali Chaudhri was born in Montreal, Quebec, Canada in 1982, and moved to the Dallas, Texas in the United States during elementary school. With his family, he lived in Philadelphia, Pennsylvania, Houston, Texas, and finally settled in Atlanta, Georgia where he attended high school and obtained a Bachelor's of Science in Applied Biology from the Georgia Institute of Technology in 2004. He served as laboratory manager for the tissue engineering laboratory of Dr. Barbara D. Boyan, Ph.D. in the Department of Biomedical Engineering from 2005 to 2007, when he began to pursue his Doctorate of Philosophy in Molecular and Cell Biology from the School of Biology at Georgia Tech. During this time, he received a fellowship from the National Institutes of Health to complete training as a Medical Scientist (TL1) at Emory University, where he also obtained a Master's of Science in Clinical and Translational Research under the mentorship of Dr. Henry Blumberg, M.D. and Dr. Thomas Ziegler, M.D.