5-2011

Cell Cycle Regulatory Roles of Estrogen Receptor alpha (ERα) in Breast Cancer Cells

Sonia JavanMoghaddam

CELL CYCLE REGULATORY ROLES OF ESTROGEN RECEPTOR ALPHA IN BREAST CANCER

by

Sonia Javan Moghaddam, M.S.

APPROVED:

Khandan Keyomarsi, Ph.D.

Janet Price, Ph.D.

Powel Brown, M.D., Ph.D.

Hesham Amin, M.D.

Sadhan Majumder, Ph.D.

George Stancel, Ph.D.

APPROVED:

Dean, The University of Texas
Health Science Center at Houston
Graduate School of Biomedical Sciences

CELL CYCLE REGULATORY ROLES OF ESTROGEN RECEPTOR ALPHA (ERα) IN BREAST CANCER

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Reguirements
for the degree of

DOCTOR OF PHILOSOPHY

by

Sonia Javan Moghaddam
Houston, Texas

May, 2011

ACKNOWLEDGEMENTS

I am heartily thankful to my supervisor, Dr. Khandan Keyomarsi, whose encouragement, guidance and support from the initial to the final point of my studies enabled me to develop an understanding of the ER and breast cancer. The amount of resources dedicated toward breast cancer research in Dr. Keyomarsi's laboratory and her unique approach to training encouraged me to develop independent thinking and trained me to try to see, touch and understand the world as a great scientist as she is.

My time at MD Anderson Cancer Center and seeing the patients has given me the enthusiasm and focus to achieve my goals and this feeling will stay with me forever.

I am also thankful to my excellent committee members throughout these years for their constant support, wisdom and guidance, who are; Dr. Hesham Amin, Dr. Powel Brown, Dr. Paul Chiao, Dr. Suzanne Fuqua, Dr. Ann Killary, Dr. Mong-Hong Lee, Dr. Raymond Meyn, Dr. Sadhan Majumder, Dr. Janet Price and Dr. George Stancel.

The members of the Keyomarsi-Hunt group have contributed immensely to my personal and professional experience. The group has been a source of friendships as well as good advice and collaboration. I am especially grateful for Dr. Hannah Wingate, Dr. Ben Mull and Dr. Kolin Debrucha, the fun group of grad students who stuck it out in grad school with me. I will not be able to thank Hannah enough for all the time she spent helping me to edit my dissertation. I would like to acknowledge Dr. Rozita Yarmand for kindly spending hours with me to discuss novel ideas and teach me Immunofluorescence techniques.

Lastly, I offer my sincere regards to my family for all their love and encouragement. For my parents, especially my mother who raised me with a love of learning and supported me in all my pursuits. For my brothers Keivan and Kamyar who have been my rock solid support. Also I would like to thank Ali Kamrani for supporting me by helping to keep our children lives in order.

And most of all my love and appreciation goes to my loving, supportive, encouraging, and patient two little boys, Arshya and Aria, their support and sharing of the time that they were completely entitled to, gave me the opportunity to pursue my doctoral degree. THANK YOU!

Cell Cycle Regulatory Roles of Estrogen Receptor alpha (ERα) in Breast Cancer Cells

Sonia Javan Moghadam, M.S.

Supervisory Advisor: Khandan Keyomarsi, Ph.D.

Previous studies have shown that Estrogen Receptor alpha (ERα) is an important indicator for diagnosis, prognosis and treatment of breast cancers. However, the question remains as to the role of ERα in the cell in the presence versus absence of 17-β estradiol In this dissertation the role of ERα in both its unliganded and liganded state, with respect to the cell cycle will be explored. The cell line models used in this project are ER-positive MCF-7 cells with and without siRNA to ERα and ER-positive MDA-MB-231 cells that have been engineered to express ERα. Cells were synchronized and the cell cycle progression was monitored by flow cytometric analysis. Using these methods, two specific questions were addressed: Does ERα modulate the cell cycle differently under liganded versus unliganded conditions? And, does the presence of ERα regulate cell cycle phase transitions?

The results show for the first time that ERα is cell cycle regulated and modulates the progression of cells through S and G2/M phases of the cell cycle. Ligand bound ERα increases progression through S and G2/M phases, whereas unliganded ERα acts as an inhibitor of cell cycle progression. To further investigate the cell cycle regulated effects of liganded ERα, a luciferase assay was performed and showed that the transcription of target genes such as Progestrone Receptor (PgR) and Trefoil protein (pS2) increased duing S and G2/M phases when ERα is bound to ligand. Additionally, complex formation between cyclin B and ER α was shown by immunoprecipitation and led to the discovery that anaphase promoting complex (APC) is the E3 ligase for both cyclin B and ERα at the termination of M phase.

Our findings suggest that unliganded ERα has an inhibitory effect on the progression of the cell cycle. Therefore, it is reasonable to speculate that the combination of drugs that lower estrogen level (such as aromatase inhibitors) and preserves ERα from degradation would provide better outcome for breast cancer treatment. We have shown that APC functions as the E3 ligase for ERα and thus might provide a target to design a specific inhibitor of ERα degradation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter I: General Introduction

In this dissertation the role of Estrogen Receptor alpha (ERα) in both its unliganded and liganded (bound to 17-β estradiol) state, with respect to the cell cycle will be explored. The model system used in this project is composed of 4 different cell lines: 1) MCF-7 (ER-positive) 2) MCF-7 siRNA-ER, 3) MDA-MB231 (ER-negative) and 4) MDA-MB231 ER (engineered to express ERα). These cell lines allow us to compare the effects of presence versus absence of ERα and also liganded versus unliganded ERα on the cell cycle duration and overall cell proliferation status of these cells. These cell lines were used to address two specific questions 1) Does ERα modulate the duration of the cell cycle differently under its liganded versus unliganded state. 2) Does the presence of ERα regulate cell cycle transitions.

Estrogen Receptor

Estrogen receptor (ER) is a hormone activated transcription factor, which plays a significant role in the development of adenocarcinomas of reproductive organs [1]. Steroid receptor expression and proliferation are strictly regulated in the normal mammary gland, but not in malignant tumors. In normal mammary gland there is a minimum expression of ERα [2] [3]. ERα expression increases when normal mammary cells are proliferating such as the case of pregnancy or puberty period [4-6]. However in breast cancer increased ERα expression appears to occur early in the premalignant to malignant progression, and these tumor cells will continue expressing ERα [7]. Additionally, ER-negative breast tumors are poorly differentiated and more aggressive [8]. The difference in ER expression between normal (low ER expression) and tumor cells (variable ER expression) raises the question as to why ERα is absent both in the normal breast epithelial cells and in the worse prognostic breast cancers while ERα is present in the breast epithelial cells in luminal cancers? Should the presence of ERα be our only marker or there are other elements to determine the prognosis of the disease. There are two possiblilites as to why lack of ER is associated with a poor prognosis 1) An ER-positive tumor has the ability to lose ER expression and in doing so be transformed into a more aggressive ER-negative tumor or 2) ER-negative tumors originate from cells

without expression of ERα. There are active investigations in support of both of these possibilites [9-13]

BREAST CANCER

According to the reports of year 2010 from the American Cancer Society, in year 2010 about 207,090 women will be diagnosed with breast cancer (new cases) and about 54,010 women will be diagnosed with an in situ breast cancer in the US [14]. The American cancer society also reported that the number of death due to breast cancer in 2010 will be about 39,840 women in the US [15]. It is expected to diagnose approximately 1,970 men with breast cancer in 2010 in the US, which is almost 1% of the total incidence of breast cancer cases. Abot 390 of these men expected to die from breast cancer in 2010 in the US. Lung cancer stands first for the number of the cancer deaths before breast cancer in women in the US [14, 16]. The most recent National Cancer Institute report states that 12.5% of all US women will be diagnosed with breast cancer [17](©2009, American Cancer Society, Inc. (404) 320-3333).

Similar to other types of cancers, in breast cancer cells growth and proliferation increase and gets out of control, which then giving rise to malignant cells originating in the breast. Breast cancer may metastasize and spread through the body. The lymphatic system is the body's primary system in which the white blood cells and other immune system cells being produced and transported through out the body. However the lymphatic system is also known for being the primary route of cancer cells to metastastasize through the body. Cancer cells come off the primary tumor, which they attached to in order to penetrate into the flow of the lymph system and finaly exit the lumph sytem to settle in their secondary sites to form a new tumor and perpetuating the disease process. Tumor cells also can metastasize through the vascular system. Detached breast tumors cells from the primary tumors slip through the wall of capillary and enter the blood stream. These floating tumor cells journey ends either with their elimination through white blood cells in the blood stream or when they get immobilized in small blood vessels of an organ to give rise to a new (secondary) cancer in that organ, which this process also called metastasis in the area. The blood circulation in the body is in a way that mainly the first destination of the blood coming out of different organs is the lungs. The blood flow system makes the lungs an organ, which is highly at risk as a secondary

organ to receive cancer cells through blood flow (metastasis). Liver is the second organ highly at risk for receiving the metastastatic growth of cancer cells [18].

Breast cancer subtypes

An unanswered question is what kind of cellular abnormalities allows cancer to grow uncontrollably and spread throughout the body? The answer to this question is currently being investigated by unraveling the profile of cancer cells at both the genomic and proteomic levels. Cancer cells exhibit a number of properties that distinguish them from normal cells. Additionally cancer cells that develop in the same tissue/organ can give rise to different forms of the disease, due to the heterogeneity of the cancer cells. For example, in breast cancer, several groups have identified that biological features of tumors correlate with different clinical behavior [19] and have identified multiple molecular subgroups [20, 21 22 23]. There are five different subtypes of breast cancer called luminal A, luminal B, HER2 type, basal type and Claudin low subtype, which all have been described in Table 1

Breast cancer subtypes	ER status	ER related gene(s) status	Other genes expression	Prognosis	References
Luminal tumors (Luminal A and luminal B type tumors)	ER-positive	PgR-positive HER-2 positive (only luminal B) High expression of: LIV-1, TFF3, GATA 3, c-myb, BTG2, topoisomerase II α, MCM2, BUB1,PCNA and cyclin D1	In Luminal A: High expression of genes related to ER and low expression of genes related to cellular proliferation In Luminal B: Higher expression of proliferation markers compared to Luminal A P53 does not seem to play a role in the luminal tumors since less than 20% of these tumors have a p53 mutation	Luminal B tumors have a worse outcome than Luminal A tumors is due to the differences in proliferation markers	Harbeck El-Tanani Prevot Hoch Perou 2000 Sotiriou 2003 Wang 2002 Tye Jallepalli Sorlie 2003
HER-2 tumors	Low level of ER	Low level of ER related genes	Over express Growth Factor Receptor-Bound Protein 7 (GRB7) High proportion of p53 mutations	Poor prognosis Involved in cell invasion and metastatic progression in numerous human cancers such as esophageal and ovarian carcinomas, gastric and breast cancers	Perou 2000 Wang-no year Sorlie 2001
Basal tumors	Lacking ER	Lacking PgR Lacking HER-2 Lacking ER related genes	Overexpressing: keratin 5, keratin 6, metallothionein 1X, and fatty acid binding protein 7, topoisomerase II α, Madp2 homolog (MAD2L1), cell division control protein 2 homolog (CDC2), and PCNA Claudin-low subtype like basal tumors express: Low levels of Claudin 3 and E-cadherin High level of markers associated with epithelial to mesenchymal transition		Reis-Filho and Lakhani 2008) Simpson,Reis-Filho et al. 2005 Perou 2000 Sotiriou 2003 Herschkowitz and Simin, 2007

Table 1: Molecular characteristics and prognosis of breast cancer subtypes.

Breast cancer risk factors

Extensive research over the last 30 years have shown different risk factors associated with higher incidence of women breast cancer (i.e. 24,000 papers listed in pubmed when the phrase “breast cancer risk factors” is interrogated). Some of the common risk factors include: age at diagnosis, genetic mutations, previous breast cancer, race, previous premalignant tumor biopsy, prior radiation treatment in the chest area, age
of mensus onset, age of menopause, (earlier onset of menstruation and late menopause increases the breast cancer risk), use of oral contraceptives, pregnancy, hormone replacement therapy, obesity, poor diet, failing to exercise, fail to breast feed and excessive alcohol intake (more than one alcoholic drink a day) [24].

Breast cancer therapy

Upon detection of breast cancer, which by The American Society of Cancer Research signs could be one or more of the listed symptoms such as: 1) Bloody discharge 2) Thickening of the skin and the underneath tissue that feels different from the surrounding area, 3) Breast lump , 4) Size or shape change of the breast, 5) Inverted nipple, 6) Peeling or flaking of the area around the nipple, 7) Redness of the skin over the breast (© American Cancer Society, Inc.)" it has a variety of treatment options available. Choice of treatment is based on patient demographics such as age, previous treatments and currently known prognostic and predictive targets for therapy (ER, PgR, HER-2and lymph node status). These characteristics help the physicians to come up with the most beneficial therapy for the patient. Surgery, to remove the primary tumor, can be followed (if necessary) by radiation, chemotherapy, hormonal therapy or any combination of these strategies.

Radiation therapy is used to destroy any remaining cancer cells that may reside after the surgery. Radiation kills dividing cells by causing severe chromosomal damage, which prevents cells from continuing to divide. Since tumor cells are frequently dividing, they are more sensitive to radiation therapy [25, 26]. The other approach to treat breast cancer is chemotherapy, which involves the use of drugs that either kills cancer cells or interferes with their ability to proliferate [27].

The first-line therapy recommended for breast cancer patients has been chemotherapy for the past fifty years [28] and is currently used in three settings including neoadjuvant, adjuvant and palliative chemotherapy. In the neoadjuvant setting first the tumor size chemotherapy is given prior to surgery in order to shrink the tumor size. In the adjuvant setting chemotherapy is given post surgery to decrease the recurrence and in palliative setting chemotherapy is given to control metastatic breast cancer. There are many choices for chemotherapy; for example the synthesis of DNA and RNA are being inhibited by anthracylcines (such as doxorubicin/adriamycin). Anthracylcines mechanism of DNA/RNA synthesis is through intercalating the strands. Anthracyclines also inhibit topoisomerase II enzyme, thus blocking DNA transcription and replication and create free oxygen radicals that damage the DNA [29]. Taxanes (such as docetaxel) function by freezing mitosis through disruption of microtubule function [30].

Modern systemic treatments continues to evolve, with use of different combination of cytotoxic chemotherapy plus endocrine therapy, or molecular targeted therapy. For example, trastuzumab is the only targeted therapy that is currently approved in combination with chemotherapy for adjuvant or neoadjuvant treatment of HER2-positive breast cancer patients. Lapatinib and bevacizumab, both are the approved drugs for treating metastatic breast cancer [31]. The preferred chemotherapeutic agent being used in these cases are anthracycline and taxane in combination [32]. The overall treatment response has been increased by the use of chemotherapeutic agents in the adjuvant and neoadjuvant settings.

Although there are many choices of chemotherapies and the combinations can be tailored according to disease stage and health of the patient, they are not highly specific to tumor cells and result in toxicities to normal cells. To solve this issue there has been new approaches such as establishment of a genetic testing to identify the biological markers for which specific therapeutic strategy is available. In this setting, tailored neoadjuvant chemotherapy will be administered to the patient based on the biological markers identification. The early tailored neoadjuvant chemotherapy treatment response will help to set the strategy for the adjuvant treatments.

Hormonal therapy

In addition to chemotherapeutic regimens, eligible patients receive endocrine therapy which falls into two treatment options 1) ER inhibitors (antiestrogens) and 2) Inhibitors of estrogen production (aromatase inhibitors). Patients will be categorized into ER-positive group versus ER-negative group based on the result of immunohistochemistry (IHC) test, which is a widely used test for assessing therapeutic biomarkers (such as ER) and has become a major part of practical diagnosis for various malignancies [33]. Not all labs use the same criteria to analyze the results of the test. One method of IHC result reporting is based on the percentage of the cells that are positive for ER. The result will be between 0% (none of the cells are stained for ER) and 100% (all the cells are stained for ER) [34, 35]. The second way to report the IHC result is based on a number between 0 and 3. Zero means that no receptors are present and 3 means that a large number of cells expressing ER [34, 35]. The third method is called Allred score, which gives numerical values between 0 and 8 [35]. The system measures the percentage of cells testing positive for hormone receptors, along with the

intensity of the stain. This information is then combined to score the sample on a scale from 0 to 8. The higher the score, the more receptors were found which were of higher intensity. An IHC score of 2 or greater (IHC of biopsy) is used to define ER positivity and as result eligibility for endocrine therapy [36]. One additional marker that is also taken into account when considering endocrine therapy is the co-expression of the PgR, which is considered as a marker of ER functionality [37] and predicts a better likelihood of responsiveness to endocrine therapy [38]. Since PgR is one of the ER's target genes, its expression suggests that ER is functional and can transcribe it's target genes [39].

The history of endocrine therapy dates back to 1896 [40], with the advent of ovarian ablation (oophorectomy) as a mode of treatment, discovered by George Beatson [41]. The rationale for oophorectomy comes from the initial observations of Dr. Beatson, who found that removal of the ovaries of the cow after calving allows for an indefinite production of milk. These observations also provided one of the first evidence of possible feed back between ovaries and mammary gland. He also found that changes (proliferation) in the mammary gland during the lactation are almost identical to those that occur in a cancerous mammary gland. He states that in both cases of lactation and breast cancer epithelial cells appears to proliferate faster to generate more cells, which the higher number of epithelial cells block the ducts and fills the acini in the gland. However the difference between the proliferation during lactation and the proliferation during breast tumor is that is that in the case of lactation cells vacuolated and then undergo fatty degeneration to form milk. In the breast cancer case epithelial cells do not go through the fattey degeneration so they grow continuesly and come short of space so the cells then start to penetrate the walls of the surrounding ducts and acini in order to make more room for hemselves, which basically during this process the tumor cells invade the surrounding tissues.

The first oopherectomy was performed by Dr. Beatson on a 33 year old breast cancer patient. He found that the cancerous tissue was reduced to a very thin layer of cells. Since, ovaries are the main gland that secret estrogen into the body, by removing this organ, the cancerous mammary gland is devoid of estrogen required for its proliferation.

The next therapeutic approach was the estrogen administration in pharmacological doses followed by hypophysectomy and adrenalectomy followed by additive therapies such as androgens, glucocorticoids and progestins in high doses. The rationale behind the estrogen

administration was that high doses of estrogen (synthetic nonsteroidal) suppress pituitary gonadotropic secretion and endogenous estrogen secretion. In fact, the anterior hypophysis controls estrogen secretion through two different pathways 1) Pituitary adrenocorticotropic hormone (ACTH) which acts on the adrenal gland to induce the release of steroid and gonadotropic hormones and 2) ovarian gland. These early methods of breast caner treatment (oopherectomy and high dose estrogen) discontinued due to the high morbidity and toxicity to the patients [42-46]. These early treatments were replaced by modern anti-endocrine therapy: 1) Selective ER Modulators (SERMs), which inhibit the activation of ER by inhibition of ER's transcriptional activity, 2) Selective ER Down regulators (SERD), which function by down regulating the levels of ER protein in the cells, and 3) aromatase inhibitors, which function by inhibition of aromatase, the enzyme involved in the production of circulating estrogen.

Selective ER Modulators (SERMs)

The SERM that is currently being used in the clinic is tamoxifen (TAM). Development of TAM as a mode of breast cancer therapy initiated in 1960s when scientists realized that oophorectomy, hypophysectomy and adrenalectomy are not viable solutions to treat breast cancer. Additionally treatment with synthetic estrogen such as cortisone, distibestrol and other hormonal agent, MER25, is not a good option either due to concerns about long-term toxicities [47]. In 1967, Dr Arthur L. Walpole who was an endocrinologists discovered the compound ICI46,474 (48). Dr Walpole discovered ICI46,474 while he was working at the Imperial Clinical Industries (ICI) Pharmaceuticals Division, which now is known as AstraZeneca [48] and performed the initial studies in rodents. This compound which was named TAM, tested on immature rat, which showed both estrogen agonist and antagonist effect while only showed a full estrogen agonist effect in the mouse. A preliminary clinical study performed by Cole et. al. in 1971 and showed that ICI46,474 (TAM) may be a good agent to treat the postmenopausal breast cancer patients, since an improvement was observed in side effect profiles capmared to the standard high-dose estrogens or androgens treatments [49]. Due to the lower side effects of ICI46,474 (TAM) in compare to high dose estrogens treatment, ICI46,474 (TAM) was chosen as the vanguard medicine for treatment of all stages of breast cancer [48, 49]. The intial lab based studies revealed that TAM blocks the binding of

E2 to ER, preventing the induction and proliferation of ER positive mammary carcinomas [50]. However increase duration of TAM treatment also results in drug resistance. Another concern regarding the use of TAM as adjuvant treatment following the surgery was revealed by an evaluation of 5 versus 10 years of adjuvant TAM administration, which showed that 10 years of adjuvant TAM treatment results in an increase in primary and secondary tumor recurrences (i.e. endometrial cancer) compared with 5 years of adjuvant therapy [51]. The reason for increased incidences of endometrial cancer following TAM treatment is due to the selective estrogenic actions of TAM in the endometrium [52]. Keoxifene, the compound that later became known as raloxifene replaced TAM since raloxifen does not show any estrogenic activity in endometrium and also enhances bone density [53].

Selective ER Down regulators (SERD)

The next generation of ER modulators are called SERDs, which have a different mechanism of action from the SERMs [54]. SERMs inhibit the activation of ER by inhibiting ER's transcriptional activity while maitnating the integrity of the ER protein. On the other hand, treatment with SERDs result in a dramatic shortening of the ER protein's half-life and down regulating the levels of ER protein in the cells [55]. The next anti-estrogen discovered, Fulvestrant (ICI 182,780) is a "pure" anti-estrogen devoid of any agonistic activities compared to TAM, and therefore unlikely to lead to an increased risk of endometrial cancer [56 57]. At the molecular level, fulvestrant competes with estrogen for binding to the ER, and prevents ER dimerization, thereby disrupting its nuclear localization [58, 59]. The mechanism of Fulvestrant's action is also different than TAM as it accelerates degradation of ER protein [60, 61].

Numerous clinical studies have been completed aimed at comparing the fulvestrant versus other agents used for endocrine therapy, such as the antiestrogen TAM and estradiol inhibitors, aromatase inhibitors (see below). These comparison studies are done in order to evaluate the efficacy and tolerability of these drugs. A study with four trials each with 2125 patients showed that there was no statistically significant difference in the overall survival and time to progression, between fulvestrant and other hormonal agents,.However in the arm of experiment with the patients receiving fulvestrant there was less incident of joint disorders compared to the patients in other arms of the trial who received hormonal agents other than

fulvestrant. [62, 63]. In another study performed by Euler et. al. the efficacy and tolerability of anastrozole (an aromatase inhibitor-see below) was compared with TAM, which have been used as the first-line therapy in postmenopausal women. There were a total of 668 patients in the trial, which 340 of them were in the anastrozole treatment arm and 328 in the TAM treatment arm. The median follow up time after the treatment was 19 months. The median time to progression was 8.2 months in the anastrozole treatment arm and was 8.3 months in TAM treatment arm. As the numbers reveal the time to progression and also overall survival were similar in both treatments. Overall patients treated with both anastrozole and TAM showed that both drugs were well tolerated however the only difference was that the incidences of thromboembolic events and vaginal bleeding were less in anastrozole treated patients compard to TAM treated patients. So Euler et. al. suggest that due to the lower observed incidence of side effects and the similar outcomes from anastrozole versus TAM, anastrozole is more beneficial to postmenopausal women with advanced breast cancer and should be considered as first-line therapy [64].

Another multinational, double-blind, randomized clinical trial aimed to compare TAM versus fulvestrant for the treatment of postmenopausal women with advanced breast cancer, who were not treated previously with any endocrine therapy was lead by Osborne et. al. In this study 587 patients were randomly assigned to each treatment (313 to fulvestrant and 274 to TAM). The results, after a median follow-up of 14.5 months, showed that there was no significant difference between fulvestrant and TAM for the primary end point of time to progression. However the objective response rate (the sum of partial plus complete responses) for the overall population was 31.6% with fulvestrant and 33.9% with TAM. Based on the objective response advantage of TAM over fulvestrant, the study concluded that there is no advantage in using a SERD (fulvestrant) over a SERM (TAM) as a first-line breast cancer chemotherapeutic or chemopreventive agent [65]. This finding suggests that with the complete elimination of ERα in the presence of SERDs, has a less favorable clinical outcome as compared to SERMs, which only inhibit the transcriptional activity of ERα without degradation of the ERα protein itself.

Aromatase inhibitors (AIs)

AIs represent an entirely different class of endocrine therapy. These agents inhibit the production of estrogen by blocking the aromatase enzyme activity, which this enzymes activity converts androgens into estrogens in peripheral tissues, such as adipose tissue, and muscle in postmenopausal women [66]. The reason that AI has shown a better result in postmenopausal women is that in postmenopausal women aromatase enzyme activity makes up the estrogen in the system rather than ovaries, which are the main estrogen producing orhan in premenopausal women. Since the circulating estrogens in premenopausal women are coming from ovaries and not from the aromatase activity so blocking the enzyme aromatase via AIs do not significantly decrease the level of circulating estrogen in premenopausal women while AIs decrease the circulating estrogens in postmenopausal women. As a matter of fact using AIs in premenopausal women not only is not beneficial but it seems that because of AI effect, which result in the decrease in peripheral estrogen a signal goes to the hypothalamus and the pituitary gland to increase gonadotropin secretion and stimulates the ovary to produce more androgen. This counteracts the effect of the AI [67]. The first generation of AIs (i.e. Aminoglutethimide) were non-selective inhibitors of adrenal steroid synthesis [68], which lacked selectivity for aromatase and inhibited biosynthesis of cortisol, aldosterone and thyroid hormone as well as aromatase. The clinical experience with aminoglutethimide treatment in women with breast cancer reveals that similar to TAM, 1/3 of women experience either complete or partial tumor regression [69]. Despite the good response rate in breast cancer [70, 71], this generation of AIs had high toxicity, which resulted in the substitution of this group of AIs with the second generation of AIs, which were more selective inhibitors of aromatase. Examples of the second AI generation are non-steroidal inhibitor fadrozole and the steroidal inhibitor formestane [72, 73]. In order to investigate the therapeutic effects of fadrozole (CGS 16949A) in women with prior treatment for metastatic breast cancer, 80 postmenopausal women with prior treatments were randomized to receive fadrozole. The overall response was 23% (10% complete response and 13% partial response), and frequent side effects (hot flashes, nausea and vomiting, fatigue and mild loss of appetite) were noted [72]. In another trial with fomestane as the AI, 409 pateients with advanced metastatic breast cancer were randomized to receive either formesatan or TAM. Formestane and TAM showed comparable responses, which lead to the conclusion that formestane is an

effective treatment [73]. However the selectivity of neither 2nd gernation AIs were complete either, and they were replaced with the third generation of AIs. These AIs, which are currently being used clinically, are more potent and selective inhibitors of aromatase and can be divided into irreversible steroidal activators (e.g., exemestane) and reversible non-steroidal imidazole-based inhibitors (e.g., anastrozole, letrozole) [74]. Disease-free survival have been noted upon exemestane treatments in postmenopausal women with ER-positive breast cancer. In this study, 4724 postmenopausal patients, who were on TAM for 2-3 years (disease-free), were randomly assigned in to two arms. Either these patients were in the arm of continue TAM (n=2372) or in the arm of switch to exemestane (n=2352) for the remainder of a 5-year endocrine treatment period. Upon a median of 55.7 months follow up on this trial the obtained results suggested that the patients in the exemestane arm showed more improvements in disease-free survival [75]. A similar study with letrozole was conducted in 4922 postmenopausal women with ER-positive early breast cancer [76]. With a median follow-up time of 71 months after randomization the results showed similar outcome in the overall survival in the women assigned in both arms of letrozole and TAM treatments. However, the studies for the long-term effects of third generation AIs on the improvements lead to real gains in survival are ongoing and there are not much information on that yet.

One can attribute the overall outcome of the SERMs (i.e. TAM) and SERDs (i.e. fulvestrant) and AIs to the fact that upon inhibition of ER activity with SERMs and/or SERDs or upon inhibition of estrogen synthesis via AIs, there will be no estrogenic activity leading to the inhibition of estrogen signaling. Overall, all three class of agents block the estrogenic activity and result in an improvement on the patient's disease free survival. However, even though the estrogenic activity resulting from the ERα binding to E2 is mitogenic and causes the tumor cell progression, the effects of liganded (with E2) versus unliganded (without E2) need to be differentiated to understand the liganded versus the unliganded activities of ERα, which does not initiate any estrogenic response. One experimental approach to address this question is to examine the effect that TAM versus fulvestrant has on the progression of the cells through cell cycle. Such an analysis will reveal if these two treatment strategies, which either results in inactivation of ER (TAM) or degredation of ER (fulvestrant) can alter cell cycle and cell proliferation of ER postive breast cancer cells differently.

THE CELL CYCLE

All cells that make up our body will eventually age and die. These cells must be replaced so that the body can continue functioning optimally. Cell cycle is the sequence of events between two cell divisions. The cell cycle occurs as a sequential progression through different phases. These four stages - G1, S, G2 and M - are collectively known as the cell cycle phases. The S phase is defined as the phase of the cell cycle when DNA synthesis takes place and the genetic materials of the parent cell are faithfully copied and passed on to the daughter cells leading to a doubling of the amount of DNA per cell. The M phase (mitosis) is the phase in which the cells divide and receive the DNA content of the cell, which has been synthesized during the S phase. The G1 (i.e Gap 1) phase is defined as the interval between the M and S phases that prepares the cells for DNA replication (Figure 1). During G1 in a diploid eukaryotic cell there are two sets of chromosomes present in the cell. If a cell does not need to divide it may enter quiescence, which, in this case it is when the cells are in the G0 phase. As shown in Figure 1, G2 is defined as the interval between the S and M phases that prepares the cells for mitosis [77]. At the end of mitosis, the cytoplasm of the cell will also be physically separated into two different cells. Passage through the cell cycle is controlled by a variety of regulatory proteins (i.e.cyclins). As their name reveals and has been shown in the Figure 1 cyclins are synthesised at specific stages of the cell cycle.

Cyclin D and cyclin E are G1 phase cyclins while cyclin A and cyclin E are S phase cyclins and cyclin A expression continues till the end of G2 and cyclin B is an M phase cyclin. Cyclins have no catalytic activity on their own and require a kinase partner, called cyclin-dependent kinases (Cdks) [78]. Cdks, which belong to a group of protein kinases (serine/threonine kinases) are activated by association with a cyclin, forming a cyclin dependent kinase complex [79]. Most cdks are constitutively expressed in most eukaryotic cells [79]. Binding of Cdks to cyclins is necessary for the activation of Cdks function, which is to phosphorylate the target proteins (i.e. substrates) on their serine and threonine residues. Phosphorylation through cyclin-Cdk can activate or inactivate target proteins in order to coordinated the cell cycle progression through the phases and entry into the subsequent phase of the cell cycle [80]. Cyclin-Cdk complexes phosphorylate specific downstream substrates (Figure 1). For example cyclin D upon binding to existing Cdk4 forms an active cyclin D-Cdk4 complex, which in turn phosphorylates the retinoblastoma protein (Rb) [81]. The Rb

protein dissociates from the E2F upon the phosphorylation. The E2F-Rb complex function by binding to the E2F responsive genes to block the transcriptions, which could results in activation of E2F. The dissociation of E2F and Rb causes the activation of E2F, which result in the transcription of various genes such as cyclin E, cyclin A and as the result progression of the cells from G1 phase to the susesuent S phase [82]. The synthesized cyclin E then binds to Cdk2, forming the cyclin E-Cdk2 complex, which activates the transition of the the cell from G1 to S phase [81]. During the G2/M phase cyclin B-Cdk1 complex formation initiates the breakdown of nuclear envelope and initiation of prophase, which results in the transition of cells from G2 into M phase [83].

The transition of each phase of the cell cycle to the next is governed by a specific set of cyclin/Cdk complexes. These complexes are are depicted in Figure 1.

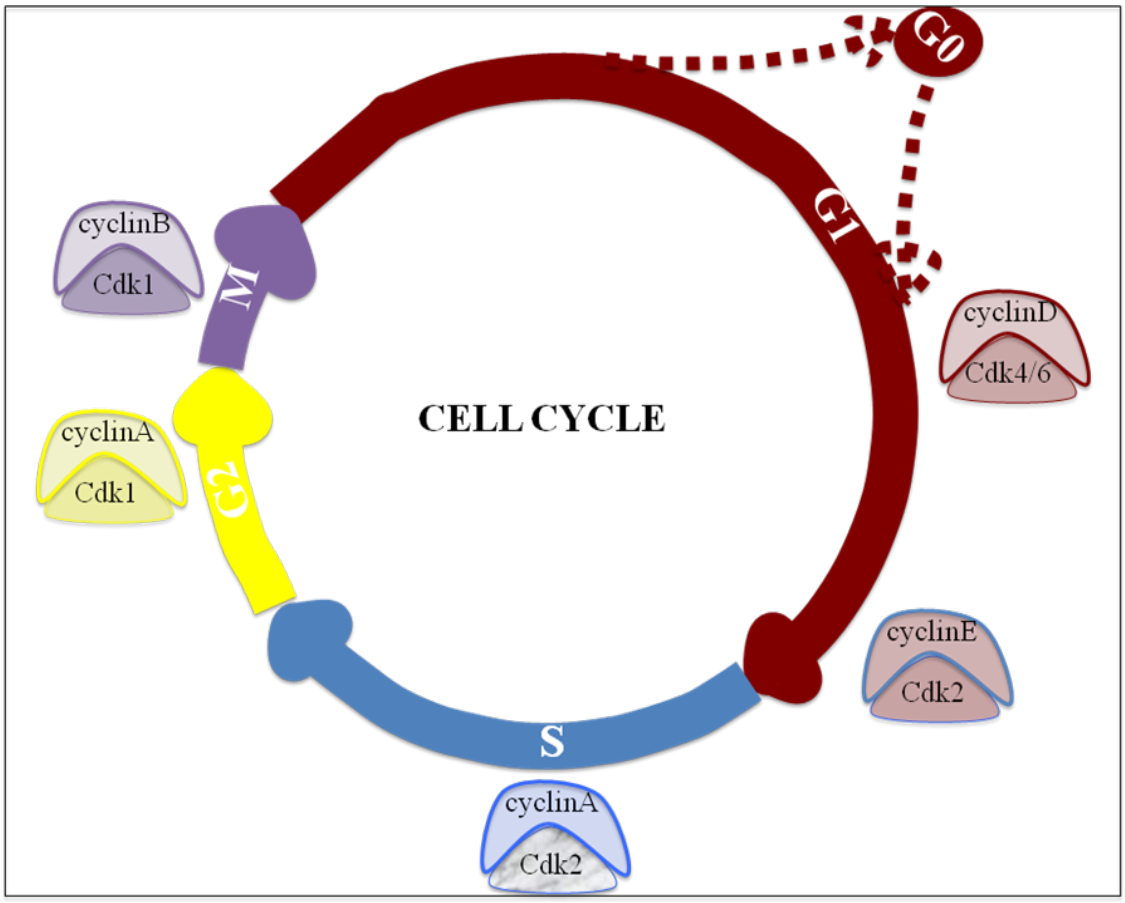


Figure 1: The cell cycle

In the cell cycle, the cells pass through a series of discrete phases in order to divide in a way that the two new daughter cells receive a complete set of genetic materials. The phases are called the G1, S, G2 and M. All these phases are collectively called the cell cycle. The key cyclin/cdk complexes governing the transition of one phase of the cell cycle to the next is depicted in this figure.

Cyclins

As the name implies, cyclins are expressed during the cell cycle cyclically. The phase specific activation of Cdks is dependent on the presence of their related phase specific cyclins. Each phase of the cell cycle can be identified by the cyclin(s) specific to that phase. The cyclin that is expressed early in the G1 phase and takes part in early G1 phase progression is cyclin D, which forms a complex with either Cdk 4 or Cdk 6. Another G1 cyclin is cyclin E and is first expressed in late G1 phase and degraded in mid S phase. During the G1 to S transition cyclin E forms a complex with Cdk2 and the cyclin E/CDK2 complex regulated G1 to S transition. Another cyclin that is also is associated with Cdk2 is cyclin A, but its association with Cdk2 occurs at S phase and continues to G2 phase when cyclin A switches partners from Cdk2 to Cdk1 [81, 84]. The cyclin regulating M phase transition is cyclin B and forms a complex with Cdk1. Cyclin B is synthesized and its expression gradually increases during the G1, S and G2 phase, reaching a peak at the end of G2 phase at which point it binds to Cdk1. The resulting Cdk1-cyclin B complex triggers the passage from G2 into M phase by regulating several events in mitosis such as phosphorylation and activation of 13S Condensin, which helps to condense chromosomes. Chromosome condensation is an important cellular process to ensure the faithful segregation of genetic information during M phase [85]. Cyclin B-Cdk1 complex is also involved in the nuclear envelope break down and necessary for the initiation of cytokinesis. Cyclin B-Cdk1 complex phosphorylate nuclear lamins, resulting in their dissociation [86]. Cyclin B-Cdk1 complex is inactive prior to mitosis because of the complex itself is phosphorylated by Wee1 kinase [87]. The complex is activated through dephosphorylation of Cdk1 by the Cdc25 phosphatases [88]. In contrast to Cdk1, which phosphorylation inactivates it, cyclin B's activation is mediated by its phosphorylation by two kinases: Polo Like Kinase (PLK) and Cdk1 [89]. Inactivated (unphosphorylated) cyclin B is abundant in the cytoplasm prior to prophase. However upon phosphorylation, cyclin B translocates into the nucleus [90]. Cyclin B phosphorylation blocks the nuclear export signal so then cyclin B is unable to exit the nucleus [91]. At the end of mitosis, cyclin B's function is completed at which point its targeted for degradation by the Anaphase Promoting Complex (APC) [92]. Once cyclin B is degraded, the cell exits mitosis.

Cdk1 is post-translationally modified by different phosphorylation and

dephosphorylation steps, rendering it active. Cdk activating Kinase (CAK)/Cdk7-cyclin H complex phosphorylates Cdk1 at a conserved threonine (activating threonine, Thr160) located on the activation segment (T loop) [93]. In addition to the CAK mediated activating phosphorylation, Cdk is also subjected to dephosphorylation on residues Thr14 and Tyr15, which lie within the presumptive ATP-binding region of cyclin B-Cdk1 (p34cdc2) [94] by the Cd25c family of phosphatases. [93, 95]. Cdc25 itself is activated through autophosphorylation of Ser 115 and Ser 320 residues by cyclin B/Cdk1 [96]. This cycle of phosphorylation of Cdc25 via Cdk1 and then dephosphrylation of Cdk1 via Cdc25 creates a positive feedback loop, which results in the activation of Cdk1-cyclin B complexes more rapidly [97].

The proper timing of expression of these cell cycle regulators and their kinases is an important aspect of the cell cycle regulation. Cyclin synthesis is regulated transcriptionally and its degradation post-translationally, via the proteasome [98, 99]. Several proteins in the cell cycle have temporally distinct positive and negative functions and are degraded accordingly at specific phases of the cell cycle [99-101]. If their destruction through the proteasome pathway does not happen on time then they will impose negative barriers on cell division. For example, mitoic progression is controlled by the sequential degradation of different proteins through APC. The signal for mitotic exit is cyclin B's destruction [102]. Similarly, destruction of cyclin D1 and cyclin E are also important in order to inhibit the premature and inappropriate transition of cells from G1 to S phase [100]. The destruction of cyclin D involves Skp1-cullin-F-box-protein complex (SCF). SCF only ubiquitinates and degrades a phosphorylated protein. Accordingly, cyclin D1's turnover is dependent on its phosphorylation on threonine 286 (T286), which is mediated by Glycogen synthase kinase 3β (GSK3β) [103-105]. Accumulation of the cyclins due to their aberrant proteasomal degradation is associated with a variety of malignancies [106-109]. For example the elevated levels, of cyclin E has been linked to poor prognosis in breast cancer. Normally, the decrease in Cdk2 activity at the G1/S transition is controlled by proteasome- mediated degradation of cyclin E, the turnover of which also depends on SCF [110]. SCF consists of four subunits: Skp1, Cdc53/Cul-1, Roc1 and an F-box protein, which determine substrate specificity [111]. The elevated level of cyclin E in the breast cancer cell line SUM149PT, is thought to be associated with the mutated gene encoding the cyclin E specific F-box protein, FBW-7 [110].

Cyclin D1 over-expression gives a growth advantage to cells, and has been associated

with numerous malignancies [112]. In ovarian cancer, over expression of cyclin D1 has been associated with decreased survival in patients. Under normal conditions, cyclin D1 levels are elevated only during the G1 phase to prepare for DNA synthesis, but then it needs to be degraded to allow for regulated transition to S phase [113]. Phosphorylation of cyclin D through GSK3β results in the export of cyclin D1 from nucleus to the cytoplasm, which follows by the cyclin D1degradation via the proteasome machinery [104, 114]. However, in rat skeletal-muscle cell line L6 and A431 (human epithelial carcinoma cell line) Ras-phosphatidylinositol 3 kinase (Akt pathway) negatively regulates GSK3β, resulting in the stabilization of cyclin D1 and a rapid G1 to S phase transition, which is a mitogenic stimulation [115, 116]. However, cyclin E, another G1 cyclin, can compensate for the cyclin D functions under conditions where cyclin D is no longer expressed [117-119]. For example, Chen et al used cyclin D knock out (KO) mice in order to study the brain development. They suggest despite the high level cyclin D expression in the embryonic brain of WT mice there is no difference between the morphology of the central nervous system (CNS) in cyclin D KO and control wild-type (WT) mice at 1, 4 and 12 months of age. These studies have shown that interestingly cyclin E expression level has increased 13.5 days post coitum in KO mice, which suggest that cyclin E may compensate for the absence of cyclin D in KO mice [118]. In a pharmacological study, it was revealed that phosphatidylinositol 3-OH-kinase (PI3K) inhibitor LY294002 can block both cyclin D1 accumulation and Cdk4 activity, resulting in inhibition of the G1 phase progression in Chinese hamster embryonic fibroblast cells (alpha-thrombin-stimulated IIC9 cells) [120]. However overexpression of cyclin E in IIC9 cells can rescure cell progression in the presence of LY294002, which suggest that cyclin E renders Cdk4 activity dispensable for G1 progression [117].

Human cyclin A degradation process during the cell cycle begins at the early pro metaphase and completes by the end of metaphase [121, 122]. However, unlike cyclin B, the protein(s) that targets cyclin A for proteolysis are still not identified. The destruction box (D-box), which is a short sequence near the N-terminus site of cyclin B is necessary for the degradation of cyclin B. D-box is involved in the ubiquitin-dependent proteolysis [123, 124]. Using affinity chromatography with immobilized D-box matrixes to identify the factors that specifically bind to the D-box in cyclin B, Yamano et al found that the APC purified from Xenopus egg extracts can bind to the D-box of cyclin B in a cell cycle-dependent manner,

whereas Fizzy (Cdc20) does not. To confirm their observations they also have performed this same experiment using mutated forms of cyclin B D-box and reveal that mutations in D-box abolished this interaction [125]. In another study Suzumori et. al. found that several proteins identified through either yeast two hybrid screening or other protein complementation assays to bind to the D box of cyclin B. These proteins included RING finger-like protein RFPL4, HR6A, which is an ubiquitin-conjugating enzyme (E2), proteasome subunit β type 1, and ubiquitin all interact with the D-box domain of cyclin B facilitating its proteasomal degradation [126]. Cyclin A also contains a D-box domain in its N-terminus region [127-129]. However, it seems that only the D-box is not sufficient for the destruction of cyclin A in human. An additional short sequence (KEN box) following the D-box has been shown to be required for cyclin A proteolysis in Drosophila [121, 122, 130]. The D-box of cyclin A is 10-20 residues longer than that of cyclin B [122]. Another difference between the mechanisms of degradation of cyclin A versus cyclin B, is their times of degradation in the cell cycle. Cyclin A degradation timing corresponds to the period when metaphase transit to anaphase, while cyclin B degradation time is right after anaphase. Geley et. al. have used microinjection of antibodies against subunits of the APC (or against human Cdc20 (fizzy) and observed that APC elimination arrested cells at metaphase and stabilized both cyclins A and B. APC is activated as cells enter mitosis. Upon APC activity during mitosis phase cyclin A is being targeted for degradation around the metaphase to anaphase transition. The cyclin B1 degradation delayed due to its role in spindle assembly checkpoint.

The degradation of cyclin B via anaphase promoting comples (APC) is necessary and occurs right after the spindle assembly checkpoint, resulting in the metaphase to anaphase passage and then exit from the mitosis [131]. APC also needs to be activated by phosphorylation via polo-like kinase (PLK) and presence of activator proteins, called Cdc20/Fizzy and Cdh1/Fizzy-related [132]. In fact mutations in fizzy have shown to block sister chromosome separation, indicating that it plays a crucial role in the metaphase/anaphase transition [102, 122, 132]. Once cyclin B is targeted for degradation it is ubiquitinated on Lys-48 in the destruction box [133, 134]. Ubiquitylated cyclin B is degraded by a constitutively active 26S proteasome, and leaving behind an inactive monomeric Cdk1 [131, 135, 136].

There are several check points in the cell cycle that keep the regulated progression of the cells from one phase to the next and help to halt the passage of the cells in a specific

phase upon damage or deregulation signals. The spindle checkpoint, which is between metaphase and anaphase, occurs before chromosome movements begin via the attached microtubules to the chromosomes, to ensure that all the chromosomes are completely lined up and will be evenly distributed in to each daughter cell [137]. DNA replication checkpoint may occur before the onset of M phase (at late G1, S and G2) to ensure that DNA synthesis has been completed successfully prior to proceeding into M phase where cell division occurs [138]. This checkpoint occurs frequently during the cell cycle in order to ensure that no DNA damage can accumulate in the process of cell division [138].

Several check point sensors have been identified, however the most established check point molecule is p53. Upon damage, the cell cycle will halt temporarily to allow the cell to repair the damage. If the damage is repaired, the cell will continue to progress through the cell cycle. However, if the damage is too severe, apoptosis can be triggered-all three events are governed by p53 [97]. Another checkpoint event (e.g. protein) is activated at the restriction point, which occurs at the end of G1 phase preventing S phase entry in the absence of mitogenic stimuli [139]. pRb, which is a key protein in the restriction point, prevents the cell from replicating a damaged DNA when it is hypophosphorylated [140]. Hypophosphorylated pRb has a tumor suppressor role and can bind and inhibit the transcription factor, E2F [141]. When E2F has not been sequestered by pRb, it activates the transition of cells from G1 into S phase. However, pRb binding to E2F and DP proteins keeps E2F-DP inactive as a transcription factor and stalls the cells in the G1 phase [142]. When E2F is inactivated, it also suppresses the next step in DNA synthesis by attracting a histone deacetylase (HDAC) protein to the chromatin to reduce the transcription of S phase promoting factors [143-145].

pRb in it's hyperphosphorylated state is inactive (147). pRb is phosphorylayed sequentially by cyclin D-Cdk4/6 and cyclin E/Cdk2 complexes through out the G1 phase [140, 146]. pRb remains phosphorylated throughout S, G2 and M phases by other cyclin/Cdk complexes active at each phase [147]. Hyperphosphorylated pRb results in the release of E2F, which can then result in transcriptional activation of proteins necessary for G1 to S transition. These proteins include cyclins E and A, and proliferating cell nuclear antigen (PCNA), among many others [140, 146, 148, 149].

Another class of cell cycle regulators are the inhibitors of cyclin dependent kinases which are generally termed as the brakes of the cell cycle and are encoded by two gene

families the cip/kip family and the INK4a/ARF family. The cip/kip family include the proteins p21, p27 and p57 and they mediate their inhibitory activities by binding to the catalytic cleft of the cdks, mimicking ATP, resulting in the inhibition of phosphorylation of substrates such as pRb and G1 arrest [150-152]. The INK4a family includes p16INK4a and p14arf proteins, which act by binding to Cdk4 and inhibiting its binding to cyclin D. The mutation on Cdk4-Arg24Cys site specifically affects the ability of Cdk4 binding with p16 wihout effecting its binding to cyclin D [153]. As such, the INK family of inhibitors antagonize the formation and activation of cyclin D-Cdk4 complexes [154]. P16 induces a G1 phase arrest by inhibition of cdk4 and cdk6 mediated Rb phosphorylation [155, 156].

Aberrant expression of proteins regulating the cell cycle checkpoints is common in cancer. For example, loss of pRb function leads to malignant phenotype due to cell cycle deregulation [157, 158]. Rb function could be abrogated via: 1) phosphorylation through cyclin D overexpression, which happens in several types of tumors such as parathyroid adenomas, B-cell lymphomas and squamous cell [159, 160], 2) viral oncoprotein binding.

In tumor cells (such as cervical cancer, mesothelioma), it has been shown that E2F gets inactivated through pRb binding to DNA tumor virus oncoprotein [161-163]. Faulty checkpoints have the same downstream effects as increased growth factor production (another characteristic of cancer cells). An excess of growth factor results in the over- production of Cdk-cyclin D complex and results in unabated G1 phase to S phase transition of the cells [164]. Ectopic expression of cyclin D in the Rb-negative osteosarcoma cells, following introduction of a function Rb, results in reversal of Rb mediated cytostatic affect. As such Hinds et. al. suggest that the cyclin D gene can function as an oncogene [165]. Additionally overexpression of cyclin D into BRK cells (rat embryo fibroblasts) resulted to increased foci formation, an indicator of oncogenic cell transformation (166). Therefore, both excessive growth factors and nonfunctioning restriction point result in untimely progression through the cell cycle.

ERα & ERβ SIGNALING

The human ER belongs to the nuclear hormone receptor family. ER is a transcription factors, which its activation is hormone dependent. Hormone (17βestradiol (E2)) upon diffuse through the membrane binds to ER, which then can initiate the transcription of genes containing ER response elements (ERE) [166]. To date, two ER have been identified, ERα and

ERβ which are differentially expressed in various tissues and thought to have different functions. The structure of the ER protein is depicted in Figure 2, which shows the known domains within the protein. These domains include the E domain. Ligand Binding Domain (LBD) or AF2 region is located in the E domain of ERα. AF2 region is an important region in initiation and activation of ER-target genes [167]. It contains the binding domains (cavities) for ligand and also coactivator or corepressor bindings [168]. Upon ligand binding to LBD at AF2 ERα forms dimers, which followed by the phosphorylation of ER (see Figure 2). The phosphorylation of ER initiates gene transcription (168,[169, 170]. The A/B domain is located at the N-terminal of ERα protein. A/B domain contains the AF-1. Similar to AF2, the AF1 region also can initiate gene transcription, however the gere transcription initiated from the AF1 region is a ligand independent phenomenon [171]. There is another difference between the gene transcriptions initiated from AF2 region compared to AF-1 region other than the Ligand dependency. The number of the genes being transcriptionaly activated through AF1 region are not as vast as genes being activated via AF2 region and also the transcriptional activity initiated from AF1 is weak [172]. The binding of ERα protein to the ERE site on DNA is done via the C domain on the ER protein. The C domain also called DNA-binding domain (DBD) (Figure 2) [173]. The domain that connects the E domain (AF-2) and C domain (DBD) is called the hinge region [171].

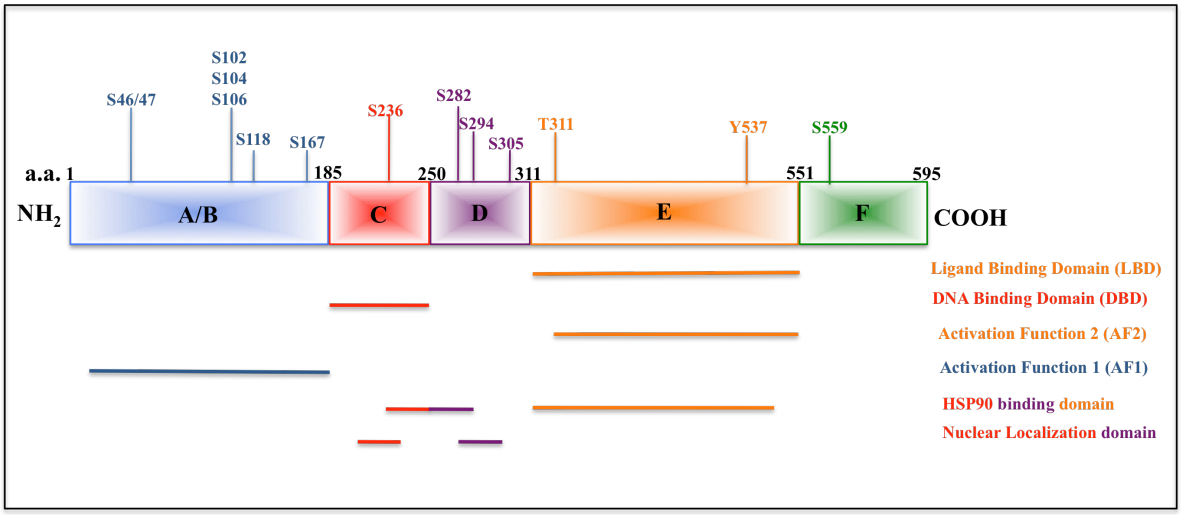


Figure 2: ERα protein structure

A schematic ERα protein, which depicts all the identified domains on the ER protein such as A/B domain (AF1) and C domain (hinge region) and D domain (DBD) which followed by E region (AF2/LBD) and last F domain. The known phosphorylation sites on each domain are marked.

Unliganded ERα is found in the cytoplasm binding to the heat shock protein of 90 kDa (hsp90) in a large molecular complex (Figure 3) [174]. The molecular chaperones binding to target protein (ie. ER) causes the maintenance of proper protein folding within the cytoplasm, which is necessary for different intracellular processes such as 1) stabilizing the nascent polypeptide chains, 2) prevention of protein aggregation 3) chaperoning and transportation of the proteins across the cellular membranes [175, 176]. E2 binding of ER follows by the dissociation of ER from the HSP90, which allows a conformation change in ER protein, and result in ERs homodimerization (Figure 3). These events trigger an estrogenic (i.e. ligand dependent) response in the cell [166], consisting of an increase (3x to 4x) in the basal level of ER phosphorylation upon treatment with estrogen as compared to un-liganded conditions [177].

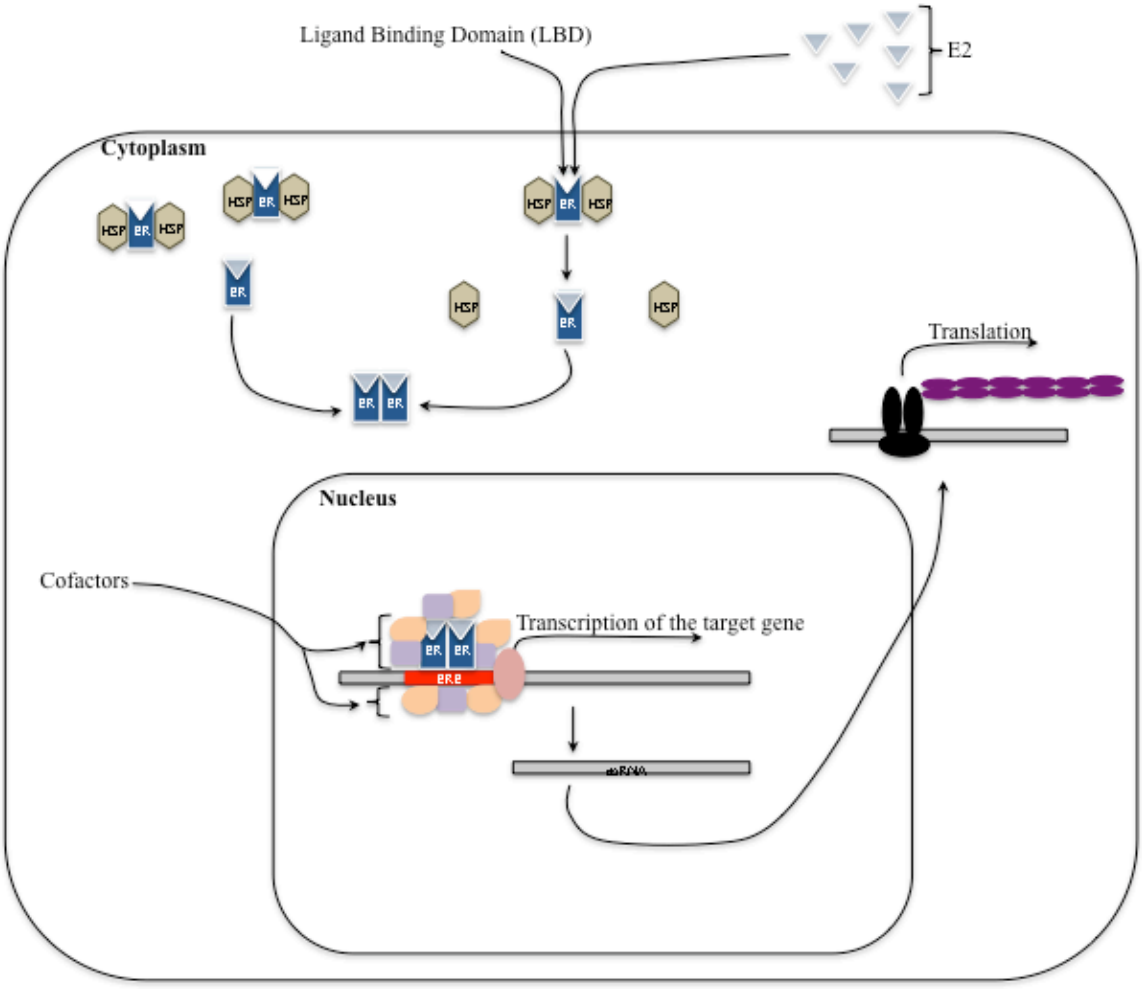


Figure 3: Estrogenic response

ERα upon binding to Estrogen (E2) dissociates from the heat shock proteins and forms homodimer and internalizes into the nucleus so it can initiate transcription from the ERE sites of the ER-target genes.

Phosphorylation of ERα is an important regulatory mechanism, which also changes, the conformation of ER exposing the protein to allow further rounds of phosphorylations, resulting in [178-182] the activation of ER and transcription of the target gene based on the specific phosphorylation [169, 183-185].

ER can also be activated by non-classical, non-genomic and/or ligand-independent manners (Figure 4) [186]. In the non-classical pathway, ligand bounded ERα forms a complex with the transcription factors (ie. SP-1 and AP-1), which are bound directly to the DNA. Through ER binding to AP-1 and SP-1 ERα is able to activates the transcription of genes that do not contain classical ERE elements [187]. Examples of non-ERE containing promoters are the ovalbumin proximal promoter, collagenase and IGF-1 genes [188-194]. In all three cases ER initiates the transcription through binding to AP-1 sites through recruitment by Jun/Fos. Under these non-ERE transcriptional activation, ER is part of the coactivator complex for Jun/Fos [195].

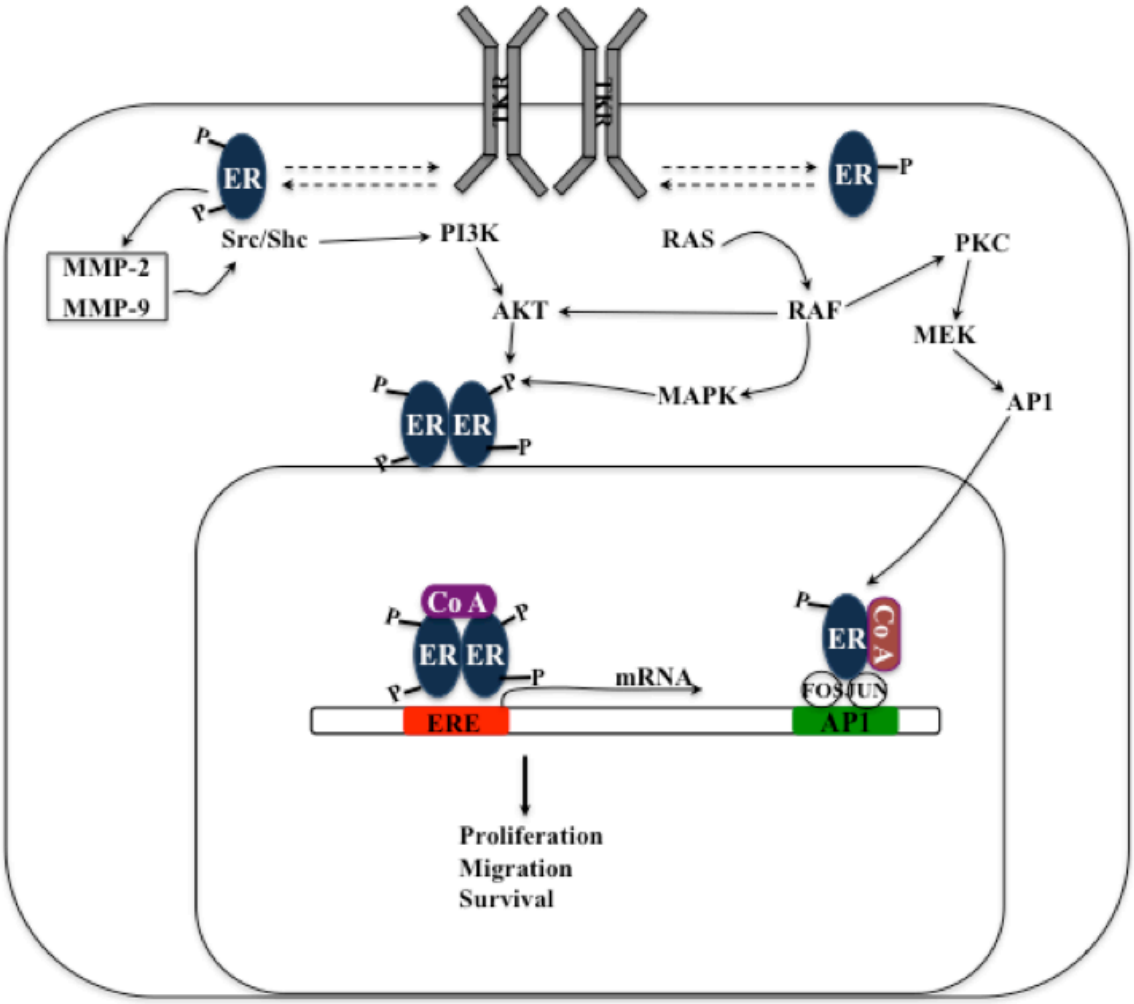


Figure 4: Non-genomic path of ERα

ERα upon stimulation of growth factors is able to initiate a signaling cascade involving the cytoplasmic pathways (i.e. MAPK and PI3K). ERα is phosphorylated in the non-genomic pathway independent of ligand and ligand binding and can result in the unregulated transcription of the downstream genes.

The non-genomic functions of ER seem to be mediated via the membrane-associated ERα. The non-genomic function of ER gives rise to the intracellular signal transduction pathways in order to generate rapid cytoplasmic signaling (Figure 4) [196-199]. Simoncini et. al. showed that "nuclear" ER also resides outside the nucleus. He mentioned that the cytoplasmic ER could have a different role in the cell compared to the nuclear ER. This group showed that ERα binds to p85-alpha subunit of the phosphatidylinositol-3-hydroxy kinase (the regulatory subunit of PI3K) in a ligand dependent manner [200]. They showed that in human vascular endothelial cells, physiological concentrations of E2 increased endothelial nitric oxide synthase (eNOS) activity. The eNOS activity upon E2 exposure is being mediated via mitogen-activated protein kinase (MAPK). Nitric oxide (NO) (a pleiotropic regulator,) functions by regulating different biological processes such as vasodilatation, neurotransmission and macrophage-mediated immunity. The NO connection with cancer has been well established [201]. ENOS activity is completely blocked using either wortmannin (PI3K inhibitor) and ER antagonist ICI 182,780 and the inactive E_2 stereoisomer [200, 202]. In this non-genomic pathway, ER initiates a rapid and transient cascade of signaling originating from the cytoplasm via direct association with signal transduction proteins such as MAPKs, PKC and guanosine triphosphate-binding proteins (G proteins) [203, 204]. In fact, treatment of human granulosa-luteal cells (hGLCs) with agents known to enhance CAMP levels, results in downregulation of ERα mRNA levels. Additional studies using adenosine 3',5'-cyclic monophosphorothioate (a PKA inhibitor), Rp-isomer, triethylammonium salt and an adenylate cyclase inhibitor (SQ 22536) also could modulate ER levels suggesting that the ER and the signal transduction pathways such as PKA or PKC are infact linked [205].

Another example is the non-genomic action of ERα is through MAPK. Treatment of MCF-7 cells with E2 results in the activation of MAPK, which is preceded by a rapid increase in cytosolic calcium. Subsequent treatment of MCF-7 cells with E2 upon treatment or no treatment with ICI 182,780 abrogates the activation of MAPK [206]. It has been suggested that the cytoplasmic or cell membrane ER is only a small subset of the classic ER, or perhaps is of a short form/spliced variant of full length ER [207, 208]. Support for this hypothesis came about when a spliced variant of ERα, with a molecular weight of 46-kDa (ERα46), was identified in human endothelial cells [208]. Confocal microscopy revealed that a proportion of both full length ERα (ERα66) and ERα46 was localized outside the nucleus capable of

binding to E2 (Figure 5). However, E2 mediated transcriptional activation by ERα46 was much less than with ERα66 and ERα46 could inhibit classical ERα66-mediated transcriptional activation (209). In other studies, the membrane ERα and the intracellular ERα were found to be closely related and originate from the same coding sequence [209-211]. Using immunohistochemical methods Watson et al found that eight distinct antibodies against full length ERα could also recognize membrane ERα, suggesting that the membrane and nuclear ERα proteins are highly related [211].

ER's activation through the ligand independent pathway is mediated by growth factor signaling which results in the phosphorylation of ER [212, 213]. Examples of ligand independent activation of ER have been documented with Epidermal Growth Factor Receptor (EGFR) and Insulin Like Growth Factor Receptor (IGFR) [214]. Signaling from EGFR activates cytoplasmic nonreceptor kinases (i.e. -Src) that can phosphorylate ERα and some of its coactivators [213]. A direct interaction between ERα and IGFR results in the activation of IGFR and downstream MAPK signaling. Specifically, upon E2 stimulation ERα binds to IGF-1R and forms a heterodimer, which results in the activation of downstream MAPK signaling [215]. In these studies, ERα was overexpressed in COS7 and HEK293 cells, which have high levels of endogenous IGFR. Treatment of these cells with E2 resulted in ERα binding to IGFR, followed by a rapid phosphorylation of IGFR, which inturn induced the phosphorylation and activation of cytoplasmic kinases extracellular signal-regulated kinases 1/2 (ERK1/2). These sequential events, triggered by ERα, were required to induce the activation of ERE-LUC in IGFR stimulated cells. Taken together, this data suggests that the ligand binding of ERα is a necessary step toward a rapid cascade of cytoplasmic signaling via IGFR (Figure 4) [215].

The cell-membrane-localized-ERα also interacts directly with Her2/neu. Yen et al. have suggested a novel mechanism by which Her2/neu coupled to the cell membrane ERα is involved in TAM resistance of breast cancer cells [216]. They have shown colocalization of membrane ERα and Her2/neu in BT-474 cells in which results in the abrogation of TAM initiated apoptosis. However, when Her2/neu was dissocated from cell membrane ER, TAM-induced apoptosis was activated [217].

E2 stimulated ERα that is G protein-coupled can also initiate signal transduction from plasma membrane through the transactivation of the EGFR/IGFR which in turn result in the

activation of c-Src and matrix metalloproteinases [218]. Briefly, E2 bound cell membrane ER has the ability to activate matrix metalloproteinase-2 (MMP-2) and MMP-9 via Src. MMP-2 and MMP-9 are type IV collagenase/gelatinase, which in mammals, degrade the collagens of the extracellular matrix and facilitate the ability of cancer cells to invade induce the ability of cancer cells to invade through basement membranes. The tyrosine kinase c-Src, leads to the activation of downstream cascades such as MAPK, PI3K, PKC. C-Src activates these cytoplasmic cascades via interactions with ion channels, G proteins, which are the membrane-associated signaling molecules. The downstream effects of c-Src via the membrane signaling molecules can elicit a number of physiological effects such as proliferation, metastasis and survival [219]. Caveolae is the plasma membrane cavity in which the signaling molecules such as ER and G-protein are enriched in and can interact [220]. Other signaling molecules, which are needed for the initiation of cytoplasmic cascades will also move to the caveolae [219]. The interaction of ERα with G protein in the caveolae can recruit c-Src, [221], Shc (Src homology complex) and p85α subunit of PI3K [222]. As a result of ERα activation, MMP-2 and MMP-9 can then activate multiple kinase cascade of signaling through the EGFR transactivation [223]. Additionally, c-Src, Shc and p85α subunit of PI3K can lead to activation of secondary signaling messengers and downstream kinase pathways such as ERK, MAPK and PDK1/AKT[216, 224-226].

COACTIVATORS & COREPRESSORS OF ERα

It is clear that ER-mediated transcriptional activity plays a significant role in proliferation of both normal and cancer cells. There are also numerous studies suggesting that ER is able to transfer extracellular signals intracellularally through it's non-genomic activity [213]. One approach that has been used to elucidate the mechanisms of E2 action is the identification of the protein partners of ER. These studies were initiated by Onate et. al. who identified a protein that interacts with human steroid receptors to enhance the transcriptional activity. Since the identified proteins stimulated transactivation of the steroid receptors such as; ER, PgR, Glucocorticoid receptor, Thyroid hormon receptor, retinoid X receptor, it has been named as steroid receptor coactivator-1 (SRC-1) [227]. Upon many different studies it is now known that there are a large number of proteins interacting with ER to regulate it's transcription. These proteins are called coactivators and or corepressors based on their effect

on the downstream transcription activity of ER at ERE [228]. Coactivators function as adaptors in a signaling pathway that transmit transcriptional responses from the DNA bound receptor to the basal transcriptional machinery [229]. For example, E2 binding to the LBD of ER induces a conformational change in the AF-2 domain (Figure 2) creating a new protein interaction site that is recognized by coactivators [179]. Alternatively, when TAM binds the conformational change at AF-2 domain is obstructed resulting in the recruitment of corepressors that can actively silence ER responsive genes (180, [229]. The presence of coactivators is essential for ER transcriptional activity [230-232]. Another co-activator of ER is p300 in which in complex with ERα has an intrinsic histone acetylase activity, resulting in the acetylation of lysine residue histone proteins [233, 234]. Like p300, other coactivators such as CBP, SRC1, GRIP1 and AIB1 bind through the coactivators' LXXLL domains and the ER's AF-2 domain (Figure 2) [235-239]. The roles of cofactors are still under investigation but so far it is believed that cofactors can either directly regulate chromatin remodeling or they can recruit factors with chromatin remodeling properties.

Another family of ERα cofactors is the p160 family of proteins, which includes SRC1, GRIP1 (SRC2) and AIB1 (SRC3), all of which have a basic helix-loop-helix (bHLH) domain. The bHLH motif, consist of two α-helices connected by a loop. One helix, which is bigger in size contains the basic amino acid residues that facilitate DNA binding and the other helix is smaller, and, due to the flexibility of the loop, allows dimerization in the form of protein-protein interaction by folding and packing against another helix [240]. bHLH domain on ER coactivator, p160, mediates the initiation of ERα's transcriptional activity [234, 241-244]. E2-bound ERα recruits HATs (p300, pCAF and CBP coactivators) to the ERα transcriptional complex via SRC1 (103, [245, 246]. CBP and p300 coactivators possess intrinsic HAT activity and can interact with all p160 family members [247-249]. P300 can also directly interact with the AF2 domain, which can further promote the transcriptional activity of ERα [236, 250-252]. P300 has not only been shown to be involved in the transcriptional initiation in breast cancer but also in the direct regulation of the acetylation state of AR leading to increased cell proliferation in prostate cancer cells [253, 254]. The increase in expression level of p300 and CBP in invasive ductal carcinoma cases [255] and in prostate cancer [256], coupled with their recruitment to the promoter of target genes underscores the role of HAT activity in regulating hormone response in these diseases [253, 254, 256-258].

Another category of ERα cofactors has histone methyltransferases function and include CARM1 (PRMT4) and PRMT1 [259, 260]. The histone tails get methylated via CARM1 and PRMT1 facilitates subsequent histone acetylation, which results in transcriptional activity [261-264]. CARM1 and PRMT1 interact with ERα indirectly through p160 or p300 cofactors [202, 265, 266]. As with p300 and CBP, the interaction between ERα and CARM1, PRMT1 could happen in the absence of E2, which might be another explanation for the role of cofactors of ERα in overcoming the transcriptional inhibition in the absence of ligand [257, 267]. CBP/p300 acetylase activity and the CARM1 methyltransferase activity can positively regulate the expression of estrogen-responsive genes (i.e. pS2) in MCF-7 cells through arginine methylation followed by acetylation of H3. CARM1 methyltransferase activity follows upon overexpression of CBP in the absence of E2 stimulation, suggesting that the acetylase function of CBP is downstream of E2 stimulation and upstream of CARM1 methyltransferase activity. Additionally the step-wise activation of CARM1 and CBP reveal how the cooperation between these two ERα cofactors can initiate the transcriptional activity without the ligand stimulation [268].

The other fairly new transcription factor, which have been introduced for estrogen receptor is FOXO3a. FOXO3a is a member of forkhead transcription factors, which are categorized in class O (FOXO). FOXO3a have been shown to have a tumor suppressor function in breast cancer[269]. FOXO3a interacts with ERα in MCF-7 cells. The new data on this group of transcription factors suggesting a crosstalk, which connects FOXO3a and ER pathways breast cancer cells expressing ERα[269].

COREPRESSORS of ERα

While HAT activity can acetylate the histone tails and mediate decondensation of chromatin and initiation of transcription, Histone deacetylases (HDACs) can deacetylate and reverse the effect of HATs on the chromatin, resulting in the condensation of the chromatin and inhibition of transcription [270] TAM-bound ERα recruits NCOR1 and SMRT, which act as adaptors for binding to proteins with HDAC activity and repress the ER transcriptional activity (HDAC3 and HDAC1) [237, 271, 272]. Corepressors were first identified in association with PgR in both yeasts and HeLa cells using wild-type and mutant PgR. This study revealed the conformational changes of PgR is different upon whether progesterone agonists or to

progesterone antagonists bind to the amino acida at the ligand binding domain of PgR [273, 274]. Hence a co-activator and a co-repressor not only bind to different regions of the receptor, but the resulting conformational change is also distinct.

NCOR1 protein levels are decreased in invasive breast cancer and in TAM-resistant mouse models. Using MCF-7 as the ER positive breast cncer cell line, investigators found resistance to TAM is associated decreased levels of NCOR1 and sequesteration of NCOR1 and SMRT into the cytoplasm [260, 275], [276]. Specifically, the investigators serum starved MCF-7 cells, in the presence of TAM followed by stimulation with insulin. Upon insulin stimulation the NCOR-ERα association was significantly decreased while the insulin treatment conversely increased the association of two coactivators, SRC-1 and p/CIP with ERα. Additionally, TAM treatment of MCF-7 cells results in the association of ERα with NCOR and SMRT while with pure antiestrogen (ICI 182780 and ICI 164384) treatment did not induce detectable association of ERα with either of the corepressors [276].

In advanced breast cancers, which have developed resistance to TAM, E2 initiation of NCOR1 degradation through proteasomal pathway could be the mechanism of NCOR suppression [277]. Perissi et. al. used embryonic stem cells to show that the two F box related proteins, which are also identified as one of the components in the NCOR complex, TBL1 and TBLR1, are involved in the recruiting the ubiquitin conjugating (19S proteasome complex) to the NCOR [277]. Table 2 has a list of ERα related cofactors and their function and activity.

Name	Coactivator/ Corepressor	Activity	Function	Reference
TAF130/250 histone acetylase	Coactivator	HAT -Acetylate the N-terminal tails of lysine residues in histones H3 and H4 leading to 'relaxed' chromatin structure	Basic component of the Pol II transcription machinery, associated with the promoter	Mizzen et.al 1996
p300/CBP histone acetylase	Coactivator	HAT-Acetylate the N-terminal tails of lysine residues in histones H3 and H4 leading to 'relaxed' chromatin structure	Associated with the Pol II holoenzyme	Nakajima et.al 1997 Bannister et.al 1996
p160 coactivators (SRC-1, GRIP1/TIF2, and pCIP/RAC3/ACTR/AIB1/TRAM1)	Coactivator	HAT-Acetylate the N-terminal tails of lysine residues in histones H3 and H4 leading to 'relaxed' chromatin structure	Associate with steroid receptors	Spencer et.al 1997
P/CAF histone acetylase	Coactivator	HAT-Acetylate the N-terminal tails of lysine residues in histones H3 and H4 leading to 'relaxed' chromatin structure	Forming protein complexes with multiple histone acetylases (p300/CBP and SRC-1)	Turner et.al 1992 Yang et.al 1996 Chen et.al 1997
Gcn5	Coactivator	HAT-Acetylate the N-terminal tails of lysine residues in histones H3 and H4 leading to 'relaxed' chromatin structure	Tightly associated with TFIID or the Pol II holoenzyme	Grant et.al 1997 Eisenmann et.al 1992
ATP-utilizing chromatin assembly and remodeling factor (ACF) & purified recombinant nucleosome assembly protein 1 (dNAP1)	Coactivator	HAT-Acetylate other transcription factors and coactivators	Purified from chromatin structure and the function is cetyl-CoA dependent	Jiang et.al 2000
Coactivator-associated arginine methyltransferase 1 (CARM1)	Coactivator	Recruit secondary coactivators	Bound to the C-terminal AD2 activation domain of p160 coactivators	Koh et.al 2001 Chen et.al 1999

Table 2: ERα coactivators and their type of activity and function-part I

Name	Coactivator/ Corepressor	Activity	Function	Reference
Protein arginine methyltransferase 1 (PRMT1)	Coactivator	Recruit secondary coactivators	Bound to the C-terminal AD2 activation domain of p160 coactivators	Koh et.al 2001 Wang et.al 2001
Brahma-related gene 1 (BRG1/hSNF2β)	Coactivator	Recruit secondary coactivators	Interact with components of various ATP-dependent chromatin-remodeling complexes	TK et.al 2003
SWI/SNF	Coactivator	Partial histone displacement may be a feature of SWI/SNFaction	Direct interaction with and stabilization of basal transcription factor binding	Hagger et.al 1998 Cote et.al 1994 Khavari et.al 1993
NCOR1	Corepressor	HDAC activity	Bind with the CoRNR box (L/I-x-x-I/V-I) to the receptor	Grignani et.al 1998 Hu et.al 1999 Chen et.al 1995
SMRT	Corepressor	HDAC activity	Bind with the CoRNR box (L/I-x-x-I/V-I) to the receptor	Hu et.al 1999 Fischle et.al 2002 Chen et.al 1995
Vertebrate Atrophin-1 (ATN1)	Corepressor	Drosophila Atrophin directly interacts with the transcription repressor “even-skipped” and is required for its repressive function in vivo	Containing CTG/CAG or CCG/CGG triplet repeats	Wang et.al 2008 Li et.al 1993 Zhang et.al 2002

Table 2: ERα coactivators and their type of activity and function-part II

ESTROGEN RECEPTOR BETA (ERβ)

A second ER isoform, ERβ, has been identified recently. The two ER isoforms have a similar domain structure as shown in Figure 5 (5). The central DNA binding domain (DBD-C region in the figure) is highly (97%) [278, 279] conserved between ERα and ERβ (Figure 5). The C-terminal ligand binding domain, which is responsible for high affinity ligand binding, dimerization, and hormone-dependent activation (AF-2- E region in the figure), is moderately conserved (59%) (283, 284). The N-terminal region contains hormone-independent activation function1 (AF-1- A/B region in the figure) and its poorly conserved in ERβ (18%) (283, 284, 23).

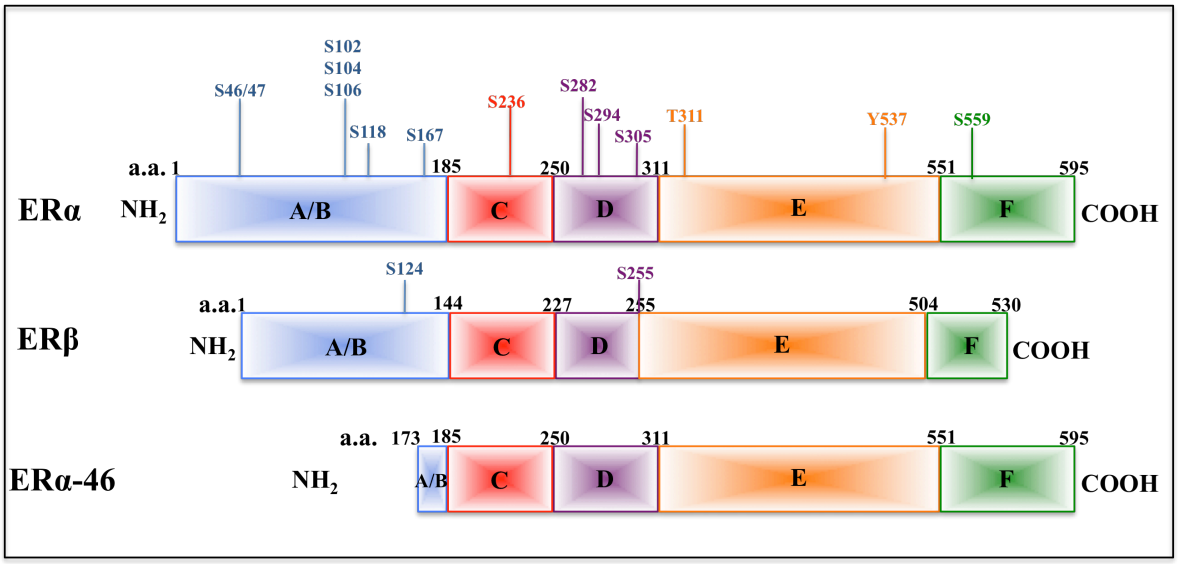


Figure 5: ERα and ERβ protein structure

ERα and ERβ show significant overall sequence homology. Both are composed of five domains (A/B=AF-1, C= DNA binding region, D= Hinge region, E +AF-2 and F domain function is not entirely clear). The 46 kDa, ERα isoform lacks 173 amino acids at the N-terminal compared to the full length 66 kDa ERα. ERα-46 is encoded by a new class of ERα transcript that lacks the first coding exon (exon 1A) of the ERα gene [280].

The current paradigm of estrogen signaling in breast cancer is that ERβ can function a repressor of ERα gene transcriptional activity [281] [282]. For example, ERβ inhibits proliferation of T47D in the presence of E2. The inhibitory effect of ERβ on the proloferation in T47D cells is concomitant with a decrease in some of the main regulators of cell cycle including; cyclin E (both mRNA and protein), Cdc25A mRNA, p45 (Skp2) protein. P45 regulates p27 proteolysis. In addition the inhibitory effect of ERβ also causes a reduced Cdk2 activity. Similarly, the expression level of the brakes of the cell sysle, such as p27 (Kip1) were increased [282]. Further evidence for the opposing action of ERα and ERß was observed when cyclin D levels were decreased in response to ERβ over expression in T47D and HC11 cells [282-285]. HC11 is derived from mouse mammary epithelial cells (282). Upon ERβ overexpression, pS2 and PgR transcription could be suppressed via binding of ERβ to the promoters of these genes in a repressive manner [286]. In this study, T47D cell line with an inducible tet-off FLAG-ERβ was generated to examine the kinetics of expression of pS2 and PgR, which were downregulated in response to ERβ induction [286]. The differences in the ERs were also evaluated in the mouse knock out models. When ERß knock out (BERKO) mice ($ER\alpha^{+/+}\beta^{-/-}$), which were generated by crossing male double-heterozygous ($ER^{+/-}ß^{+/-}$) mice with female double-heterozygous ($ER^{+/-}ß^{+/-}$) mice [287], were examined for bone loss the results revealed that female mice with ERβ knockout were partly protected against age-related trabecular bone loss. Peripheral Quantitative Computed Tomography (pQCT, a test to measure bone density) was used to measure trabecular volumetric BMD (Bone Mineral Density) in the metaphysis of the distal femur and in the proximal tibia in both the ERα knock out mice (ERKO) and BERKO. The pQCT results showed that BERKO mice do not show any significance change in the BMD, while ERKO mice showed decreased BMD as well as radial skeletal growth. Thus, ERα but not ERβ is essential for the maintenance of bone mass in female mouse [288].

One possible mechanism by which ERβ represses ERα activity could be through preventing of the recruitment of particular cofactors of ERα. For example, a CHIP assay targeting ERß depicted a marked reduction in the recruitment of AP-1 factors to both the pS2 and PgR promoters. Additionally, upon ERβ expression the recruitment of c-Fos to the PgR

promoter is abrogated in part due to abrogation of E2-dependent induction of c-Fos mRNA [286].

The function of ERβ in breast cancer remains unclear as current data suggests different function and pattern of expression of ERß as compared to ERα. ERβ is expressed in surrounding stromal cells rather than in epithelial tissue of the mammary gland, which is the case for ERα (145). The comparison between the distribution of ERα and ERß mRNA levels in different tissues of a normal rat revealed that ERβ mRNA is expressed in organs such as: 1) Prostate, 2) Ovary, 3) Lung, 4) Bladder, 5) Brain, 6) Uterus, 7) Testis. However they showed that ERα mRNA, is mainly expressed in 1) Uterus, 2) Testis, 3) Pituitary, 4) Ovary, 5) Kidney, 6) Epididymis, 7) Adrenal [289]. The lack of ERβ protein affects tissues such as ovaries, uterus, bladder and lung, while mammary glands have minimal functional defects as a result of ERβ deficiency [287, 290, 291].

The ability of ligand binding and activity have been compared between the two receptors based on different levels of homology of DBD and LBD found in each receptor (see Figure 5). For these analysis, the binding affinity of diethylstilbestrol, TAM,17ß-E2 and ICI-164384 to each receptor was measured using radioligand upon treatment with different doses of unlabeled competitors. These studies revealed that the ligand binding (set as arbituary values with that for E2 being 100 for each receptor) was different for each receptor. For example, the ligand binding values for diethylstilbestrol is 468 and 295, for TAM is at 6 and 7, for ICI-164384 is 85 and 166 for ERα and ERβ respectively [289]. Hence, these results reveal that in addition to the differences enumerated above, there are also significant differences in ligand binding affinities between ERα and ERβ. It is thought that the reason patients with the same ERα levels respond differently to endocrine therapy may be due to differences in the level and activity of ERβ.

ERα IN NORMAL MAMMARY TISSUE

The female mammary gland is in a state of quesante until puberty, which at that point the cells division increases substantially. Upon onset of puberty during the adult life some cyclical changes (estrous cycles) are observeable in female's mammary gland cells. These cyclical changes contain the proliferation of the cells, which followed by the involution [292]. The expression pattern of hormones during estrous cycle is depicted in Figure 6. In

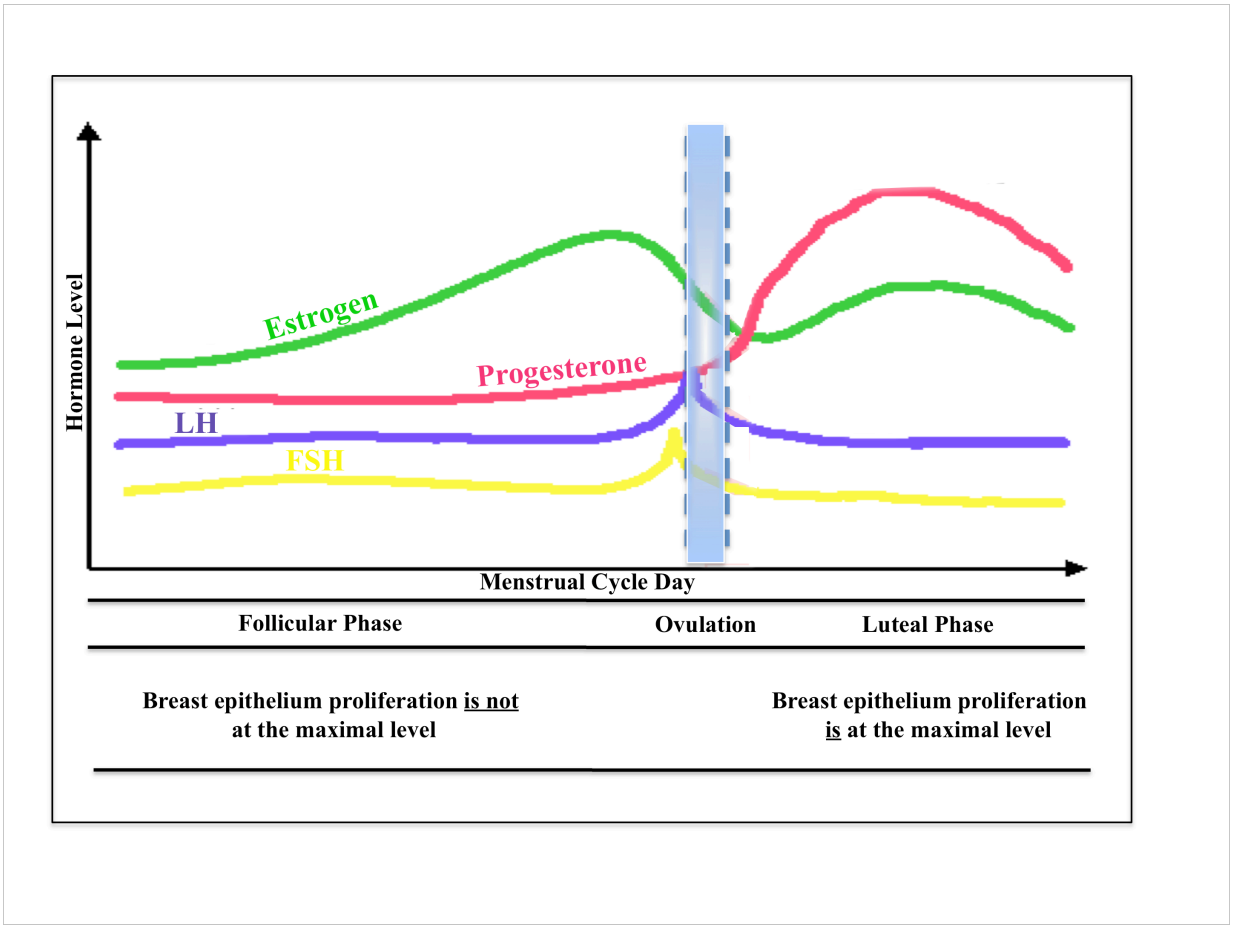


Figure 6: Menstrual cycle in humans

The menstrual cycle is a recurring cycle of hormonal fluctuation, which begins at menarche and ends at menopause. During each cycle a series of physiological changes occurs in order to prepare the female body for the purpose of fertilization and ovulation. Unless pregnancy occurs, the cycle will repeat.

the adult breast, the luteal phase marks the phase of the estrous cycle that the proliferation of breast epithelial cells are maximal, which the exact timing is a week after ovulation. Both hormones, E2 and progesterone, are being secreted through corpus luteum during luteal phase while during follicular phase only the E2 level is high [293-300].

However, normal human breast tissue proliferation appears to be solely dependent on E2 with no obvious effects of progesterone [301]. To examine the specific function of E2 versus progesterone in the normal mammary gland proliferation, pieces of normal human breast tissue were implanted into athymic nude mice (subcutaneously) [301]. Slow-release E2 and/or progesterone pellets were also inserted subcutaneously into these mice and the rate of proliferation of the implanted tissues was assessed by thymidine uptake. The results revealed that E2 pellet increased the thymidine labeling index from a median of 0.4% to 2.1% after 7 days while progestrone pellet had no effect on the thymidine labeling index (306). Additional evidence for the importance of E2 in maintenance of a healthy breast is the association of the breast cancer risk with the longer duration of E2 exposure.

For example early onset of menarche and late menopause are both associated with greater cancer incident risk [302]. During the proliferative stages of a normal breast, which are both puberty and the estrous cycle (Figure 6), the majority of proliferating cells do not express ER or express ER in a very low level in terminal end buds and ducts [303]. The level of differentiation of mammary parenchyma determines the mammary epithelium proliferative activity [302]. In post-pubertal women the lobule type 1 (Lob 1) is the most undifferentiated structure. Lob 1 is also known as Terminal Ductal Lobular Unit (TDLU). Lob 1 progresses to Lob 2, which are more morphologically complex morphology and are more differentiated when compared to Lob 1. Lob 1 and Lob 2 progress to Lob 3 and Lob 4 during pregnancy under hormonal influence [304]. Upon the full differentiation of Lob 1, Lob 2 and Lob 3 into Lob 4 during pregnancy, the proliferative activity of mammary epithelium is reduced [292].

The content of ERα and PgR in the lobular structure are directly proportional to the rate of cell proliferation. The ERα/PgR content of epithelial cells in Lob1 is much higher (14%) than Lob 3 (0.5 %) due to the higher proliferation activity of cells in Lob 1, which could explain the higher susceptibility of Lob 1 to transform and become the site of origin for ductal carcinomas [4-6]. Lob 1 contains at least 3 different cell types based on their ER and proliferative ability as masured by Ki67 expression: A) $ER^-/Ki67^+$, B) $ER^+/Ki67^-$, C)

$ER^+/Ki67^+$. These three cell types are also regulated by positive and negative feed back loop mediated by estrogen signaling. For example, E2 stimulation of group B could release certain growth factors, which through paracrine pathways can increase the proliferation of group A. The group C cells could be the precursor of ER-positive tumors. The groupings into the three different cell types is dependent on two proteins, Ki-67 and ER. Ki-67 is a potent tool for evaluating the proliferation index of cells in the three different goups. The antibody against ki-67 recognize the nucleare antigen ki-67, which is only expressed in cycling and not G0 cells [305]. Using an antibody to ER to determine the precise level of expression of ER is critical since, the ER content of the tumor cell dictates if a patient will respond to endocrine therapy. Additinally, there is some controversary regarding the possibility that ER-negative cells maybe capable of reverting to ER-positive cells [306, 307]. To address this question, a study was initiated to examine whether cellular expression of ER occurrs on a clonal basis or as a function of the differentiation process. For these studies MCF-7 cells were subjected to soft agar colony assay in the presence or a TAM followed by immunoperoxidase staining with monoclonal ER antibody. The results revealed that there was heterogeneity in ER expression among cells within the same clone or different clones in agar cultures. TAM significantly reduced clonal growth. The ERexpression was so low in the proliferating clones , which were unresponsive to the antiestrogen (TAM). Based on these results, some investigators propose that change of ER expression occurs concommitantly with differentiation of cells within clones [306]. They suggest that ER-positive colonies may arise from ER-negative progenitors. There is also evidence that the proliferation state of the cells can be independent of the ER/PgR content of the cell. Knabbe et. al. suggest that adjacent cells can control the proliferation of adjacent cells through autocrine/paracrine function. [308-310]. To this end they co-cultured MCF-7 cells with MDA-MB231 cells and found that secretion of biologically active Transforming Growth Factor-β (TGF-β) from MCF-7 cells can result ininhibition of cell proliferation in MDA-MB231 cells [308-310].

40% of the epithelial cells in the prepubertal rats express ER in the nucleus. The level of ER expression in these cells decreased to 30% upon puberty. The level of ER expression decreased even more to 5% upon pregnancy to only 5%. However there was a huge induction in the nuclear ER protein level during lactation, which was up to 70%. Studies show that about 90% of ERβ expressing cells do not proliferate (2). Also studies have shown that 55-

70% of thecells, which are dividing express neither ERα nor ERβ (2). These data shows that none of these receptors are a prerequisite for estrogen-mediated proliferation [2]. Similarly in proliferating MCF-7 cells treatment with any of the SERMs (i.e. TAM) results in increase of ERα levels. The increase in ERα results in the increase in p27, which is a marker of non-proliferative cells [276].

In another model system, which is done in the cells from the rat mammary gland, the stage of mammary gland development is dependent on the percentage of ERα expressin in the nuclei Shyamala, 1990 #706}. ERα is expressed in 30% of the cells during puberty. The ERα expression level goes down to 5% at day 14 of pregnancy. The ERα expression in the nuclei increase during lactation till the expression level reaches to 70% by day 21. Through out pregnancy cells do not express ERα or express a very low level of ERα , which no or low level of ERα has been shown (discussed in my current study) to be a marker of proliferative phenotype. However, ERα reexpress during lactation in 60% of breast epithelial cells. These cells are non-proliferative and E2-insensitive [311]. These studies, using normal mammary gland, highlight the paradoxical role of ER has on cell proliferation.

Unlike normal mammary epithelial cells containing ER/PgR, which do not proliferate in response to estrogens, human preadipocytes are stimulated to proliferate by estrogens. When human preadipose cells are treated with estrogens, c-myc and cyclin D1 expression are induced, suggesting that they may be mediators of estrogen stimulated proliferation in these cells [312]. The pathway that seems to be activated in these cells is the autocrine/paracrine pathway. Proliferating cells are often located adjacent or in close proximity to non-dividing cells that contain ER/PgR [292, 303, 313, 314]. ER/PgR positive cells can stimulate proliferation in adjacent ER/PgR negative cells via paracrine signals through E2-induced genes such as PgR, pS2 and genes that encode the growth factor amphiregulin. Amphiregulin binds to EGFR and mediates signaling through intracellular pathways such as MAPK, JAK and STAT to stimulate proliferation through Myc, Myb and ETS and cyclin D1 [315].

Another example of the paracrine/autocrine cross talk was revealed upon examination of proliferation rate of lobules in non-tumor bearing women throughout the menstrual cycle. The proliferation rate in these women showed a correlation with different satges of the menstrual cycle, which the details of this study have been explained below. This study started by enrolement of 83 women to this study. Breast tissue samples were collected from these

women at different stages of menstrual cycle. Samples were analyzed for the proliferation and apoptosis rates. Interestingly there was a sequential cell multiplication (mitosis) and cell deletion (apoptosis) during each menstrual cycle (Figure 6). There was a higher levels of mitosis and apoptosis in the second half of menstrual cycle [295]. The higher level of mitosis and apoptosis at the second half could be due to higher E2 and progesterone level at this half of the menstrual cycle (Figure 6). There was a cyclical changes between the mitosis and apoptosis throughout the 28-day menstrual cycle. The peak for apoptosis was 3 days after the peak for mitosis. Breast, is a target tissue for E2 and progesterone, so the abundance of these hormones at the second half of menstrual cycle cause the higher proliferation rate which is followed by apoptosis to control the cell number and homeostasis of the tissue.

ERα AND ITS INTERACTION WITH CELL CYCLE MEMBERS

Dearegulated expression of key cell cycle regulators can trigger a cascade of events leading to mammary tumorigenesis [312]. Both ER receptors have been shown to influence cell proliferation and cell cycle. A well established example linking the ERα with the cell cycle is through its interaction with cyclin D1 [312]. Cyclin D1, a key cell cycle regulator, binds to the Retinoblastoma protein (Rb), which directs Cdk4 and Cdk6 to Rb, allowing for its efficient phosphorylation and resulting in the passage of cells from G1 to the S phase of the cell cycle [164] (Figure 7). Cyclin D1 is required for normal breast cell proliferation and differentiation associated with pregnancy. However, cyclin D1 also have shown to be an important factor in the breast cancer development (Figure 7) [316]. To evaluate the oncogenic potential of cyclin D1, Schmidt et al. generated transgenic mice over expressing cyclin D1, which overexpression of cyclin D1 gave rise to the malignant mammary cell proliferation, which end up to the development of mammary adenocarcinomas [317]. Additionally, cyclin D1 highly express in majority of human breast adenocarcinomas [318, 319]. Estrogens can induce the expression of cyclin D1 via the binding of the liganded ERα to the cyclin D1 gene promoter to activate its transcription [320, 321]. When MCF-7 cells were cultured in an E2-free condition about 72 hours and restiumulated with E2 and subjected to ChIP assays, results reveated that ERα protein binds to a site downstream from the cyclin D gene, important for its transactivation [272]. Moreover, E2 increased p300 and Forkhead box protein A1 (FoxA1, an ERα transcription factor) recruitment to the cyclin D regulatory regions, which prepares the

site for transcriptional activation of cyclin D (Figure 7). Furthermore, cyclin D mRNA and protein expression levels increase upon ERα, FoxA1 and P300 binding to the promoter while cyclin D transcription is disrupted upon downregulation of each of these factors [321].

Cyclin A is another key cell cycle regulator, which has been linked to the ER pathway. Cyclin A shares several features with cyclin D, such as phosphorylating Rb protein upon binding to it. Due to its function in phosphorylating Rb it is normal to expect that the modulation in cyclin A expression results in the irregulation of the G1 to S progression [82, 322]. The phosphorylation of serine residues located at amino acids 104 and 106 of ERα protein via cyclin A-Cdk2 complex causes the increase in the transcriptional activity of ER [323]. Additionally cyclin A over expression leads to increased ER transcriptional activiation independent of E2 or TAM treatment [323].

Additionally, as shown in Figure 7 ER may influence cell proliferation by direct protein-protein interactions with regulatory proteins such as p27. p27 is the main inhibitor of the cyclin A-Cdk2 complex resulting in the arrest of cells at S phase and activation of apoptosis (221).

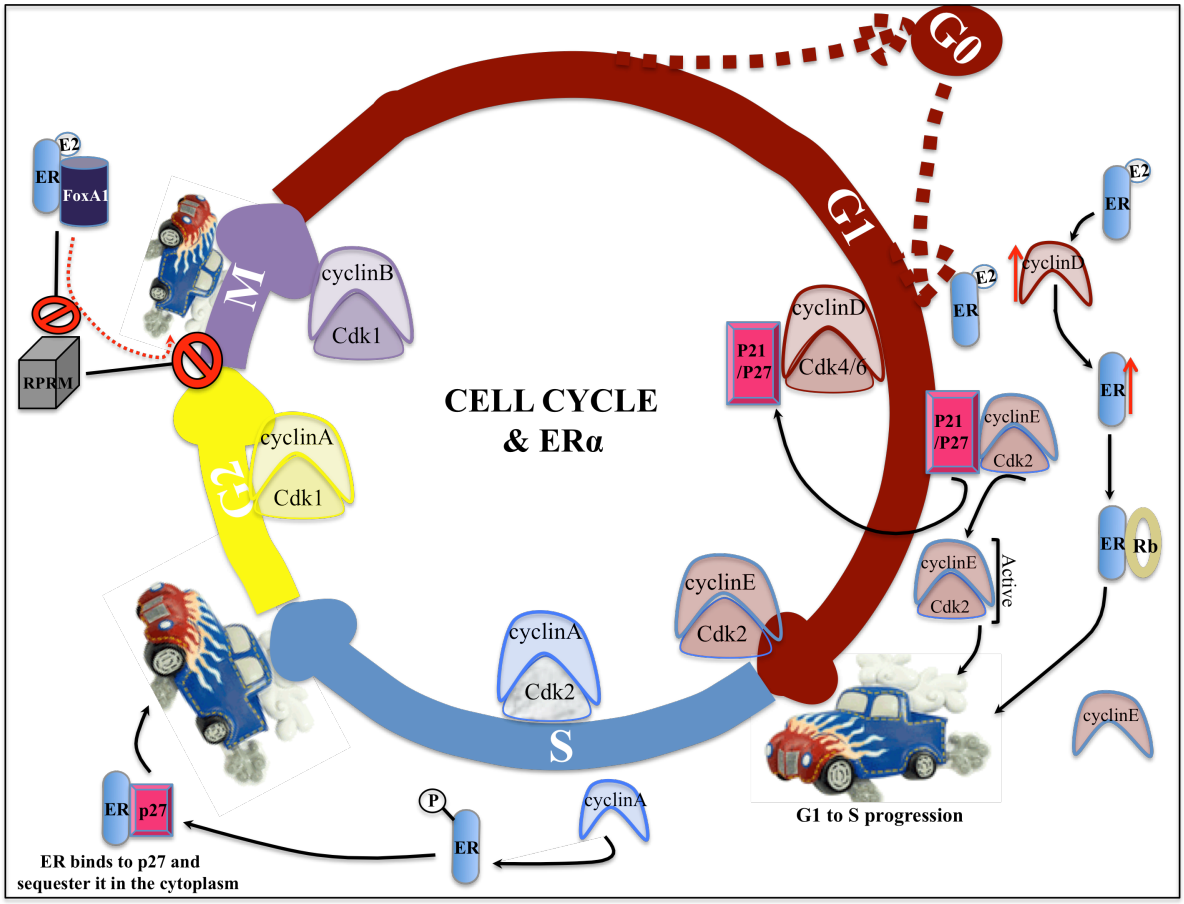


Figure 7: ERα involvement in cell cycle

Role of ERα during different phases of the cell cycle, and identification of interactions between ERα and cell cycle elements, such as FoxA1 and Rb during G1 phase and ER interaction with p27 during S phase.

ERα is capable of binding to the C terminal region of p27 to sequester it to the cytoplasm, which interrupt p27's inhibitory activity in the cell cycle (Figure 7) [324].

Other members of the cell cycle regulatory pathway are also indirectly involved in ER function or its regulation. For example, when MCF-7 cells are serum starved and stimulated by E2 to reneter the cell cycle, cyclin E-Cdk2 is activated which leads to the phosphorylation of the Rb and the progression of the cell cycle from G1 to S phase (Figure 7) [325]. E2 (1nM) was sufficient to induce Cdk2-associated histone kinase activity and Rb kinase activity 8 and 5 fold respectively higher than that in growth-arrested cultures [326]. Additionally, levels of cyclin D and E, proteins were increased sequentially post E2 treatment of growth-arrested MCF-7 cells [325].

It has been reported that the E2 mediated transactivation of cyclin D which results in overexpression of cyclin D also results in the shift of p21 complexing with cyclin E-Cdk2 to cyclin D-Cdk4. The dissociation of p21 from cyclin E-Cdk2, results in the activation of cyclin E-Cdk2 complex. Additionally, association of p21 with cyclin D-Cdk4 has been shown to activate this complex as well [325]. These results support the notion that E2 can manipulate the cell cycle progression and in a bigger picture the proliferation rate of the breast cancer cells by modulating the G1 phase cyclin, cyclin D and also regulating the activities of G1 cyclin-dependent kinases [325].

Another way that E2 can modulate the transition of cells from one phase of the cell cycle to the next is by the inhibition of the negative regulators of the cell cycle. For example, E2 can repress Reprimo (RPRM), a cell cycle inhibitor (Figure 7) [327]. RPRM is a protein known to be induced following irradiation in a p53-dependent manner. RPRM was first identified following a screen of genes induced by x-ray irradiation in wild-type as compared to p53-/- MEF cells. RPRM mRNA levels were induced in wild-type MEFs and not in p53-/- MEFs following x-ray irradiation [328]. Over expression of RPRM in various cell lines, including HeLa, MCF7 and mouse NIH3T3 cells resulted in G2 arrest of the cell cycle by inhibition of Cdk1 activity and also interfering with the nuclear translocation of the Cdk1-cyclin B1 complex. In double thymidine synchronized HeLa cells, transduced with RPRM adenovirus, resulted in the cytoplasmic accumulation of cyclin B and inhibition of key M phase events such as chromosomal condensation. (333, [329-331]. Formation of a complex between ERα and histone deacetylase 7 and FOXA1 is the necessary first step to inhibit

RPRM [327] (Figure7). FOXA1, protein expression (IHC score greater than 3) in breast cancers has been associated with ERα positivity, luminal A subtype and better predictor of survival compared to PgR [332].

While the examples listed up to this point underscore the importance of E2 in activating cell cycle progression, there are also examples of how modulation of E2 leads to inhibitory affects on cell cycle. The genes that are repressed by E2 include genes encoding proteins such as CD24, E-cadherin, Breast Cancer And Salivary gland Expressed Gene (BASE), Interleukin-6, Insulin Receptor (IR), pRb, ERBB2, vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), and CD36 [333-340]. Below I have highlighted the repressive action of E2 onRb and IL-6.

There have been studies that link increased risk for breast cancer in the mothers of children suffering from retinoblastoma and osteosarcoma [341]. In fact, MCF-7 cells treated with 10^{-9} M E2 for 48 hours lead to decreased expression of Rb protein by 70%, and its mRNA by 50% [336].

Cytokine interleukin-6 (IL-6), is a key mediator of immune system, and acts as both a pro-inflammatory and anti-inflammatory cytokine [342]. The role of estrogens on the activity of IL-6 were examined in endometrial adenocarcinoma cell line Ishikawa which were treated with phorbol esters to induce the activation of the IL-6 promoter [339]. In these experiments Ishikawa cells were transfected with IL-6/luciferase in the presence or absence of ER. Eighteen-hours post-transfection cells were treated with E2 in the presence or absence of phorbol ester. E2 treatment showed an ER-dependent inhibitory effect on IL-6 promoter, an affect that was abrogated when mutant ER transcripts were used. These experiments suggest that ER requires both its DBD and HBD to repress IL-6 while residing on the IL-6 promoter region. Similarly, the direct interaction of ER with the transcription factors NF-IL6 and NF-kappa B results in their inability to bind DNA, and provides a plausible mechanism by which estrogens lead to repression of IL-6 gene expression (344).

Another cell cycle related gene that is repressed by E2 is the G1 cyclin called cyclin G2. Similar to cyclin E, cyclin G2 expression site is in highly differentiated tissues, such as heart, muscle, and brain [343]. The function of these cyclins despite the other "established" cyclins, which are mainly involved in progression of the cells through cell cycle phases, is associated with the inhibition of the cell cycle progression by negatively controlling the cell

cycle progression and maintaining the cells in a quiescent state [344]. In fact, cyclin G2 was identified in microarray analysis as an E2 down-regulated gene in MCF-7 [345]. Cyclin G2 mRNA levels are repressed in response to E2 treatment with very rapid kinetics. ChIP assays also revealed that E2 treatment results in the recruitment of ER to the cyclin G2 promoter region, followed by detachment of the RNA polymerase II detaches, and formation of a complex containing N-CoR (Nuclear Receptor Corepressor), and histone deacetylases (i.e. HDAC1). These steps act coordinately to repress the cyclin G2 transcription [346]. Due to inhibitory effect of cyclin G on the cell cycle it's repression, augments the proliferation and survival of the tumor [346]. The one common modality in transcription repression is the recruitment of repressive complexes such as NCoR SMRT (Silencing Mediator of RAR and TR), HDAC1 and CtBP1 (C-terminal-binding protein 1) to the promoter region of the target gene.

TAM-bound ERα associates with the corepressors, NCOR and SMRT, which result in the repression of transcription [345]. However, silencing of both corepressors (NCOR and SMRT) led to TAM induced stimulation of the cell cycle [271]. Silencing of NCOR and SMRT in MCF-7 cells treated with with E2 or TAM did not alter the activation of the ER target genes such as c-myc, cyclin D1 or stromal cell-derived factor 1, however, XBP1 was markedly elevated in these cells [271]. Additionally, in MCF-7-derived TAM-resistant cells XBP-1 expression was elevated 3 fold compared to the parental cell line [347]. What is missing from these studies is role for XBP-1 in E2- or TAM-mediated cell proliferation. These examples suggest that in breast cancer cells NCOR and SMRT prevent TAM from stimulating proliferation through repression of XBP-1.

The role of ER in the G2/M phase of the cell cycle has not been explored as thoroughly as its role in G1 phase, which as discussed is the mediator of cell proliferation. However, there are several studies, which show that there might be link between the ER-E2 pathway and G2/M phase. The key regulator of the G2/M transition is the cyclin B-Cdk1 complex, which resides as an active complex until initiation of metaphase. In order for proliferating cells to enter into anaphase, cyclin B needs to be degraded via the APC [202]. Mitotic Arrest Deficient 2 (MAD2) protein, which interacts with APC, is one of the kinetochore proteins present on the chromosomes during cell division and is involved in the attachment of the chromosomes to the mitotic spindle upon anaphase onset. MAD2 inhibits the activity of

APC, which results in the blockade of anaphase. ERβ interacts directly with MAD2 and increases MAD2's activity. Moreover, the ERβ/MAD2 complex helps to correct chromosome orientation in the mitotic spindle through binding of MAD2 to the kinetochores [348]. These studies suggest that ERβ possesses an ability to delay cell division and activate apoptosis. So based on the ERβ and MAD2 relationship and the opposite activities of ERα and ERβ, one can conclude that ERβ could have an inhibitory effect on the cell cycle during G2/M, by regulation of chromosomes attachment to the mitotic spindle prior to anaphase entery. The studies described in the following chapters of my thesis have interrogated the effects of ERα on G2/M phase and reveal that ERα hastens the passage of cells from S and G2/M by upregulating ER target genes, which result in increased proliferation. Based on the disparate roles of ERα and ERß, one can predict that while ERα leads to the rapid progression of cells from S and G2/M, that ERß may result in the slowing down of the G2/M transition.

There have been also been reports providing evidence for cross talk between ERα and cyclin B. In one study aimed at examining how RanBP-type and C3HC4-type zinc finger-containing protein 1 (RBCK1), a protein kinase C1 (PKC1) interacting protein, result in the progression of cell cycle by driving the transcription of both ERα and cyclin B1 in ER-positive breast cancer cells [349], ChIP analysis on parental MCF-7 cells revealed that RBCK1 is recruited to the major ERα promoter region, which results in induction of ERα mRNA levels. No RBCK1 was detected on the cyclin B promoter. However, when RBCK1 was silenced, both both ERα and cyclin B mRNA and protein levels were reduced and cells were arrested in the G2 phase. Addition of cyclin B, but not ERα, released the cells from G2 arrest. This study connects both ERα and cyclin B to the progression of cell cycle. The importance of cyclin B for the cell cycle progression is not a new concept while the connection of ERα to the cell cycle progression is new and supports our observation in this study.

ERα LOCALIZATION

ERs are classically viewed as nuclear receptors, and reside in the cytoplasm in the absence of ligand. Heat shock protein 90 (HSP90) is the chaperone protein binding to

unliganded ER in the cytoplasm (54). Ligand binding triggers a conformational change and the subsequent translocation of the receptor into the nucleus where it functions transcriptionally. There have been reports suggesting that ERα is also localized to the cytoplasm and cell plasma membrane [223, 350]. The studies were initiated by immunofluorescence staining which detected ER in the cytoplasm [350]. Others have shown that the cytoplasmic ER has non-genomic functions (Figure 4) [186, 351]. ER localization to the nucleus versus cytoplasm could explain, at least in part, the possibility of existence of multiple complimentary and overlapping mechanisms that might be working to influence ERα interactions in the cell. Membrane-associated ERα is G-protein linked and E2 binding to ER has been shown to activate many signal transduction pathways that emanate from G-protein activation, such as MAPK and PI3K pathways as shown in Figure 4 [352]. Although the ERα that resides in the cell is localized to different regions and mechanistically be different (signaling versus transcriptional transactivation), the cell biological roles are likely to overlap or be complementary [225, 353-355]. For example E2 bound ERα can rapidly activate MAPK in breast cancer cells. MAPK activation involves the phosphorylation of Shc (Src-homology and collagen homology), which in turn results in Shc-Grb2 (growth factor receotor protein-2)-Sos (son of sevenless) complex formation in MCF-7. The biological consequence of the activation of the down stream proteins in the MAPK pathway is the transcriptional activation of ERE (Estrogen Response Element) and SRE (Serum Response Element), which results in a higher proliferation rate of the cells [225].

Even though these studies support the presence of a functional ERα in the plasma membrane, other groups, suggest that the ERα present in the cell membrane is not the full length protein, but the amino-terminal truncated product (ER46) of full length ERα (ER66), which is detectable by the ERα antibody and capable of transducing E2-triggered signaling (Figure 4,5) [207]. For these studies ER46 was stably overexpressed in immortalized human endothelial cells (EA.hy926), cells were separated into subcellular fractions, and the expression of ER was monitored in each cytosolic fraction. The results revealed that ER46 is found in the plasma membranes, and the components (Figure 8) [207]. ER46 is devoid of the N-terminus, which contains the A and B domain (AF-1) of ERα protein. ER46 is capable of binding to ER66 and forming heterodimers in order to activate the estrogenic response. Additionally, upon binding to ER66, ER46 serves as a competitive inhibitor of ER66 for

DNA binding. However since ER46 is missing the AF-1 domain it can only function as a competitive inhibitor when transactivation is mediated through the AF-1 domain [280, 356, 357].

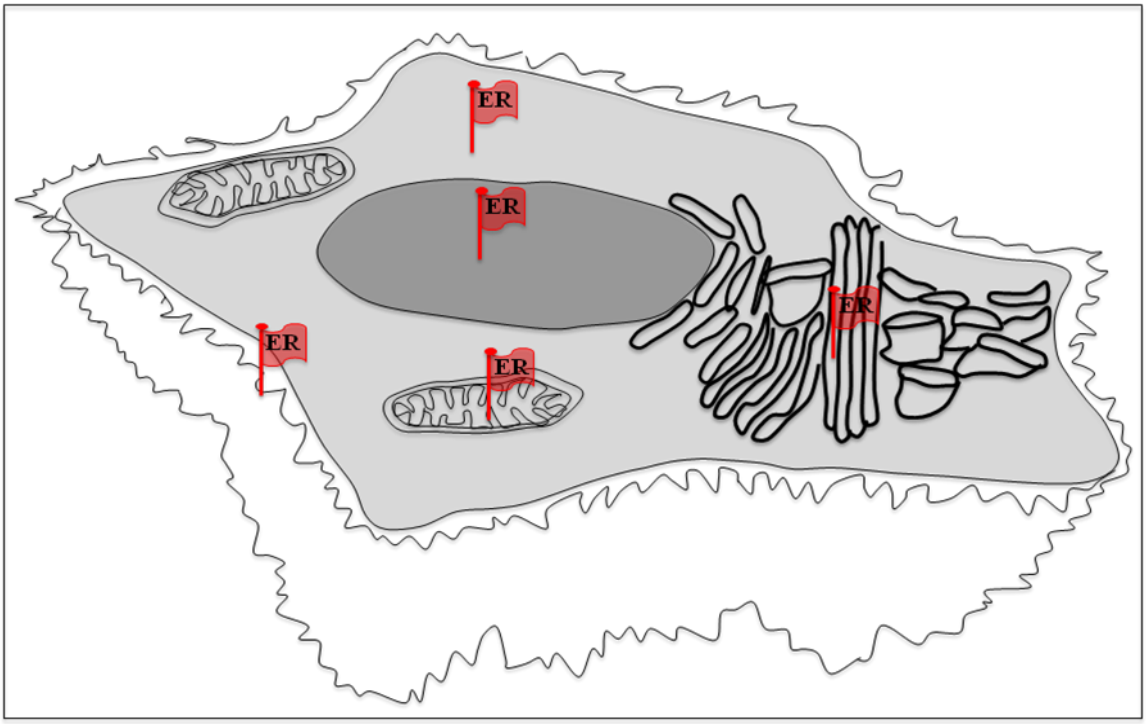


Figure 8: ERα localization in the cell

ERα is primarily located in the nucleus. However, a fraction of the ERα is localized to the plasma membrane region, which mediates signal transduction in a non-genomic manner. There are other studies showing the presence of functional ERα in the cytoplasm, endoplasmic reticulum, mitochondria and microsomes. The precise structural features and different actions of ERα that result from the different localization of this protein have not been determined.

To this end a a series of cotransfection experiments of ERα66 and ERα46 with different ratios were performed in HeLa and the HepG2. These cell lines were ideal for these experiments since they are very specific in the manner in which ER transactiation occurs. In Hela cells the ERα transactivation is mainly through the AF-2 domian while in HepG2 ERα signaling is dominant through the AF-1 domain [358-360]. The results revealed that while ERα46 is a potent competitive inhibitor of ERα66 in HEPG2 cells resulting in the complete suppression of ERα66 activity, that in HeLa cells ERα46 did not inhibit the transactivation of ERα66 [280].

The differential localization of ERα could also be organ and gender dependent. For example in uterine Syrian hamster myometrium (SHM) cells ER is mainly perinuclear [361]. SHM cells are primary uterine leiomyosarcoma cells, which are stimulated by both chronic E2 and androgen treatment. In lung adenocarcinomas, whether the cells are derived from a female (H1793) or a male patient (A549), the localization of ER is changed [362]. Both cell types have equal ER expression howover the H1793 and not the A549 cells respond to E2 by transcriptionally activating proliferative genes. When both cell lines are cultured in the presence of E2, only in H1793 but not A549 cells, the phospho-serine-118-ERα is increased concommitatnly with cyclin D-both in the nucleus as measured by dual-label immunofluorescence staining and fractionation. These experiments, in just one pair of cell lines do provide evidence supporting the gender dependent localization of ER. It is important to note that the addition of E2 in H1793 activates the non-genomic activities of ER through MAPK signaling, which could be responsible for the sex-dependent differences in the incidence of lung adenocarcinoma. Additionally, since there is a two fold higher risk of lung cancer in females as compared to males, one can propose that there may be ER mediated sex-dependent factors, which could cause the differences in the etiology of lung cancer [363].

In addition to the nuclear, perinuclear, cytoplasmic and memberane localization of ER, there is evidence ERα also localizes to the nuclear matrix complex [364]. These studies show that 16α-hydroxyestrone, a natural metabolite of E2, is able to interact with ERα covalently sequestering to the complex to the nuclear matrix as performed by frationiation, which followed by the analysis of the samples via western blot on treated MCF-7 cells (Figure 9). The levels of 16α-hydroxyestrone metabolite of E2 was also measured in 10 normal and 33 breast cancer patients and the results revealed that the level is higher in breast cancer

patients, which is statistically significant (14.9%) compared to normal women (9%) [365, 366]. Additionally in patients with breast cancer and the high-risk women, the enzyme involved in formation of 16α-hydroxyestrone from E2 (estrogen 16 alpha-hydroxylase) is shown to be at higher level compared to normal women.

The translocation of ER by pharmacological agents has also been examined to assess if the signaling of ER either through its genomic or non-genomic pathways is altered. To this end, several different cell lines from different tissues, COS-7 (monkey kidney-derived cell line), Mouse osteoblastic cell line, which is called MC3T3-E1 and MCF-7 cells all were transfected with ERα-GFP. Upon ERα-GFP transfection cells were treated with either E2 or Raloxifene (RLX), a SERM and ER localization was monitored microscopically. The results showed that in 80% of both E2 and RLX-treated MCF-7 cells there was nucleolar translocation of ERα. The transcriptional activity of ERα was also examined under E2 and RLX treated conditions in ERE-Luciferase and ERα transfected cells. The results revealed that in contrast to E2 treated MCF-7 cells, RLX treated cells show an inhibition of the ERα mediated transcriptional activity [367]. Overall the results here show that RLX induces its inhibitory effect on the growth of mammary gland cells by inducing the nucleoli translocation of ERα.

ERß is also subject to multiple cellular localization. ERß is localized to the mitochondria and co-localizes almost exclusively with a mitochondrial marker (MitoTracker Red) in rat primary neurons, primary cardiomyocytes, and a murine hippocampal cell line. Microscopically, the ERβ staining was distributed predominantly in the cytosol in a punctuate form similar to that of the MitoTracker Red staining. To examine the response of ERß to ligand, the primary cardiomyocytes were cultured in an E2 containing media followed by western blot analysis with ERß and MnSOD (mitochondrial marker) and Histone H1 (a nuclear marker). The results revealed that in response to the ligand, ERß did not translocate to the nucleus [368].

ERß has also been detected in mitochondria as a protection against apoptosis. When C2C12 cells (murine skeletal muscle cell line) were treated with E2 (10^{-8} M) for 45 minutes prior to the apoptotic stimulus of H_2O_2, apoptosis (as measured by staining for nuclear and DNA fragmention) was decreased significantly as compared to non E2 treated cells-from 73% in the absence of E2 to 8% apoptosis in the presence of E2 [369]. Analysis of the apoptotic

pathways revealed that the phosphorylation of BAD and PI3K/AKT signaling activation has been modulated in response to E2. Western blot analysis revealed that while there was no change in phosphorylation of Akt upon treatment with only H_2O_2 that the E2 pre-treated cells showed an increase in AKT phosphorylation. Additionally in the presence of H_2O_2 phosphorylation of BAD was not observed, while E2 pre-treated group showed phosphorylation of BAD (376). Collectively, this data suggests that the mitogenic effect of E2 is partly due to the anti-apoptotic action of ERβ-E2 in the mitochondria [370].

Studies on the binding affinities of estrogens in different normal tissues and tumor cell lines have revealed that a population of estrogen-binding sites have been observed in the microsomes in the uterine and anterior pituitary cells of rat [371 372]. When purified microsomal portions of the anterior pituitary as well as the uterine were compared to MCF-7 cells, the results showed that the normal tissues had equivalent high affinity binding estrogen sites as those in breast tumor cells [371 372]. Other tissues, such as lung and diaphragm from adult female rats showed that these organs were devoid of specific microsomal E2 binding sites. The nature of the microsomal binding moieties with high affinity for E2 was examined by treating the microsomes with different detergents that can degrade either DNA, RNA or protein. These studies revealed that the E2 binding to these moieties is sensitive to pronase (proteinase), but not to ribonuclease or deoxyribonuclease, suggesting that neither RNA nor DNA are involved in the microsomal E2 binding. The main macro-molecules that are involved in the E2 binding affinity in the microsomes are proteins [371 372]. The function of the microsomal-protein with high affinity for E2 has not been examined, however one can speculate that microsome binding to E2 can provide another pathway for the activation of the non-genomic functions of ER in the cytoplasm.

The localization of the different male and female sex steroid hormone receptors have also been examined in the normal human mammary gland. The localization of ERα, ERβ, Progesterone Receptor A (PgRA), Progesterone Receptor B (PgRB) and Androgen Receptor (AR) on samples derived from reduction mammoplasty tissue of 17 premenopausal women have been determined by IHC [373]. The results revealed that all steroid receptors examined were mainly localized in: 1) The inner layer acini lining (epithelial cells) 2) Intra lobular ducts (epithelial cells) 3) The external layer of interlobular ducts (myoepithelial cells) 4) Stromal cells. However, ERβ is the most widespread steroid receptor and was also detected

in epithelial cells and myoepithelial cells of acini and ducts as well as stromal cells. It is highly possible that the direct or indirect interaction of steroid hormones on epithelial and stromal cells in human mammary gland affects both the normal function and/or malignant function of the steroid receptors in the breast.

E2 METABOLISM, STRUCTURE AND FUNCTION

In addition to ovaries, which are the main source for estrogen production, there are other tissues in the body such as: 1) Ovarian granulose cells, 2) Brain ,3) Adipose/skin fibroblasts, 4) Bone 5) Placental syncytiotrophoblast,, that can synthesize estrogens from androgens. [374]. What all these tissues/organs have in common is the enzyme (aromatase), which is involved in androgen conversion to estrogen. Aromatase is an estrogen synthase, which is categorized as a cytochrome P450 enzyme (CYP19). The C18 estrogens synthesizes from C19 androgens via the activity of CYP19 enzyme [375]. Follicle-stimulating hormone (FSH) induces the formation of P450-aromatase through formation of cyclic AMP and its regulation is transcriptional [376 377].

It is thought that differential pattern of distribution of fat in men versus women is due to the different concentrations of CYP19 enzyme distribution in different organs. Measurement of the aromatase activity by [1β-^{3}H]-androstenedione in abdominal subcutaneous (Sc) adipose tissue from male and female patients revealed that, aromatase activity increases in females from 11.5 to 28.0 pmol/mg·h by 10^{-6} M cortisol. However aromatase activity is inhibited in males from 19.4 pmol/mg·h to 7.5 by 10^{-6} M cortisol. Western blot analysis using an antibody against the aromatase enzyme (CYP19) confirmed the enzymatic results. Since the level and activity of CYP19 in tissues generates estrogen which can induce preadipocyte cell proliferation, it suggests that, CYP19 can regulate adipose tissue mass and its distribution differently in men and women [378].

To understand the role of CYP19 in generating estrogen, one needs to understand the estrogen metabolic pathway, schematically presented in Figure 9. There are three metabolites of estrogens - E1, E2 and E3. E1 (estron) is the least powerful and most abundant, which accounts for 80-90% of human estrogen in the body. E2 (estradiol) is the most powerful and most carcinogenic (17-ß estradiol and 4-hydroxy-17-ß estradiol induce oxidative damage and are carcinogenic). E2 is mainly produced by the ovaries in non-

pregnant females, and breaks down to estrone in the liver [374]. E3 (estriol) has similar properties to E2 but is considerably less biologically active. Estriol is also the primary estrogen during pregnancy (week 20 to 40) [379].

First through a17ß oxidation activity E2 converts to estrone, which this conversion is reversible. Then the next step is the irreversible conversion of estrone to 2-hydroxyestrone or 16alpha-hydroxyestrone through an oxidation process. As the figure 9 shows 2-hydroxyestrone and 16alpha-hydroxyestrone are the initial metabolites formed [380].

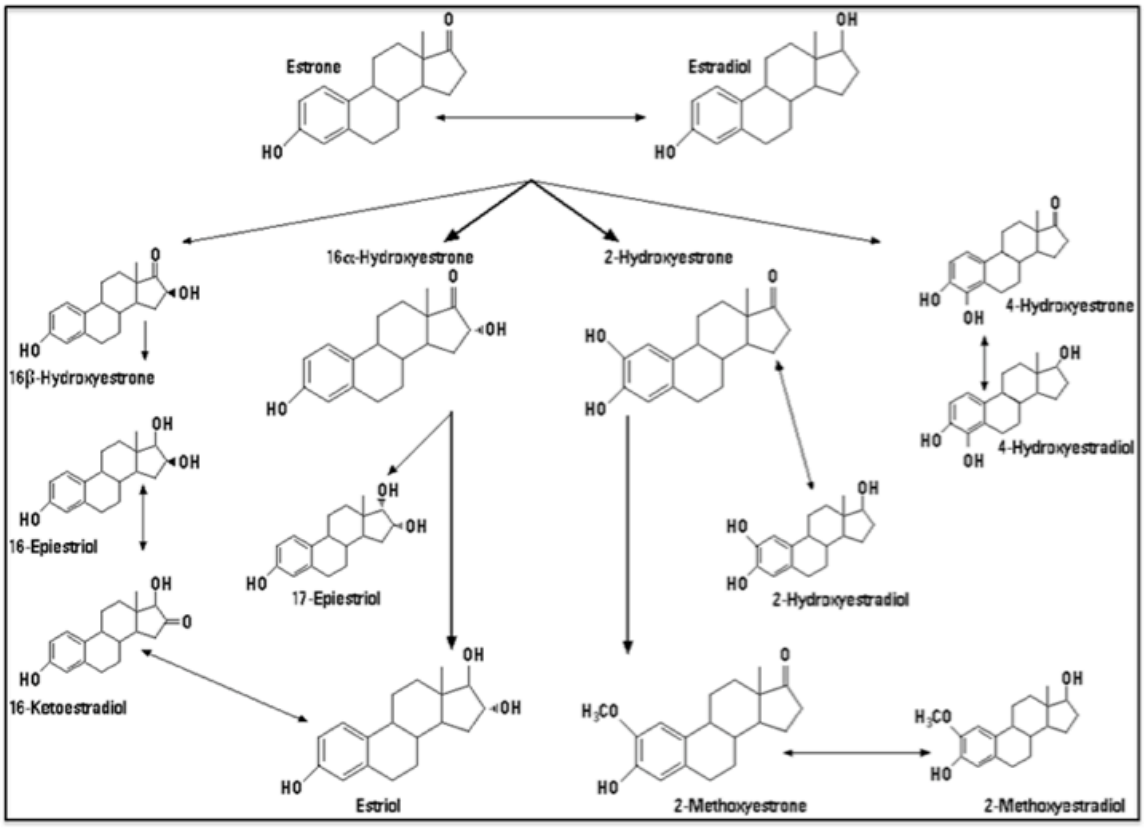


Figure 9: Estrogen Metabolism[380]

2-hydroxyestrone and 16alpha-hydroxyestrone are the two starting products that initiate the two dominant pathways for estrogen metabolism. The ratio of estriol to estrone, which gives rise to these metabolites, varies in women. It is thought that the imbalance between their ratios is associated with an increased risk for breast cancer.

The balance between the two converted metabolites from E2 is a very important phenomenon. Alteration of the balance between 2-hydroxyestrone and 16alpha-hydroxyestrone could be the reason for the proposed risks of certain pesticides, herbicides, plastics, and other xenoestrogens (foreign estrogens) for breast cancer incidence [365, 381]. In fact, 16alpha-hydroxyestrone is elevated in C3H mouse strain, assoicated with high tumor incidence, while its levels are lower in C57BL mice, associated with low tumor incidence. Cross-breeding between the two mice results in progeny who had inherited estradiol 16 alpha-hydroxylase as an autosomal dominant resulting in tumor incidence similar ot the C3H mice [382].

Unlike 16alpha-hydroxyestrone ,2-hydroxyestrone is a very weak estrogen. The affinity of 2-hydroxyestrone to 2-hydroxyestradiol for binding to cytosolic ER is also different. 2-hydroxyestradiol has twice the affinity for tumor cytosol receptors in dimethylbenzanthracene (DMBA)-induced rat mammary tumors as compared to 2-hydroxyestrone. Additionally, 2-hydroxyestradiol, but not 2-hydroxyestrone, can translocate tumor cytosol ER to the nucleus [383]. The levels of these two estrogen metabolites are also different in human breast tumors. 16alpha-hydroxyestrone is five times more active in urine of patients with breast cancer compared to women without cancer [380].

Early on, it was discovered that the disruption of the aromatase gene could have dire consequences on disease development including cancer. The generation of the aromatase knockout mouse (ArKO) revealed that the resultant female mice had pubertal failure, cystic ovaries, hypergonadotropic hypogonadism, virilization (the development of male physical characteristics), , delayed bone age, which could be the reason behind tall stature phenotype [384]. Female ArKO mice showed an undeveloped external genitalia and uteri at 9 weeks. ArKO mice also showed numerous follicles with abundant granulosa cells in the ovaries due to the arrested antrum formation. However, aromatase deficiency in male mice does not have any remarkable effect in childhood development except that aromatase deficient male mice show a tall staure due to the continued linear bone growth throughout puberty, which is due to the failure of epiphyseal closure in these mice. Aromatase deficient male mice also show osteopenia (delayed bone age). In the male ArKO mice there was evidence of enlarged male accessory sex glands wihtout affecting their fertility (391).

A very limited number of human cases (six females and three males) with aromatase deficiency have been reported, which these cases show mutations in the CYP19 gene. The mutations observed include: 1) two single base changes in the coding region of the P450 gene [C to T mutation at the base pair 1303 and G to A mutation at the base pair 1310], resulting in codon changes of R435C and C437Y, respectively; 2) insertion of 87 base pairs, which the insertion encodes the aminoacids which are located from exon 6 to intron 6 of the normal aromatase gene with no termination codon [385-387]. These mutations highlighted the importance that estrogen effect has on the height (due to its role in epiphyses) and male bone density [388-390]. The absence of aromatase activity also causes androgen accumulation, which inturn results in virilization of the female at birth (males are not affected) [386] and if the deficiency occurs in in the placenta of pregnant mother it can result in the virilization of mother [385]. Absence of aromatase activity in female causes amenorrhea [391]. Both female and male individuals lacking estrogen show a tall stature due to the failure of estrogen function to close the distal ends of the bones [388].

E2 IN BRAIN

E2 is not only involved in the regulation of function of reproductive tissues but is also important in other organs such as the brain. E2 is critical for neuronal plasticity and protection of neurons in the hippocampus and prefrontal cortex, both of which are involved in the explicit and working memory [392]. The role of E2 in female brain has been the topic of several studies and the implication is that E2 increases the functioning of this major organ. The studies examining the role of E2 included post menopaual women (46) who were treated with E2 (2 rounds) over a 3 weak period, at which point brain activation patterns using MRI were performed and showed that treatment with E2 increased activation in the inferior parietal lobule, the area in the brain critical for storage of verbal material [393]. Other studies concluded that E2 is a key element for learning and memory in women, and that the cognitive decline and age-related dementia in women could be due to the decline of E2 synthesis [394]. One key unanswered question is what are the brain specific consequences of endocrine therapy in breast cancer patients. One could speculate that endocrine therapy could result in the lack of E2 availibility to the nervous system resulting in lethargy, a common side affect of this therapy.

ENVIRONMENTAL E2

During the last several years, scientists have learned that new synthetic products and materials not only have brought more convenience to our lives but have also been a detriment to the environment by releasing of chemicals with hormone-like effects. For example, nonylphenol, used in plastics manufacturing, which can be released from plastic has estrogenic activity. To examine the carcinogenic functions of nonylphenol, it was extracted from plastic products, purified by HPLC and used to treat MCF-7 cells. Nonylphenol treated MCF-7 cells resulted in higher rate of cell proliferation and an increase in ERE transcriptional activity, which upregulated PgR. Nonylphenol also triggered endometrial mitotic activity in the rat endometrium [395]. Bisphenol-A (BPA) is another compound with estrogenic activity released from plastic in high temperatures [396]. MCF-7 treated with BPA had increased PgR expression higher rate of prolifearation.

Pesticides also release metabolites with estrogenic activity including dichloodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE). Exposure to DDT early in life increases breast cancer risk. A prospective study of young women who were exposed to DDT and DDE using using blood samples obtained during 1959-1967 revelaed that women who were heavily exposed to DTT in young age contained a high levels of serum DDT, which the high DTT serum level predicted a 5 fold higher breast cancer risk [397-399].

The impact of the environmental contamination with these man-made products (i.e. plastic, DDT, pestisides) is vast and affects both men and women. For example, low sperm counts were found in workers in a plant producing Kepone containing DDT [400] and feminized male fish were found near sewage outlets which contain Alkyls phenols, a product of degradation of detergents during sewage treatments [401]. The use of DDT was banned in the1970s and 1980s in most of the developed countries. However there are different products in our daily use at the present time that has been found to be a health hazard such as BPA in plastic products. BPA has been found in the content of canned food due to the polycarbonate lining in the can placed to isolate the food content of the can from the aluminium [402].

ERα TURN OVER

The ubiquitin-proteasome pathway is the major route for the degradation of the short-lived regulatory proteins in eukaryotes. Protein degradation through the proteasome pathway requires the ubiquitination of the targeted protein for degradation. The binding is covalent. Ubiquitin is a highly conserved 8.6 kDa protein, which binds to the lysine residue of the targeted protein for degradation [403, 404].

Ubiquitination involves three classes of enzymes, E1, E2 and E3. E1 (UBA) is the ubiquitin activating enzyme. E1 activity requires ATP. The ATP dependent function of E1 results in a thioester bond between the cysteine residue of E1 with the carboxyl-terminal (glycine residue) of UBC. E2 (UBC) is the ubiquitin conjugating enzyme, which receives the ubiquitin from the UBA. Unlike E1, there are several E2 enzymes, which are all involved in the transfer of ubiquitin from E1 to E3. E3 is the ubiquitin protein ligase, which is specifically is involved in the transfer of the ubiquitin (from E2) to the specified target protein [403-408]. More than 35 UBCs and more than 100 E3 ligase enzymes have been identified to date [409, 410].

One example of how a cell cycle protein is degraded through the ubiquitin pathway is cyclin B. Proteasomal degradation of cyclin B at the end of mitosis requires the formation of ubiquitin-cyclin B conjugates as a sequential action of E1, E2 and E3 [411]. The initial studies used the clam oocyte system [412], where M phase extracts were fractioniateded and separated by high speed centrifugation. One band was the result of fusion of cyclin B to E1 enzyme. The other fraction was the n-terminal portion of cyclin B bound to ubiquitin. This fragment was only found in the extracts harvested from the M phase of the cell cycle and not found in The G2 extracts. They also found a novel species of E2, which they called it E2-C. E2-C is "specifically" involved in the ligation of ubiquitin to cyclin B (419). The E3 ligase that is involved in the cyclin B degradation is APC, consisting of a group of subunits such as Cdc20 and Cdh1 [413, 414].

Ubiquitination of proteins prepares them for degradation. However, the protein degradation is not the only fate of the ubiquitinated proteins. In some cases, ubiquitination may serve a regulatory function by redirecting the sub cellular localization of the ubiquitinated protein [405, 415].

For example, IκBα activates the transcription factor NF-κB by phosphorylation, at

which point IκBα is targeted for degradation. The degradation of IκBα is depends on the phosphorylation of IκBα on serine residues 32 and 36, which then targets this protein to the proteasome pathway. The kinase that phosphorylates IκBα requires ubiquitination too and is a pre-requisite for specific phosphorylation of IκBα. Thus, ubiquitination serves a novel regulatory function that does not always result in proteolysis.

The degradation of ERα is also through the ubiquitin-proteasome pathway and occurs in an E2-dependent fashion [416]. Following transcriptional activation of ERα by E2 it is targeted for degrades through proteasome pathway. In the original study documenting ER mediated proteasome degradation, HeLa cells were transfected with both ERα and an ERE-luciferase reporter construct. Following transfections the cells were treated with E2 and and MG132 (proteasome inhibitor), at which point they were subjected to luciferase assay. The results revealed that ERα protein levels were decreased (i.e. degraded) upon E2-stimulated transactiovation while in control (not treated with E2) ERα remained intact. Additionally MG132 treatment attenuated luciferase activity while in untreated cells (with E2 present) luciferase activity was high. This study provided the first evidence that the ubiquitin-proteasome dependent degradation of ERα requires ligand binding and regulates transcription at ERE sites (423, 311). Additionally, ERα coactivators (SRC-1, SRC-2 or CBP) are also subject to degradation through the proteasome pathway, contributing to ERα transcriptional activity [416].

Other groups have also shown that SRC3, one of the most well studied coactivators of ERα, degrades through proteosome pathway [417]. They suggest that the basic helix-loop-helix (bHLH) domain on SRC3 contains a bipartite nuclear localization signal domain. These residues (K17 and R18) are required for the turn over (proteasome dependent) and nuclear localization and also regulation of SRC3 transcriptional coactivator capacity [417].

The studies showing the importance of E2 in the stability of ER dates back to the 1970s when Gorski et al. revealed that the half-life of the mammalian ER in the absence of hormone is almost five days. However, in the presence of E2, the half life of ER dramatically declines to one hour [418]. For these studies cells (which ones) were treated with cycloheximide for different time intervals to block protein synthesis and an E2 binding assay was used to estimate the turnover of ER. In other studies E2 treatment of ovariectomized rats resulted in a 60% drop in the ER levels in uterine tissue [419]. While these studies all show that ligand

binding to ERα is a requirement for the turn over of ERα, others provide evidence that unliganded ERα is also capable of degradation through proteasome pathway. To this end, Hela cells were used to purify the ubiquitin ligase complex for unliganded ERα, by using the (GST)-fused LBD domain of ERα in the presence or absence of E2 as the bait. The protein products were then subjected to SDS–PAGE, and peptide mass fingerprinting which identified a 35 kDa protein, carboxyl terminus of Hsc70-interacting protein (CHIP). Hsp/Hsc70 itself, is a 70 kDa chaperone protein [420]. CHIP is an E3 ubiquitin ligase, which can specifically bind to unliganded ERα and conjugate it to ubiquitin [421-424]. The ability of CHIP to degrade wild-type ERα was compared to ERα (HE82), which has no ability to bind to DNA due to three amino-acid substitutions in the DNA-binding region [425]. These studies revealed that ERα (HE82) gets degraded by CHIP, while ligand binding of wild-type ERα causes the dissociation of CHIP from ERα. These results suggested that the CHIP-dependent degradation is specific for unliganded and misfolded ER proteins [426]. These results also highlight that the turnover of ERα is regulated by two independent ubiquitin-proteasome pathways. One pathway is necessary for the transactivation of ERα and the other pathway is involved in the quality control of ERα (433).

Even though many studies suggest that ERα down regulation is essential to ERα transcriptional activity [416], 433, 434), there are other studies, which provide an alternate view. For example, in studies aimed to examine the dependency of ERα transcriptional activity to its degradation revealed that proteasomal degradation of ERα is not always necessary for its transcriptional activity. In this study HeLa cells were transfected with ERα and ERE-pS2-Luc and then blocked either ubiquitination (cotransfection of Hela cells with construct expressing the mutant Ubc12 (pcDNA-Ubc12C111S) or proteasomal degradation (addition of MG132). The results show that both ways of inhibition of ER degradation markedly increased E2-induced ER transactivation at ERE. When this experiment was repeated in MCF-7 cells with endogenous ERα, similar conclusions were reached since an increase in PgR (an endogenous target of ER transactivation at ERE) levels were observed [427].

There are several signaling pathways that can affect the faith of ERα in the cell either through proteasomal degradation or stabilizing the ER protein [380]. One example is the Glycogen Synthase Kinase-3 (GSK3) which phosphorylates ERα on Ser-118 in an E2-

induced fashion and results in increased ERα transcriptional activity and also stabilizes ERα by protecting it from proteasomal degradation [428]. When GSK-3α and GSK-3β were silenced in MCF-7 cells it resulted in a modest (35%) of ER protein in the absence of E2, while E2 treatment of the GSK-3/ß silenced cells resulted in a pronounced downregulation of ER protein (90%). In control MCF-7 cells, E2 treatment resulted in only a 50% decrease of ER protein level. GSK-3 silencing results in the increased proteasomal degradation of ER. as analyzed by CHX treatment. Collectively, these results suggest that GSK-3 phosphorylates ERα, protecting it from from degradation through proteasome, allowing enough stable ERα in the cells to continiously initiate the transcriptional activation at ERE [429-432].

ERα AND BREAST CANCER

Steroid receptor expression is strictly regulated in the normal mammary gland, but not in malignant tumors. Increased ERα expression appears early in the pre-malignant to malignant progression, as it is detectable in ductal hyperplasia [7]. Elevated levels of ERα are detected in two thirds of all breast cancers as compared to the normal adjacent tissue. Tumor formation and metastasis rise due to imbalance between mammary epithelial proliferation and death (apoptosis) [433]. ER participates in different stages of tumorigenesis and is associated with uncontrolled cell proliferation and low apoptotic potential of the cells (176). ERα is therefore a positive prognostic marker in breast cancer, and consequently breast cancers that are positive for ER are generally well differentiated, have low rates of proliferation and respond well to anti-estrogens. ER-negative breast tumors on the other hand are poorly differentiated, have a deregulated signaling pathway, higher proliferation rate and do not respond to anti-estrogens. [8].

ERα knock down (ERKO) mice have helped to shed light on mammary gland development and tumorigenesis. The mammary glands in ERKO mice can form tumors despite the absence of ER following the introduction of oncogene, wnt-1. Wnt-1 MMTV transgenic (TG) mice were engineered to produce the secretory glycoprotein, Wnt-1, resulting in the development of mammary hyperplasia and neoplasia. ERKO mice were then crossed with Wnt-1 TG mice to examine the oncogenic potential of Wnt-1 transgene in the absence of ERα. The results revealed that Wnt-1 TG/ERKO mice were capable of forming tumors, suggesting that at least in this mouse model system, ERα is not required for

oncogenesis [434]. Addition of E2 to this system results in an accelerated tumor formation. E2 was administered using the "Clamp" method which consists of small silastic tubes filled with E_2/cholesterol mixtures at various levels: those representing postmenopausal level of E2 in women (5 and 10 pg/mL), representing E2 at early follicular phase in women (80 pg/mL) and representing E2 at midluteal phase in women (240 pg/mL). The implants containing the hormones surgically placed under skin of the back of the mice. The implants were changed every other months. The results showed that E2 at the levels of follicular and midluteal phase levels accelerated tumor formation ERKO/Wnt-1 animals. Consequently, when oophorectomy was performed or when these mice were treated by an aromatase inhibitor (Letrozole) delayed tumor development occurred [435].

The results of both in vivo studies suggest an ER-independent action of E2, which can influence breast tumor development in mice. Additionally, based on the results of my own project, described in subsequent chapters of this thesis, I suggest that even though ER has a key role in the development of reproductive tissue that it is not a strong driver of mammary hyperplasia-instead E2 is more effective. As such, I suggest that the blocking of E2 synthesis would be a more effective approach to prevent breast cancer rather than the current treatments such as antiestrogens, which only block ER-mediated effects. There are several epidemiological studies that also illustrate the importance of E2 as a key contributing factor to initiation and progression of breast cancer. E2 administration in various animal models results in breast cancer. The key study was performed in a DMBA treated rat model. In this experiment 63 female Sprague-Dawley rats were randomized into the following groups 1) no treatment 2) oophorectomy and 3) TAM, 10 mg/kg/week 2 weeks prior DMBA treatment. All animals received 20 mg DMBA. At the end of the 16 weeks post treatment, 78% of the untreated rats developed tumors, while only 22% of TAM treated rats developed tumors. However, none of the rats in the oophorectomy group developed tumors [436, 437].

The role of E2 in human cancer development has also been investigatod. For example, exogenous E2 in the form of contraceptive pills and hormone replacement therapy have been linked to higher cancer incidence in women [438]. Alternatively, oophorectomy before age 35 reduces the risk of breast cancer by 75% [439, 440]. Additionally, high levels of circulating E2 due to obesity and long-term hormone replacement therapy are also factors associated with a higher risk of breast cancer incidence. A prospective cohort study within 11,508 women, who

had a hysterectomy and reported information on E2 use at baseline in 1980 and follwed for 22 years (cohort 28,835) revealed a high incidence (30% increase) of invasive breast cancer in women being exposed to E2 versus those who did not take E2 in any form [441-444].

The mechanisms by which E2 acts as a driver of oncogenesis, while studied extensively, still remains controversial. The most commonly proposed hypothesis is that cell proliferation is being stimulated upon ligand (E2) binding of ERα [445]. During each round of cell cycle despite the tight checkpoint and restriction points there are still chances of error. These errors could result in point mutations, which accumulate over time and give rise to neoplastic transformation [446, 447]. These sequential events rush the cell through transcriptions in which the DNA fidality has not been preserved due to the mutations during the replication, coupled with the inability of the DNA repair mechanism to repair mutation due to the rapid passage of the cells through cell cycle mediated by E2. The incidence of both ER-positive and ER-negative breast cancers abolish upon ovarectomyThis observation suggest that ovarian hormones are also important for the initiation and progression of the ER-negative breast cancers [448].

The model system used to examine the role of E2 in tumor formation was the human xenograft HMLE-Ras^{hi} cells which were injected in both pregnant and nulliparous female mice [449, 450]. HMLE-Ras^{hi} mammary tumors formed with high efficiency in mice injected following pregnancy and also in nulliparous mice. However the difference between these two groups was that the tumors developed in pregnant mice 2 to 4 weeks before their nulliparous counterparts. When a weaker version of the oncogenic cells, the HMLE-Ras^{lo} cells were injected in both pregnant and nulliparous mice they found that preganat mice formed palpable mammary tumors within 8 weeks while nulliparous mice did not form any mammary gland tumors and only formed benign epithelial structures in their mammary glands [448]. Examination of the stromalization of the tumors formed in HMLE-Ras^{hi} and HMLE-Ras^{lo} pregnant and nulliparous mice revealed that tumors in mice injected with both HMLE-Ras^{lo} and HMLE-Ras^{hi} following pregnancy included a large proportion (44%) of stromal cells within the tumor tissue while this percentage was 9% in the nulliparous mice [448].

E2 has also been implicated as a ligand for other receptors besides ER. The G protein-coupled receptor (GPR30) is a candidate receptor, which binds to E2 yet differs from classic

ERs. The role of GPR30 in ER-positive breast cancer remains unclear; however, it is known that GPR30 can mediate nongenomic signaling. GPR30 is involved in early response to E2, which results in transactivation of EGFR, activation of MAPK and PI3K signaling pathways (Figure 4) [352, 451]. GPR30 can also modulate proliferation and tumor progression in hormone-dependent tumors such as endometrial, ovarian and breast cancer tumors. For example, the ability of E2 and G-1 (GPR30 ligand) to activate transcriptional activity of c-fos by ERE was examined in BG-1 cells. BG-1 cells, derived from a patient with stage III ovarian adenocarcinoma express ERα and GPR30 but lack ERβ. The results revealed that E2 induced transcriptional activity at the ERE site while G-1 induced the transcription of c-fos promoter but not the ERE site. They also found that both G-1 and E2, were able to result in up-regulation of c-fos protein levels, likely through transcriptional activation of c-fos. Consequently, the c-fos protein induction was abrogated by the inhibitors specific for EGFR kinase (tyrphostin AG 1478), MAPK (PD 98059), of the Src family tyrosine kinase (PP2). However the inhibitor to PI3K (wortmannin) did not alter c-fos protein level. These results suggest that both G-1 and E2 signal through the EGFR/ERK signaling pathway but not through PI3K to induce the expression of downstream transcriptional effectors such as c-fos [452-459]. GPR30 can also induce the expression of ERα46, variant of ERα (ERα66) that is responsible for the non-genomic activities and can also be expressed in ERα66 negative breast cancer (see Figure 4) [460]. GPR30 knockdown in SKBR-3 breast cancer cells results in down regulation of ERα46 while the ectopic expression of GPR30 into results in the increase of endogenous ERα46 expression by transcriptional activiationof GPR30. The activation of ERK1/2 in response to E2 and G-1 were also examined and the results revealed that .E2 induced phosphorylation of the MAPK/ERK1/2 in both ERα46 and ERα66 cell lines but not in the control cells transfected with the empty expression vector [461]. These results suggest that E2 can exert its mitogenic activity through ERα46 and ERα66 and also through GPR30. E2 effects through ERα46 and GPR30 result in the activation of non-genomic pathway of ERα, which inturn initiates the mitogenic cytoplasmic pathways such as MAPK/ERK1/2, resulting in deregulated proliferation (Figure 4).

GAP OF KNOWLEDGE:

Previous studies have shown that the presence or absence of ER in breast cancers is an important indicator for diagnosis, prognosis and treatment. Expression of ERα is necessary for response to endocrine therapy. However, not all ER-positive cancer cells respond to endocrine therapy. Some ER- positive cells keep their sensitivity to therapy while some others develop resistance.

There are controversial reports regarding the advantages versus disadvantages of the presence of ERα in breast cancer cells. The important question that these studies raise, is what is the function of ERα in the presence versus absence of E2? Does unliganded ERα have an inhibitory effect on the cell proliferation and, as a result, on the cell cycle? If so, could modulation of ERα activity provide new opportunities to exert influence on proliferation and cell cycle progression? Could unliganded ERα be utilized as an inhibitor of the cell proliferation in cancer cells?

The goal of this study is to determine the differential effects of liganded ERα versus unliganded ERα on cell cycle progression and ERα transcriptional activity. Our findings thus far suggest that unliganded ERα has an inhibitory effect on the progression of the cell cycle. However, the liganded ERα (bound to E2) could cause a rapid progression of the cell through the cell cycle. Increased knowledge of ERα activity during the cell cycle will enhance our understanding of the causes, potential treatments, and mechanisms to prevent breast cancers via new approaches.

Chapter II: Estrogen Receptor Alpha is a Cell-Cycle Regulated Protein and has different effects on the cell cycle depending on its ligand status (liganded versus unliganded ER).

INTRODUCTION

The function and involvement of estrogen and ER have been extensively studied through the years both in the normal mammary gland development as well as in the malignant transformations, which have been described in Chapter 1 of this dissertation [5]. Cumulativly the data suggests an important role for ERα in proliferative state of the breast epithelial cells both in normal (during puberty and pregnancy) and also in breast tumor cells. However the mechanism of function of ERα in regulation of cell proliferation under both normal and cancer remains unclear. To address the role of ERα in cell cycle, I have examined the following in this chapter: (1) The pattern of ERα expression in relation to the cell cycle and (2) the role of liganded versus unliganded ERα on the progression of the cells through cell cycle. These investigations will help elucidate the role of ERα on the progression of the cells through different phases of the cell cycle in the presence versus absence of the ligand, estradiol. The results of these studies show the novel findings that: (1) ERα is cell cycle regulated and modulates the progression of cells through the cell cycle by affecting S and G2/M phases of the cell cycle and (2) ligand bound ERα functions to increase cell cycle progresion, whereas unliganded ERα acts as a cell cycle inhibitor.

MATERIALS & METHODS:

Chemicals

Lovastatin (Mevinolin/Lovastatin), FW: 404.25, LKT Laboratories, Inc.
To make a 10mM stock solution of lovastatin in 70% ethanol, 0404g of lovastatin was added to 7mL of 100% ethanol (EtOH) in a tube. The solution was vortexed to dissolve the powder completely and 3mL of filtered dd H20 was added to the tube and 1mL aliquots of the 10mM solution were stored at -20°C for several month.
Mevalonate (DL-Mevalonic Acid Lactone), FW: 130.1, Sigma.

To make a 0.5M solution of mevalonate, 1 gram of mevalonate was added to 7.7mL of 1M NaOH and incubated overnight at room temperature. Next day, the pH of the solution was adjusted to 7.0 with HCl and the volume was adjusted to 15mL with H20, making a final concentration of 0.5M mevalonate. The solution was filtered through a 0.22 micron filter to sterilize and then aliquotted in 1mL cryovials and stored at -20°C for couple months.

Nocodazole (Methyl N- (5-thenoyl-2-benzimidazolyl) carbamate, FW: 301.32, Sigma. A stock solution of 3.3mM nocodazole is made by dissolving 10mg of Nocodazole per mL of DMSO (nocodazole is not H2O soluble) and aliquotted in cryovials and stored at -20°C for up to two months.

Aphidicolin from Nigrospora sphaerica, FW: 338.48, Sigma.
Aphidicolin was solubilized in EtOH at a concentration of 1mg/mL and is stable for 1 week in ethanol in 4°C. Solublized aphidicolin is stable for six weeks at -20°C.

Thymidine (1-(2-Deoxy-β-D-ribofuranosyl)-5-methyluracil, 1-(2-Deoxy-β-D-ribofuranosyl) thymine, 2′-Deoxythymidine, dT, Thymine deoxyriboside), FW: 242.23, Sigma. A 0.2 M stock solution of thymidine was generated by adding 2.42g of thymidine to 50 mL H2O. To facilitate solubilization, the solution was incubated at 37°C for 10 minutes followed by vortexing and the solubilized mixture was sterilized using a 0.2 micron filter and aliquotted for storage in -20°C for up to 2 weeks.

Cell Culture Conditions

The cell lines (MCF-7, MDA-MB231, ZR75-T, ZR75-1, MDA-MB468, 293T) were acquired from the depository of American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were authenticized using the short tandam repeat (STR) method. Prior to authentication, 40 vials of each cell line were stroed in liquid nitrogen. Once the cells were authenticized, each vial would remain in culture for only 6-7 passages to ensure that the same cell population is used in all experiments. To examine the effect of E2 on MCF-7 cells, MCF-7 cells were kept in E2-free media for three weeks to clear estrogen completely from the cells. E2-free media is supplemented with 10% charcoal dextran-treated FBS (Hyclone, South Logan, Utah), which is stripped of estradiol. In order to prevent the slight estrogenic activity of phenol red in the media, phenol red-free IMEM (Biosource, Carlsbad, CA) was used to culture the MCF- 7 cells as described [462]. The phenol red-free, E2 free media, with 10% charcoal dextran treated FBS was also supplemented with 1M Hepes, 200mM Glutamine, 10mg/mL cipro (Ciprofloxacin), 1mg/mL Insulin from bovine pancreas (Sigma, 15500), 1mg/mL hydrocortisone (Sigma, H4001), 25µg/mL EGF (Recombinant human epidermal growth factor, Millipore, Bedford, Massachusetts), 1% of nonessential amino acids and 1% sodium pyruvate.

Early passage (from ATCC) MCF-7 cells were cultured in 37°C in a humidified incubator containing 6.5% CO_2. The MCF-7 cells were passaged at a 1:10 when 70-80% confluency was reached and harvested by incubating with 0.25% trypsin-EDTA (Sigma) for 5 minutes, followed by inhibition of trypsin using 3.8 g/L of trypsin inhibitor (Sigma). The cells were then pelleted and plated as the next passage at a 1:10 ratio.

Following three weeks of E2 starvation, MCF-7 cells were seeded at a density of 1×10^6 mL in 100mm plates. For all experiments, cells were plated in duplicates to compare the effects of the presence versus the absence of estradiol (10nM) on MCF-7 cells. Therefore, the experiments testing the E2 positive and negative conditions were always run parallel to each other.

To culture cells in non-E2 free conditions, (MCF-7, MDA-MB231, ZR75-T, ZR75-1, MDA-MB468, 293T) cells were cultured in complete alpha medium supplemented with [1% of 1M HEPES (Sigma, St. Louis, MO), 10% of FBS (Atlanta Biologicals, Atlanta, GA), 1% of non-essential amino acids (Sigma, St. Louis, MO), 1% of 200mM Glutamine (Sigma, St. Louis,

MO), 1% of Sodium pyruvate (Sigma, St. Louis, MO), 0.1% of 1mg/mL Insulin (Sigma, St. Louis, MO), 0.1% of 1mg/mL Hydrocortisone (Sigma, St. Louis, MO), 0.05% of 25μg/mL of EGF (Invitrogen, Carlsbad, CA), 1% of 10mg/mL Cipro] and incubated as before in 37°C in a humidified incubator containing 6.5% CO_2

Plasmids

ERα plasmid was subcloned into a pcDNA3.1 backbone and used in all transfection studies described with MDA-MB231 and MCF-7 cells . For some experiments I also used HA tagged ERα, also in pcDNA3.1 backbone, which was kindly provided by Dr. Susan Fuqua. HA-ERα was expressed through CMV promoter in this plasmid. HA-ERα was used to transfect MCF-7 cells in order for us to be able to differentiate the endogenous ERα from the expressing exogenous ERα. pERE-tk Luciferase (containing the estrogen-regulated element) was on pcDNA3 back bone.

ERE-Luc plasmid used in order to transfect MCF-7 cells and measure the ERE transcriptional activity. Myc tagged Ubiquitin (myc-Ub) was used for an invitro ubiquitination assay in order to measure the ubiquitination of ERα.

Synchronization of cells

Four different arresting agents were used to synchronize cells in different phases of the cell cycle (Figure 10). These include Lovastatin, which arrests cells at early G1 phase through inhibition of the proteasome. As a result of proteasome inhibiton, p21 and p27 levels increase and inhibit Cdk2, which halts the cell cycle at G1 Phase[463]. Double thymidine block which results in the inhibition of DNA synthesis by inhibiting nucleotide synthesis caused by an imbalance of the nucleotide pool [464, 465], therefore inhibiting cells in early S phase. Aphidicholin arrests cells in late G1 phase by inhibiting DNA polymerase of eukaryotic cells (i.e. DNA polymerase α) [466]. Nocodazole causes microtubule stabilization inhibiting cells from pulling away from the metaphase plate and arresting the cells in mitosis [467, 468].

Lovastatin (Fgure 11A): 1×10^6 cells from each cell line (MCF-7, MDA-MB231, ZR75-T, ZR75-1, MDA-MB468, 293T) were plated on 100mm tissue culture dishes in E2-free media. Twenty-four hours post plating, the media was changed to fresh E2-free media containing

10μM lovastatin (2) for 36 hours, at which media was changed to E2-free media containing 1mM mevalonate (to release the cells from lovastatin arrest). At the point of mevalonate addition (considered time zero), cells were harvested every 4 hours thereafter for 44 hours and prepared for western blot and FACS analysis. For the group of cells that were to be given E2 (10nM), it was added at the same time as mevalonate (at the release from arrest).

Double Thymidine (Figure 11B): A double treatment of thymidine provides a method for synchronizing cells at the G1/S border [469]. 1x 10^6 exponentially growing MCF-7 cells were plated in 100mm plates. Twenty-four hours post plaing media was removed and replaced with media containing 2mM thymidine and incubated for 24 hours. The thymidine-containing media was removed from the cells by washing three times with thymidine free media and cells were cultured for an additional 12 hours in thymidine free medium (E2-free medium, supplemented with 10% charcoal dextran treated FBS and growth factors). At the end of the 12 hours, cells were cultured for an additional 24 hours with a second dose of thymidine at a final concentration of 2mM. The cells were washed three times before adding normal growth media to the cells, at which point cells were harvested every 4 hours thereafter for 44 hours and prepared for western blot and FACS analysis.

Aphidicholin (Figure 11C) MCF-7 cells were maintained in E2-free media for three weeks before they were treated with 4 μg/mL of aphidicholin for 24 hours. Cells were released from arrest by washing with drug free media at which point cells were harvested every 4 hours thereafter for 44 hours and prepared for western blot and FACS analysis. As with other synchronization methods, E2 (10nM) was added to the E2-positive group at the time of release from arrest.

Nocodazole (Figure 11D) Nocodazole is widely used to arrest cells at the M phase of the cell cycle because of its ability to induce mitotic arrest through stabilizing microtubules[468].

To arrest MCF-7 cells with nocodazole, cells were maintained in E2-free media for three weeks and were then treated with increasing concentration of nocodazole (0 - 0.4 μg/mL) for 24 hours. The goal of treating the cells with various concentrations of drug was to optimize the percentage of cells arrested in the G2/M phase with the least amount of toxicity. After optimizing the nocodazole concentration, MCF-7 cells were treated with the selected concentration of nocodazole for 24 hours. Cells were then released from arrest

by three repeat washes with drug free media. E2 was added (10nM) to the cells in the E2-positive group at the time of release. Cells were collected at regular intervals to be analyzed by both flow cytometry and western blot analysis to examine the ERα expression and the cell cycle status in the presence or absence of the ligand E2.

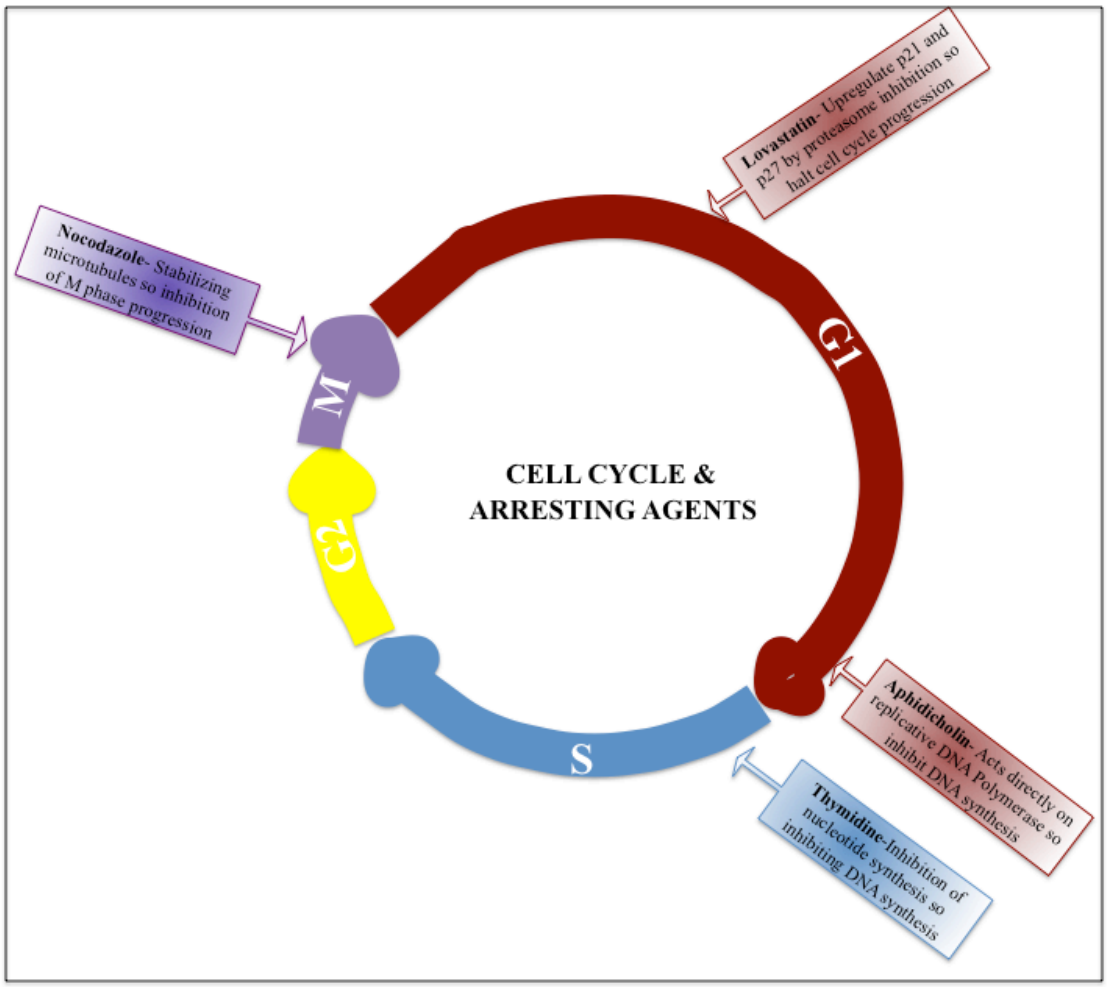


Figure10: Arresting agents used to synchronize cells in different phases of the cell cycle.

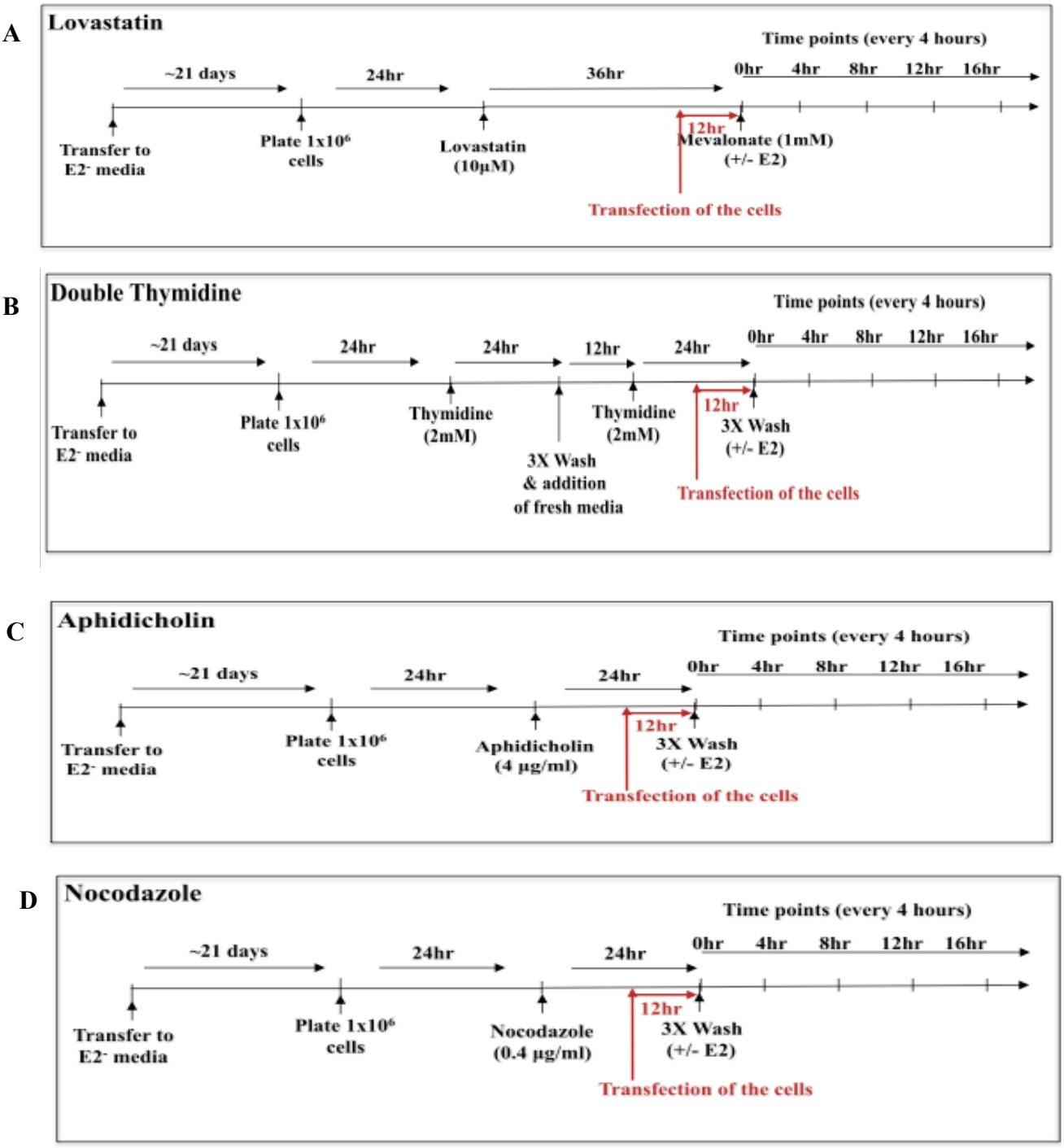


Figure 11: Flow chart of different synchronization schedules (A) Lovastatin, (B) Double Thymidine, (C) Aphidicolin, (D) Nocodazole

Transient transfections in combination with lovastatin synchronization

MDA-MB231 cells were cultured in E2-free media for 3 weeks, then synchronized with lovastatin as described above with the following modifications. Briefly, $1x10^6$ MDA-MB231 cells were plated in 100mm. The next day, 10μM of lovastatin was added to the cells. Lovastatin remained on cells for 36 hours. After 24 hours of cell exposure to lovasatin (12 hours before taking off the lovastatin), cells were transfected with the vector of interest (HA-ER, ERE-Luc or Ub-myc). The media on the cells was not changed at the time of transfection, however, 12 hours following the transfection (36 hours after lovastatin treatment), fresh media containing 1mM mevalonate was added. Depending on the conditions being tested, E2 was added (or not) at the time of mevalonate addition. Twelve hours of transfection proved to be enough time to take up the introduced DNA and start expressing the protein. Samples were harvested and prepared for further experiments.

Generation of Stable Pools and Clones

Generation of stable clones of MCF-7 cells with shERα: The ERα shRNA was inserted into pSilencer 2.0-U6 vector according to the protocols provided by the manufacturer. The DNA oligos for ERα shRNA vector construction were synthesized by SigmaGenosys (Houston, TX). The sense oligo sequence for ERα shRNA is: sense
5'GATCCCGCGCTCTAAGAAGAACAGCCTTCAA
GAGA GGCTGTTCTTC TTAGAGCGTT TTTTGGAA-3', and the antisense oligo is:
5'AGCTTTTCCAAA
AAACGCTCT AAGAAG AACA GCCTCTCTTGAAG GCTG TTCTTCTTAG AGCGCG G-3'.

For ERα knockdown using the ERα shRNA, MCF7 cells were cultured on a 100mm culture dish (for immunocytochemical staining, one cover slip was put in each dish) and grown to 70% confluency in DMEM medium with 10% FBS. Premixed with 15μL of FuGene6 (Roche, Switzerland) and ERα shRNA/or negative control vector (Ambion, USA) at 10μg/dish were added to each well. Cells were harvested at 24, 36, and 48 hours after ERα shRNA transfection. Western blot analysis and immunocytochemical staining was performed to ensure the down regulation of ERα.

For stable down regulation of ERα, $1x10^6$ MCF-7 cells were plated in 100 mm plates and then transfected with fugene6 (Roche, 1 814 443) and 5μg of the vector per plate. The next day, the transfected cells were selected by culturing with medium supplemented with 1μg/mL of puromycin for 14 days. At the end of 14 days, colonies were picked microscopically and individual colonies were transferred into each well of a 96 well plate already containing media with 1μg/mL of puromycin. After the cells reached 70% confluency in 96 well plates, they were sequentially transferred to 6 well plates and 100mm plates upon reaching 70% confluency. The puromycin was kept in the media as cells were sequentially passaged. Some cells were harvested upon each passage to assess the ER expression

Immunocytochemical staining: $5x10^4$ cells were plated on sterilized cover slips that had been placed inside each well of a 6 well plate. At the time of harvest cells on the coverslips were fixed using 4% paraformaldehyde for five minutes at room temperature (RT), then washed with PBS for 3x 5minutes, followed by blocking (3% BSA containing 0.1% NP-40 in PBS) for 1 hour at RT. Primary antibodies were added at a dilution of 1:200 in blocking solution and were incubated on the slides at 4°C overnight. The next day, the slides were washed for 3 X 10 minutes with PBS and were incubated with fluorescent labeled secondary antibodies for three hours at RT. Slides were washed again with PBS for 3 X10 minutes and covered with 4',6-diamidino-2-phenylindole (DAPI) containing anti-fade solution (Vector, Burlingame, CA). Slides were then analyzed using an Olympus DSU spinning disc confocal microscope fluorescent microscope.

Irradiation of MCF-7 cells

MCF-7 cells were plated at a density of $5x10^5$ cells per 100mm plate. This density allowed cells to be about 50% confluent the next day when the media was changed and cells were irradiated using increasing grays of raditation: 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 grays. Cells recovered in the incubator for 24 hours after the irradiation, at which point the cells were harvested and prepared for FACS and western blot analysis.

Cell Cycle Analysis

The DNA content of cells was determined by flow cytometry. For each preparation, 1×10^6 cells were harvested,washed with cold PBS and centrifuged to pellet the cells. The cell pellets were then fixed overnight with 60% ethanol. The next day, the cells were washed twice with PBS and then stained with a solution containing propidiom iodide (PI) (10μg/mL), RnaseA (20μg/mL), 0.5% Tween-20 and 0.5% bovine serum albumin (BSA). The solution to PI staining made in PBS. All staining reagents were from Sigma-Aldrich (St. Louis, MO). Cells were filtered 24 hours after the staining and then subjected to analysis using a fluorescent activated cell sorter (FACS) machine, FACscalibur (Becton Dickinson, Franklin Lakes, NJ). Events (2×10^4) were acquired within a cellular region of forward scatter versus side scatter plot using Cell Quest software (Becton Dickinson). These events were then statistically analysed for the percentage of cells residing within different phases of the cell cycle using Modfit software (Verity Software House, Topsham, ME). A diploid model was selected and the position and range of the marker placement was assessed. The program's "fit" option was selected to calculate the relative cell cycle distribution of the acquired cells.

Growth Curve and Doubling Time Analysis

To examine the doubling time of MCF-7 and MDA-MB231 cells in alpha media and in E2-free media, $1x10^4$ cells were plated in each well of a six-well plate. Enough wells were plated to analyze triplicate wells every other day for two weeks. The media was changed every other day. Doubling time was calculated using the cell counts from the exponential growth phase using the following formula:

Doubling time $=[(0.301\ \Delta t)] : [\mathrm{Log}_{10}\ (N/N_0)]$

Western Blot Analysis

Protein samples were obtained from cells that had been harvested and resuspended in 1x protease/phosphatase inhibitor (ppi) [250μg/mL of Leupeptin, 25μg/mL of Aprotinin, 10μg/mL of Pepstatin, 1mM Benzamidine, 10μg/mL of soybean trypsin inhibitor, 0.5mM phenylmethylsulphonyl fluoride (PMSF) made in dimethyl sulfoxide (DMSO), 50mM sodium floride (Naf), 0.5mM sodium ortho-vanadate] and kept in -80°C. To this end the cells

were lysed by sonication with a sonicator fit with a horn adaptor for three rounds of six minutes. After the sonication, samples were centrifuged at 45,000rpm for 45 minutes in a table top ultracentruge (100,000 x g). The supernatant was removed from the pelleted cell debri and was ready for storage in -80°C and protein assay. BioRad protein assay was performed to determine the concentration of the protein extracted from the cells using optical density at 595nm.

Expression of different proteins using samples from synchronized cells was determined by subjecting cells to western blot analysis. To this end 50μg of protein lysate diluted in 0.4μg/μL in PBS and 10μL of sample buffer (500μL 20% SDS, 106μL β-mercapthoethanol, 208μL crystal violet, 207μL 80% glycerol) were denatured by boiling for 10 minutes, kept on ice for 5 minutes and then put in a 37°C H2O bath for five minutes. The samples were vortexed and centrufuged briefly to bring the tubes content down and then loaded on each well of different percent SDS-PAGE gels. Following electrophersis, the protein content of the gels were transferred into pre-soaked PVDF in the cold room for 90-120 minutes using 100 volts. The blots were incubated in to a bloking solution, blotto (5% nonfat dry milk in 20mM tris, 137mM Nacl, 0.25% Tween, PH 7.6) overnight. The next day the blots were washed in TBST (20mM tris, 137mM Nacl, 0.25% Tween, PH 7.6) and incubated in primary antibody for 2.5 hours. The monoclonal antibodies used were: ERα (NCL-ER-6F11, Novocastra Laboratories, UK), cyclin A, cyclin B, cyclin E, cyclin D1 (all from Santa Cruz Biotechnology, Santa Cruz, CA), Cdk1 (POH1, cell signaling Technology) and the polyclonal antibodies used were ERα and cyclin B both from Santa Cruz Biotechnology, Santa Cruz, CA. All primary antibodies made in blotto at a concentration of 1μg/mL. After the incubation with the primary antibody, blots were washed with TBST for 10 minutes for a total of 5 washes, then incubated for 60 minutes with secondary antibody in blotto. Secondary antibodies (horseradish peroxidase conjugate, Pierce biotechnology, Rockford IL.) were used at the dulution of 1:5000. After an hour in secondary antibody, blots were washed with TBST for 10 minutes 6 times and then developed in chemoiluminescence reagent (NEN Life Science Products, Boston, MA) as directed by the manufacturer.

RESULTS

Lovastatin mediated synchronization of breast cancer cells arrests them reversibly in the G1 phase of the cell cycle.

To study the role of ERα during each phase of the cell cycle, a system was devised to evaluate the percentage of cells in each phase of the cell cycle and the duration of the cell cycle phases under various conditions. To this end, cells were synchronized in their cell cycle be arresting the cells at a specific point of the cell cycle. Once most of the cells were arrested at the same point, the cells were released from arrest in order for cells to enter the cell cycle phases sycnhronously. The cell cycle phase duration and the pattern of regulatory proteins in each phase were examined. Lovastatin was used to arrest the cells in the G1 phase [470]. Synchronization by lovastatin arrests many cell types, including human breast cancer cells MCF-7 and MDA-MB231 cells, reversibly in the G1 phase of the cell cycle. Lovastatin (10μM) mediates an 85% accumulation of MCF-7 and MDA-MB231cells in the G1 phase of the cell cycle (Figure 12). The cells were released from the G1 arrest and entered into late G1, S and G2/M phases of the cell cycle synchronously by addition of mevalonate (at 100X the lovastatin concentration). Western blot analysis was performed to detect the expression of key cell cycle proteins. Cyclins A and B were used as markers specific to the S and M phases, respectively, to indicate the synchronous transition through phases of the cell cycle following the release from G1 arrest by lovastatin. As Figure 12 shows, at time zero (release from arrest) 85% of the MCF-7 cells were coming out of a G1 phase arrest. As the MCF-7 cells exited G1 phase around 15 hours after the release, the percentage of cells in S phase increased. Similarly, as the cells exited from S phase 24 hours after the release, the population of the cells in the G2/M phases of the cell cycle increased.

The percentage of cells in G2/M phase was the highest 31 hours after release from lovastatin induced arrest (Figure 12). At approximately 33 hours after relase, cells exited G2/M phase, and entered G1 phase of the subsequent cell cycle, shown by an increased percentage of cells in G1 phase again. Within the 44 hours that samples were collected, one complete cell cycle plus the G1 phase of the subsequent cell cycle was observed. A high reproducibility of data was seen in the triplicate lovastatin arrest experiments shown in Figure 12 and labeled as I, II and III depicting three different experimental replicates.

Similar to what was observed with MCF-7 cells, lovastatin resulted in a high percentage of MDA-MB231 cells (an ER-negative cell type) to be arrested at G1 phase. Upon the release of cells with mevalonate, MDA-MB231 cells progressed through the cell cycle synchronously. In experiment one (shown as I in Figure 12) of lovastatin induced synchronization on MDA-MB231 cells, 85% of the cells arrested in G1 phase. MDA-MB231 cells exited G1 phase 17 hours after release from arrest, at which point the population of cells in S phase increased and peaked at 28 hours after release. After 29 hours, cells exited S phase and entered G2/M phase. The peak of the percentage of cells in G2/M phase occurred at the 36 hour time point. By 38 hours, cells exited G2/M phase and entered the G1 phase of the next cell cycle. Similar results were observed in experiments II and III of lovastatin synchronized MDA-MB231 cells (Figure 12). As shown, the cells progressed through the cell cycle synchronously after release from lovastatin induced arrest and the lengths of G1, S, and G2/M phases can be extrapolated from the FACS data (Figure 12). The increasing slopes of the graph as the cells enter each cycle phase represent the start of each phase, and the declining slopes represent the end of each phase (Figure 12). Due to the high level of synchrony of the cells, this method of extrapolation was used throughout this study to determine the length of the cell cycle phases.

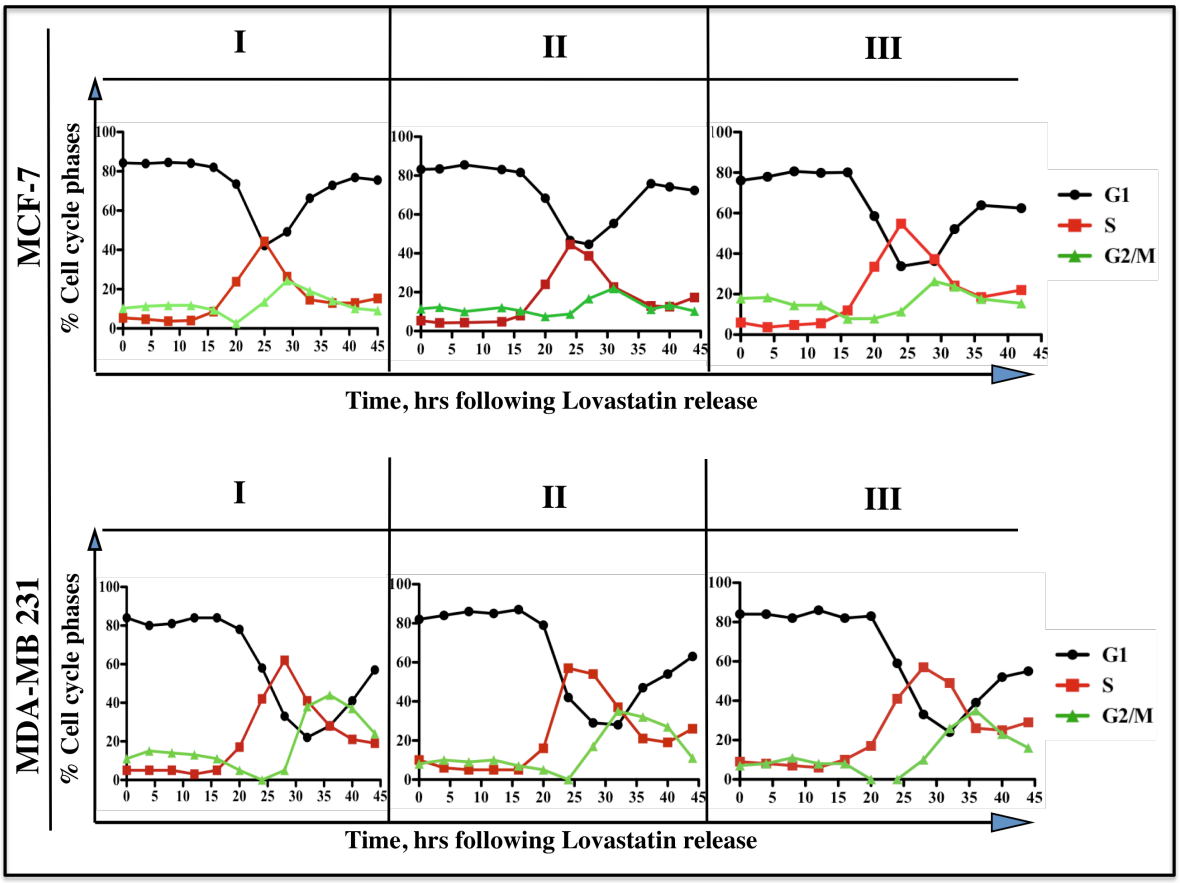


Figure 12: Lovastatin arrests MCF-7 and MDA-MB231 cells in G1 phase of the cell cycle. FACS analysis of MCF-7 (top row) and MDA-MB231 (bottom row) cells synchronized with lovastatin from three separate experiments (I, II and III) for each cell line.

ERα expression is cell cycle regulated in synchronized MCF-7 cells.

To investigate whether ERα is a cell cycle regulated protein and whether the presence or absence of estradiol affects the cell cycle distribution pattern of ERα, we studied the expression pattern of endogenous ERα using MCF-7 cells. MCF-7 cells, which were maintained in E2-free media, were synchronized with lovastatin as described above. In this experiment there were two parallel groups, one group in which lovastatin synchronized cells did not receive 17β estradiol (E2) at the time of release and the other group which did receive 17β estradiol (10nM). Cells were harvested at 4-6 hour time points after the release and then prepared for western blot and FACS analysis. When exposed to E2 at the time of release from arrest, the level of ERα protein increased at the 24 hour time point, peaked at 28 hours and then decreased at 40 hours after release (Figure 13, left panel). A similar pattern of expression was observed for cyclin B. Cyclin B protein expression also increased at 24 hours, peaked at 28 hours and was down regulated at 40 hours after release from lovastatin arrest. The levels of cyclin A protein showed an increase at 20 hours, peaked at 28 and was also down regulated at 40 hours after release. Therefore, ERα and the cell cycle markers cyclin A and cyclin B showed a similar pattern of cell cycle expression. This data suggests that in the presence of E2, ERα expression coincides with the expression of cyclin A and B, concomitant with entrance of cells into late S and G2/M phases of the cell cycle.

The expression pattern of ER was different under E2-negative conditions. When cells were synchronized in the absence of E2, the expression of ERα protein increased at 28 hours after release and peaked at the 40 through 46 hour time points. However, no decreases were observed in the ERα protein level even at the last taken time point. A similar pattern was observed for cyclin B levels. Cyclin B was up regulated at 24 hours, peaked at 32 hours, but did not decrease within the time points observed. The levels of cyclin A had increased by 28 hours and stayed up through the 46 hour time point. These results suggest that the endogenous ERα is subject to cell cycle regulation with its expression level peaking at the S-G2/M phase of the cell cycle (Figure 13). It was also evident from the western blot and densitometric analysis that E2 free conditions caused a slower down regulation of cyclins A and B and ERα compared to the sharp decline in their expression at the end of G2/M phase in E2-positive (liganded ERα) conditions.

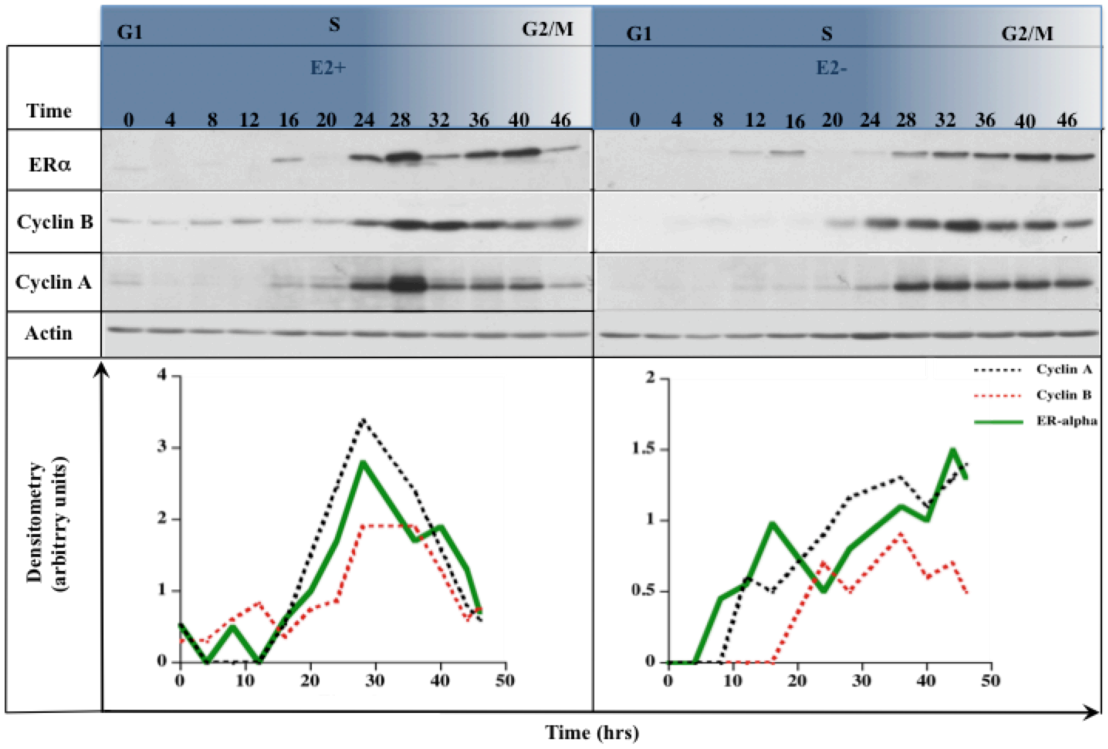


Figure 13: ERα is a cell cycle regulated protein in MCF-7 cells. The upper panels show western blots of endogenous ERα, cyclin A and cyclin B expression at 4-6 hour timepoints after release from lovastatin induced arrest in the presence (left panel) and absence (right panel) of E2. The densitometric values of the ERα, cyclin A and cyclin B western blot data were then graphed (lower panel).

Liganded ERα modulates cell cycle progression by decreasing the time that MCF-7 cells spend in S and G2/M phases of the cell cycle.

The data so far shows that ERα expression is cell cycle regulated under both E2-positive and E2-negative conditions and is predominanty expressed during the G2/M phases of the cell cycle. The question arises as to the role of liganded and unliganded ERα during S and G2/M phases. To address this question, the cell cycle profile of ERα expression was examined in MCF-7 cells in the presence and absence of ligand (17β estradiol) to allow for a quantitative analysis of cell cycle progression. MCF-7 cells were maintained in E2-free media and then synchronized with lovastatin; meanwhile, 17β estradiol (10nM) was added to one group of cells while another group remained E2-free.

Cells were harvested at four-hour intervals after release from arrest and were subjected to flow cytometry. As shown in Figure 14, 88% of MCF-7 cells were arrested in G1 phase and then released synchronously into the cell cycle phases and the experiment was repeated three different times and labeled as I, II and III. In experiment I, the cell cycle profiles of the two groups of MCF-7 cells (presence and absence of E2) were compared. Synchronized MCF-7 cells exited from G1 phase 17 hours post release in both conditions. By the time cells entered the proceeding G1 phase, the cells in the presence of E2 had altered cell cycle profiles compared to the cells in the absence of E2. Specifically, MCF-7 cells in the E2 negative conditions showed a delayed entrance to the subsequent G1 phase of the next cell cycle compared to cells under E2-positive conditions. The comparison of S phases between the E2-positive and E2-negative conditions of experiment I showed that cells entered S phase approximatley 4 hours earlier under E2-positive conditions compared to E2-negative condition, with 17 and 21 hour entry times respectively.

The decline of the S phase population of MCF-7 cells was much faster and sharper in E2-positive compare to E2-negative conditions. It follows that the entrance of the cells to the subsequent S phase of the cycle was earlier under E2-positive compared to ER-negative conditions. MCF-7 cells entered G2/M phase 7 hours earlier in E2-positive conditions (20 hours post release) compared to E2-negative conditions (27 hours post release). Also, the population of cells in G2/M peaked at 29 hours in E2-positive condition compared to the cells in E2-negative conditions, which was at 31 hours. The exit of the cells out of G2/M was

again faster in E2-positive conditions compared to E2-negative conditions. The entrance of cells in to the subsequent G2/M phase of the next cell cycle was detectable under E2-positive conditions, but not seen in the E2-negative condition due to the slow cell cycle progression of the cells (Figure 14).

In experiment II, there was not as much of a difference in the timing of the G1 phases between the two conditions as there had been in experiment I, however the effect of E2 was seen later in S phase. Cells exited S phase faster under E2-positive conditions (at 31 hours post release) compared to E2-negative conditions (35 hours post release). The cells in E2 media that exited S phase faster also entered G2/M faster (24 hours post release) compared to those in E2-negative conditions (29 hours). Again the G2/M phase of the subsequent cell cycle was not observed in E2-negative conditions; while in E2-positive conditions, the entry of cells in to the next G2/M was observed (Figure 14). In experiment III, the profile of cells in G1 phase did not differ much between the two conditions. There was a sharp increase in the percentage of S phase cells at 17 hours after release from lovastatin arrest under E2-positive conditions compared to the broader peak of S phase cells at 20 hours after release in the E2-negative conditions. The effect of E2 was more pronounced in G2/M phases. Cells sharply entered into G2/M phase at 25 hours in E2-positive conditions, compared to E2-negative condition where the incline started at 29 hours. The cells in G2/M under E2-positive conditions peaked at 30 hours, while the percentage of cells in G2/M phase did not peak until 37 hours under E2-negative conditions (Figure 14).

Overall, the results of the second and third independently performed experiments support the results from experiment I. Flow cytometric analysis clearly revealed that liganded ERα in MCF-7 cells modulated cell cycle progression by shortening S and G2/M phases of the cell cycle (Figure 14).

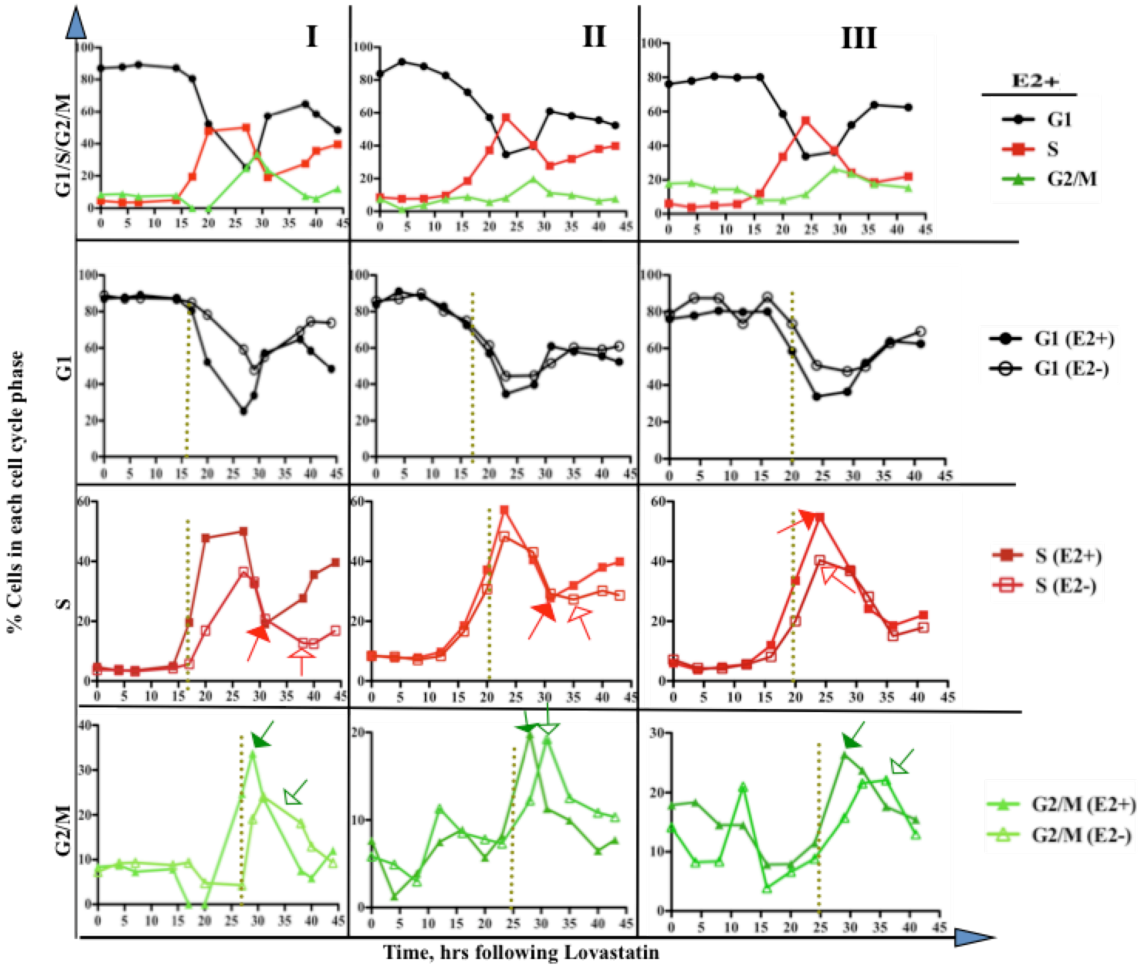


Figure 14: Liganded ERα hastens the passage through S and G2/M in MCF-7 cells.

The percentage of cells in each phase of the cell cycle as determined by FACS analysis of lovastatin synchronized MCF-7 in the presence (E2+) or absence (E2-) of estradiol. Three separate repeats of the experiment are shown (I, II and III). The arrows indicate the peak percentage of cells in each phase and the point at which cells start to exit the phase of the cell cycle. The time spent by MCF-7 cells in each phase of the cell cycle under E2-positive versus E2-negative conditions can be extrapolated from the graphs. The hatched vertical lines in each panel depict the start of the specific phase of the cell cycle.

Data used for experiment III are the data used in experiment III in figure 12.

Table 3 shows the results of analysis of the duration of S and G2/M phases of the MCF-7 cell cycle in the presence versus absence of estradiol. Treatment of the MCF-7 cells with estradiol resulted in a shortening of S phase by 3.3 and of G2/M phase by 2.3 hours in the in the presence of the ERα ligand (E2). The total time for the cell cycle of MCF-7 cells in E2-free condition is 36.5 hours while the total cell cycle time of MCF-7 cells in the presence of E2 is 32 hours making the difference between E2-positive and E2-negative conditions 4.8 hours, which is significantly different ($p<0.05$). The differences between the duration of each cell cycle phase was measured by calculating the hours that cells spent between the times that the percentage of cells started to increase in that phase (ascending line) until the time that the percentage of cells started to decrease in that particular phase (descending line) (Figure 14). The measurment of each phase of the cell cycle was done on three different replicates of the experiment and then averaged. The significance of the difference between each cell cycle phase in E2-positive and E2-negative conditions were measured using two-tailed t-test. The results are depicted in table 3.

	G1 (hrs)	S-Phase (hrs)	G2/M (hrs)	Total Cell Cycle (hrs)	S+G2/M (hrs)	
Liganded (E2+)	15.8 ± .4	9.7 ± 1.2	6.5 ± 2	32±2.1	16.2±1.9	Δ = 4.6 hours p<0.05
Unliganded(E2-)	15.7± .8	12 ± 1.4	8.8 ± 1.5	36.5±1.7	20.8±1.8	

Table 3 -Analysis of the duration of G1, S and G2/M phases (in hours) of MCF-7 cell cycles in the presence versus absence of E2 as determined by FACS. Data shown is the average from three different experiments (Figure 14).

Regulation of endogenous ERα expression is observed in MCF-7 cells synchronized with aphidicolin.

We have shown that ERα expression is cell cycle regulated upon synchronization of cells in the G1 phase of the cell cycle by lovastatin. To confirm that these results are not unique to the method of synchronization used, cells were also synchronized using aphidicolin (Figure 15). Aphidicolin arrests the cells in G1/S phase through inhibition of DNA replication by selectively inhibiting the cellular replicative DNA polymerase alpha [471]. MCF-7 cells, which had been maintained in E2-free media, were used for aphidicolin synchronization. As with the lovastatin synchronization experiments, two parallel groups (one receiving 10nM 17β estradiol at the time of release and the other group not receiving any ligand) were used for aphidicolin experiments. Cells were harvested in three hours time intervals and the samples were then prepared for FACS and western blot analysis.

Aphidicolin synchronized 78% of MCF-7 cells at the border of G1/S phase (Figure 15, bottom panel). The synchronized cells were all arrested at the end of G1 phase, therefore the cells synchronously progressed through S and G2/M phases and also the G1, S and partially through G2/M phase of the subsequent cell cycle quickly (by about 12 hours) upon release from aphidicolin release. It was immediately clear by examining the FACS data that MCF-7 cells exit the S and G2/M phases faster in the presence of E2 compared to the absence of E2 (Figure15, bottom panel).

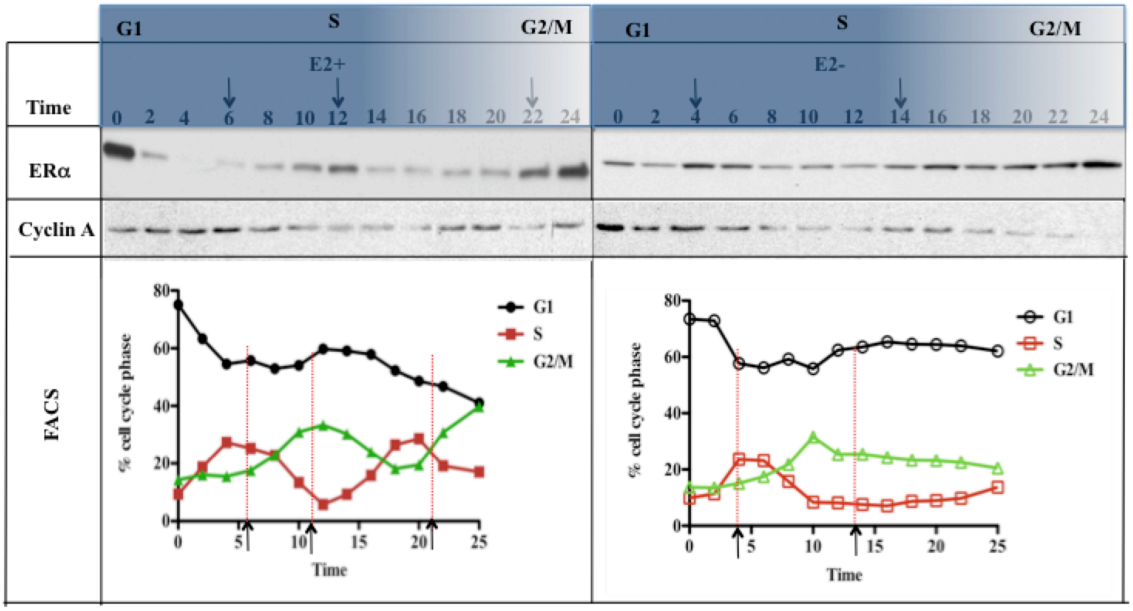


Figure 15: Aphidicolin synchronized MCF-7 cells confirm that ERα is cell cycle regulated. Top panel shows the western blot analysis of aphidicolin synchronized MCF-7 cells. Arrows indicate the times where ERα expression is increased. The bottom panel shows the FACS data graphed as the percentage of aphidicolin synchronized MCF-7 cells in each phase of the cell cycle. The dotted lines correspond to timing of ERα protein expression determine by the above western blots.

The faster progression of cells through S and G2/M phases in E2-positive condition compared to E2-negative condition possibly could explain why there were two cell cycles in the E2 treated cells within the time frame studied while in E2-negative conditions only one cell cycle was observed (note the 2 S phase peaks in E2-positive conditions and only one S phase peak in E2-negative conditions in figure 15), In E2-positive conditions, the cells had progressed through one entire cell cycle, and the G1, S and part of G2/M of the second cell cycle. Meanwhile, in E2-negative conditions, only a prolonged G1 phase of the second consequent cell cycle was observed (Figure 15). The western blot analysis of MCF-7 cells synchronized with aphidicolin and released under E2-positive conditions (top left) showed that there were two peaks of ERα expression, as shown by arrows in Figure 15. The first peak of ERα expression started at 6 hours and ended by 12 hours after release from arrest. The second peak was between the 22 and 24 hour time points. Interestingly, the duration of both of these peaks correlated with the peaks of cell populations in S-G2/M phases in two consecutive cell cycles. Similar to the observed peaks of ERα, there are two peaks in the protein expression of cyclin A in the E2-positive condition. The first peak of cyclin A expression starts from time point 0 and continues until time point 8. The second peak starts at time point 18 and continues until time point 22. The timing of the increases in cyclin A expression corresponds to S phase. In the cells released from aphidicolin arrest under E2-negative conditions (Figure 15, right side), there were again two peaks for the level of ERα protein. The first peak started at the four hour time point and declined at 8 hours. The timing of this peak coincided with the S and G/M phases of the first cell cycle. The second peak of ERα level started at 14 hours after release and remained to the end of the experiment. Similarly, there were two observed peaks for the protein expression of cyclin A. The first peak started at time point 0 and continued until time point 6, which corresponded to the S phase. The second peak of cyclin A expression, which is not as strong as the first peak, started at time point 14 and declined at time point 18. Both peaks of cyclin A corresponded to the S phase, however because the population of cells in the second S phase is not as high as the first S phase in the E2-negative condition, the amount of cyclin A protein expression is lower.

The FACS data was analyzed to explore what the effects were on the progression of the cell cycle as the ERα levels remained elevated. The FACS data showed no observable

incline, peak or decline in the percentage of cells entering S and G2/M phases of the second subsequent cell cycle. From 14 hours after release from arrest (when ERα expression peaked the second time) through to the end of the experiment (30 hours) there were still 40% to 50% of the cells residing in the S and G2/M phases. In summary, in the absence of E2, progression through the cell cycle was abrogated compared to the presence of E2. Collectively, the results to this point show that ERα is cell cycle regulated in MCF-7 cells that have been synchronized by arresting cells with lovastatin (Figure 13) and aphidicolin (Figure 15). The increase in ERα protein expression level correlates with the time points at which the cells are in S-G2/M phase of the cell cycle.

To better quantitate the effects of liganded versus unliganded ERα in MCF-7 cells synchronized with aphidicolin, three independent replicates of the experiment were performed (Figure 16). As shown in the left panel of Figure 16 (experiment I), 78% of MCF-7 cells were arrested in G1 phase by aphidicolin and released synchronously into the cell cycle phases. Aphidicolin synchronized MCF-7 cells exited G1 phase at the 4 hour time point in both conditions, and entered the subsequent G1 phase at 11 hours after their release. The percentage of cells in the second G1 phase peaked at 14 hours after release in the presence of E2, while the peak percentage of cells in G1 in the absence of E2 was not until the 20 hour time point. In addition, the exit from the second G1 phase appears much faster in E2-positive conditions compared to E2-negative conditions (Figure 16).

When comparing the percentage of MCF-7 cells in the S phase between the E2-positive and E2-negative conditions, the results from experiment I showed that cells entered and exited the S phase of the first cell cycle at the same time after release from arrest. In both the presence and absence of E2, the MCF-7 cells entered S phase at 2 hours and exited 8 hours after relase from aphidicolin arrest. Despite this synchronous S phase progression, the pattern of cells progressing through the S phase of the subsequential cell cycle is different when E2 is present compared to when it is not. In E2-positive conditions, cells entered the S phase of the subsequent cell cycle at 15 hours, peaked at 18 hours and exited 20 hour post release.

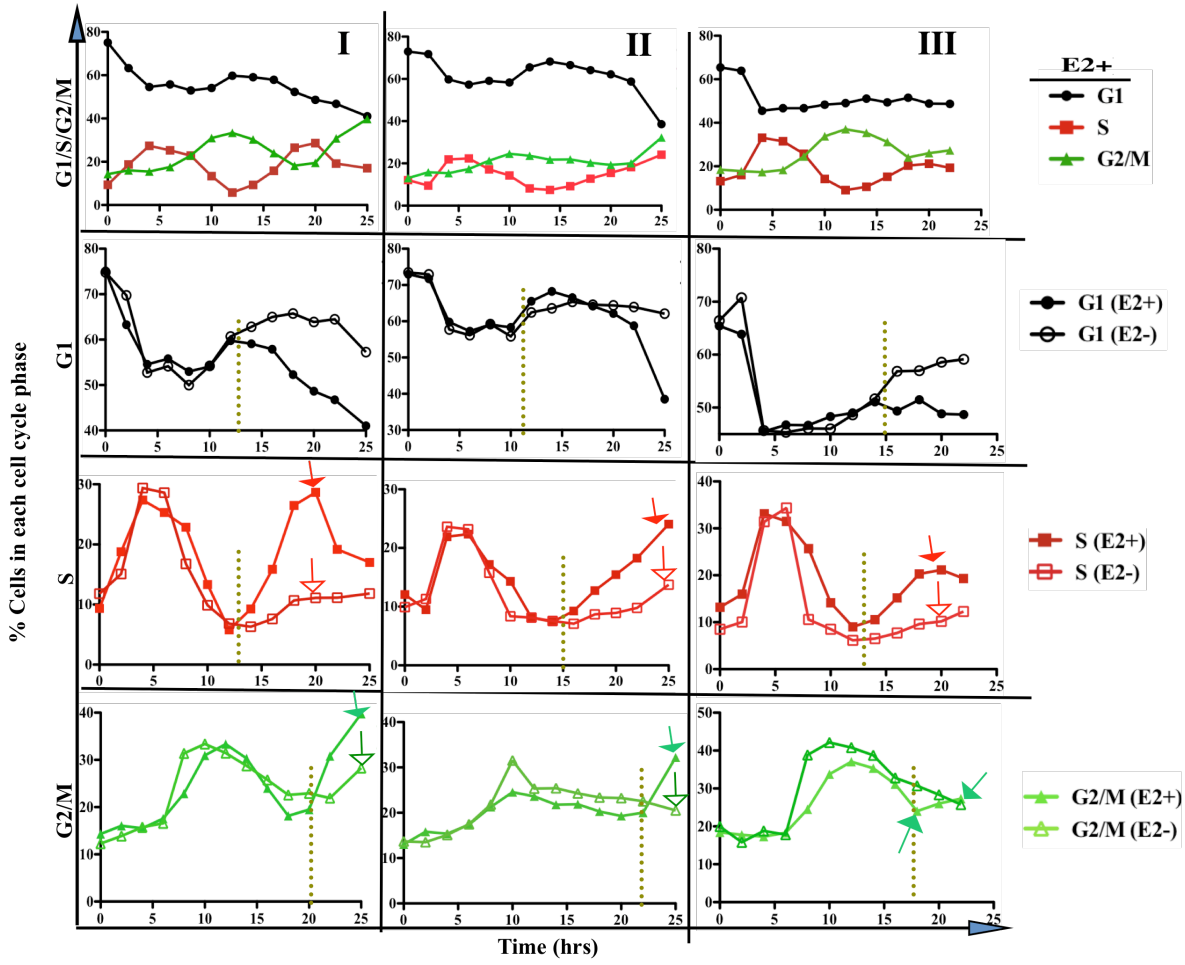


Figure 16: 17β estradiol hastens the passage through S and G2/M phases in aphidicolin synchronized MCF-7 cells. FACS analysis of aphidicolin synchronized MCF-7 cells in the presence (E2-positive) or absence (E2-negative) of 17β estradiol. The data is collected from three independent experiments. Comparsion of the profiles of G1, S and G2/M phases of experiment I has been shown in the left column (I), those of of experiment II have been shown in the middle column (II) and experiment III have been shown in the right column. The hatched vertical lines in each panel depict the start of the specific phase of the cell cycle. The data used for ploting the all cells cycle phases FACS data shown for experiment I in this figure has been also used to plot the E2-positive graph in figure 15.

In contrast to the 18 hour peak of S phase in E2-positive conditions, under E2-negative conditions, the population of cells only entered the S phase of subsequent cell cycle at 18 hours after release and never formed a sharp peak. This suggests that cell cycle progression is delayed in the absence of E2.

The MCF-7 cells entered and exited the G2/M phase of the first cell cycle after release from arrest at the same time whether E2 was present or not. The cells entered G2/M at 7 and exited G2/M at15 hours after being released from arrest. Cells released under E2-positive conditions entered the second G2/M phase at 20 hours after release. The percentage of cells in G2/M phase peaked at 25 hours, which was the last harvested time point of this experiment. As expected after a delayed S phase, cells released from arrest did not show progression in to a second G2/M phase by 25 hours in E2-negative condition (Figure 16). The results from the second (II) and third (III) repeats of the experiment supported the results described for experiment I. In order to calculate whether the changes between the S and G2/M phases upon E2-treatment is statistically significant compared to untreated cells, the percentage of the cells at the peak of each phase were used in order to measure the difference between the quantity of the cells present in each phase under E2-positive condition versus E2-negative condition. The numbers for each phase and condition were average of the three repeats of the experiment. Two-tailed t-test was used in order to measure the p-value. The results are depicted in table 4.

MCF-7 + Aphidicolin	% cells in G1	% cells in S-Phase	% cells in G2/M	Total % cells in S+G2/M	
Liganded (E2+)	55.23	22.68	33.11	55.79	Δ p<0.05
Unliganded (E2-)	62.01	10.34	24.85	35.19	

Table 4 -Analysis of the percentage of the aphidicolin synchronized MCF-7 cells present at the G1, S and G2/M phases (consequent cell cycle) in the presence versus absence of E2 as determined by FACS. Data shown is the average from three different experiments (Figure 19).

In summary, flow cytometric analysis of the aphidicolin synchronized MCF-7 cells clearly showed that liganded ERα modulates cell cycle progression by accelerating the progression of the cell cycle. The effect of liganded ERα on cell cycle progression is most pronounced in the S and G2/M phases. The major difference between the results generated from lovastatin mediated arrest compared to that of aphidicolin is that E2 modulated the first cell cyle after release from lovastatin arrest, but the second cell cycle was affected by E2 after aphidicolin mediated arrest. The reason for the delayed effects of E2 in aphidicolin treated cells is because ERα expression did not increase until 8 hours after release from arrest and the MCF-7 cells treated with aphidicolin had already completed the first cell cycle.

ERα expression is regulated at the protein level and not at the transcriptional level.

The expression of ERα throughout the cell cycle has been examined by western blot analysis, providing insight in to the levels of expressed ERα protein. However, it is not known whether the expression of ERα mRNA is cell cycle regulated at the transcriptional level. To determine the levels of ERα mRNA expression throughout the cell cycle, Quantitative Real Time Polymerase Chain Reaction (qRTPCR) analysis was performed on lovastatin synchronized MCF-7 cells. Synchronized MCF-7 cells were collected at 3 hours intervals between 0 hours (when the cells were released from arrest using mevalonate) and 40 hours and RNA samples were extracted from each sample. After reverse transcription, qRT-PCR was performed using sense and antisense oligos for ERα. Figure 17 (top panel) shows that lovastatin synchronization arrested 75% of the cells in G1 phase. Upon release of the MCF-7 cells from arrest using mevalonate, they synchronously progressed through the cell cycle phases. FACS data shows that lovastatin synchronized MCF-7 cells exited G1 phase at 15 hours post arrest and entered S phase, peaking in S phase at 20 hours after release. The cells entered G2/M phase at 25 hours post release, reached a peak percentage of cells in G2/M phase at 30 hours and exited G2/M at 35 hours after being released (Figure 17). The middle panel shows the graph of values obtained from densitometry of western blot analysis of ERα protein expression (Figure 17).

As observed previously (Figure 13), ERα protein showed a cell cycle regulated pattern. The densitometry results showed that ERα protein level increased from 15 to 28

hours after release and declined at 35 hours. Alignment of the western blot data and the FACS analysis shows that the increasing ERα protein expression coincided with the S phase and the highest ERα protein level coincides with the highest percentage (around 70%) of cells residing in S and G2/M pahses. ERα protein expression declined at 35 hours, the same time that FACS analysis showed the cells had exited S and G2/M phases and had entered the G1 phase of the successive cell cycle (Figure 17).

In contrast to the western blot data that shows fluctuations in protein levels with the cell cycle, qRT-PCR results showed that mRNA levels of ERα did not change throughout the cell cycle (Figure 17). Furthermore, the qRT-PCR analysis also showed that ERα mRNA levels are not affected by E2 as the values were equivalent for both groups of cells (presence and absence of E2 and repeated twice under both conditions). The quantification of ERα mRNA via qRT-PCR revealed that the cell cycle dependent changes in ERα expression are not transcriptionally regulated. We deduced that the decreased ERα expression during the G1 and early S phases are due to post-translational mechanisms.

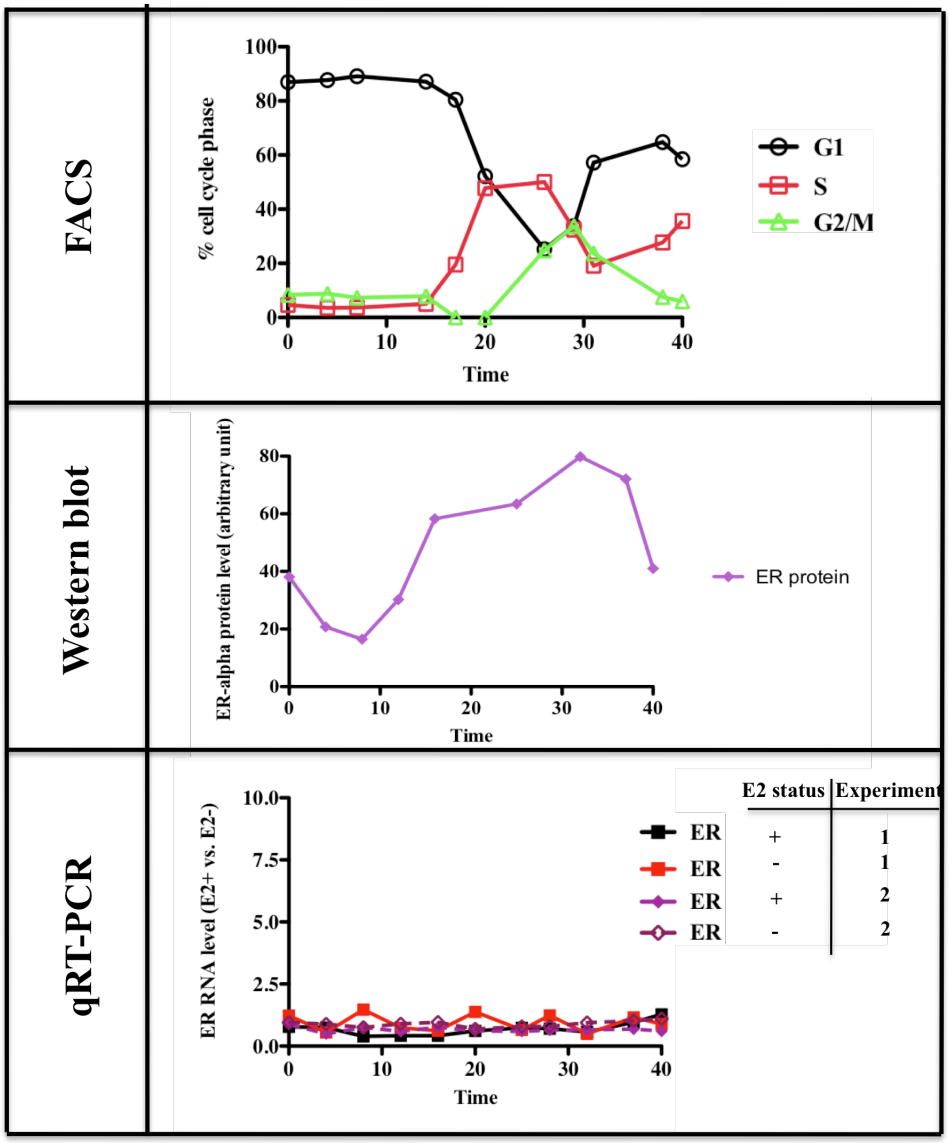


Figure 17: ERα is not regulated at the transcriptional level. Top panel shows the FACS analysis of the lovastatin synchronized MCF-7 cells. The middle panel shows the graphed values of densitometry from western blot analysis of ERα in lovastatin synchronized MCF-7 cells. The bottom panel shows the values obtained from qRT-PCR analysis of lovastatin synchronized MCF-7 cells from two separate experiments. The data used for ploting the all cells cycle phases FACS data in this figure has been also used to plot the experiment I in figure 14.

ERα expression is also cell cycle regulated in the ER-positive breast cancer cell line ZR75-1

To address whether ERα is cell cycle regulated in other ER-positive breast cancer cells, the ZR75-1 cell line was used to validate the generality of the ER cell cycle regulation in other cell lines. The ZR75-1 cell line has phenotypic characteristics similar to MCF-7 cells. For example, both ZR75-1 and MCF-7 cells are ER/PgR-positive and both cell lines have low invasive and metastatic potential [472].

ZR75-1 cells were maintained in E2-free media and then synchronized with lovastatin as described earlier for MCF-7 cells [463]. Lovastatin synchronized 95 % of ZR75-1 cells in G0/G1 (Figure 18, bottom panel). Furthermore, the lovastatin synchronization was reversible with mevalonate, which means that upon release of the cells from lovastatin arrest ZR75-1 cells progress through the cell cycle synchronously. ZR75-1 cells were harvested every four hours from zero hours (release from arrest) through 44 hours after addition of mevalonate. Lysates were collected for western blot analysis and cells were fixed and stained for flow cytometric analysis.

ZR75-1 cells exited G1 phase at 15 hours when the population of cells in S phase increased and reached a peak at 20 hours. As Figure 18 shows, there is not a sharp decline of the cells out of S phase, however at 25 hours, a fraction of the cells (<5%) exited S phase. The G2/M phase of lovastatin arrested ZR75-1 cells peaked at 25 hours after release and was prolonged until 38 hours after release (Figure 18).

ERα protein expression was up regulated in synchronized ZR75-1 cells at 24 hours after release from arrest and stayed high until 40 hours (Figure 18, upper panel). Cyclin B was also up regulated between the 20 and 40 hour time points. Based on FACS analysis of these cells, the percentage of cells in S and G2/M phases also peaked between 20 and 40 hours after release from arrest. Cyclin B was used as a cell cycle marker for late S and G2/M phase and showed a similar pattern of cell cycle expression compared to ERα. These data suggest that the endogenous ERα in ZR75-1 cells, like the MCF-7 cells, was subject to cell cycle regulation with expression levels peaking at the S and G2M phases of the cell cycle (Figure 18). From this series of experiments, we can conclude that cell cycle regulation of ERα is not a cell type specific phenomenon.

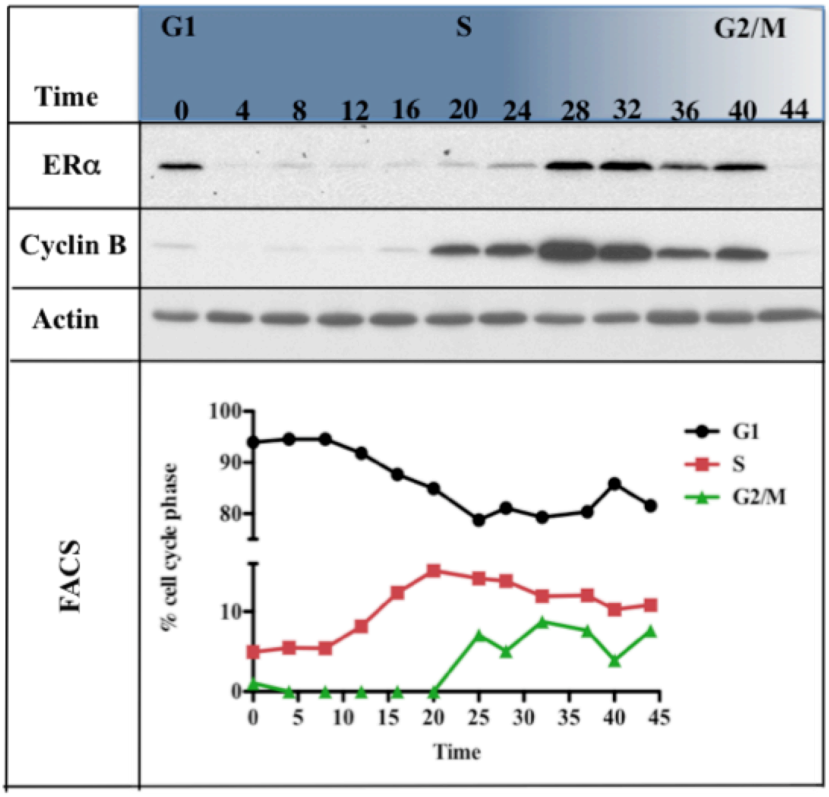


Figure 18: The cell cycle regulation of ERα protein is not cell type specific.

Western blot analysis of ERα and cyclin B expression in lovastatin synchronized ZR75-1 cells (top panel). Actin is shown as a loading control. FACS analysis of lovastatin synchronized ZR75-1 cells graphed as the percentage of cells in each cell cycle phase (bottom panel).

Radiation causes a dose dependent accumulation of MCF-7 cells in G2/M with concomitant ERα expression

It is known that gamma-irradiation arrests cells in the G2/M phase of the cell cycle. We have shown that the timing of ERα peak expression correlates with the G2/M phases of the cell cycle. Therefore, we next investigated whether gamma-irradiated cells express a high level of ERα in G2/M phase. ER-positive MCF-7 breast cancer cells were plated on day zero, and fresh medium was added the next day with either 10nM estradiol for the E2-positive condition or without any estradiol for plates in the E2-negative conditon. The cells were irradiated with a range of doses (from 0-7 grays). Cells were harvested and prepared for FACS and western blot analysis 24 hours after the exposure to radiation to assess ERα expression and cell cycle profile. As the bar graph in Figure 19A shows, there is a radiation dose dependent accumulation of cells in G2/M phase of the cell cycle. Cells also accumulated in S phase upon exposure to lower doses of radiation. The percentage of cells in S, G2 and M phases combined was highest (40%) when cells were irradiated with 0.5, 1, 6 or 7 grays. The western blot analysis of lysates from cells arrested through irradiation revealed that the level of ERα protein was also the highest in the cells irradiated with 0.5, 1, 6 and 7 grays. The FACS data of the control (0 gray) shows 40% of the cells reside in S and G2/M phases and that is why a high expression of ERα is observable in western blot. Cyclin B expression was used a marker to indicate when cells are late S and G2/M phases. A western blot for cyclin B expression revealed the exact same pattern as for ERα, where the protein is up regulated after irradiation with 0.5, 1, 6 and 7 grays.

In summary, irradiation resulted in the accumulation of cells at S and G2/M phases and showed increased ERα protein expression in the samples enriched in S and G2/M (Figure 19). These data support our previous findings that ERα is a cell cycle regulated protein with expression peaking at S and G2/M phases of the cell cycle.

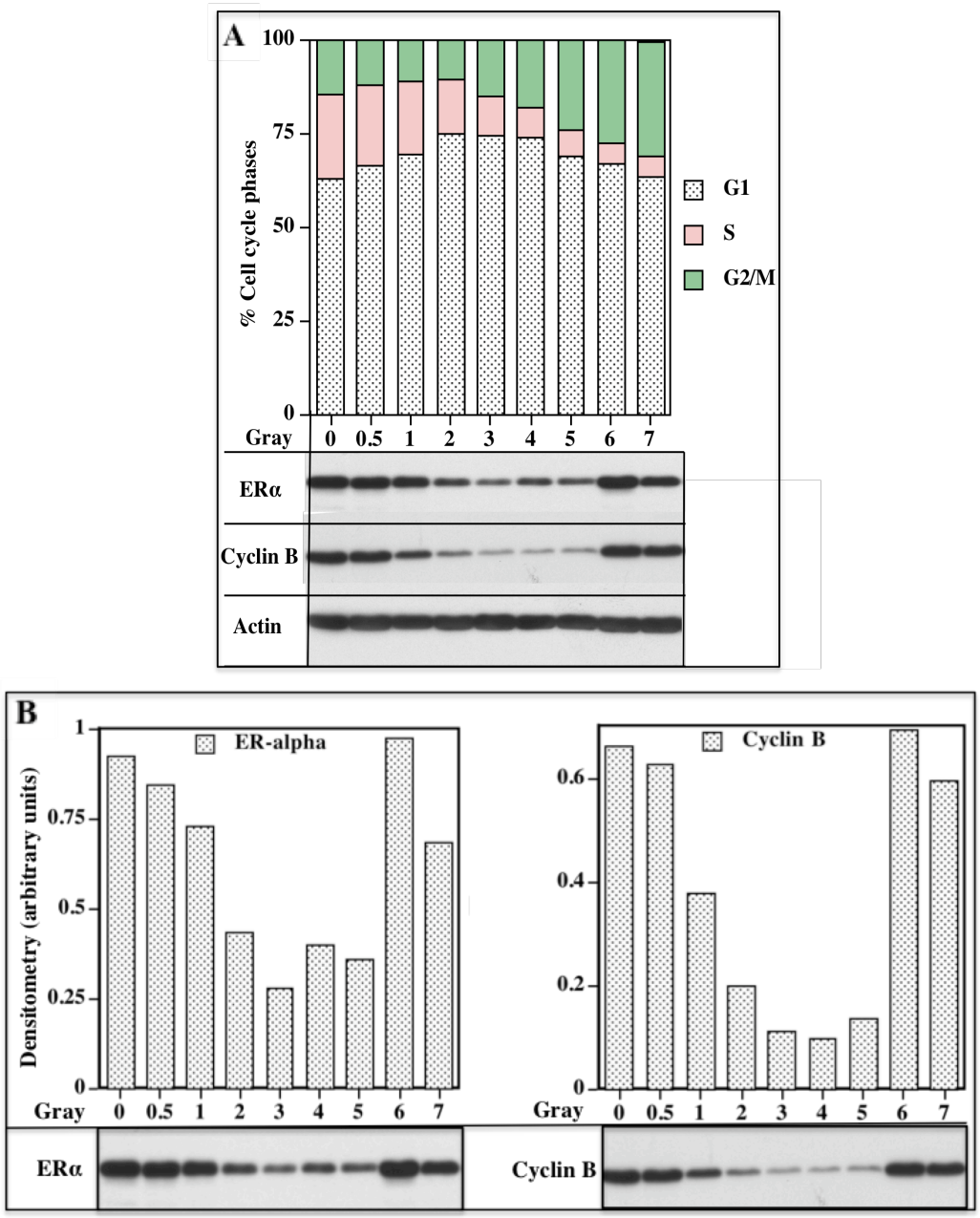


Figure 19: Accumulation in S and G2/M phases due to irradiation correlates with increased ERα expression. MCF-7 cells were exposed to increasing doses of radiation. (A) The percentage of cells in each phase of the cell cycle was determined by FACS analysis and is shown as a bar graph. Western blot analyses of lysates collected from radiated cells were blotted for cyclin B, ERα and actin expression. (B) The densitometric values of ERα and cyclin B expression were derived from the western blots perfomed on MCF-7 cell lysates after treatment with various doses of radiation.

ERα depletion accelerates mitotic exit.

The data to this point shows that cells arrested in G2/M phase of the cell cycle have increased expression of ERα. The next step in understanding the role of ERα during the G2/M phase of the cell cycle was to determine whether the downregulation of ERα in MCF-7 cells would affect the cell cycle progression specifically during G2/M phase. The method, which was employed to downregulate ERα expression, was use of shRNA.

First, an shRNA was generated using the U6 promoter based vector due to its ability to maintain the secondary structure of the short hairpin RNAs. This vector has been shown to effectively induce sequence specific gene silencing in mammalian cells and could be used to stably suppress target genes [368]. To confirm the efficiency of ERα shRNA, western blot analyses were performed at 24, 36, and 48 hours after transfection of MCF-7 cells with ERα shRNA (Figure 20A). The vector containing shRNA-ERα did not have a selection marker; therefore, a plasmid with puromycin gene was cotransfected with the shRNA-ERα. The selection of stable clones started 24 hours after the transfections and selectable, isolated colonies were observed 15 days after transfection. The stably expressed shRNA resulted in ERα expression that was silenced completely for the first two passages. However the ERα expression came back after three to four passages, which made the system impractical for use during lovastatin synchronization. For a successful synchronization experiment, MCF-7 cells need to be passaged 3-4 times to obtain enough cells needed for western blot and FACS analysis. Due to the unsuccessful generation of stable ERα silenced pool we decided to deliver shRNA-ER transiently at the time of synchronization. In order to examine the efficiency of shRNA- ERα in silencing ERα after the transient transfection we transiently transfected MCF-7 cells with shRNA-ERα and harvested the cells in 24, 36 and 48 hours after the transfection. Western blot analysis showed that the decrease in the level of ERα protein occurred within 24 hours after the transfection of MCF-7 cells with shRNA to ERα (Figure 20A). Furthermore, the ERα level remained reduced at 36 and 48 hours after the transfection (Figure 20A).

ERα protein levels remained constant, as expected, in the cells transfected with scrambled RNA or empty vector. However, upon transfection of MCF-7 cells with shERα, the expression of ERα decreased (Figure 20B).

ERα silenced MCF-7 cells were generated by transfecting the cells at the same time as plating the cells for lovastatin synchronization. Upon release of the cells from lovastatin arrest, cells were harvested at time intervals coinciding with S and G2/M phases (17-32 hours) and were subjected to western blot analysis with ERα and FACS analysis. Western blot analysis of lovastatin synchorinzed parental (untransfected) cells showed that ERα protein levels increased at 21 hours, peaked at 26 hours then decreased by 33 hours after release from arrest (Figure 20C). The MCF-7 cells in which ERα has been silenced by transfection of shRNA to ERα did not show a detectable level of ERα protein for the duration of G2/M phase (Figure 20C). To determine the effects of ERα silencing on the progression of cells through G2/M phase of the cell cyle, FACS analysis of the parental and shRNA treated synchronized MCF-7 cells was performed. The cells with down regulated ERα entered G2/M phase at 29 hours, peaked at 34 hours and then encountered a sharp decline with a complete exit from G2/M by 38 hours after release (Figure 20D). In contrast, parental MCF-7 cells entered G2/M phase at 29 hours, had a peak percentage of cells in G2/M at 36 hours, but then underwent a slow exit from G2/M phase until 42 hours after release (Figure 20D). The arrows on the graph in Figure 20D indicate the estimated time of the cells exiting G2/M phase. There was a six hours delay in exit from G2/M in the case of parental MCF-7 cells compared to the ERα-silenced MCF-7 cells (Figure 20D).

In summary, ERα down regulation results in a faster exit from G2/M phase, suggesting that ERα plays a role in delaying the exit of cells from G2/M phase. The ability of cells devoid of ERα expression to progress through the cell cycle more rapidly than those with ERα expression could explain why ER-negative breast cancer cells are more proliferative and aggressive than ER-positive breast cancer cells.

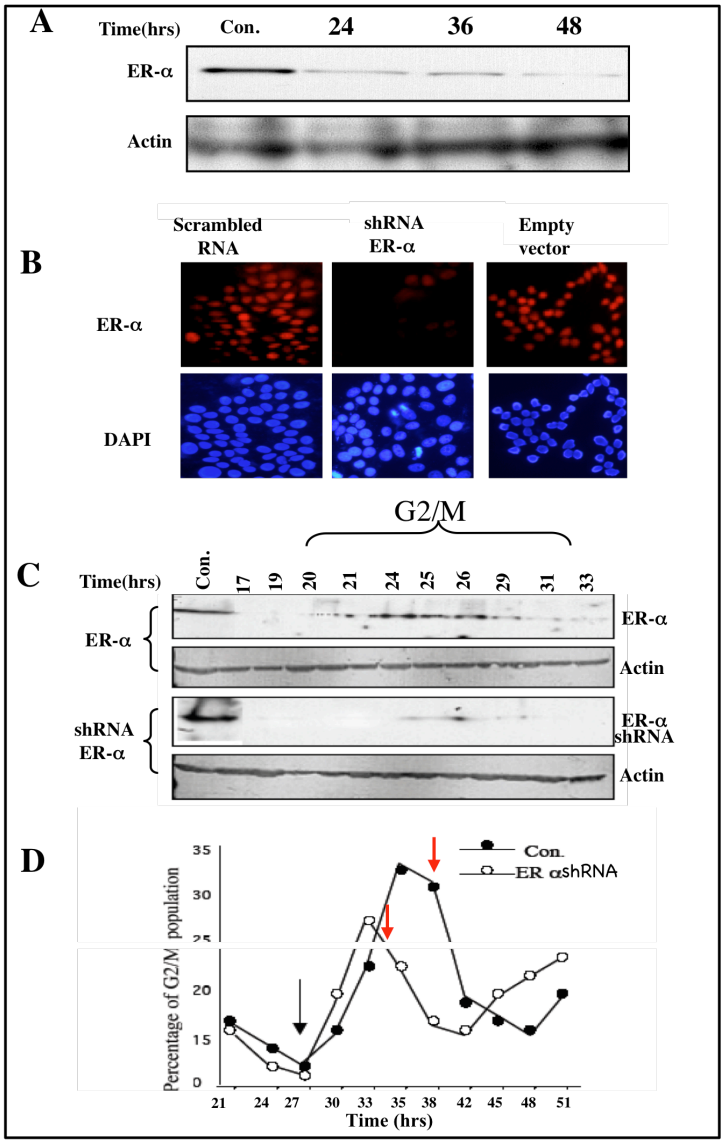


Figure 20: Downregulation of ERα in MCF-7 cells increases passage through G2/M.MCF-7 cells were transfected with shRNA against ERα, a scrambled control shRNA or an empty vector and subjected to (A) western blot analysis and (B) immunoflouresent staining to assess the efficiency of ERα silencing. The ERα is fluorescently labeled red and the nucles was stained using DAPI in MCF-7 cells treated with shERα compared to the controls (scrambled RNA and empty vector). MCF-7 cells were then synchronized, transfected with shRNA and again subjected to(C) western blot analysis and (D) FACS analysis to determine the effects of ERα downregulation on the cell cycle.

Exogenous ERα elongates the S and G2/M phases of MCF-7 cells.

ERα down regulation in MCF-7 cells resulted in a rapid exit from G2/M phase compared to parental MCF-7 cells. To investigate wether this effect was mediated by ERα, HA-ERα was next over expressed in breast cancer cells and again, the progression of cells through the cell cycle phases was assessed. The hypothesis was that unliganded ERα has an inhibitory effect on the progression of cell cycle compared to liganded ERα. To test this hypothesis, exogenous ERα was transfected in to lovastatin synchronized ER-positive MCF-7 cells using an HA-ERα construct to generate ERα-overexpressing MCF-7 cells.

Upon overexpressing exogenous ERα in MCF-7 cells FACS analysis of the cell cycle profiles showed that 80% of MCF-7 cells arrested in G1 (time zero). Upon the release from arrest at time zero, cells entered the cell cycle synchronously (Figure 21, upper panel). The experiment was repeated twice and is shown as experiments I and II. Percentage of cells in each phase of the cell cycle was evaluated over a 24 hour period for the parental and HA-ERα transfected MCF-7 cells to compare the duration of G1, S and G2/M phase. The FACS results for experiment I showed that in both conditions (untransfected and HA-ERα overexpressed) cells exited G1 phase at approximately 18 hours after release and entered S phase by 20 hours. However the parental MCF-7 cells had the highest peracentage of cells in S phase at 25 hours, while HA-ERα-cells formed a peak in S phase at 25 hours (Figure 21). In parental cell, S phase declined sharply starting at 25 hours post release with a complete exit out of S phase by 31 hours. Meanwhile, the HA-ERα cells showed a prolonged exit from S phase. Additionally, parental MCF-7 cells entered the S phase of the subsequent cell cycle, but ERα-overexpressing MCF-7 cells showed no observable second S phase within the 45 hour time frame studied (Figure 21). The parental MCF- 7 cells then enter G2/M phase at 25 hours, peaking at 28 and exiting completely from G2/M by 31 hours after release from arrest. After a slow exit from S phase, the ERα-overexpressing MCF-7 cells entered G2/M phase at 25 hours, peaked at 34 hours and exited the G2/M phase at 39 hours after release from arrest.

The outcome of the repeat of this experiment (II) was similar to experiment I (Figure 21, right column). Together, the data showed that ERα-overexpressing MCF-7 cells exhibit prolonged S and G2/M phases compared to parental MCF-7 cells, resulting in an

approximately six hours delay in the cell cycle (Figure 21). These results support our hypothesis that unliganded ERα has an inhibitory effect on the progression of the cell cycle.

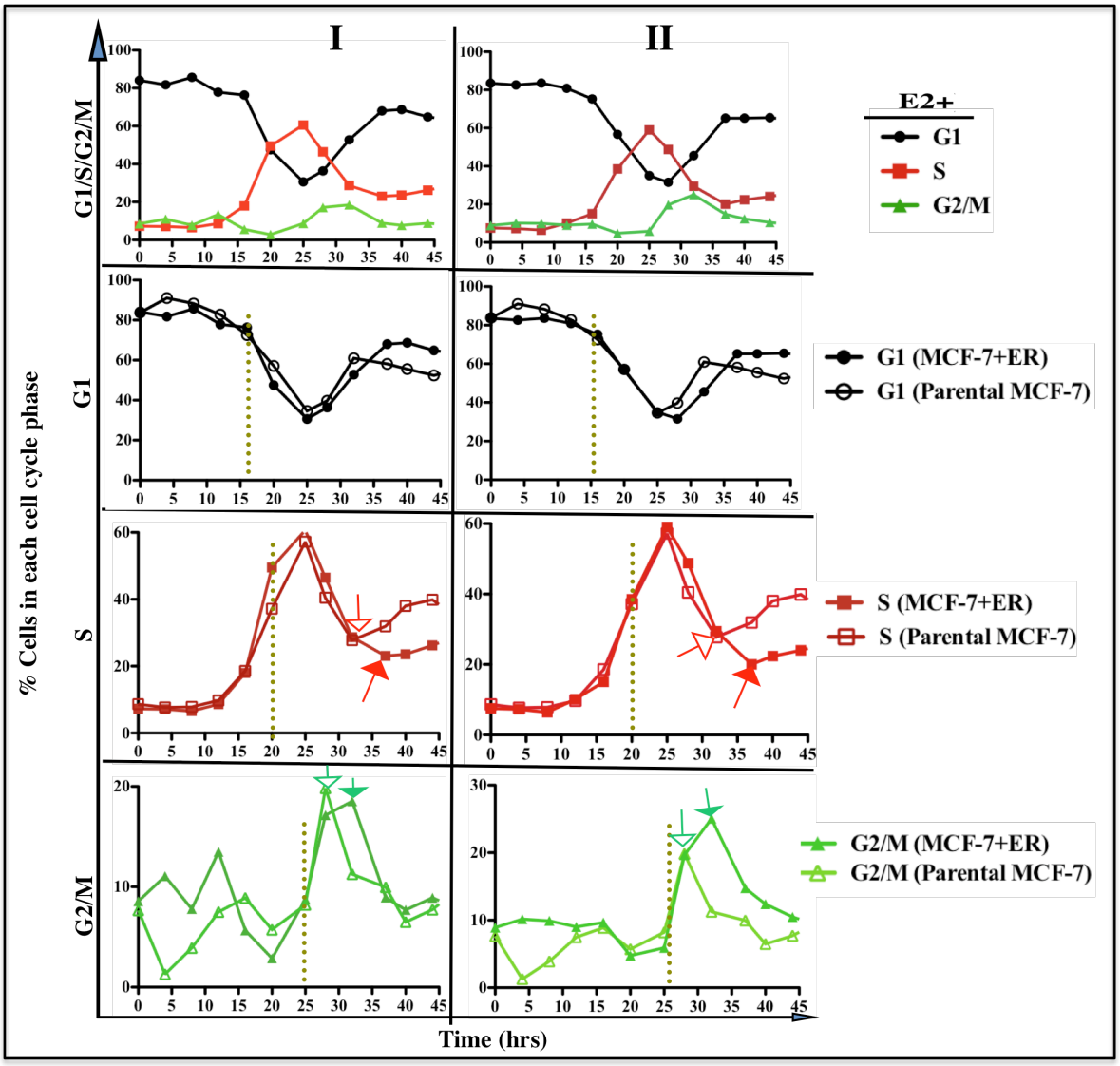


Figure 21: Overexpression of ERα elongates the S and G2/M phases. FACS analysis was performed on parental MCF-7 cells and HA-ERα-overexpressing MCF-7 cells to compare the effects of exogenous ERα on the phases of the cell cycle. Left column shows the results of experiment I, while the right column shows the duplicate experiment II.

Exogenous ER is cell cycle regulated and expressed similarly to endogenous ERα

We next performed a similar experiment to HA-ER overexpression in MCF-7, by overexpressing ER in an otherwise ER negative cell line, MDA-MB231. To this end, we transfected MDA-MB231 with ERα cDNA and initially examined the timing of ER expression following transfection by harvesting cells every six hours for 36 hours following transfection. ERα expression was first detected 12 hours after the transfection of ERα into the unsynchronized MDA-MB231 cells (Figure 22A). MDA-MB231 cells were also synchronized using lovastatin and ERα was transfected in to the cells at time point zero (mevalonate mediated release from arrest) and cells were harvested every 4 hours to extract lysates for western blots (Figure 22B). Exogenously transfected ERα resulted in low levels of expression at 12 hours, which had decreased by 16 hours and was completely undetectable by 20 hours after release. However, the expression of ERα was again observed at 24 hours and for the duration of the experiment (Figure 22B).

The data suggest that ERα expression observed at 12 and 16 hours is due to the transcription and translation of the transfected construct (as this is what we observed in the unynchronized cells (Figure 22A). However, in the synchronized cells, the exogenous expression was immediately down regulated in part since the majority of the cells reside in G1 phase at at 12 hours after release from arrest for synchronization. Based on the previous data, ERα is cell cycle regulated is expressed predominantly at the S and G2/M phases (Figure 15). Therefore the cell cycle regulatory mechanisms that are responsible for the expression of ERα in G2/M and/or the repression of ERα in G1 phase are down regulating ERα expression at the 12, 16 and 20 hour time points. ERα re-expression at the 24 hour time point correlates with the time point where the majority of cells are expected to reside in the late S and G2/M phases. Hence, the transfection of both synchronized and unsynchronized MDA-MB231 cells with ERα showed that ERα is under tight cell cycle regulation that results in expression during G2/M phases whether the protein is exogenously or endogenously expressed. Furthermore, by monitoring the timing of expression of the transfected ERα, it was determined that cells required 12 hours to process the transfected DNA into expressed protein; therefore, for future experiments that require transient transfection, the cells will be transfected 12 hours prior to their release from arrest so that by the zero time point the transfected ERα is expressed.

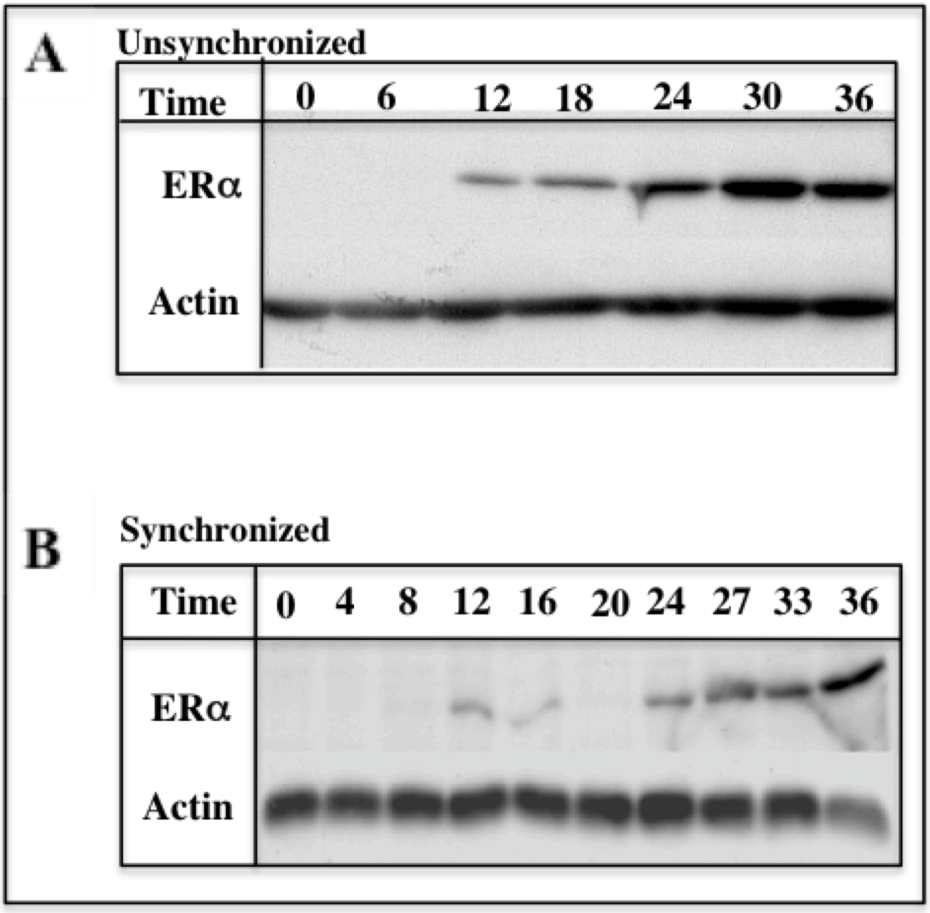


Figure 22: The timing of ERα expression in unsynchronized and synchronized MDA-MB231 cells after transfection with ERα. Western blot analyses of (A) unsychronized MDA-MB231 cells and (B) synchronized MDA-MB231 cells, which have been transfected with ERα and harvested every four to six hours to extract lysates.

ERα expression is cell cycle regulated in an ER-negative cell line

Both exogenous and endogenous ERα are cell cycle regulated in ER positive cell lines MCF-7 and ZR75-1 cells (Figures 22, 15 and 18). The next objective was to determine whether ER-negative cells could regulate exogenous ERα expression similar to that of endogenous ERα in ER-positive cells. A pcDNA3.1 vector containing the human ERα gene was generated for these experiments.

MDA-MB231 cells were synchronized in the same manner as MCF-7 cells previously, with lovastatin. As shown in Figure12 MDA-MB231 are synchronized very effectively with lovastatin with 80 to 85% of the cells arresting in G1 and released synchronously once lovastatin is removed. ERα was transfected into the MDA-MB231 cells 12 hours prior to the release of the cells from lovastatin arrest (as determined in Figure 22). Following synchronization, cells were harvested at four hour time intervals and subjected to flow cytometry to determine the cell cycle distribution and western blot analysis with ERα and key cell cycle regulators (cyclin E, cyclin A and cyclin B) to ascertain the cell cycle expression pattern of ERα.

Cyclins E, A and B demonstrated a sequential expression pattern of cell cycle expression, which coincided with the cell cycle phase that they are known to regulate (Figure 23A). For example, cyclin E expression was observed between 12 and 24 hours, which corresponded with G1 phase from the FACS data (compare G1 phase with cyclin E expression). Cyclin A was induced at 20 hours post lovastatin release, and the FACS data showed that this time point corresponded to early S phase or the time point in which the percentage of cells entering S phase is increasing (Figure 23B). Lastly, cyclin B expression was up-regulated at 27 hours, which corresponded to the entrance of the cells to G2/M phase based on the the FACS data. The percentage of cells in G1 phase had declined sharply by 24 hours after entering the cell cycle (Figure 23B) and expression of exogenous ERα protein levels were also upregulated at 24 hours, after which it remained high. Therefore, the timing of ERα expression coincides with the entrance of cells in to S and G2/M phases (Figure 23B), which is also consistent with the expression of cyclin A and cyclin B (Figure 23A). In summary, the ER negative cell line MDA-MB231 has the ability to regulate the expression of ERα in a cell cycle dependent manner similar to that of ER positive cells, with expression coinciding with the S and G2/M phases of the cell cycle.

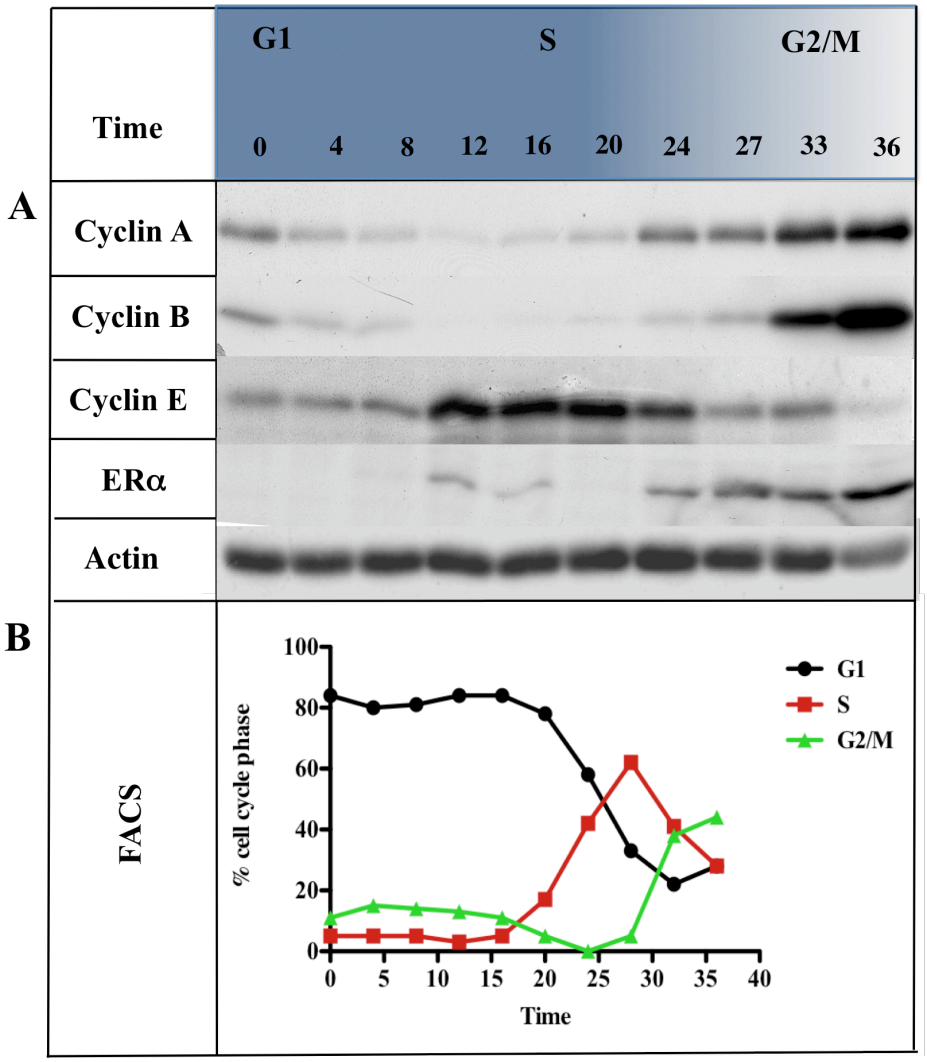


Figure 23: Exogenous ERα is cell cycle regulated in the ER negative, MDA-MB231 cells. MDA-MB231 cells were synchronized with lovastatin and lysates were subjected to (A) western blots to assess ERα, cyclin A, cyclin B and cyclin E expression. (B) FACS analysis was also performed on the cells to determine the percentage of cells in each cell cycle phase. The data used here has also been used to plot the experiment I (MDA-MB231 cells) figure 12.

ERα shortens the S and G2/M phases of ER-negative, MDA-MB231 cells.

In MCF-7 cells, liganded ERα has been shown to significantly decrease the time that cells spend in S and G2/M phases compared to unliganded ERα which delayed cell cycle progression (Figure 14). Therefore, the effect of exogenous ERα on the cell cycle phases in otherwise ER-negative cells was also examined. Lovastatin synchronized MDA-MB231 cells were transfected with ERα twelve hours prior to the release of the cells from arrest, at which point the E2-positive group received E2. Samples were then harvested and prepared for flow cytometry.

Figure 24 shows duplicate experiments of lovastatin synchronization of MDA-MB231 cells. In experiment I, lovastatin arrested 80% of the cells at G1 phase. Upon release of the cells from lovastatin arrest, the cells entered into and progressed through the cell cycle synchronously (Figure 24-expeiment I). The comparison of G1 phases between the E2-positive and E2-negative conditions showed that the cells began to exit G1 phase at the same time (17 hours) under both conditions. However, cells in the presence of E2 exited G1 faster (they have a sharper drop-off in the percentage of cells in G1 phase) than those in the absence of E2.

Under E2-positive conditions, cells exited G1 at 25 hours, whereas under E2-negative conditions, cells exited G1 phase at 31 hours following release. In both condtions, the cells progressed through the remainder of the cell cycle until they again entered into the G1 phase of the subsequent cell cycle. The population of cells entered S phase at 18 hours, had the greatest percentage of cells in S phase occurred at 22 hours and then underwent a sharp decline, showing cells exiting S phase by 28 hours in the E2-positive condition. However in E2-negative conditions, cells entered S phase at 20 hours, peaked at 30, and exited S phase at 35 hours after release. Lastly in the comparison of the G2/M phases, it is apparent that MDA-MB231 cells treated with E2 exhibited a premature G2/M phase compared to those in the absence of E2. Specifically, cells in the presence of E2 entered G2/M phase at 21 hours, formed a peak at 26 hours and exited G2/M at 34 hours after release. The cells kept in E2-negative conditions entered G2/M phase at 25 hours, formed apeak at 36 hours, and finally exited G2/M at 42 hours. The combined S and G2/M phases demonstrated 6-8 hours of delay in cell cycle progression in the absence compared to the presence of E2 (Figure 24-experiment I). The next column on the right shows a second repeat (experiment II) of

lovastatin synchronization on MDA-MB231 cells to compare the G1, S and G2/M phases of liganded exogenous ERα (E2-positive condition) and the unliganded exogenous ERα (E2-negative condition). The results from the repeated experiment also confirmed the results from experiment I. Table 5 summarizes the results and quantifies the change in duration of S and G2/M phases in MDA-MB231 cells with ERα in the presence or absence of ligand. The total time spent by MDA-MB231 cells under E2-free conditions in a cell cycle was 37.5 hours, while the MDA-MB231 cells in the presence of E2 spent 35.75 hours in one round of the cell cycle. Treatment of the MDA-MB231 cells with estradiol resulted in a faster progression of cells through S phase and G2/M phase. Due to faster progression of the cells through cell cycle phases in E2 teated conditions (specifically through S and G2/M phases) cells form S phase peak 6.25 hours earlier and G2/M phase peak 3.25 hours earlier in the E2-treated condition (Table 5).

MDA-MB231 (+ER)	G1 (hrs)	S-Phase (hrs)	G2/M (hrs)	Total Cell Cycle (hrs)	S+G2/M (hrs)	
Liganded (E2+)	18	8.5	11	37.5	19.5	Δ = 4.75 hrs
Unliganded(E2-)	19	13.75	10.5	43.25	24.25	

Table 5 – Analysis of G1, S and G2/M phases of MDA-MB231 cells upon transfection of ERα in the presence versus absence of E2. The change in the time for completion of one cell cycle in the presence or absence of E2 is shown along with statistical analysis.

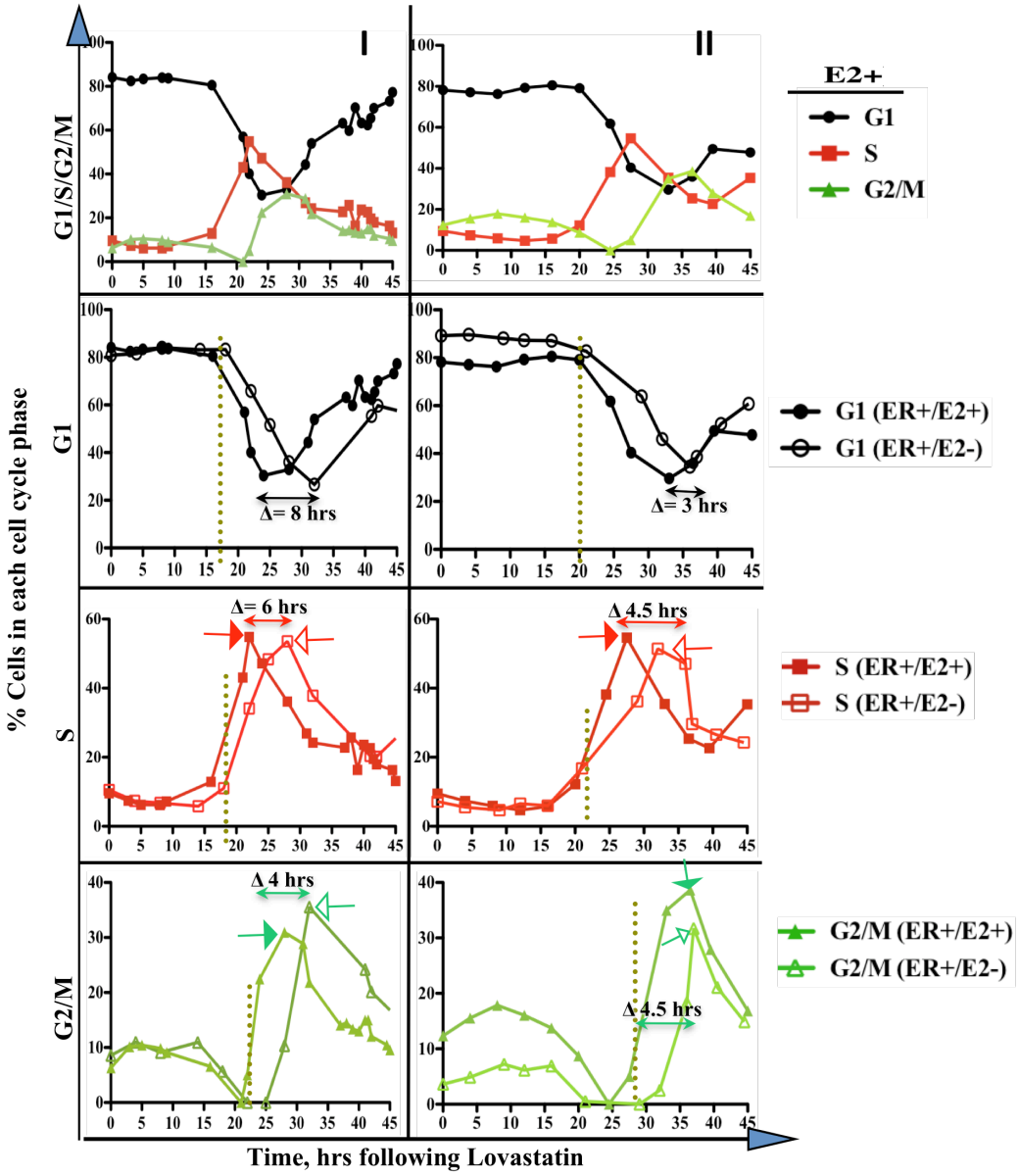


Figure 24: Exogenous ERα hastens the passage through S and G2/M in the presence of E2 in MDA-MB231 cells. FACS analysis data of two experiments (I and II) in which MDA-MB231 cells expressing exogenous ERα were synchronized by lovastatin in the presence or absence of E2. The percentage of cells in each phase of the cell cycle is shown for the time following release from arrest. The filled circle show liganded ERα compared to open circles showing the E2-negative condition. The time differential in each phase of the cell cycle between E2-positive and E2-negative conditions are noted on the figure and again in table format in table 5

Unliganded, exogenous ERα prolongs the S and G2/M phases of the ER-negative cell line MDA-MB231

In MCF-7 cells, liganded ERα has been shown to significantly decrease the time that cells spend in S and G2/M phases compared to unliganded ERα (Figure 14). However, overexpression of ERα in MCF-7 cells had an inhibitory effect on cell cycle progression compared to parental cells with only endogenous levels of ERα (Figure 21). To further examine the effect of the receptor (unliganded ERα) on the cell cycle, exogenous ERα was introduced in to otherwise ER-negative MDA-MB231 cells that had been synchronized using lovastatin.

The length of the cell cycle phases was examined by FACS analysis to examine the effects of ERα expression on MDA-MB231 cells. The parental MDA-MB231 cells exited G1 phase at 20 hours after entering the cell cycle after arrest, while the ERα-overexpressing MDA-MB231 cells exited G1 phase at 23 hours (Figure 25-experiment I). Parental cells then entered S phase at 18 hours and peaked in S phase at 28 hours, while the ERα-overexpressing MDA-MB231 cells entered S phase 20 hours and peaked at 32 hours after release (Figure 25-experiment I). Parental cells entered G2/M phase at 25 hours Peaking at 33 hours compared to the ERα-overexpressing MDA-MB231 cells that entered G2/M phase at 33 hours, peaking at 37 hours after release (Figure 25-experiment I).

The middle column shows a repeat (experiment II) of lovastatin synchronization on MDA-MB231 cells and the right column shows the third replicate (experiment III) to compare the duration of G1, S and G2/M phases in ERα-overexpressing MDA-MB231 cells and parental MDA-MB231 cells. The results from experiments II and III supported those of experiment I. In summary, the introduction of exogenous ERα to MDA-MB231cells resulted in prolonged S and G2/M phases compared to parental MDA-MB231 cells, which did not express any ERα (Figure 25). These observations suggest that overexpressed ERα, in the absence of E2, has an inhibitory effect on the progression of the cell cyle that is similar to that of endogenous ERα in MCF-7 cells (Figure 21).

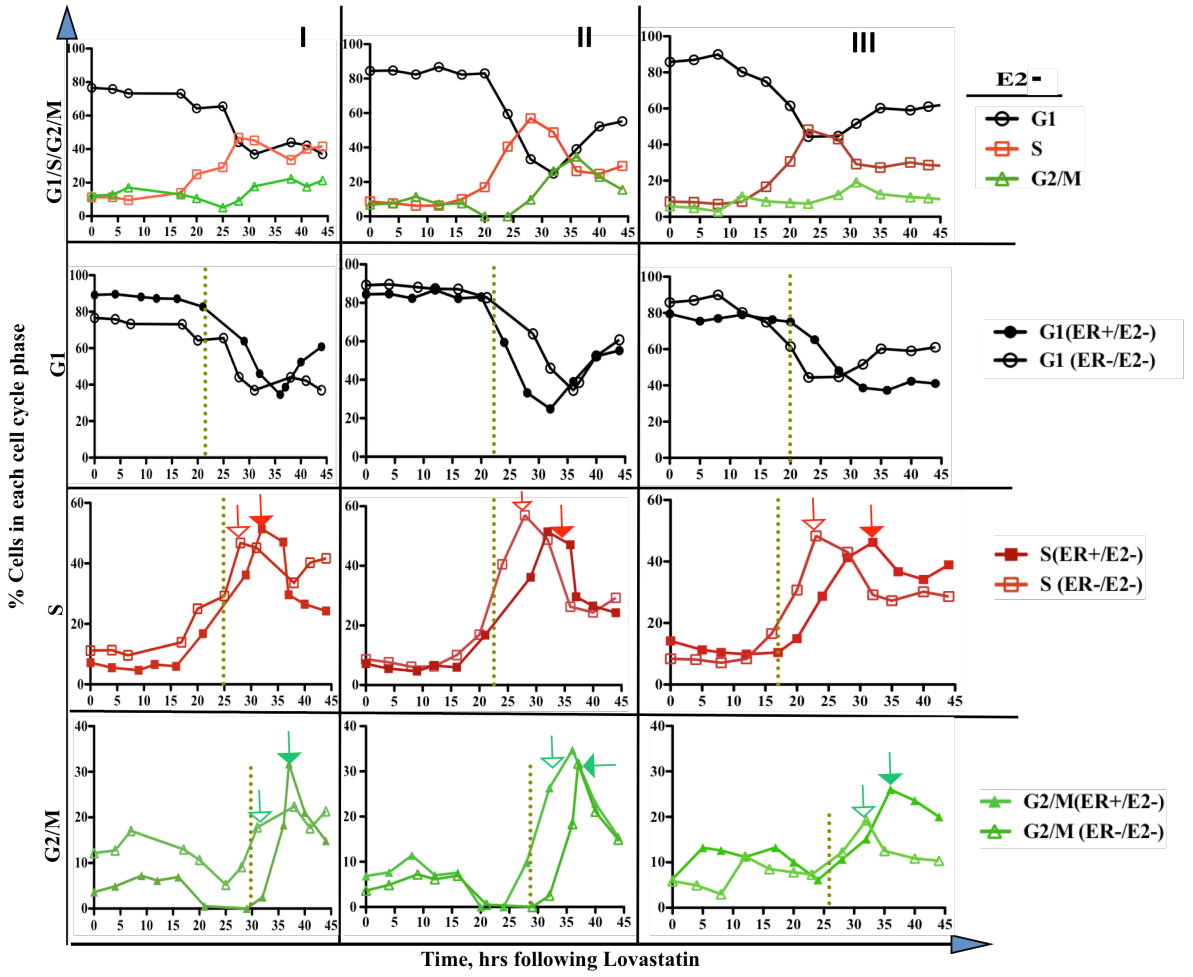


Figure 25: Exogenous ERα inhibits the cell cycle of MDA-MB231 cells, similarto the effect of endogenous ERα on the cell cycle in MCF-7 cells. FACS analysis of lovastatin synchronized MDA-MB231 cells is shown as the percentage of cells in each cell cycle phase over a 50 hours period. Three replicate experiments are shown (experiments I, II and III).

Differential effects of Tamoxifen and fulvestrant on cell cycle progression could be due to their unique interactions with ERα

Tamoxifen and fulvestrant are both antiestrogens used extensively in the clinic that exert their inhibitory effect on tumor proliferation by blocking the estrogenic activity through ER. Upon binding to ERα, both fulvestrant and tamoxifen inhibit the transcription of estrogen sensitive genes. However the binding of fulvestrant to ERα increases the rate of ER degradation and subsequently reduces the amount of ER protein in the cell, whereas tamoxifen has no effect on the stability of ERα.

The results presented thus far show that 1) unliganded ERα has an inhibitory effect on the cell cycle compared to liganded ERα and 2) down regulation of ERα increases progression through the cell cycle. The fact that fulvestrant degrades ERα while tamoxifen maintains the integrity of ERα protein, provided the experimental tools and the rationale to compare the effect of tamoxifen (intact ERα) versus fulvestrant (degraded ERα) on the progression of cells through the cell cycle. The hypothesis we tested here is that fulvestrant would hasten the passage of cells through the cell cycle due to the degradation of ERα, however treatment of cells with tamoxifen would not cause changes in cell cycle regulation due to its inability to downregulate or degrade ERα. To address this hypothesis, MCF-7 cells were treated with various concentrations of fulvestrant and tamoxifen and the time for progression through the cell cycle phases was determined (Figures 26A and 27A).

All concentrations of fulvestrant tested (2.5nm, 5nM, 10nM, 20nM, 40nM and 80nM) resulted in ERα degradation in MCF-7 cells (Figure 26A). In contrast, none of the doses of tamoxifen tested (2μM, 4μM, 6μM, 8μM and 10μM) affected the ERα protein stability in MCF-7 cells significantly (Figures 27A). The treatments with 8μM and 10μM tamoxifen degraded ERα slightly. As a result of the downregulationo of ERα at high concentrations of tamoxifen, 5μM tamoxifen was used for comparison against 5nM fulvestrant in the lovastatin synchronization experiments.

To compare the effects of tamoxifen with fulvestrant on the transition of cells from the phases of the cell cycle, MCF-7 cells were synchronized with lovastatin and then treated with either tamoxifen (5μM) or fulvestrant (5nM) in parallel experiments. The results depicted in Figure 26B show that MCF-7 cells in the presence of fulvestrant entered S phase

at 8 hours, while the untreated MCF-7 cells entered S phase at 12 hours. The percentage of cells in S phase then formed a peak 15 hours in fulvestrant compared to 20 hours in untreated cells. Cells exited S phase at 22 hours after treatment with fulvestrant, which was 6 hours earlier than the exit at 28 hours by untreated cells (Figure 26B). Cells entered G2/M phase of the cell cycle at 17 hours and reached a maximum at 25 hours regardless of whether they have been treated with fulvestrant or not. The fulvestrant treated MCF-7 cells, however, declined sharply and exited G2/M phase by 28 hours, while the untreated cells did not exit G2/M phase until 32 hours after release. Therefore, fulvestrant affected the S and G2/M phases of the cell cycle by promoting the progression of the cells through S and G2/M phases. These results support the previous finding that down regulation of ERα in ER-positive cells causes progression of the MCF-7 cells through G2/M phase (Figure 20).

In contrast to the increased progression through the cell cycle observed with fulvestrant treatment, tamoxifen treatment slowed down the progression of lovastatin synchronized MCF-7 cells through S and G2/M phases compared to untreated cells (Figure 27B). Untreated MCF-7 cells entered S phase at 8 hours and peaked at 15 hours, while the tamoxifen treated cells entered S phase at 12 hours and peaked at 20 hours after release from arrest (Figure 27B). Similar results were observed for the progression of treated versus untreated cells through G2/M phase. Untreated cells entered G2/M phase at 17 hours, while tamoxifen treated cells entered G2/M phase at 20 hours. The tamoxifen treated cells showed an overall slower progression through G2/M phase and a delay of 5 hours entering the G2/M phase of the subsequent cell cycle compared to untreated cells (Figure 27B).

In summary, fulvestrant treatment of synchronized MCF-7 cells resulted in a shorter S and G2/M phases compared to tamoxifen treatment (Figure 28). These differences in cell cycle progression could be accounted for by the effects of tamoxifen and fulvestrant on the stability of the ERα protein as the results with fulvestrant treatment, which mirror those from the shERα experiments in which ERα was silenced in MCF-7 cells (Figure 20). The MCF-7 cells treated with tamoxifen have a cell cycle profile similar to that of MCF-7 cells and MDA-MB231 cells with over expressed ERα (Figures 21 and 25). Tamoxifen treatment leaves the ERα protein intact, therefore the changes in transcription downstream of ER play a role in stalling the S and G2/M phases of the cell cycle upon treatment with tamoxifen.

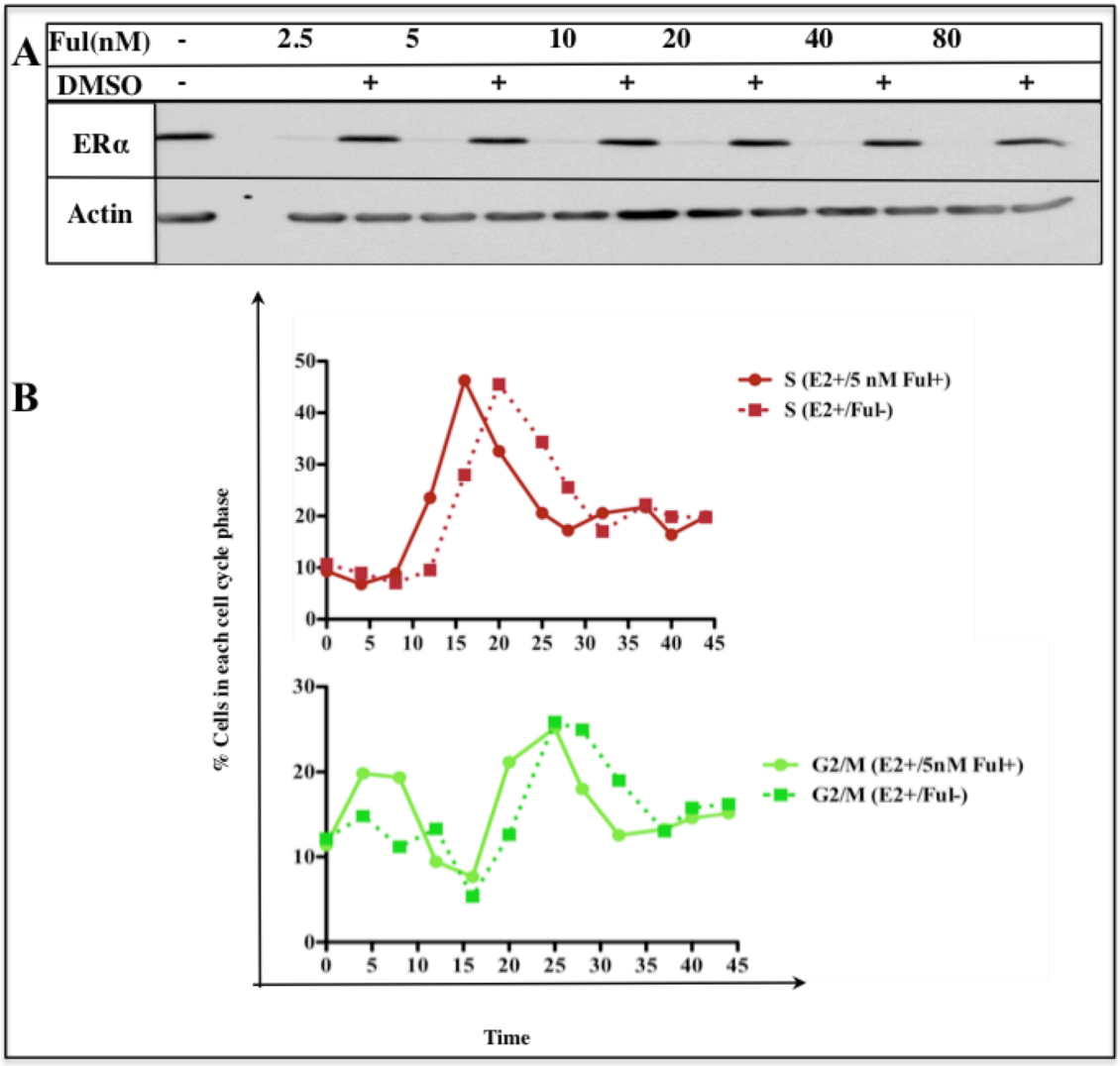


Figure 26: Fulvestrant down regulates ERα in MCF-7 cells, which results in progression through S and G2/M phases. (A) MCF-7 cells were treated with increasing concentrations of fulvestrant and lysates were subjected to western blot analysis of ERα expression. (B) MCF-7 cells were synchronized and cultured in the presence or absence of 5nM fulvestrant. At the indicated intervals, samples were collected and subjected to FACS analysis to determine the percentage of cells in S phase (upper graph) or G2/M phase (lower graph).

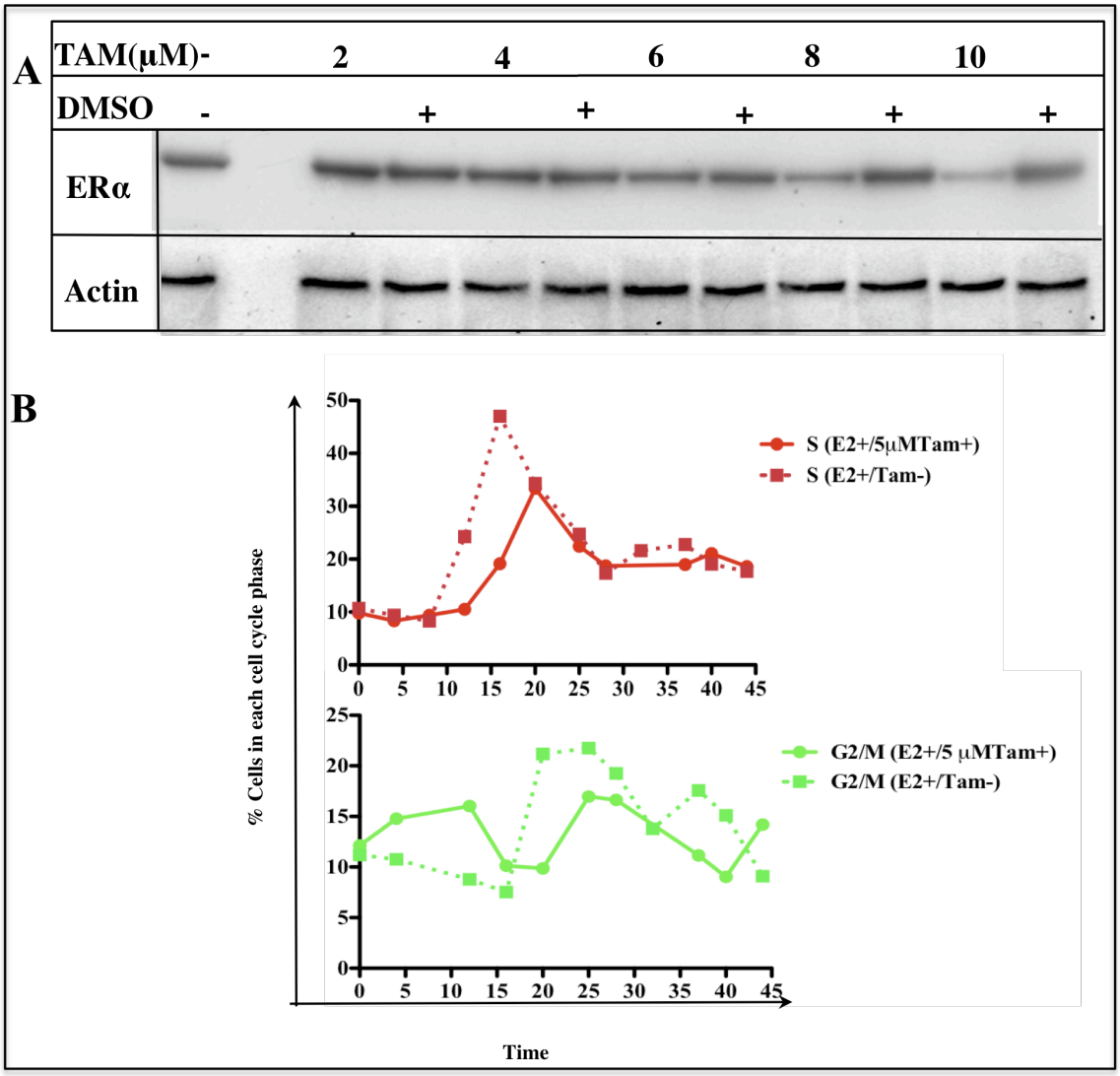


Figure 27: Tamoxifen preserves ERα protein resulting in an inhibitory effect on the progression through S and G2/M phases of the MCF-7 cell cycle. (A) MCF-7 cells were treated with different concentrations (0 - 10μM) of tamoxifen (TAM) and lysates were run on a western blot and probed for ERα. (B) MCF-7 cells were synchronized and cultured in the presence or absence of 5μM tamoxifen. At the indicated intervals, samples were collected and subjected to FACS analysis to determine the percentage of cells in S phase (upper graph) or G2/M phase (lower graph).

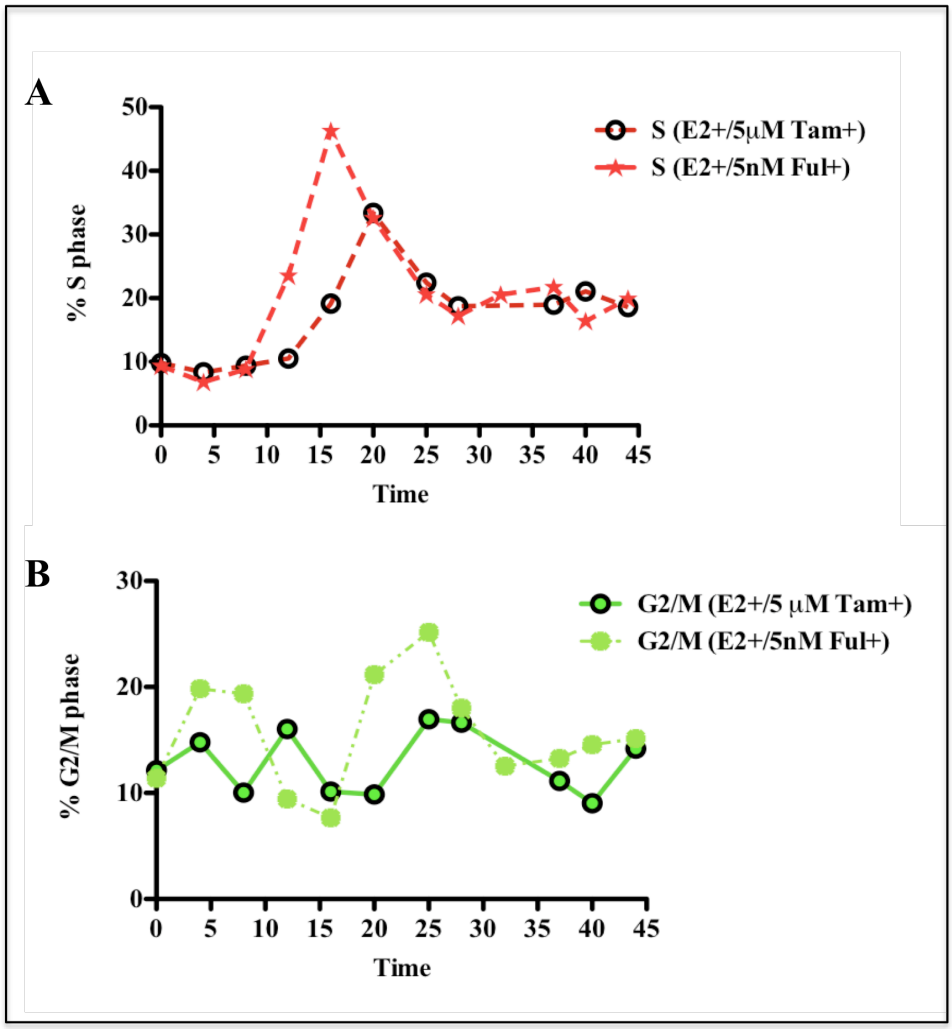


Figure 28: Fulvestrant treatment of MCF-7 cells results in premature progression through S and G2/M phases of the cell cycle compared to tamoxifen treatment. Lovastatin synchronized MCF-7 cells were treated with tamoxifen or fulvestrant and subjected to FACS analysis. The percentage of cells in (A) S phase and (B) G2/M are shown over the 50 hour period after release from arrest are compared between the two different treatment groups.

DISCUSSION

ERα is an important regulator of growth and differentiation in normal breast tissues [473]. Normal mammary cells do not express ERα, except when needed for proliferation during puberty and pregnancy [302]. However, there is considerable evidence that ERα also plays an important role in the development and progression of breast cancer [5]. For example, clinical experience has established that ER-positive breast tumors have a more favorable prognosis compared to tumors that have little or no expression of ERα [292]. Therefore, the functional role of ERα expression in breast cancer etiology is paradoxical; the expression of ERα is associated with good prognosis, but ERα is low in normal mammary cells and absent in triple negative tumors, which have an extremely poor prognosis. Because it is not clear whether the presence or absence of ERα provides a mechanism for cancer cell growth, one should not categorize it as an oncogenic factor. Unlike ER, a typical protein with oncogeneic function, is the, Low Molecular Weight forms of cyclinE (LMW-E) which is associated both with poor prognosis and increased deregulated proliferation in tumor cells and tissues [474].

The question that I set out to address in this chapter was whether the presence or absence of ERα provides the means that could cause progression of breast cancer. Previous reports suggest that the presence of ERα could have an inhibitory effect on the proliferation of breast cancer cells [475, 476]. However, the effects of ERα on the machinery of proliferation (the cell cycle) have not been shown.

The experiments described in this chapter are the first (to my knowledge) to address the effects of ERα on different phases of the cell cycle. The model system consisted primarily of MCF-7 cells, an ER-positive cell line, cultured in media supplemented in the presence or absence of the ER ligand E2. However, to show that the results are not cell type specific, ZR75-1 cells, which are also ER-positive, were used to validate the generality of the results obtained with MCF-7. Lovastatin was used to arrest the cells at G1 phase of the cell cycle and then the cells were released synchronously into the cell cycle by addition of mevalonate, which allowed for evaluation of cell cycle expression of ERα. To study the effects of ligand on ERα, synchronization experiments were performed in duplicate arms, the arm that received E2 is considered liganded and the second arm is considered unliganded ERα as no

E2 was added to culture medium. To compare effects of the presence versus absence of ERα on the cell cycle, MCF-7 cells with endogenous ERα expression or silenced ERα were utilized. Silencing ERα in MCF-7 cells provided a good model to compare the presence of versus absence of ERα in the same cell line. MDA-MB231 cells, which is an ER-negative cell line, was also used to examine whether exogenous ERα could mirror the cell cycle effects that were observed in MCF-7 cells with endogenous ERα.

The data clearly shows that ERα is a cell cycle regulated protein (Figure 13). Western blot analysis of the samples harvested at time points taken through the different phases of the cell cycle revealed that ERα protein expression is low at G1 and is induced to its maximum level during S and G2/M phases of the cell cycle (Figure 13). . To confirm the cell cycle regulation of ERα, ZR75-1 cells were also synchronized using lovastatin and cells were then released through the cell cycle phases. ERα showed the same pattern of cell cycle regulation in ZR75-1 as in MCF-7 cells (Figure 18). Furthermore, the cell cycle regulation of ERα is independent of the ligand, 17-βestradiol (E2) as both liganded ERα and unliganded ERα are expressed during S and G2/M phases. Eventhough the presence versus absence of 17β estradiol had no effect on the expression of ERα during S and G2/M phases, the presense of ligand resulted in a sharp rise and fall of the protein expression for ERα, which coincided with cyclin A and cyclin B expression during S and G2/M phases of the cell cycle (Figure 13). On the other hand, in the absence of the ligand, the S and G2/M phases are prolonged (Figure 13 & 14). FACS analysis confirmed the prolonged duration of S and G2/M phases in cells in the presence of unliganded ERα compared to the presence of liganded ERα (Figure 14). Interestingly, despite seeing consisnent shortening of G2/M phase of the cell cycle of MCF-7 cells in the presence of estradiol, we did not observe a change in the proliferation of MCF-7 cells over a 10 day period (Supplemental Figure 1).

Next, we asked if ERα cell cycle regulation is at the level of transcription. To answer this question, we harvested RNA at different time intervals following synchronization of MCF-7 by lovastatin, followed by qRT-PCR for ER. The results show that the level of ERα RNA expression remians constant and does not change during the cell cycle and is therefore not regulated at the level of transcription.

ER negative cells also have the machinery necessary to regulate ER expression. Exogenous ERα was introduced into the ER-negative cell line MDA-MB231 and was expressed during S and G2/M phases similar to the endogenous ERα of MCF-7 cells (Figure 22). These experiments raise the question of whether ectopic expression of ER result in a different timing of expression compared to the cell cycle regulated expression of endogenous ERα. So initially we transfected ERα into asynchronous MDA-MB231 cells and examined when ER protein can be detected upon transfection (Figure 22A). The results clearly reveal that ERα is expressed within 12 hours of the transfection. However in synchronized cells ERα is predominantly expressed at 24 hours following transfection, which coincides with the exit of cells from S phase and entrance into G2/M phase of the cell cycle. It should be noted that in these synchronized cells, low levels of ERα are still detectable 12 hours following the transfection, which is diminished by 16 hours, most likely due to the cDNA expression time after the transfection. The tight cell cycle periodicity in the transfected MDA-MB231 cells is very similar to that seen with endogenous ERα in MCF-7 cells and ZR75-1 cells, which also coincided with the expression of cyclins A and B. We also interrogated the role of unliganded versus liganded exogenous ERα in modulating the cell cycle in MDA-MB231 cells. Liganded ERα shortened the S and G2/M phases compared to non-transfected MDA-MB231 cells in the presence of E2.

To ensure that cell cycle effects observed in MCF-7 cells were due to ERα, and not the method by which the cells were synchronized, we used a different method to synchronize cells by using aphidicholin as an arresting agent (Figure 15). The ERα expression was again examined along with the cell cycle profile after arresting cells with aphidicolin. Aphidicholin arrests cells at the end of G1 phase, therefore after release from arrest cells enter S phase immediately. As a result, two cell cycles could be follwed during a 44 hour time span. Two peaks of ERα expression were observed, which corresponded with an increase in the S and G2/M population of the cells. The FACS data confirmed the results acheived with lovastatin. In both cases, liganded ERα hastens the progression through S and G2/M phases of the cell cycle compared to unliganded ERα, which has a reverse effect and prolongs the S and G2/M phases duration compared to parental cell line (Figures 14 and 16). However the change was observed in the second cell cycle phase after treatment with

aphidicolin. It is possible that upon traetment with aphidicolin, the cells arrest slightly later in the cell cycle and the machinery regulating ERα expression are not active until the second S and G2/M phases. These data emphasize the notion that ERα is cell cycle regulated and that ERα, in turn, modulates the cell cycle. Other arresting agents can also be used to effectively synchronize MCF-7 cells, such as double thymidine block and using this third method of synchrony we also observed a difference in the way presence of ligand would affect the cell cycle progression. The results of double thymidine synchronization of MCF-7 in the presence and absence of the ligant is shown in Supplemental Figures 2 and 3.

We next directly addressed the role of ERα in mediating the changes in cell cycle profile by ligand in MCF-7 by downregulating it and alternatively by overexpressing it in both MCF-7 and MDA-MB231 cells. The rational for these series of experiments came from the fact that increased ERα expression appears early in the pre-malignant to malignant progression of breast cancer [7] while the more aggressive form of breast cancer do not express ER[477]. At this point the question was solely the role of ERα on cell cycle progression. ERα was silenced in MCF-7 cells and also overexpressed in both MDA-MB231 and MCF-7 cells. ERα depletion showed acceleration of mitotic exit whereas ERα overexpression resulted in a prolonged S and G2/M phase. These data show that even without taking the ligand E2 in to account, the receptor has cell cycle effects. The presence of unliganded ERα inhibits the cell cycle and the absence of ERα increases cell cycle progression. The proliferative respsonse to a lack of ERα may partially explain the aggressive phenotype of the ER-negative tumors.

Tamoxifen and fulvestrant are anti-estrogens, which antagonize the ERα signal in different ways. Tamoxifen does not affect the integrity of ERα protein while fulvestrant results in the down regulation of ERα. These agents provided tools to modulate the expression of ERα in vitro as is done in the clinical setting. ERα degradation after fulvestrant treatment caused a faster cell cycle progression through S and G2/M phases compared to the untreated cells. There was a striking difference in the cell cycle profiles of cells treated with fulvestrant compared to tamoxifen because tamoxifen treatment which resulted in the elongation of S and G2/M phases compared to the fulvestrant treated cells. These data support the findings that shERα also shortened the duration of S and G2/M phases

There are increasing numbers of reports showing that proteasome inhibitors are effective for certain types of cancer [478]. The effectiveness of proteasome inhibitors for breast cancer is yet to be seen [479]. However, our data suggest that the inhibition of proteasome mediated ERα degradation may be effective to restrain proliferation of breast cancer cells. There is some data showing that the proteasome inhibitor, PS-341, inhibits the growth of MCF7 cells [480]. The traditional antiestrogen, tamoxifen, not only binds to ERα to antagonize it, but also inhibits ERα's degradation [481]. Therefore it would be interesting to be able to tease apart these two effects of tamoxifen to determine whether inhibition of ERα's transactivation and/or inhibition of the degradation of ERα accounts for its antiproliferative role in ERα-positive cancer cells.

In summary the results described here show that ER is cell cycle regulated at the G2/M phase of the cell cycle. Further, upon removal of ER from an ER-positive cell line, the length of G2/M is reduced, while overexpression of ER inhibits cell cycle progression during G2 and M phase in both ER-positive and ER-negative breast cancer cells. These data imply that ERα is an important factor in modulating the cell cycle, which could lead to inhibition or progression of the tumor cells. These data also provide the rationale for the hypothesis that ERα has opposing effects on the progression of cell cycle in G2/M phase, under non-liganded and liganded conditions, suggesting that estradiol increases cell cycle progression. The next chapter of my dissertation will address the mechanisms responsible for changes in ERα expression throughout the cell cycle and the pathways downstream of ERα, which modulate cell cycle progression.

Chapter III:

The cell cycle dependent regulation of ERα and the resulting modulation of G2/M phase are due to an interaction of ERα with cyclin B.

INTRODUCTION

In chapter II we showed that ERα is expressed in a cell cycle regulated manner in ER-positive cell lines and when exogenously expressed in ER-negative cell lines (Figures 13 & 22). Furthermore our data also showed that the changes in the ERα expression throughout the cell cycle were not regulated at the level of transcription (Figure 17). We also showed that the addition of estradiol to the cells shortened the transition of cells from S into G2/M phase of the cell cycle. Lastly we showed that exogenously expressed ERα in ER-negative cells (MDA-MB231) slows down the cell cycle progression while silencing ERα in ER-positive cell (MCF-7), speeds the cell cycle progression.

In this Chapter we set out to investigate if the turnover of ER is responsible for the progression of cells through the cell cycle. It is well established that turnover of ERα protein is mediated through the ubiquitin–proteasome pathway [482]. Specifically, it has been shown in an in vitro setting that ER degradation depends on E1 (ubiquitin-activating enzyme) and E2 (ubiquitin-conjugating enzymes) [483]. The results from Chapter II suggested that the regulation of ERα during S and G2/M phases of the cell cycle is at the protein level (Figure 7). These important and novel findings led to the hypothesis that this cell cycle turnover of ERα is also regulated by proteasomal degradation. Chapter III addresses the question of whether ER protein is regulated at the level of proteasomal degradation in a cell cycle manner and if so, what cellular process targets ERα for degradation.

While analyzing the changes in cell cycle protein expression, it was observed that ERα is down regulated at the end of G2/M, which coincided with the timing of cyclin B down regulation leading to the hypothesis that ERα could share the E3 ligase, Anaphase Promoting Complex (APC), with cyclin B. The first question to address was whether ERα is ubiquitinated upon exposure to APC. In collaboration with Dr. Weihua Zhang (University of Houston), it was observed that ERα became ubiquitinated at the end of M phase upon

exposure to APC along with E1 and E2 enzymes. To directly show that APC targets ERα to the proteasome, the next step was to determine whether ERα binds to the components of APC and determine if the inhibition of the proteasome by MG132 could lead to the down regulation of ERα at the end of M phase. The results showed that ERα and cyclin B both bind to cdc27, which is one of the components present in the APC complex. Based on this novel finding we hypothesized that ERα and cyclin B bind together directly. Through immunoprecipitation experiments cyclin B/Cdk1 was identified as an ERα interacting protein.

We have previously shown that liganded ERα causes increased progression through S and G2/M phasse of the cell cycle. Therefore, the next question was how liganded ER confers the increased progression through S and G2/M phase of the cell cycle compared to unliganded ER. The results showed that ERE site is transcriptionally active during S and G2/M phases in the presence of liganded ERα compared to the unliganded ERα. The transcription of well established ERα targets genes such as PgR and pS2 [484, 485] were also examined in collaboration with Dr. Powel Brown's laboratory (UT-MDACC). The induction of PgR and pS2 transcription during S and G2/M phases were reported using qRT-PCR. In summary this chapter will further investigate the mechanisms behind the cell cycle regulation of ERα and how liganded ERα results in a fast progression of cells through S and G2/M phases of the cell cycle while unliganded ERα prolongs these phases.

MATERIALS & METHODS

Immunoprecipitation Assays

MCF-7 cells were cultured in E2-free media and were synchronized with lovastatin as described in Chapter II. Cells were harvested using trypsin and were resuspended in ppi (protease/phosphatase inhibitor- components listed under western blot analysis in chapter II) for sonication. After performing a protein assay to determine protein concentration, lysates were kept in -80°C. For immunoprecipitation (IP), protein lysates were thawed on ice. 300-500μg of protein lysate was aliquoted for each IP and the volume was adjusted to 50μL with 1x PBS. 30μL of 0.1g/mL sepharose A beads (Amersham/GE Healthcare- Piscataway, NJ) were aliquotted for each sample. The beads were resuspended in an equal volume of 1x buffer [11.2ml of 5x lysis buffer (3mL of 1M Tris HCl, pH 7.5, 3mL of 5M NaCl, 300μL of 20% NP-40 and 5.7 mL of ddH_2O), 7mL of 10x ppi and 39.6mL of ddH_2O). The beads in buffer were left on ice for 15 minutes to swell. The swelled beads were centrifuged for 3 minutes at 6000rpm at 4°C. The buffer was aspirated and the process of adding fresh buffer followed by centrifugation was repeated 3 times to wash the beads. 36μL of 10x5x10 buffer (225μL of 10x ppi, 450μL of 5x lysis buffer, 22.5μL of 0.1M DDT and 202.5μL of ddH_2O) was added to each tube and antibody was added to each tube of protein lysate samples. For IP, 3μL of polyclonal cyclin B (Santa cruz, H-20, SC594) or 5μL polyclonal ERα antibody (Santa cruz, HC-20, SC543) was used per sample. The samples (lysates and antibody) were incubated for an hour in the cold room (4°C) with rocking. At the end of the first hour incubation, 30μL of beads was added to each sample and were mixed thoroughly. The beads with protein and antibody were then incubated in the cold room for 3-4 hours with rocking. After incubation, the samples were centrifuged for 3 minutes at 6500rpm at 4°C. The supernatant was carefully removed without disturbing the beads and 300μL of 1x lysis buffer was added to wash the samples by centrifuging at 6500rpm for 3 minutes at 4°C. The wash was repeated four times. After the last wash the samples were subjected to western blot analysis as described in materials and method section of chapter II. Blots probed with anti-ERα (Novocastra Laboratories-UK) or anti-cyclin B monoclonal (GNS-1) antibodies at 1μg/mL in blotto and developed as described in chapter II.

GST Pull-down Assay

The GST-ERα construct was provided to us by Dr. Kumar's lab [486]. The construct contained a gene for ampicillin (Amp) resistance. The GST-ERα construct was transformed into BL21 gold competent cells in order to amplify the plasmid and also to make bacterial stock. For transformation, the BL21cells were thawed on ice and 100μL of the competent cells were aliquotted into pre-chilled polypropylene tubes. 50ng of GST-ERα was added to each reaction tube containing cells, which was then swirled gently and incubated on ice for 30 minutes. Meanwhile, SOC medium (Super Optimal Broth) [2% tryptone 0.5% yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulfate, 20mM glucose] was preheated in a 42°C water bath. In the final step of cell transformation, the use of S.O.C. Medium results in maximal transformation efficiency of E. coli [487]. Each transformation reaction was heat-pulsed by placing it in 42°C water bath for 20 seconds, followed by incubation on ice for 2 minutes. Next, 0.9mL of preheated SOC medium to 42°C was added to each reaction and then incubated at 37°C for an hour while shaking at 250rpm. The cells were centrifuged for 5 minutes before lawn streaking the entire transformation reaction onto a single LB agar plate containing 100μg/mL of ampicillin. The plates were then incubated over night at 37°C. The next day, a single isolated colony was picked for amplification by inoculating 5mL of LB broth plus Amp and incubated over night again at 37°C. The following day, 50μL of the 5mL culture was transferred to 50mL of LB plus Amp and again incubated over night at 37°C. On day three following transformation, the 50mL culture was added to 500mL of LB broth with Amp and incubated at 30°C for 3-4 hours, while checking the optical density (OD) at 600nm every 30 minutes. The desired OD, signifying the optimum amount of density of the bacteria for the purpose of GST-pull down, is 1.2-1.6. Once the OD was within the optimal range, the sample was spun down at 7000rpm for 15 minutes at 4°C. The resulting pellet was resuspended in 10mL of lysis buffer (20% Glucose, 10% Glycerol, 2mM $MgCl_2$, 50mM Tris pH 8.0, ddH_2O to final volume of 100mL, 1M DTT and 10mL fresh ppi). The sample was sonicated for 2-3 minutes, pulsing at the 40-50 setting using a portable hand sonicator. The lysates were set on ice for 20-30 minutes before centrifugation at 10,000rpm for 10-15 minutes at 4°C. The

supernatant was removed and added to 0.5mL of pre-soaked GST-beads [Glutathione Sepharose 4B, Amersham Pharmacia Biotech (Piscataway, NJ)] in PBS or NP-40 (50mM Tris, 150mM NaCl, 0.5% NP-40, Glycerol, $MgCl_2$, EDTA, ddH_2O to final volume of 500mL). The beads and sample were rotated at 4°C for three hours before washing them three times with 8-10mL of NP-40 buffer and spinning at 3500rpm. The beads and attached proteins (GST- ERα) were then transferred to a 1.5mL eppendorf tube and kept at -80°C.

To ensure that the ERα-GST fusion protein was not degraded during the process of purification, the integrity of the samples were analyzed on a coomasie blue stained SDS-PAGE gel. For in vitro binding assays, cyclin B protein was generated using TNT® quick-coupled transcription/translation system (Promega, Madison-WI) for in vitro analysis of ERα binding to cyclin B. To this end, cyclin B was cloned into a pGEM vector, which is a vector containing a multiple cloning region and also SP6 and T7 polymerase promoters. The pGEM vector containing an SP6 promoter in front of the cyclin B gene was used for translation using the TNT kit. All samples and reagents were kept on ice at all times to prevent degradation of the translated protein.

TNT® SP6 quick master mix, [^{35}S] Methionine (1000 ci/mmol at 10 mCi/mL) and PCR-generated DNA template (0.5μg) were combined together and incubated at 30°C for 60-90 minutes. The integrity of the cyclin B translation was monitored by its mobility on SDS-PAGE gel. To examine the in vitro binding of ERα and cyclin B, 10μL of translated cyclin B was added to 2μg of either full-length or truncated GST purified ERα protein. The truncated forms of ER protein were GST-AB (AB domain/AF1,), GST-C (C domain/DBD,), GST-D (D domain/Hinge region), GST-F (F domain/AF2), all fused to GST] or GST beads alone and 400μL of protein-binding buffer (1M Tris, pH 7.5, 1M NaCl, Glycerol, 1% NP-40 also 20μL/mL of sodium vanadate and 40μL/mL of fresh protease inhibitor), in each tube. The samples were incubated at 4°C for 4 hours followed by six washes with protein binding buffer. After centrifugation, the supernatant was removed and 10μL of 4x SDS dye was added prior to loading the samples for SDS-PAGE analysis. The proteins were then transferred, stained with ponceau stain and developed by phoshpho-imager. Ponceau staining allows for visualization of total protein and phospho-imager shows the specific proteins of interest by radio-labeling.

RNA Extraction

Total RNA was extracted from synchronized MCF-7 cells by Cesium Chloride separation and centrifugation. This method provided a high yield of pure RNA. Cells were cultured for each condition as indicated in the results section and for each experimental condition using 150mm plates cells were homogenized by addition 3mL of GIT (93.53g Guanidine Isothiocyanate powder and 1.67mL of 3M sodium acetate (NaAc) solution brought up to 200mL with DEPC water, filtered and kept in a dark container). Before addition of GIT to each plate, 37.5μL of fresh β-mercaptoethanol was added. The cells detached from the plate and were scraped for transfer to a labeled 15mL conical tube. The harvested cells were vortexed vigorously and DNA was sheared by passing the solution through a 18 gauge needle several times at which point 2.7 ml of RNA containing GIT solution was layered on top of a 1.9mL of CsCl solution. The tubes were centrifuged at 38,000rpm overnight (up to 16 hours) at room temperature. The next day the RNA was visible as a clear pellet at the bottom wall of the Beckman tube. The supernatant was removed gently, without disturbing the pellet, which was then washed from the wall of the tube with 170μL of 0.3M sodium acetate (NaAc) and incubated in 37°C for 10 minutes.

After the incubation, 500μL of a 1:1 mix of phenol/chlorophorm was added to each tube, vortexed and centrifuged at room temperature at 14,000rpm for 3 minutes. The supernatant was transferred to a second labeled tube and the process of washing with phenol/chlorophorm was repeated 3 more times. After the washes, RNA was precipitated by the addition of 1mL of 100% ethanol to each tube and placed in liquid nitrogen for an hour, followed by pelleting the now purified RNA via centrifugation and washing the pellet with 80% ethanol to remove any residual salt. The pellet was left to dry for an hour in a laminar flow hood then the RNA was reconstituted with 0.1X TE in DEPC water. The quantity and quality of RNA samples were assessed by determining the OD at 260/280. The RNA prepared by this methodology can be stored at -80C for years without any degradation.

ERE-Luciferase Assay

The transcriptional activity of ERα at the ERE site was measured in lovastatin synchronized MCF-7 cells to examine the ERα associated activity during S and G2/M phases. To this end MCF-7 cells, which had been kept in E2-free media for three weeks were exposed to lovastatin for 36 hours. Twenty-four hours after the lovastatin treatment (12 hours prior to mevalonate treatment) cells were transfected with the construct containing ERE-Luciferase. Transfection took place without changing media on the cells in order to keep lovastatin on the cells. The negative controls used for these experiments were MCF-7 cells, which did not transfect with ERE-Luc and also MDA-MB231 cells, which had been transfected with ERE-Luc. Thirty-six hours post lovastatin treatment (twelve hours after the ERE-Luc transfection) cells received mevalonate. At the time of mevalonate addition the E2-positive group received 10nM E2 while the E2-negative group did not receive any E2.

After harvesting the cells, samples were prepared for luciferase assay. 500μL of passive lysis buffer (Promega- Madison, WI) was added to each well of the six well plate that MCF-7 cells had been cultured in. The contents of the plates were mixed by rocking for 15 minutes at room temperature. Then the cells in each well were scraped (triplicates for each time point and condition) and collected in labeled centrifuge tubes. The tubes were frozen and thawed three times using liquid nitrogen and a 37°C H2O bath to ensure complete cell lysis. After centrifuging the tubes for 10 minutes at 14000rpm, 20μL of each sample was transferred into the specific tubes used (BD Monolight™ Luminometer Cuvettes, 12X75 mm, Sparks, MD) 80μL of Luciferase Assay Reagent II (LAR II) (Promega- Madison, WI) was added to the 20μL of the sample in the tube. Using an illuminator machine the firefly-luciferase activity in each sample was quantified.

ImmunoFluorescent staining

$5x10^4$ cells were plated on sterilized cover slips that had been placed inside each well of a 6 well plate. At the time of harvest cells on the coverslips were fixed with 4% paraformaldehyde in PBS at room temperature (RT) for five minutes, then washed with PBS for 3x 5minutes, followed by blocking (3% BSA containing 0.1% NP-40 in PBS) for 1 hour at RT. Primary antibodies were added at a dilution of 1:200 in blocking solution and were incubated on the slides at 4°C overnight. The next day, the slides were washed for 3 X 10 minutes with PBS and were incubated with fluorescent labeled secondary antibodies for three

hours at RT. Slides were washed again with PBS for 3 X10 minutes and covered with 4',6-diamidino-2-phenylindole (DAPI) containing anti-fade solution (Vector, Burlingame, CA). At the very last step the coverslip were flipped on a microscope slide so the side with the fixed/stained cells has direct contact with the slide. The slides and coverslips were sealed with a clear nail polish. Slides were then stored in dark in 4°C, and analyzed microscopically within 2 weeks of storage. Slides were then analyzed using an Olympus DSU spinning disc confocal fluorescent microscope. All the pictures were taken using a 60X lens.

***In vitro* ubiquitination assay**

The main components of this assay were purified ER, APC, E1 and E2. 100 μg of recombinant human ERα (Panvera, Madison, WI) was further purified by immunoprecipitation with 200μL protein G conjugated Sepharose beads prebound to 10μg of rabbit polyclonal ERα antibody (MC20- Santa Cruz, CA). ER-bound beads were eluted with 0.1M glycine and neutralized with 5mM Tris buffer to pH 7.0. The eluted ERα was then subjected to dialysis with saline at 4°C for 2 hours. The purified protein concentration was measured spectrophotometrically (OD 260) following dialysis.

For APC, we used anti-cdc27 antibody (one of the components of this E3 ligase) to purify it from cell extracts. To this end, 100μL protein G conjugated sepharose beads, pre-bound with10μg of goat anti-cdc27 antibody (Santa Cruz, CA), was used for APC purification through immunoprecipitation on 1mg of cell extracts from synchronized MDA-MB231 cells, which correspond to G2/M phase. E1 activating and E2 conjugating enzymes were purchased from Boston Biochem (Cambridge, MA).

The *in vitro* ubiquitination assays were conducted in 30μL of reaction buffer (10mM Tris-HCl, 10mM NaCl, 30mM $MgCl_2$, 1mM dithiothreitol, 0.1mM ATP, and 1.0mM Ubiquitin, pH 7.5) at 37°C with gentle rocking for 1 hour. The samples used in this assay were: 1) ERα protein/cdc27 antibody pre-bound protein G beads, 2) ERα/ E1/cdc27 antibody pre-bound protein G beads, 3) ERα/ E1/ E2/cdc27 antibody pre-bound protein G beads and 4) ERα/ E1/ E2/protein G beads with precipitates of APC from G2 phase of MDA-MB231 cells. The reactions were terminated with SDS sample buffer and subjected to western blot

to analyze the ability to form polyubiquitin chains on ERα using monoclonal anti-ERα antibody.

Reverse-Phase Protein Array (RPPA) analysis

RPPA analysis was performed to examine an array of different protein expression in MCF-7 cells in the presence versus absence of E2. To prepare for RPPA, MCF-7 cells were plated in 100mm plates and incubated in 37°C at 6.5% CO2 in E2-free medium. Twenty four hours after plating, cells were synchronized with lovastatin as described in chapter II and were harvested in four hour intervals upon release from lovastatin by addition of mevalonate. Cells were harvested using the same procedure as for western blot analysis (see Chapter II). Lysates were stored in -20°C until all samples had been acquired for RPPA.

Lysates (～1 nL of protein lysate was used to print the sample on the nitrocellulose glass slide) were first printed on the slides. Non-specific binding events were blocked using a blocking buffer and then the slides were probed with primary followed by secondary antibodies. In order to amplify the signal slides go through a series of incubations, which are first incubation with streptavidin-biotin-peroxidase complex and then this incubation follows by biotinyl-tyramide/hydrogen peroxide incubation and finally incubation with streptavidin-peroxidase. Using hydrogen peroxide the slides develope and then scaned by using DakoCytomation catalyzed signal amplification (CSA) system. The values obtained were ploted as the graphs shown in Figures 40 and Supplemental Figure 4.

RESULTS

ERα induces transcriptional activity through an ERE site during S and G2/M phase in MCF-7 cells.

Previous western blot and FACS analyses showed that ERα protein is maximally expressed in G2/M phase and causes rapid exit from the cell cycle in the presence of E2 (Figures 13 and 14). These data provided the rationale to examine the transcriptional activity of ER by measuring the ERE activity in cells that express ERα, to determine whether maximal expression of ERα at G2/M phases can also modulate the transcriptional activation at ERE site during G2/M. To this end, we measured the transcriptional activity at ERE sites in lovastatin arrested MCF-7 cells in complete medium without starving for E2. A dual luciferase assay was used to investigate the transcriptional activity initiated by ERα at ERE sites during different phases of the cell cycle. A schematic diagram of the experimental design used to synchronize the cells and introduce the ERE sites to MCF-7 cells is shown in Figure 29. The cells were then processed for FACS and luciferase assay, which were performed twice each. Luciferase activity revealed that ERE is activated at 20 hours after cells enter the cell cycle, which overlaps with the time that cells are entering S phase. The ERE activity reached peaked at 30 hours, then dropped quickly (Figure 30B) which coincided with the peak and then decline of the percentage of cells in G2/M phase of the cell cycle (Figure 30A). The experiment was repeated twice and the results were similar for both experiment I and II. In summary, the ERE driven luciferase assay showed increased ERE mediated transcriptional activity during S and G2/M phases, which coincided with the timing of ERα expression during the cell cycle. The next question was whether the transcriptional activity of ERE initiated by ERα protein is affected by the presence or absence of E2.

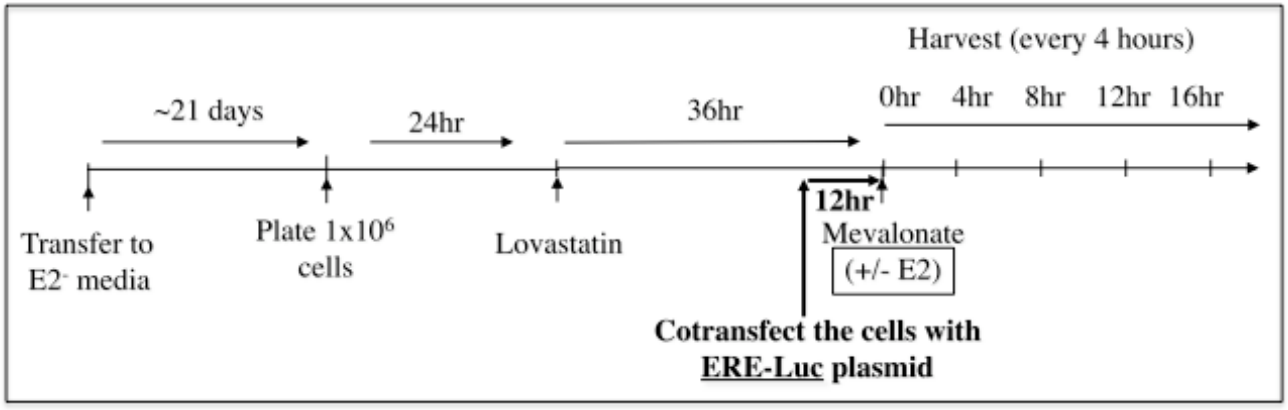


Figure 29: Experimental design used to test ERE activity. MCF-7 cells were cultured in E2 free media and synchronized using lovastatin. Twelve hours prior to release from arrest using mevalonate, the cells were transfected with ERE-Luc plasmid. Cells were then harvested for analysis by FACS and luciferase assays.

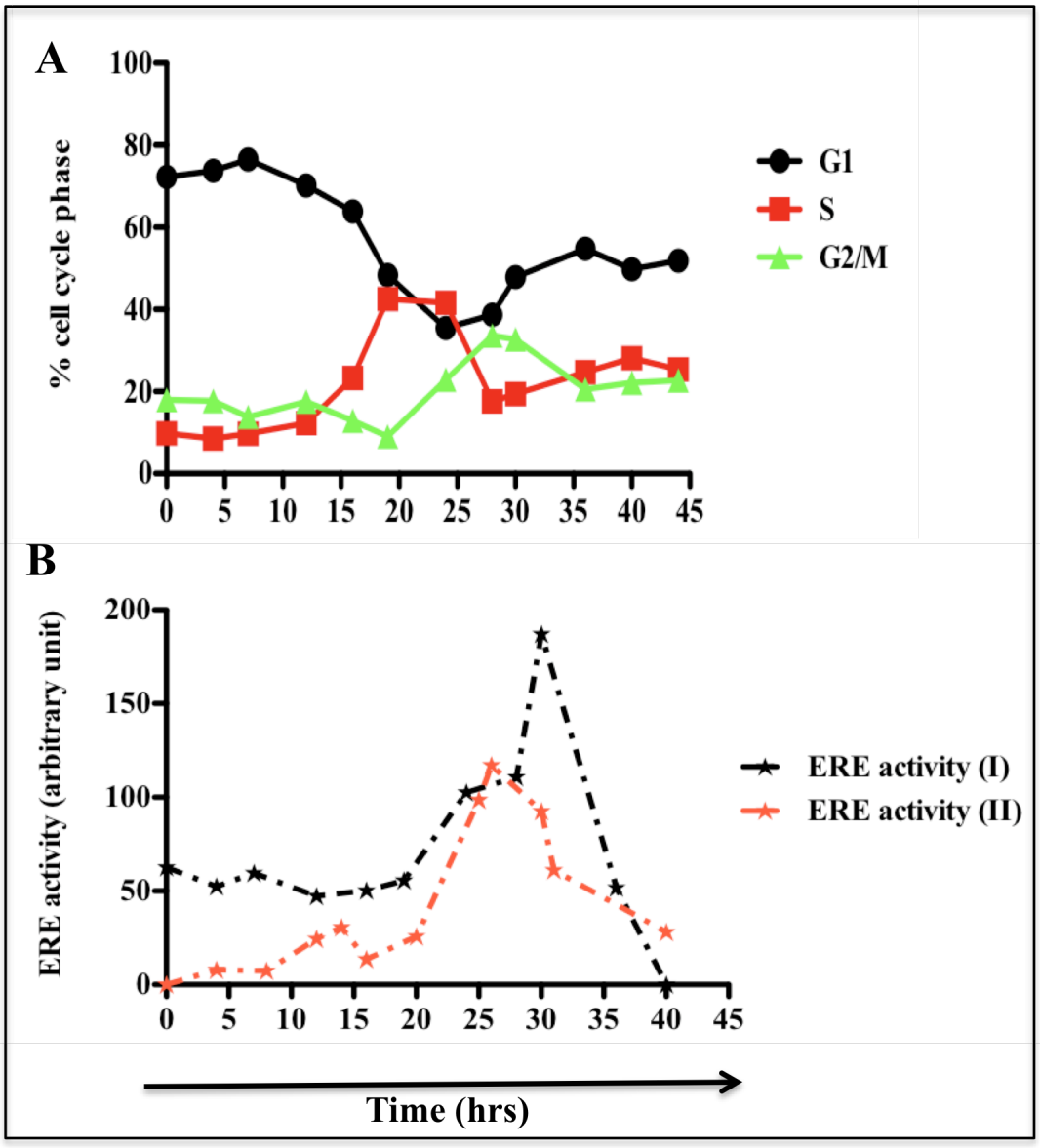


Figure 30: ERE activity is elevated during S and G2/M phases in MCF-7 cells.

(A) The percentage of cells in each phase of the cell cycle was determined by FACS analysis of lovastatin synchronized MCF-7 used for the luciferase assay. (B) ERE activity was assessed using a luciferase assay. The results from two different experiments are provided. The data used here has also been used to plot the graphs in figure 17.

The Transcriptional activity of ERα that is regulated by ERE sites is highest during S and G2/M phases coinciding with the expression of ERα protein.

In chapter II we showed that in a synchronized population of MCF-7 cells, ERα expression is cell cycle dependent. The ERα expressed during S and G2/M phases modulates the exit from S and G2/M phases of the cell cycle. Furthermore, the modulation of the cell cycle by ERα is different in the presence versus absence of E2. These findings led to the hypothesis that ER may modulate cell cycle progression from S to G2/M as a function of ER transcriptional activity at ERE site and that the genes activated by ERE are different in the presence versus absence of E2. To test the hypothesis the ER activity was examined using an ERE-Luciferase construct (ERE-Luc) under both E2-positive and E2-negative conditions in synchronized population of cells. MCF-7 cells, which had been cultured in E2-free media for three weeks, were synchronized with lovastatin and transfected with ERE-Luc plasmid twelve hours before the release from arrest. At the time of release, E2 was added to one group of cells. Samples were harvested every 4 hours and prepared for FACS analysis and luciferase assay to examine the transcriptional activity of ERα and cell cycle profile of the cells.

The results shown in Figure 31 are from biological replicate (3 times) assays using the lysates from cells transfected with ERE-Luc. In E2 treated group of experiment I, the ERE activity increased 6 hours after the release, until it peaked at 16 hours and then decreased to a minimum at 28 hours after release. ERE-Luc activity in E2-negative group shows a similar trend to the E2-positive group. However the amount of ERE activity in this group is much lower. This low ERE activity in E2-nagative group could be due to the basal level of ERE activity in MCF-7 cells. The results of experiments II and III show the same trend and confirm the time frame for ERE activity. The time span in which ERE was active (hours 6-28) corresponds with the S and G2/M phases of the cell cycle (Figure 31).

In summary, the results from three independent experiments, revealed an increase in luciferase driven by ERE at the end of S and throughout and G2/M phase of the cell cycle, which also correlates with the timing of the ERα expression in the cell cycle (Figure 13).

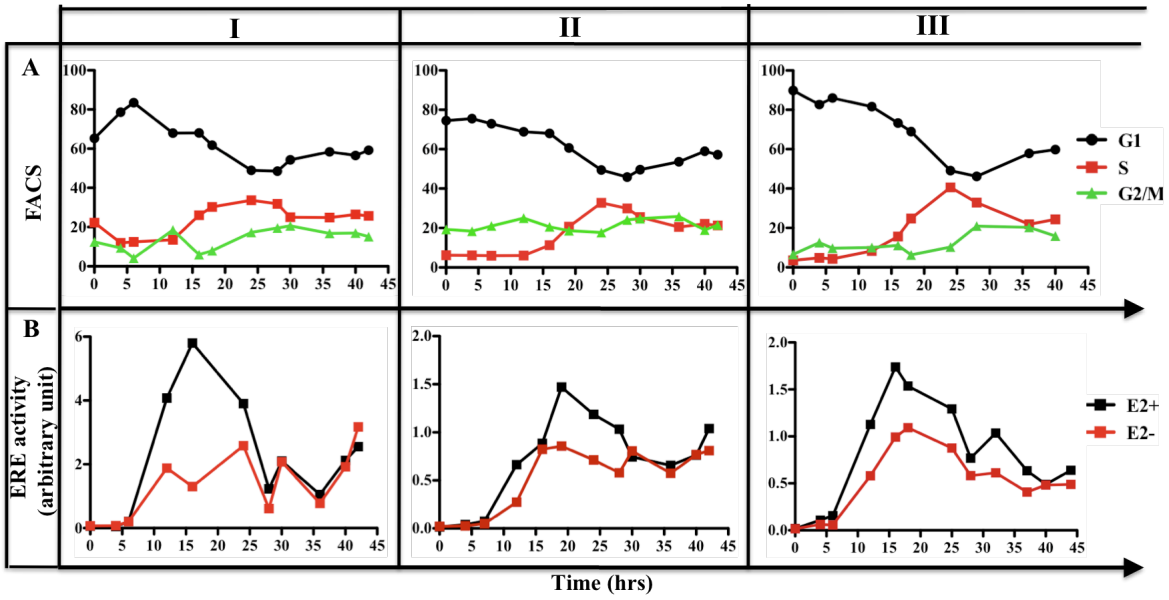


Figure 31: ERE site is highly activated during S and G2/M phases in the presence of liganded ERα compared to unliganded ERα. (A) FACS analysis of three synchronization experiments in the presence or absence of E2 is shown as the percentage of cells in each cell cycle phase. (B) Luciferase activity driven by ERE in the presence or absence of E2 is graphed over a 42 hours time frame.

qRT-PCR analysis of ERα target genes shows increased expression during S and G2/M phase.

Since the systemic ERE activity modulated by ER is cell cycle regulated (Figure 31), we next set out to examine the cell cycle regulation of specific ER target genes. To examine the role of ERα in gene regulation during G2/M phases of the cell cycle, the expression of ERα target genes PgR and pS2 was quantified by performing qRT-PCR. PgR and pS2 have previously been shown to be cell cycle regulated [488].

Lovastatin synchronized MCF-7 cells, released either in the presence or absence of E2, were harvested at time points throughout the 40 hours after the release and processed for flow cytometric analysis to ensure the cell cycle status at each time point and also for qRT-PCR by extracting RNA from cells at each time point. FACS analysis showed the sequential entrance and exit of the cells through the cell cycle phases starting at G1 phase. Two representative experiments show that ERα mRNA expression does not change during the cell cycle, confirming previous results (Figure 17) that ERα is not being regulated at the transcriptional level in either the presence or absence or E2 (Figure 32). The qRT-PCR analysis of PgR expression showed that in E2-positive conditions PgR expression began to increase at 12 hours, formed a peak at 25 hours and then decreased slowly through to 38 hours after release from arrest (Experiment I, Figure 32). In experiment II, the RNA levels of PgR started to increase at 5 hours, peaked around 25 hours after release, but then a decrease in the RNA levels of PgR were not observed in the presence of E2. In both trials, the expression of PgR did not increase from baseline levels in the absence of E2. Under E2 positive conditions, the RNA levels of pS2 also increased at 5 hours and kept increasing through the remainder of the cell cycle. However, under E2-negative conditions, the RNA levels of pS2, like PgR remained at a basal level (Figure 32). The time points at which PgR and pS2 transcripts were expressed coincided with the S and G2/M phases of the cell cycle based on the FACS data (Figure 32).

In summary, qRT-PCR results showed that the transcription of two targets of ERα, PgR and pS2, increase during S and G2/M phases in the presence of E2. These results corroborate the previous finding that ERE driven activity is highest at S and G2/M phases and further shows that gene expression is regulated by liganded ERα.

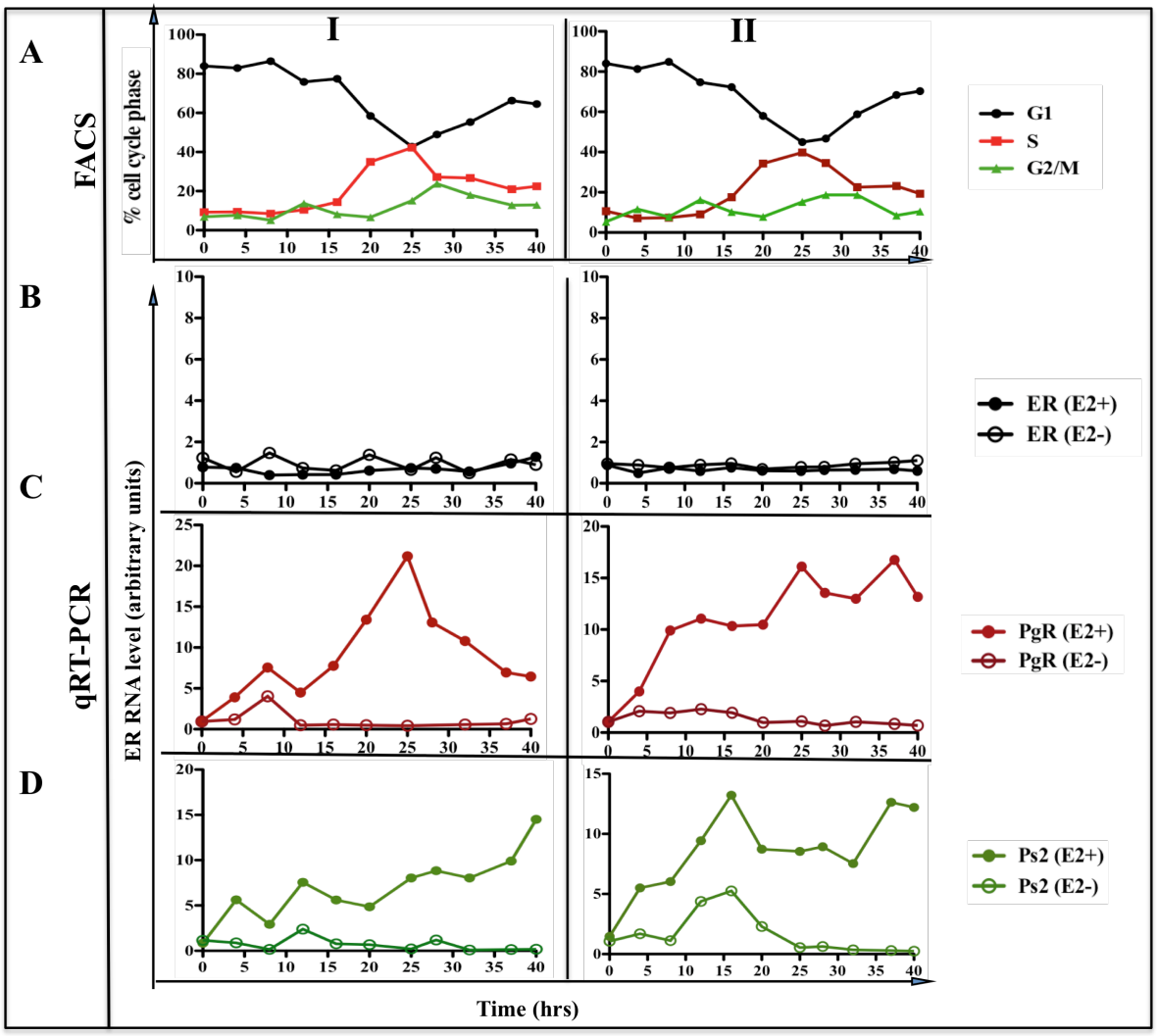
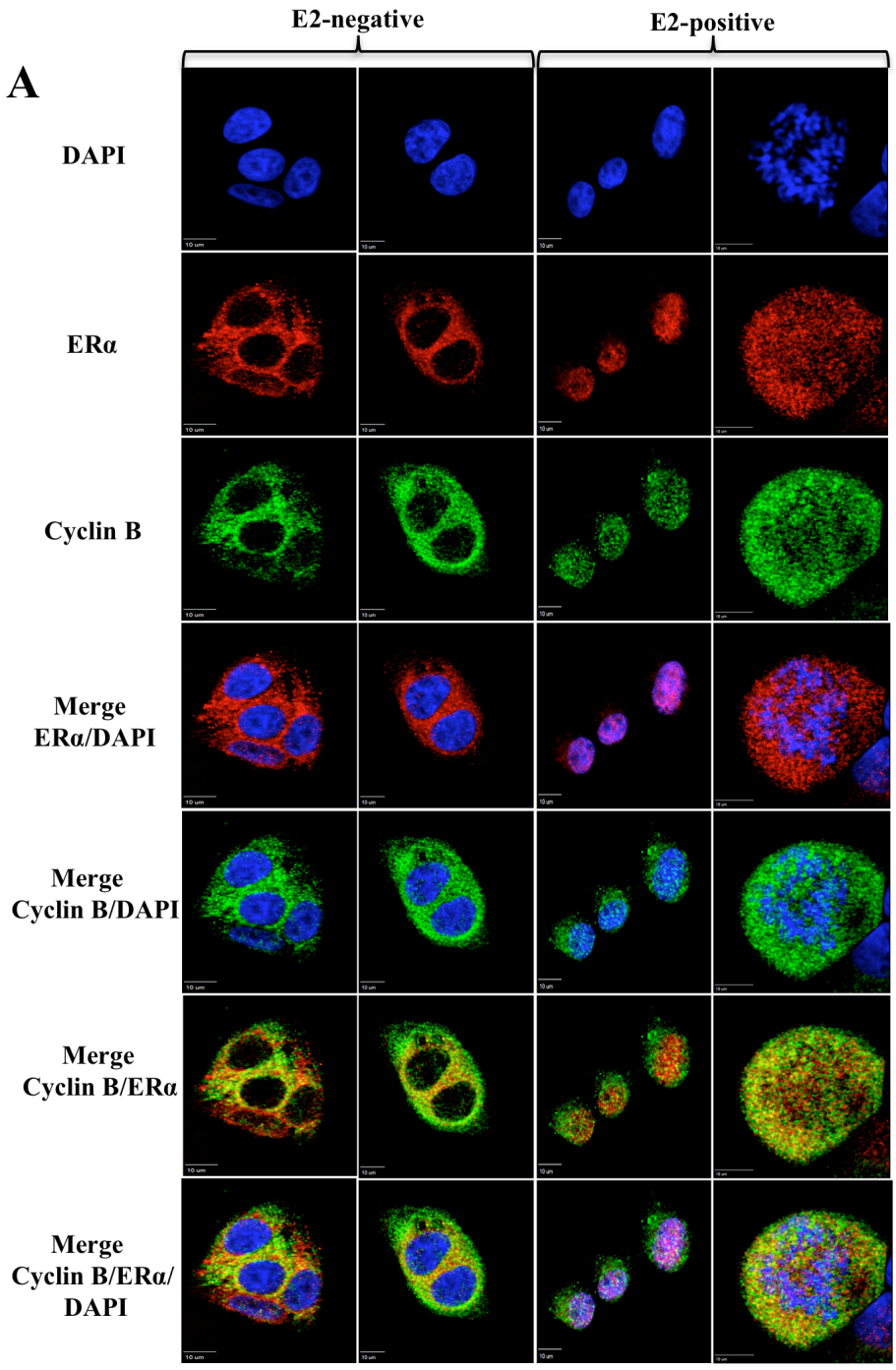


Figure 32: PgR and pS2 transcripts are up regulated during S and G2/M phases in the presence of liganded ERα. MCF-7 cells were synchronized and released in the presence or absence of E2 and samples were collected for (A) FACS analysis to assess cell cycle profile of the cells. qRT-PCR was performed to measure the transcripts of (B) ERα, (C) PgR, and (D) pS2. Two representative experiments are shown. The data used here to show the ERα RNA level has also been used to plot the graphs in figure 17.

Localization of ERα is cell cycle regulated

Although ERα is known as a nuclear protein there are recent studies that show both cytoplasmic and cell membranous localization of ERα [350]. Therefore, the next objective was to determine whether the cellular localization of ERα differs throughout the cell cycle. To examine the localization of ER at each point in the cell cycle, MCF-7 cells were plated on cover slips, synchronized with lovastatin. After 36 hours of lovastatin treatment, mevalonate were added to the wells while the E2-positive group received 10nM E2 at the time of release with mevalonate and E2-negative group only received mevalonate. At the time of harvest first 4% paraformaldehyde were added into each well and the plate left in -20°C for 20 minutes. Then after 3X washed with 1X PBS, coverslips were taken out of each well of six well plate using 18 gauge needle. For the purpose of blocking and then staining of the coverslips I made a very smooth platform and would place a drop of antibody and then flip the coverslip over and keep it in dark and room temperature for the staining process. Cells were triple-stained with an ERα antibody, DAPI (for nuclear staining) and cyclin B (Figure 33A). The slides were analyzed under the fluorescent microscope to examine the localization of ERα and cyclin B compared to the DAPI staining. Two hundred and thirty different pictures were taken of the cells under the fluorescent microscope. The data revealed that ERα resides both in the cytoplasm and in the nucleus (Figure 33A). In Figure 33A the red staining is for ERα while the green staining is for cyclin B and blue staining (DAPI) marks the DNA. Four representative pictures for each condition are depicted in Figure 33A. These images mainly showed a colocalization of ERα and cyclin B (Figure 33A-merge cyclin B/ERα). Our results also showed that the addition of E2 resulted in the nuclearization of ERα and cyclin B (Figure 33B). The co-localization of ER and cyclin B is most likely mediated by E2 treatment, which as we have shown in Chapter II, can hasten the progression of cells into the G2/M phase of the cell cycle. Since cyclin B is one the key regulators of G2/M entry and exit, its co-localization with ER is further conformation of the transport of ERα from cytoplasm to nucleus at G2/M mediated by E2. The measured p value for the difference between the nuclear versus cytoplasmic cyclin B was 0.0001 and for ERα was 0.1 (Figure 33B).


A
E2-negative
E2-positive
DAPI
ERα
Cyclin B
Merge
ERα/DAPI
Merge
Cyclin B/DAPI
Merge
Cyclin B/ERα
Merge
Cyclin B/ERα/
DAPI

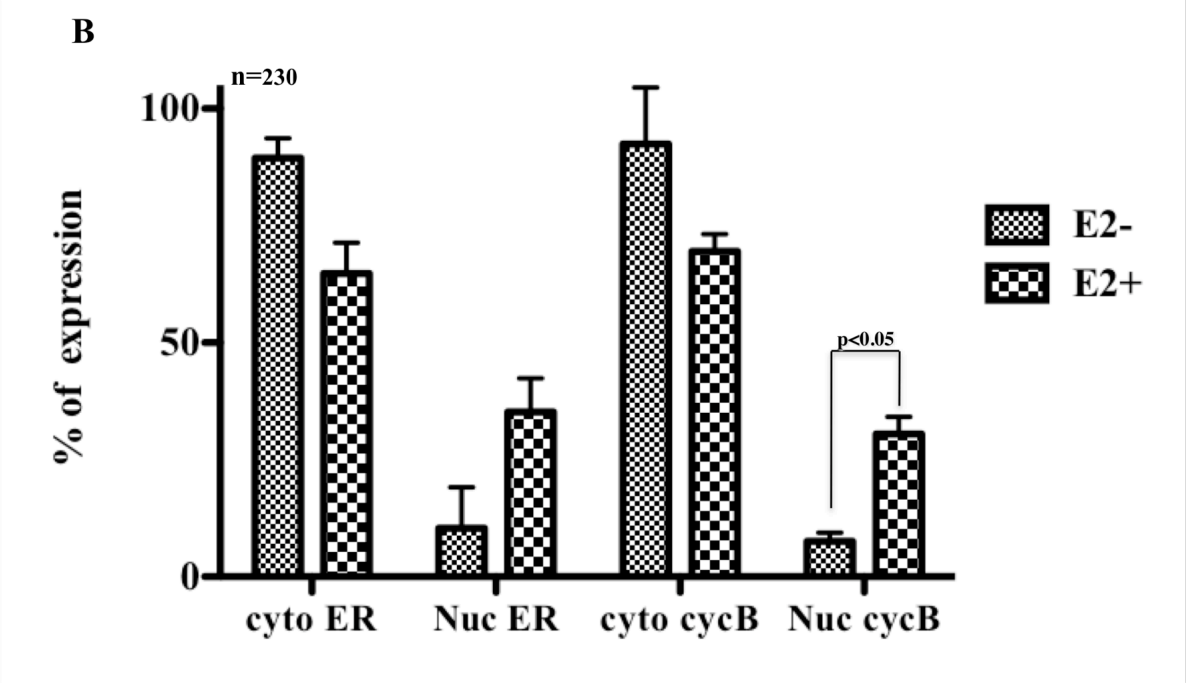


Figure 33: ERα and cyclin B have similar localization and their movement to the nucleus is E2-dependent. (A) The location of ERα and cyclin B in MCF-7 cells with or without E2 was compared to that of cyclin B and DAPI (nuclear) in 230 frames of photographs taken with the fluorescent microscope (magnification: 60X). (B) The results were graphed as the percentage of cells found either in the nucleus or cytoplasm for ERα or cyclin B, as indicated. Error bars represent the standard error from the mean. P value was measured using a two tailed t-test.

Cyclin B and ERα proteins form a complex, which also contains Cdk1.

Our results thus far show that cell cycle progression (specifically through S and G2/M phases) is faster in MCF-7 cells with liganded ERα compared to unliganded ERα. Therefore, the next objective was to determine potential mechanisms employed by liganded ERα that are responsible for the progression through S and G2/M phases of the cell cycle. The Cdk1 protein is present throughout the cell cycle, however its activity is tightly regulated by protein-protein interactions with cyclin B and changes in its phosphorylation status. The two regulatory partners of Cdk1 are the cyclins involved in S and G2/M phases of the cell cycle, cyclins A and B. Cdk1, when partnered with either cyclin A or cyclin B positively regulates progression through mitosis. One regulatory function overseen by Cdk1 to allow cells to proceed through mitosis is the phosphorylation of Anaphase Promoting Complex that promotes binding to cdc20. Upon binding of phosphorylated APC to cdc20, cyclins A and B are targeted for degradation and cells exit mitosis in to anaphase [489].

Based on our findings that ER and cyclin B are both cell cycle regulated with similar patterns of cell cycle expression and also that they are co-localized and that both their co-localization is modulated by E2, we hypothesized that liganded ERα could contribute to the proliferative phenotype of cells through its binding to the cyclin B-Cdk1 complex. To determine whether liganded ERα binds to cyclin B-Cdk1, MCF-7 were synchronized with lovastatin and released from the arrest in the presence or absence of E2. Samples were taken at 3 hours intervals and were prepared for immunoprecipitation and FACS analysis. The samples, which coincided with the timing of S and G2/M phases were examined for binding of ERα with cyclin B by immunoprecipitation assay (Figure 34). First, cyclin B was immunoprecipitated from lysates of synchronized cells and the presence of ERα and Cdk1 were examined for immune complex formation. Then, the reverse experiment was performed by immunorecipitating with ERα, followed by western blot with cyclin B and Cdk1 to determine if all three proteins are found in the same complex.

The results show that ERα protein was immunoprecipitated with cyclin B in a cell cycle dependent fashion with peak complex formation detected at 24-44 hours after release from synchronization. The FACS data in the lower panel confirms that this complex is forming during the times that the cells are in S and G2/M phases of the cell cycle. In the reverse experiment (Figure 34, second panel from the top), cyclin B is again associated with the

immunoprecipitated ERα. Cyclin B expression is evident as part of a complex with ERα between the 24 and 44 hour time points. Likewise, Cdk1 protein is detected at 24 hours and remains to the 44 hour time point upon immunoprecipitation with ERα. Together, these data show that ERα, Cdk1 and cyclin B are complexed during the S and G2/M phases of the cell cycle.

The bottom two panels of Figure 34 show the IP/western blot analysis to assess cyclin B and ERα protein levels that were IPed with monoclonal antibody and immunoblotted with polyclonal antibody to ensure that we have captured both proteins as they are expressed during the cell cycle. The cell cycle regulation of both ERα and cyclin B is evident, as the proteins are expressed beginning at 16 and 12 hours, respectively. The expression of both ERα and cyclin B peak between 24 and 28 hours and then decrease by 44 hours. These peaks of ERα and cyclin B levels overlap with the S and G2/M phases of the cell cycle as determined by FACS analysis (Figure 34). This is the first time that the presence of ERα in the same complex with cyclin B and Cdk1 is being reported.

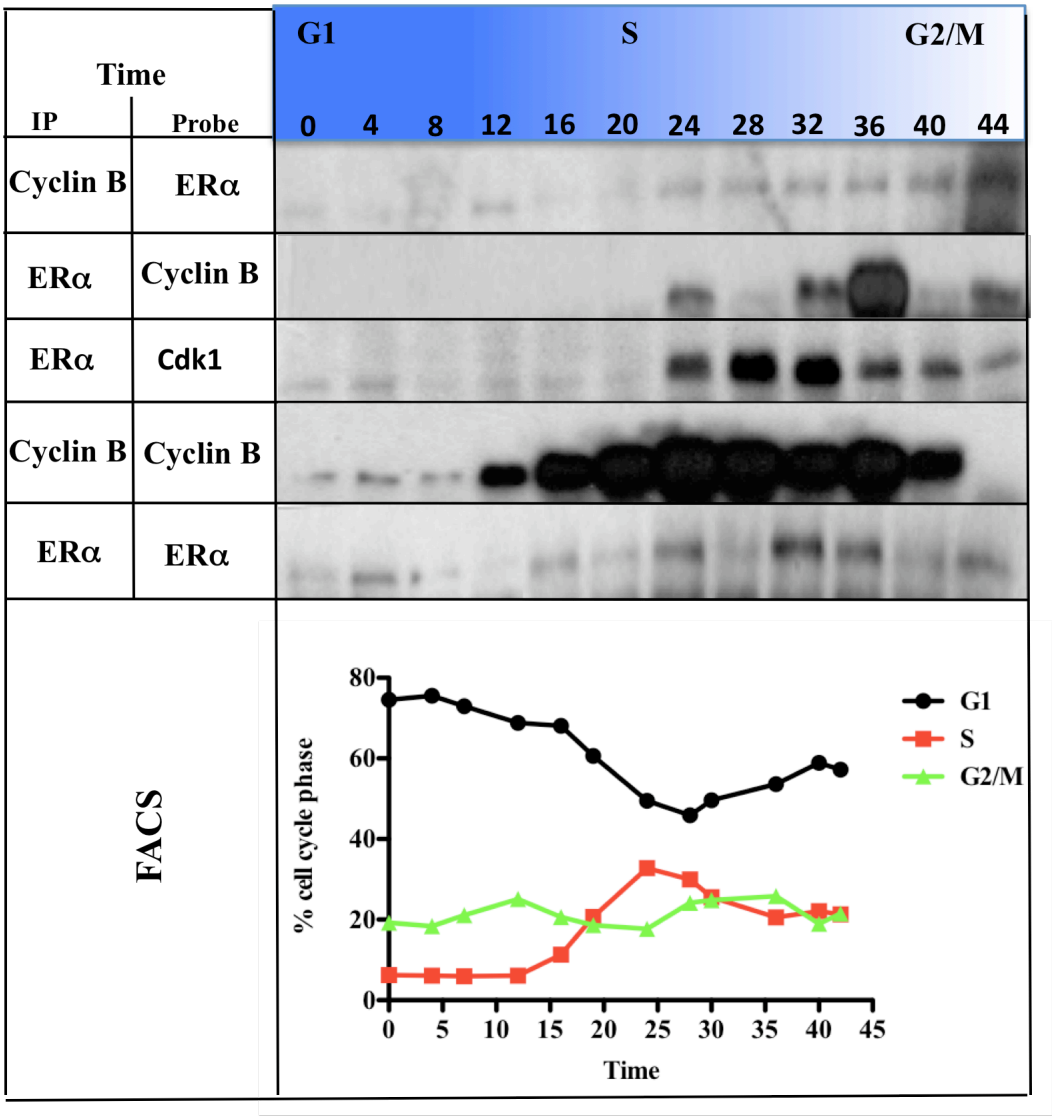


Figure 34: ERα, cyclin B and Cdk1 are found in the same complex during S and G2/M phases of the cell cycle. IP westerns using the indicated antibodies for immunoprecipitation followed by probing the western blot are shown. Controls for the efficiency of immunoprecipitation are shown by IP and probing with antibodies to the same protein (for both ERα and cyclin B). The bottom panel shows the percentage of cells in each phase of the cell cycle from FACS analysis of the lovastatin synchronized MCF-7 cells used for immunoprecipitation.

ERα binds to cyclin B the F domain

Using MCF-7 cells we showed that ERα and cyclin B bind to each other, therefore we next set out to determine if this binding is direct or whether it requires other proteins for their complex formation. To this end, we used GST pull down assay to show the binding of ERα and cyclin B *in vitro*. A series of constructs encoding GST-fusion proteins with mutated AB, C, D and F domains of ERα were used to verify the binding of ERα and cyclin B and to determine the domains within ERα that mediate the binding. A diagram of the domains within ERα is shown in Figure 35A.

Figure 35B shows the results of the phosphor imager detection of the cyclin B product from TNT kit, run on an SDS PAGE blot indicating that the in vitro translated cyclin B is the right size and was not degraded. However, Figure 35C shows the ponceau staining of purified GST-fusion proteins run on SDS-PAGE and indicates that the full length ERα has been degraded. The degradation is likely due to the fact that the fusion between GST and full length ERα generates a large protein (92 KDa) that is hard to synthesize stably in vitro and is easily degraded during the in vitro purification process. However the other ER constructs were not degraded and the size on the gels corresponded to the predicted size.

ERα GST fusion constructs were transfected in to bacteria and lysate was incubated with in vitro synthesized ^{35}S labeled cyclin B (TNT®) to analyze binding. Proteins isolated on glutathione-sepharose beads were eluted and subsequently processed for analysis by western blot and phosphor image detection. Ponceau staining shows the presence of purified intact fusion proteins; GST-truncared AB-ERα protein/ GST-truncated C- ERα protein/ GST-truncated D- ERα protein and GST-truncated F- ERα protein in the same reaction with synthesized ^{35}S labeled cyclin B (Figure 35D).

There was a direct interaction between ERα and cyclin B (Figure 35E) shown by the ^{35}S labeling in the lysates from transfection with the AB, C, and D truncated forms of ERα. However, no ^{35}S labeling was detected in the lysates containing ERα with a truncation in the F domain. The results suggest that the F domain of the ERα protein is involved in binding of cyclin B to the C-terminus of ERα. Even though functional role for the F domain has not been fully characterized to date but there are some reports suggesting that F domain is involved in responsiveness to antiestrogens (11). Mutations in the F-domain can confer

agonist activity to tamoxifen [490]. Based on our data here it is possible that F domain have more function in the regulation of cell cycle. Binding of cyclin B to the F domain may interrupt the normal F domain functions and interactions.

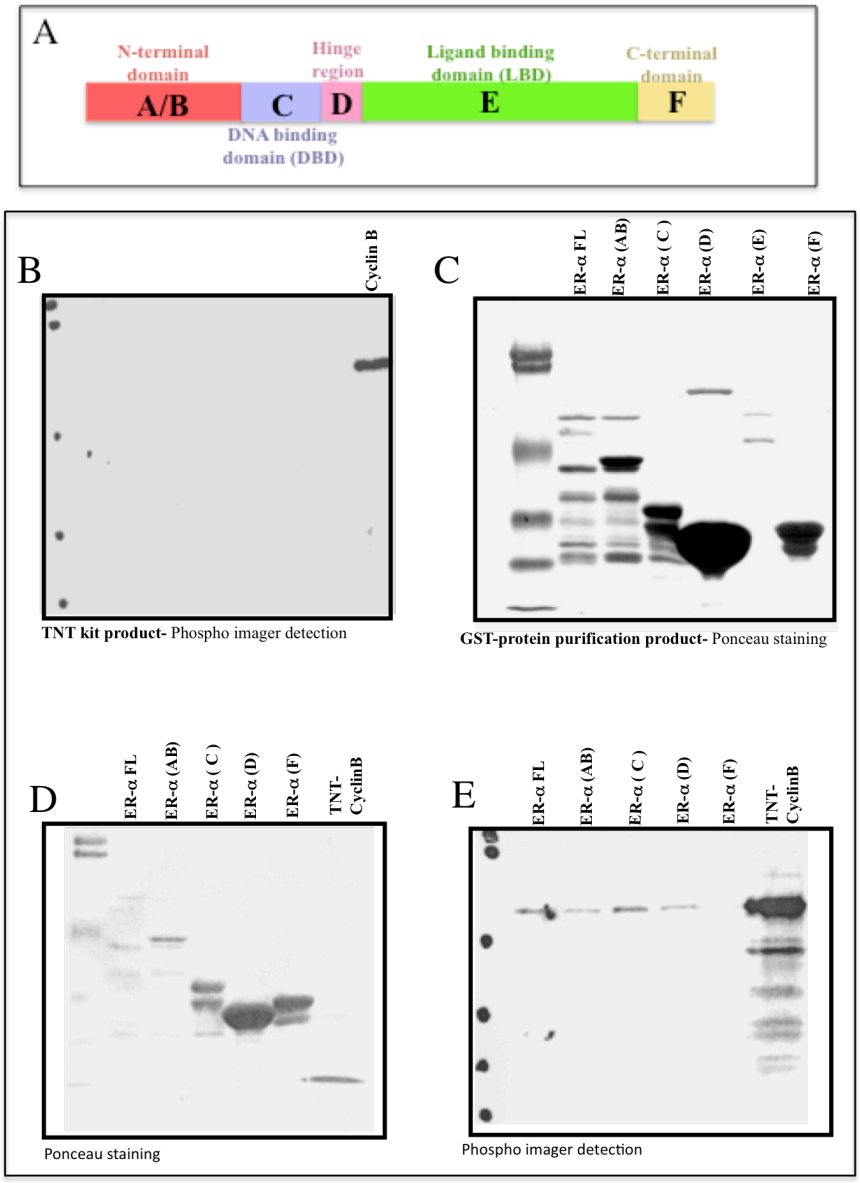


Figure 35: In vitro analysis of ERα and cyclin B binding. (A) A schematic representation of the ERα protein showing the domains that were mutated in the GST-fusion constructs. Western blots were run to confirm the expression of (B) ^{35}S labled cyclin B protein generated by in vitro translation and (C) truncated forms of purified ERα protein (constructs of GST fusion truncated forms of ERα at AB, C, D, E and F domains) upon transformation. After incubation of cyclin B with the ERα truncated proteins, a western blot was run and analyzed by (D) ponceau staining to ensure protein loading and (E) phosphor imager to detect radioactivity associated with protein products eluted from GST pull down of ERα.

The E3 ligase APC targets ubiquitin to ERα.

Several studies have shown that ERα is ubiquitinated and degraded via the proteasome pathway [483]. In our studies, the down regulation of ERα at the end of G2/M phases observed by western blot analysis suggest that ERα could be degraded at the end of G2/M phase of the cell cycle. Furthermore, the expression of ERα throughout the cell cycle expression is similar to that of cyclin B. The turnover of cyclin B is mediated via the E3 ligase APC, therefore we hypothesized that the turnover of ERα may also be mediated by the APC. To address this hypothesis, the direct interaction between liganded versus unliganded ERα and the APC complex was examined.

To address whether there is ubiquitination of ERα upon APC exposure, an in vitro ubiquitination assay using purified ERα was performed in the presence and absence of ubiquitin-like activating enzyme 1 (E1) and ubiquitin-like conjugating enzyme (E2) and APC, an E3 ligase (Figure 36). The purified ERα showed background levels of ubiquitination, which was not affected by the addition of E1. However, the addition of both E1 and E2 increased the ubiquitination of ERα slightly. The most dramatic increase in ubiqutination of ERα occurred when APC, E1 and E2 were added together to purified ERα (Figure 36). These results suggest that the E3 ligase APC could be responsible for unliganded ERα turnover during the cell cycle. However, this experiment was done in an *in vitro* system and had to be interrogated using MCF-7 to investigate its physiological relevance. To determine if there is a direct interaction between ERα and the APC complex, lysates of S and G2/M phase enriched MCF-7 cells were examined for an interaction of ERα with cdc27 of the APC complex.

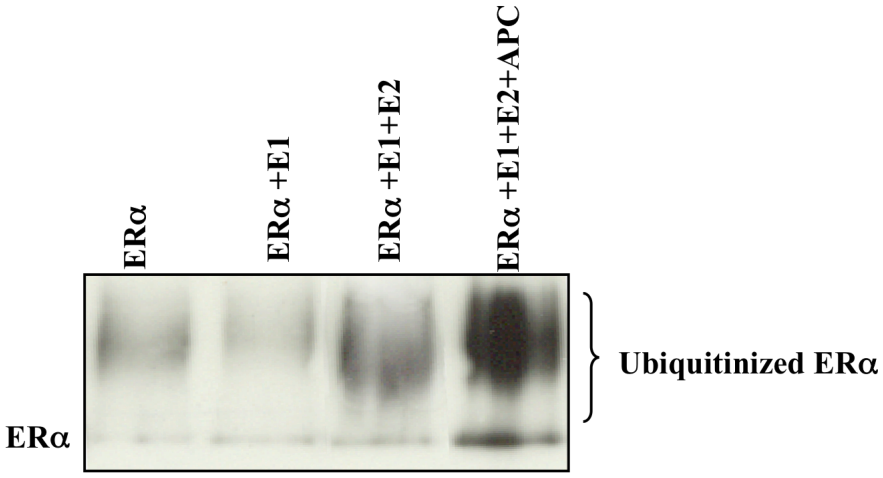


Figure 36: Ex vivo ERα ubiquitination assay. Western blot analysis of ERα expression upon incubation of purified ERα with or without E1, E2 and E3 ligase (APC). The upper bands show the ubiquitinated ERα (Ubq-ERα).

APC binding to ERα and cyclin B during S-G2/M transition and also the E2-dependent degradation of ERα and cyclin B at S-G2/M transition

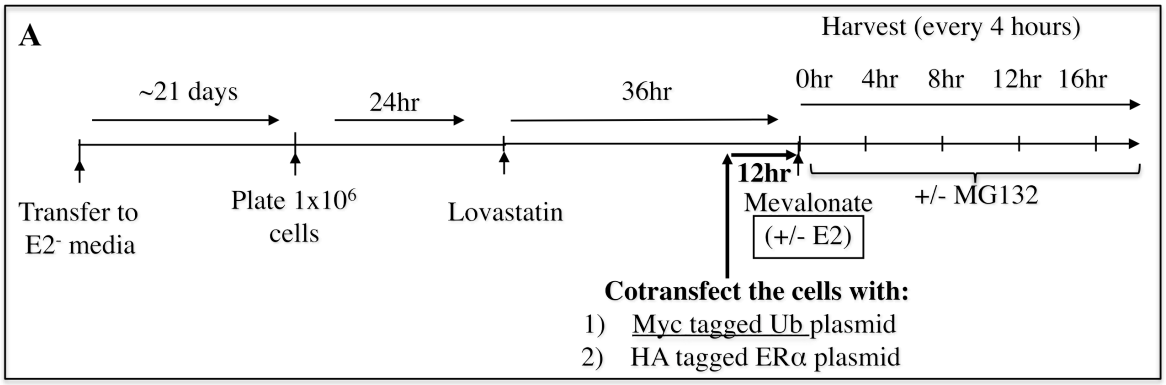
The lovastatin synchronization of MCF-7 cells was performed as depicted in Figure 37A. MCF-7 cells treated with lovastatin for 36 hours. Then cells treated with mevalonate in order to release the cells from arrest. However twelve hours prior to mevalonate release (24 hours after the lovastatin treatment) cells co transfected with myc-Ub and HA-ER constructs. Cells were also treated with MG132 to inhibit the proteasome. There are 4 arms to this experiment: Twelve hours post transfection and at the time of release, cells were treated with 1) E2 and MG132; 2) E2 alone; 3) MG132 alone; or 4) PBS. The cells were then harvested and subjected to IP/western studies to address two different questions. First we asked if both ERα and cyclin B can bind to cdc27 (a component of APC) during S-G2/M transition, The binding of ERα and cyclin B to cdc27 (APC) will provide in situ evidence that APC is the E3 ligase for not only cyclin B but also for ERα. Secondly we asked if the turn over of ERα and cyclin B is dependent of proteasome (the MG132 arms of the study) (Figure 37B).

Upon addition of mevalonate (and E2 into the E2-positive group) cells were harvested every 3 hours and samples were prepared for FACS analysis to gauge their position in the cell cycle and for immunoprecipitation and western blot analysis. In the groups, which required MG132, 10μM of MG132 were added three hours prior to the harvest. The lysates from the cell samples harvested for IP were immunoreceipitated using rabbit polyclonal cdc27 and the presence of ERα was assessed in the same complex by western blotting with ERα.

Under E2 positive conditions, MG132 treatment showed an overall increase in expression of cdc27, ERα and cyclin B (Figure 38). When ERα or cyclin B were immunopreciptated from cells that were treated with E2, cdc27 could be found associated with the complex at all times except for the 36 hour time point which is the G2/M peak (Figure 38). These data suggest that that upon binding to cdc27, ERα and cyclin B are degraded by the proteasome at G2/M.

Under E2-negative conditions, the increased protein expression resulting from MG132 treatment is not as pronounced as in the presence of E2. Densitometry was performed and quantitatively shows that proteasomal inhibition affects E2 treated cells to a greater extent than untreated cells (Figure 38). Immunoprecipiation with both ERα and cyclin B shows

complex formation with cdc27 from 8 hours until 36 hours after release under E2-positive conditions. The decreasing presence of cdc27 bound to ERα and cyclin B coincided with the end of G2/M phases (Figure 38). Without MG132 treatment, there was not a detectable level of cdc27 for densitometry. These data suggest that ERα and cyclin B form a complex, and upon binding, are targeted with ubiquitin by cdc27 for degradation by the proteasome (Figure 38).



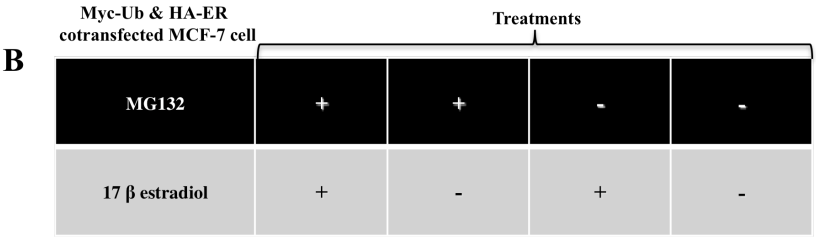
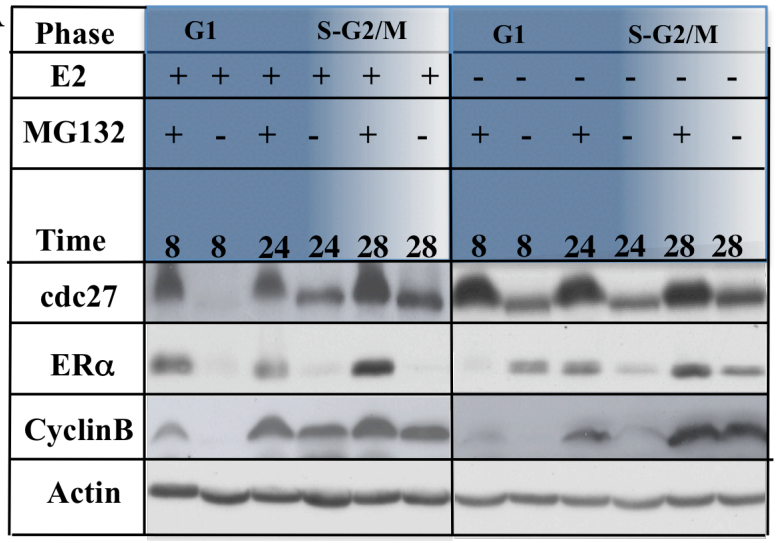
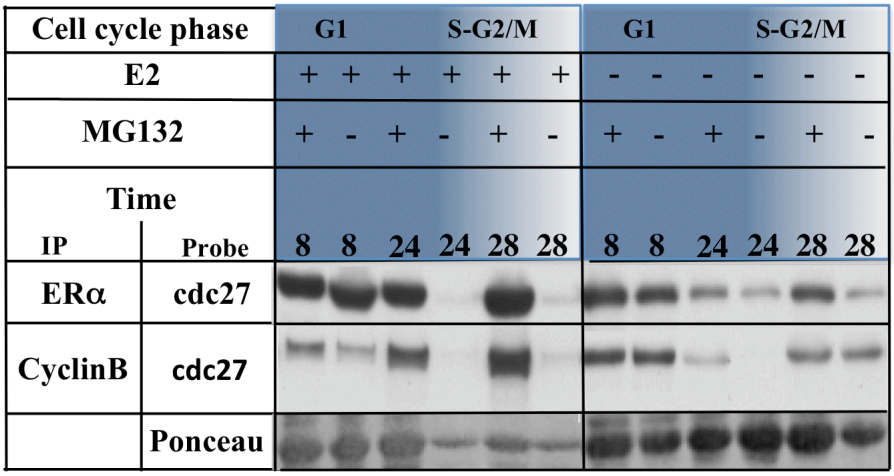


Figure 37: A schematic diagram to show the (A) schedule of the transfection and treatments of the MCF-7 cells with myc-Ub, HA-ERE2 and E2 and or MG132. (B) Depicts the arms of this experiment including treatment steps.

A


Phase
G1
S-G2/M
G1
S-G2/M
E2
+ + + + + + - - - - - -
MG132
+ - + - + - + - + - + -
Time
8 8 24 24 28 28 8 8 24 24 28 28
cdc27
ERα
CyclinB
Actin

B


Cell cycle phase
G1
S-G2/M
G1
S-G2/M
E2
+ + + + + + - - - - - -
MG132
+ - + - + - + - + - + -
Time
IP
Probe
8 8 24 24 28 28 8 8 24 24 28 28
ERα
cdc27
CyclinB
cdc27
Ponceau

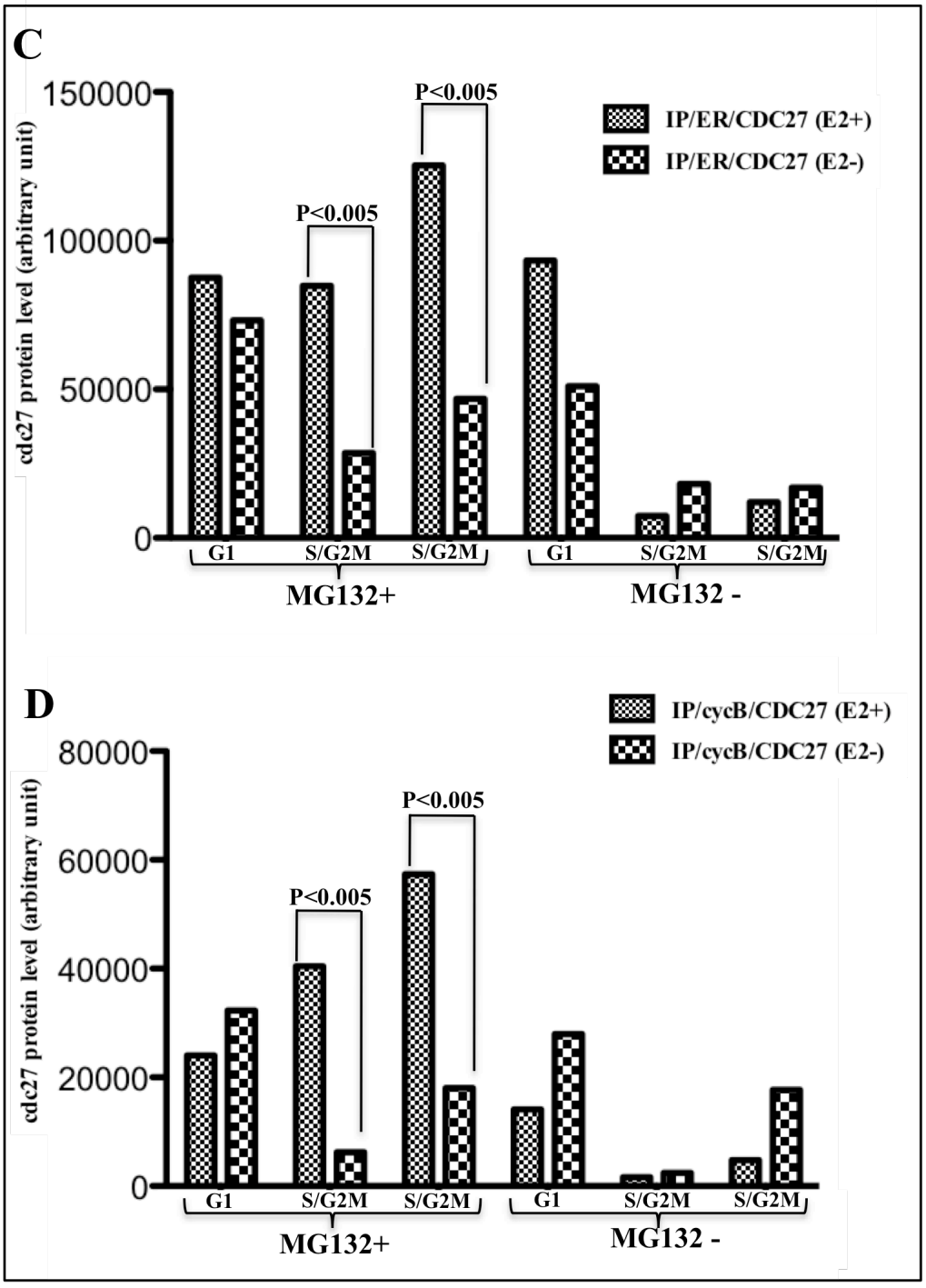

C
150000
100000
50000
0
cdc27 protein level (arbitrary unit)
P<0.005
P<0.005
IP/ER/CDC27 (E2+)
IP/ER/CDC27 (E2-)
G1
S/G2M
S/G2M
G1
S/G2M
S/G2M
MG132+
MG132 -
D
80000
60000
40000
20000
0
cdc27 protein level (arbitrary unit)
P<0.005
P<0.005
IP/cycB/CDC27 (E2+)
IP/cycB/CDC27 (E2-)
G1
S/G2M
S/G2M
G1
S/G2M
S/G2M
MG132+
MG132 -

Figure 38: Both ERα and cyclin B bind to cdc27. Lysates of synchronized MCF-7 cells treated with or without E2 and MG132 were subjected to SDS-PAGE analysis followed by western blotting with the indicated antibodies followed by densitometry of the cdc27 bands. (A) Western blot analysis of ERα, cyclin B and cdc27 expression upon treatment of MCF-7 cells with E2 and MG132 (the arms of this experiment have been shown in Figure 37. (B) IP westerns using the indicated antibodies for immunoprecipitation followed by probing the western blot are shown. (C) cdc27 protein level upon IP with ERα on samples, which treated (left panel) or untreated (right panel) with MG132, (D) cdc27 protein level upon IP with cyclin B on samples, which treated (left panel) or untreated (right panel) with MG132.

Nocodazole treatment arrests ER positive cells in G2/M phase while ER-negative cells are capable of returning to the cell cycle.

Throughout this thesis we have shown reproducibly that ERα is expressed during the S and G2/M phases of the cell cycle. Therefore, we predicted that if we were to treat the cells with nocodazole, which arrests cells in G2/M phase, would allow for the detection of ERα when it is first expressed. To perform nocodazole synchronization of cells, an optimal nocodazole concentration was identified by initially treating cells with increasing concentrations (0-0.4μM) of nocodazole for 24 hours (Figure 39). The lowest concentration of nocodazloe that would give rise to a G2/M arrest would be used in the next series of experiments. For these experiments we used both MCF-7 (ER-positive) and MDA-MB231 (ER-negative).

Following 24 hours of nocodazole treatment, cells were harvested (zero time point), and again at six and twelve hours after the treatment for flow cytometryic analysis. After 24 hours of nocodazole treatment, 50% to 60% of MCF-7 cells and 90% of MDA-MB231 cells were in G2/M phase. The percentage of MDA-MB231 cells in G2/M phase had drastically decreased by 6 and 12 hours after treatment due to the synchronous exit of the cells from G2/M phase upon release from nocodazole arrest. By 12 hours after release from arrest, only 25% of MDA-MB231 cells were in G2/M phase. This data shows that MDA-MB231 cells not only can be arrested in G2/M following nocodazole arrest, but that they are also released from arrest upon removal of this arresting agent. With MCF-7 an entirely different profile was observed. Upon removal of nocodazole from MCF-7 cells, which were already 50-60% arrested in G1, the cells were not released to enter G1 and as a result, the percentage of cells in G2/M phase remained fairly constant, not allowing for the meaningful assessment of cell cycle dependent protein expression (Figure 39).

These results that were specific to nocodazole suggested that MCF-7 (ER-positive) cells could not recover from the nocodazole arrest to enter the cell cycle again, while MDA-MB231 (ER-negative) cells could come out of the nocodazole induced arrest. Hence, we can predict from these results that ER has a very profound role in the G2/M exit and when cells that are ER positive are arrested in G2/M, the levels of ER reach its maximum and most likely mediate negative signaling pathway to inhibit proliferation (Figure 39).

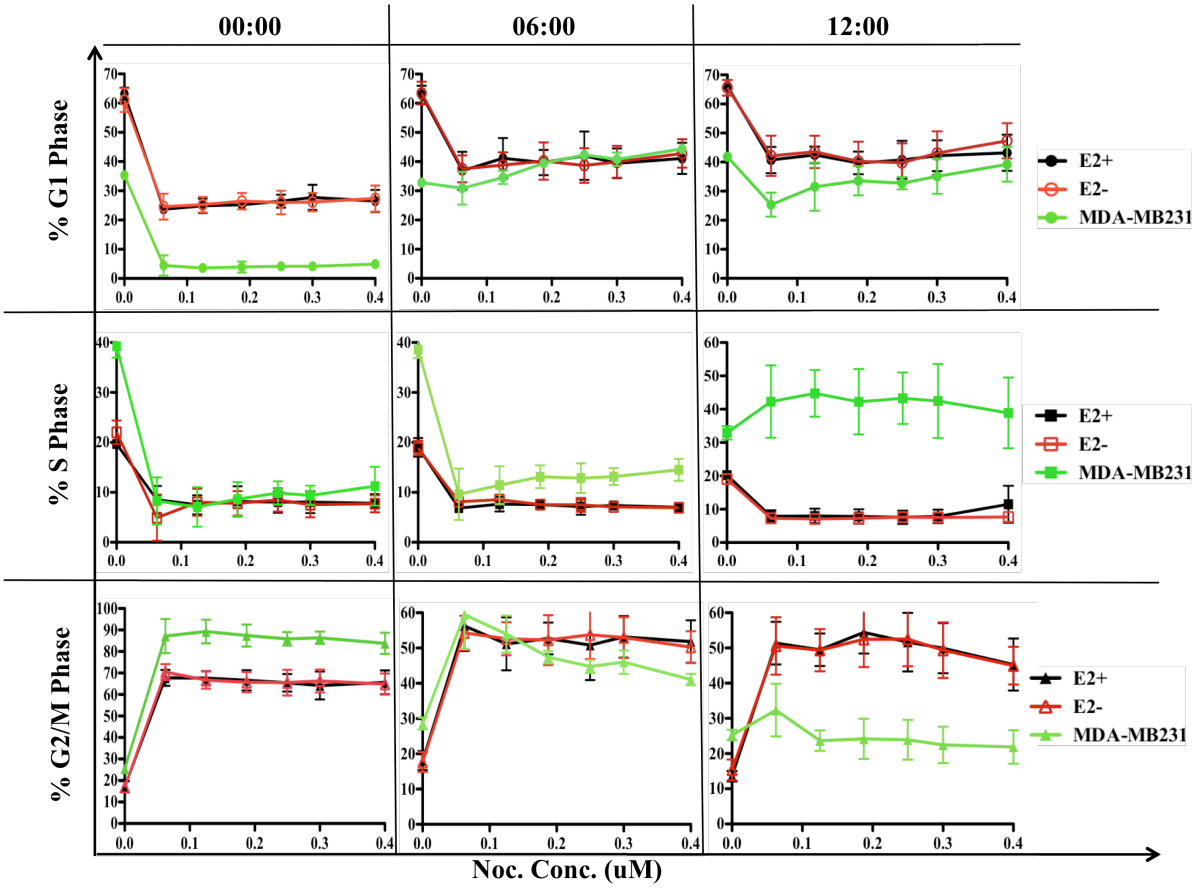


Figure 39: ER-positive cells could not exit nocodazole induced G2/M phase arrest, while ER-negative cells entered the cell cycle upon release from nocodazole arrest.

FACS analysis of MDA-MB231 (green) and MCF-7 (red and black) cells synchronized using increasing concentrations of nocodazole. The percentage of cells in each phase of the cell cycle is shown.

RPPA can be used to screen proteins activated by the ERα pathway.

Reverse-Phase Protein Array (RPPA) analysis is an ideal method to examine the expression of a series of proteins in parallel. RPPA is a method to measure the protein expression levels (just like western blot) in a large number of biological samples using high quality antibodies [491]. RPPA is also subject to accurate quantification as serial dilutions of the test lysates are printed on each slide alongside with known amounts of protein as controls. RPPA was performed in collaboration with Dr. Gordon Mills' laboratory. The expression pattern of a series of proteins (the list of all proteins examined is in supplemental Figure 4) during the cell cycle was examined in relation to cell cycle phase and ERα expression. Lovastatin synchronized MCF-7 cells, which had been divided in to two groups of E2-treated versus the E2-untreated were harvested and diluted in ppi (protease/phosphatase inhibitor-components listed under western blot analysis in chapter II) and sample buffer to the final concentration of 1μg/μL and subjected to RPPA analysis in the Mills' laboratory The samples were immobilized (printed) on individual spots on as many microarray slides as there are antibodies. Then each slide was incubated with a single specific antibody in order to detect the specific target protein expression on all the samples simultaneously. Detection was performed using a primary and a secondary labeled antibody. Chemiluminescence was used to detect the expression. The values resulting from intensity of antibody detection were plotted in graphs in (Figures 40 and supplemental Figure 4). Using this analysis we found that three of the proteins examined had a very different pattern of cell cycle expression depending on whether or not E2 was present. The proteins that are modulated by E2 include ERp118, MAPKp, and AKTp473 (Figure 40). ERp118 expression shows a very different pattern for E2-positive compared to E2-negative condition. In E2-positive condition ERp118 expression increased approximately 8 fold the expression peaked at 12 hours and declined by 18 hours after release from arrest. These time points coincide with the G1 phase of the cell cycle. There was no change in ERp118 under E2-negative conditions. MAPKp also showed a very different pattern between E2-positive and E2-negative conditions.

MAPKp is highly expressed in E2-negative conditions starting at 4 hours and continuing throughout the cell cycle while MAPKp expression in E2-positive conditions remains at baseline through the cell cycle phases. AKTp473 expression increased at 10 hours, peaked at 20 hours and decreased very suddenly, reaching zero by 28 hours. There was another incline

in expression between 32 and 40 hours. The time points that showed AKTp473 expression corresponded to G1 and G2/M phases. Under E2-negative conditions, the expression of AKTp473 increased from baseline between 10 and 18 hours after release from arrest. Based on the differences in expression profiles of different signaling molecules as shown in the pathway in Figure 41, one can speculate that the cell cycle regulated ERp118 could be the reason for the observed changes in S and G2/M phases of the cell cycle under E2-positive condition since ERp118 shows an increase under E2-positive condition in a cell cycle regulated manner. There is no detectable change in ERp118 level in E2-negative condition compared to E2-positive condition.

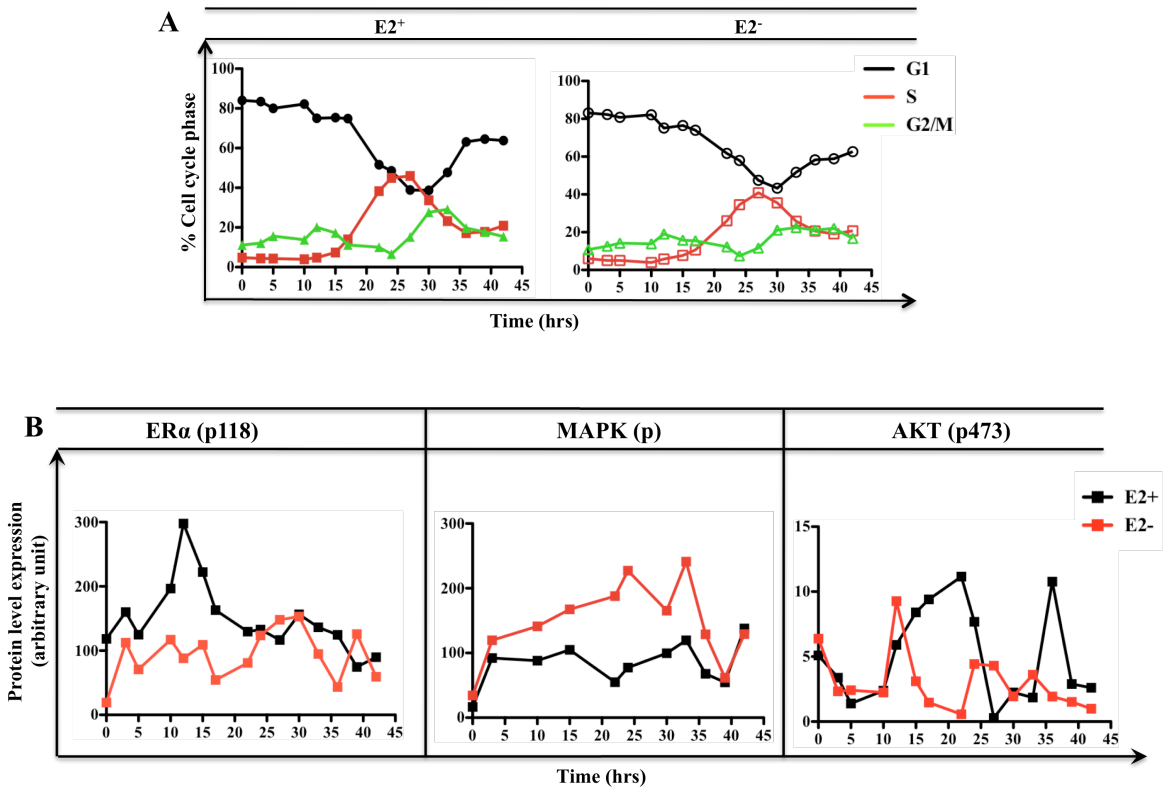


Figure 40: RPPA detects differential protein expression in key signaling proteins when ERα is liganded to E2 in MCF-7 cells. RPPA analysis was performed on lovastatin synchronized MCF-7 cells in the presence and absence of E2. (A) FACS analysis shows the percentage of cells in each cell cycle phase after release from arrest. (B) Lysates from each time point were subjected to RPPA to detect the expression of ERp118, MAPKp and AKTp473 in the presence of E2 (black) and absence of E2 (red).

DISCUSSION

In this study, we found that the expression of ERα is cell cycle regulated. The peak of ERα expression level coincides with late S and G2/M phases in a ligand independent fashion and levels decrease at the end of G2/M phase in a ligand dependent fashion. The data reported in chapter III of this study also suggests that the down-regulation of ERα is mediated by the proteasome pathway, in which APC functions as the E3 ligase scaffold.

The correlation between the expression of ERα during late G2 phase and the G2 arrest of the ERα-expressed MDA-MB231 cells suggested that ERα may have yet unknown functions in regulating G2/M phase. These functions are likely to induce inhibition of proliferation. A role for ERα in regulating the cell cycle would be consistent with other's reported data that the constitutive expression of ERα in ER-negative breast cancer cells (MDA-MB231) caused G2 arrest in the presence of estrogen [492]. Additionally, our data shows that ER-negative cells that have been arrested and synchronized in G2/M by nocodazole can re-enter the cell cycle upon release from nocodazole arrest. However, ER-positive cells remain arrested in G2/M phase upon nocodazole treatment, unable to re-enter the cell cycle following removal of nocadazole. These observations emphasize that ERα function is important for the G2 and M phase transition.

To understand the function of ERα in relation to the genes it regulates, we, as well as others, have assessed whether the target genes are downregulated or upregulated upon exposure to liganded versus unliganded ERα. Microarray (cDNA) studies revealed that liganded ERα is able to downregulate almost as many genes as it upregulates [345, 493, 494]. One of the genes that is downregulated by liganded ERα is cyclin G2. Northern blot analysis revealed that cyclin G2 mRNA is tightly regulated and oscillates through the cell cycle with peak expression in late S phase [495, 496]. The induction of cyclin G2 has an inhibitory effect on the cell cycle independent of p53 [495]. Horne et.al even suggests that cyclin G2 may be a key negative regulator of cell cycle progression [497]. Stossi et.al have shown the mechanism underlying the downregulation and eventually inhibition of cyclin G2 activity is through the recruitment of liganded ERα to the promoter region of cyclin G2, which results in the dismissal of RNA polymerase II. Additionally, liganded ERα also recruits Nuclear Receptor

Corepressor (N-CoR) and histone deacetylases, leading to the downregulation of cyclin G2 [346]. Therefore this data is one example as to how liganded ERα may downregulate cyclin G2, which leads to the uncontrolled cell cycle progression. Another cell cycle brake that is downregulated via the activity of liganded ERα is Reprimo (RPRM)[327]. Ohki et.al identified RPRM through its upregulation upon x-irradiation. They also showed that RPRM is a downstream mediator of p53 activity. The function of RPRM was revealed by overexpressing RPRM in HeLa cells, which resulted in G2 arrest of these cells. The RPRM mediated mechanism of G2 arrest is through inhibition of Cdk1 and also inhibition of the nuclear translocation of cyclin B-Cdk1 complex [484]. These interactions of ERα with cyclin G2 and RPRM demonstrate the ability of liganded ERα to accelerate the progression of the cell cycle by inhibition of the built-in cell cycle brakes. Conversely, there are other examples of liganded ERα accelerating the progression through cell cycle by upregulating genes that are involved in facilitating cell cycle progression [488, 498-501]. For example, Frasor et.al took advantage of having the human genome sequence along with microarray technology in order to study the gene expression profile of breast tumors. In this study, liganded ERα resulted in the upregulation of cyclin A2, which is cell cycle regulated with its peak expression during early S until M phase, can form an active complex with both cdk2 and cdk1 resulting in the regulated progression of cells through S and G/2 M phases [498, 502, 503]. The upregulation of cyclin A2 mediated by liganded ERα suggests that these two proteins work cooperatively to allow cells progress through S and G2/M phases.. Another example is survivin a cell cycle regulated protein, which is expressed during G2/M phase of the cell cycle and whose levels are induced by liganded ERα. Survivin regulates the function and dynamics of microtubules of the mitotic spindle and has an antiapoptotic effect [504, 505]. The overexpression of survivin by liganded ERα may overcome an apoptotic checkpoint and favor aberrant progression of transformed cells through mitosis [504].

Reports from other groups suggest that ERα may have essential functions in the cell beyond the classical mechanisms of ligand binding and growth factor regulated transcription. For example, Fan et al. show that the degradation of ERα is not necessary for its transcriptional activity [427]. To this end they show inhibition of ubiquitination and proteasome pathway prevents the degradation of ERα upon ligand binding, allowing ERα to induce transcriptional activity at the ER-responsive gene [427]. Other groups suggest that ERα can be

activated in a ligand independent manner [498, 506]. This group suggests that the basal level of ER protein can dictate the ability of cells to migrate. They show that the ER protein levels in, MCF-7, ZR75-1 and T47D cells is inversely correlated with cell migration [498, 506]. Another example of the role of unliganded ER is through the work of Maynadier et.al [507].who suggests that unliganded ERα binds to the Cdk inhibitor, p21, an interaction that is disrupted upon estradiol treatment resulting in the binding of p21 to the cyclin E/Cdk2 complex. This group has not done the FACS analysis to reveal the status of the cell cycle, but their data shows that unliganded ERα arrests the cells at the end of S phase. The data by Maynadier et al. is very similar to the data that we generated during this study, showing that unliganded ERα exerts its inhibitory effect on the cell cycle during S and G2/M phases. This group also shows that the binding of unliganded ERα or the variant of ERα only containing the interaction region (amino acids 184-283) with p21 results in significant increases of p21 expression while silencing of ERα reduces p21 levels. Additionally they show that silencing p21 in cells with unliganded ERα abrogates the inhibitory effect of uniganded ERα [508]. Together, these studies could explain the better outcomes of ER-positive breast tumors compared to ER-negative tumor and suggest that the key players in the ER mediated cell cycle progression are unliganded ERα and p21 which could inhibit cell cycle progression at S phase [507]. Our data is consistent with these findings since it provides new evidence as to how ERα is regulated in the cell cycle and what are the downstream events of ER expression in the presence and absence of E2 ligand.

Initially we examined the transcriptional activity of ER during the cell cycle in the presence and absence of ligand. This analysis showed a higher ERE activity during S and G2/M phases for liganded ERα compared to unliganded ERα. For example, the transcription of pS2 and PgR (two downstream effectors of ER) were increased during S and G2/M phases when ERα was bound to E2 (Figure 32). Li et.al, in one of their early studies, showed that there is a sequences on the pS2 protein, that contains several S/TPXX amino acid motifs, which are often found in DNA binding proteins and are potential target sites for regulatory kinases such as the Cdk1[497, 509]. When they examined the intranuclear trafficking of pS2 protein, they found that it is associated with interphase kinetochores and centrosomes, which are involved in cell cycle regulation and mitosis [509]. Additionally, mutant pS2 causes chromosome missegregation during mitosis [483]. These results suggest that pS2 is involved in

cell cycle regulation specifically during M phase of the cell cycle through its effect on the chromosome segregation function. Our own analysis, qRT-PCR in Figure 32, shows that liganded ERα induces the expression of pS2 during S and G2/M phases, which probably facilitates the chromosomal segregation and a fast passage through G2 and M phases. In addition to PS2, PgR has also been identified as a target of ERα target gene, which is also induced upon the binding of estradiol to ER [484, 499].. PgR contains multiple phosphorylation sites that are potential substrates for Cdks, suggesting that PgR activity might be regulated during the cell cycle. In fact, PgR is phosphorylated on these sites and becomes transcirptionall active in a cell cycle regulated fashion with its peak activity occurring during S phase. [488, 510]. Similar to our data with PS2, we have been able to show that the PgR transcript is also cell cycle regulated increasing during S and G2/M phases upon estradiol treatment. In another study Narayanan et. al also showed that PgR activity is stimulated by cyclin A/Cdk2 [500]. Collectively both published work and our own results suggest that PgR, a target gene of ERα, is cell cycle regulated at S and G2/M phases, and that through this cyclical pattern of expression, estradiol can exert its mitogenic effects specifically at S and G2/M phases.

Musgrove et al. have shown that PgR affects the cell cycle by accelerating the passage of cells through the first cell cycle and then induces an arrest at the G0/G1 phase of the subsequent cell cycle [501]. In agreement with this data, we made the observation that ERα expression resulted in cell cycle modulation in the second subsequent cell cycle (rather than the first cell cycle) after aphidicolin treatment (Figure 15). Since the activity of PgR is regulated not only by progesterone but also by modulators of various cell signaling pathways in the cytoplasm, more experiments are required in order to explain this cell cycle arresting phenomenon [511, 512].

Additionally, the localization of ERα during different phases of the cell cycle was examined in this study to help to explain the functions of ERα. ERα showed a similar pattern of subcellular localization as cyclin B. The cytoplasmic versus nuclear localization of ERα and cyclin B was assessed by immunnofluorescent analysis (Figure 33) and colocalization could be seen both in the cytoplasm and nucleus of both these proteins, which were also altered coordinately by the addition of E2. The possibility that ERα and cyclin B form a complex was examined by IP/western analysis of the two proteins using synchronized MCF-

7 cells. The results showed that ERα was in the same complex not only with cyclin B, but also with Cdk1 (Figure 34). Cyclins, which are expressed in a cell cycle-dependent manner, can regulate steroid receptor function and this function could be independent of the kinase partner. There are several studies that have shown cyclin regulation of steroid receptors. For example, cyclin D forms a complex with the androgen receptor and inhibits its transactivation ability [513]. Another example is that cyclin A/Cdk2 acts as a PgR coactivator [500]. Neuman et.al have shown that cyclin D can stimulate ERα transcriptional activity independent of cdk4 [514]. Lastly, cyclin E associates with androgen receptor by binding directly to the C terminus portion of androgen receptor. Cyclin E then functions as a coactivator for androgen receptor and enhances androgen receptors transactivation activity [515]. In all these cases the involvement of cyclins with the different members of the nuclear receptor family provides a potential means for integrating the regulation of these nuclear receptor's activity with cell cycle progression. In the cases that both cyclin and its Cdk are being involved in binding to the nuclear receptor (i.e cyclin A/Cdk2 recruitment to PgR), the elevated level of kinase activity in the complex containing the nuclear receptor may result in the phosphorylation of coactivators and therefore facilitate the promoter targeting and subsequent transcriptional activities.

To confirm the data generated by IP that cyclin B,cdk1 and ERα form a complex, an *in vitro* GST-pull down assay was performed. ^{35}S labeled cyclin B was incubated with wild-type and truncated forms of ERα (AB, C, D and F deletion constructs). The results of GST-pull down assay confirmed the IP data showing that ER and cyclin B can directly bind to each other. The GST-pull down assay was performed using various mutant forms of ERα, thus giving us the opportunity to examine the specific site of ERα binding to cyclin B. As Figure 35 shows, when expressing the F-domain mutant, no binding between cyclin B and ERα were detected while all the other mutants bound to cyclin B. From this data it is concluded that cyclin B specifically binds to the F domain of ERα (Figure 35). Peters et. al have shown that both E and F domains of ER have a role in dimerization and interaction with coactivator Receptor Interacting Protein (RIP-140) [516]. It is possible that cyclin B can directly bind to the F domain or it could require the binding of another coactivator to the F domain.

The degradation of ERα is known to be mediated by a proteasome through a ubiquitination pathway. It has been reported that E1 and E2 were able to ubiquitinate ERα in vitro [483]. Our data support the findings that ERα could be ubiquitinated by E1 and E2, however the addition of APC dramatically increased the ERα ubiquitination *in vitro*, suggesting that APC is an E3 ligase for ERα ubiquitination. This is a new finding, but is not unusual as many of the substrates for APC are cell cycle related, such as securin and cyclin B1 [517]. Since we already showed that cyclin B and ER are co-localized and are found in the same complex, this led to the hypothesis that through its binding with cyclin B, ERα is also degraded by the same E3 ligase as that for cyclin B. When we addressed this hypothesis, our results show that ERα can interact with cdc27, which is one of the protein complexes in the APC. The timing of cdc27 bound to ERα was similar to the APC- cyclin B binding (Figure 38). These results suggest that as a result of the APC directed ubiquitination, ERα is ubiquitinated at the end of S and G2/M phases of the cell cycle and degraded through the proteasome (Figures 36 and 38).

The cell cycle dependent degradation of ERα in the absence of ligand is similar to that of the cyclins and further supports a role for ERα in G2/M phase progression. Additional evidence for the role of ER in G2/M was presented in the RPPA analyses, which depicted cell cycle regulation of ERp118, which was induced by the addition of ligand. These results suggest that liganded ERα results in its phosphorylation at serine 118 (ERp118), which in turn could down regulate cell cycle breaks such as RPRM. The other hypothesis based on the RPPA data could be that ERp118 interrupts the function of FOXO transcription factor family members, which have been shown to have regulatory effects during G2/M phase of the cell cycle [518, 519]. Another protein, which the RPPA showed to be cell cycle regulated and its regulation is modulated by the addition of E2 is AKTp473, whose levels are high during G1 and S phases in E2-positive condition. It has been shown that E2 induces phosphorylation of AKT at ser473 (AKTp473), which in turn results in the phosphorylation of FOXO1 and FOXO3a to inactivate them [510, 520]. The FOXO transcription factors are important in regulating different aspects of cellular homeostasis and apoptosis [521] while AKTp473 plays an important role in cell survival [522]. The RPPA results show that under E2-positive conditions ERp118 expression is increased approximately 8 fold in G1 phase of the cell cycle compared to other time points corresponding to the S and G2/M phases. There was no change

in ERp118 under E2-negative conditions. Phosphorylation of ER α at serine-118 results in activation of ER α in regulation of genes containing an estrogen response element in their promoters [172]. The well-known kinase involved in the phosphorylation of serine 118 is MAPK [172]. MAPK is active during all phases of the cell cycle in E2-positive condition (MAPK does not show increases activity in E2-negative condition). The MAPK during G1 could be involved in phosphorylation of ERp118. The resulting active ERp118 may be responsible for the rapid progression of the cell cycle specifically at S and G2/M phases, following the phosphorylation of ERα at G1. One interaction that the RPPA suggests and that needs further investigation is the E2-dependent interaction of ERα (ERαp118) with MAPK and AKTp473 during the cell cycle. If ERα interacts directly with MAPK and/or AKTp473 then we can speculate that the ERp118, which is cell cycle regulated is being phosphorylated through these kinases and as the result the active ERp118 could accelerate the cell cycle progression. The relationships of these processes are depicted in a model in Figure 41.

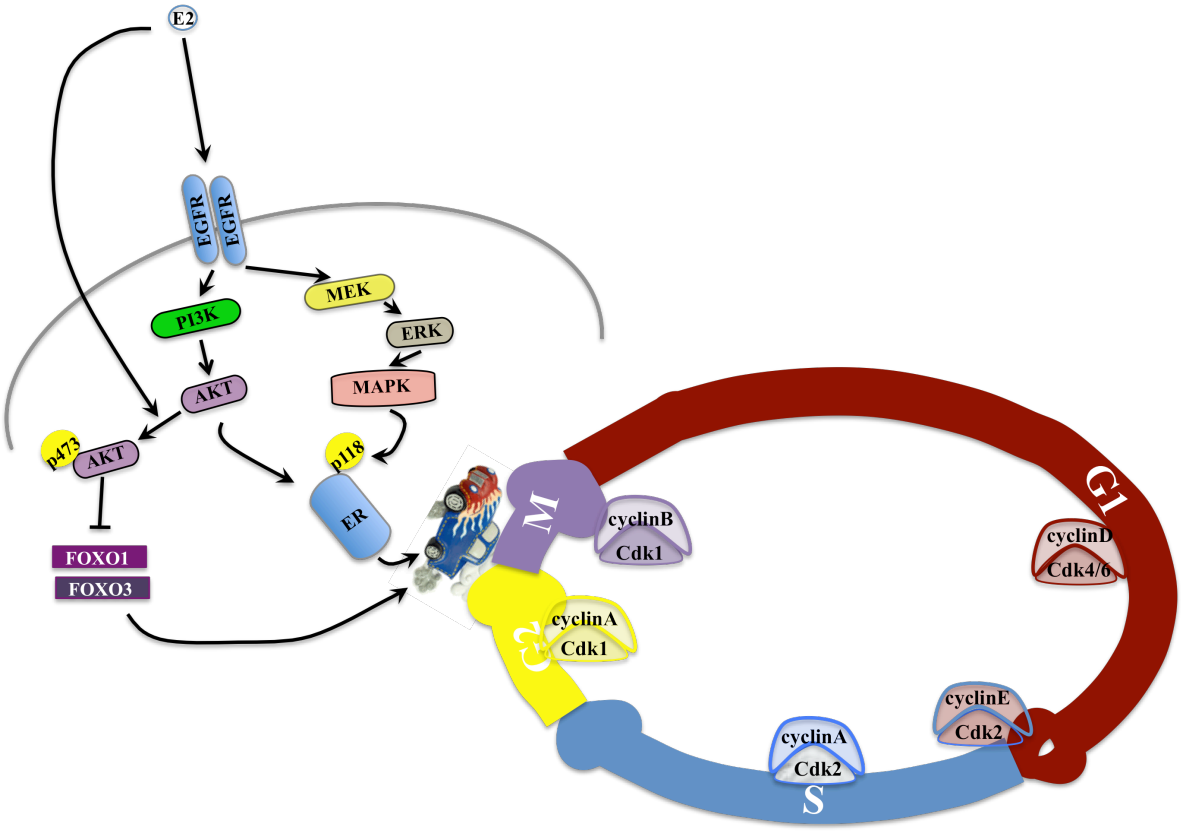


Figure 41: A schematic diagram showing the possible pathways that could cause ERα phosphorylation and progression through cell cycle (especially at S and G2/M phases). E2 stimulation could result in the activation of both MAPK and Pi3K pathways, which in turn can phosphorylate ERα at serine 118, which activates ERα. Additionally E2 can directly result in the phosphorylation of AKT at serine 473. The AKTp473 inhibits the activation of FOXO1 and FOXO3a, which both are involved in the regulation of cell cycle passage especially at S and G2/M phases.

In conclusion, the experiments presented in this chapter shed light on the possible function and mechanism of unliganded versus liganded ERα in the cell. We have shown that ERα and its transcriptional targets with ERE sites are regulated during the cell cycle. Additionally, we have identified cyclin B and cdc27 as binding partners of ERα and showed localization and ubiquitination status of the resulting complex. Very importantly we show that the E3 ligase that may be responsible for the degradation of ER is APC. This information not only adds to our knowledge of cell biology but also can impact future research in breast cancer therapeutics.

Significance and Future Directions

Patients with ER-positive breast tumors have a more favorable prognosis than those with ER-negative tumors. In fact, several reports have revealed that the overexpression of ER in ER-negative breast tumor cells could result in the inhibition of the proliferation of these tumor cells (38, 39). The reason for such inhibition of cell proliferation has remained unclear.

In this study we showed that ERα upregulation alone could prolong the duration of cell cycle specifically in S and G2/M phases compared to parental cell line or the ER silenced counterpart. Our findings also suggest that unliganded ERα has an inhibitory effect on the progression of the cell cycle, specifically the exit of cells from mitosis. Other groups have also reported the inhibitory effect of unliganded ERα on breast cancer cells. They have shown that unliganded ERα in cells could provide protection against tumor spreading and metastasis [507] and involved in the inhibition of breast cancer cell growth as well as tumor formation in nude mice[523]. However, the liganded ERα (bound to E2) causes a rapid progression of the cell through the cell cycle. Considering breast cancer cell proliferation needing the degradation of ERα through coupling of ERα to E2, it would be reasonable to speculate that the combination of a drug that lowers estrogen level (such as using aromatase inhibitors) and preserves ERα from degradation (inhibitors of ubiquitin-proteasome pathway) would provide better outcome for breast cancer treatment. Our finding that APC functions as the E3 ligase for ERα might help to design more specific inhibitor for ERα degradation. Similarly, since ERα protein is predominantly expressed during S and G2/M phases of the cell cycle, it suggests that combination of, taxol (which arrests cells at G2/M by inhibiting

the microtubule depolymerization and hence stabilizes microtubules) with tamoxifen and aromatase inhibitors to enrich for ERα and maintain its expression while eliminating its ability to bind ligand would be a potential treatment strategy for patients with ER positive breast cancer..

To better understand the importance of this research and to put it in to perspective of the field of endocrine therapy, it is necessary to give a brief history of endocrine therapy. The first person to suggest endocrine therapy was Dr. Beatson, who realized that the hormonal function of ovaries affect the function of mammary glands. His approach to endocrine therapy was oophorectomy, which resulted in a quick regression of the cancerous tissues in patients. The reason behind this observation is that by oophorectomy he was eliminating the source of estrogen, which the cancerous tissues in the breast were dependent on. In the following years other methods of treatment were being used such as hypophysectomy and adrenalectomy. In all these treatment strategies, the main purpose was elimination of the source of estrogen in the body. Hypophysis controls the estrogen secretion from both adrenal glands and ovaries and results in the shut down of these organs. High doses of synthetic nonsteroidal estrogen (i.e. Diestibestrol) were also used to suppress pituitary gonadotropic secretion and endogenous estrogen.

By eliminating estrogen from the body, regression of the cancerous tissue was achieved. However, the use of diestibestrol was discontinued due to its extreme toxicity. At this point the endocrine therapy shifted from inhibition and elimination of estrogen to inhibition and elimination of ER. Scientists started using SERMs and SERDs in order to inhibit the estrogen signaling. Antiestrogens are still being used in the clinic but primarily in post menopausal women, which have much less estrogen production. What my research has shown is that estrogen elimination (block) could be a much better and promising method of treatment rather than elimination of ER activity by using antiestrogens. In this dissertation, we have shown that the presence of ERα can function to inhibit cell cycle progression. In the absence of estrogen, ERα is essential as an inhibitory factor in tumor cells. Antiestrogens (SERDs) actually degrade ERα and thus provide the same conditions as an ER-negative tumor, in which ER is absent and is not able to function. It is important to keep the integrity of the ERα protein intact. In this dissertation I have shown that APC is the E3 ligase essential for the degradation of ERα. Therefore, an APC inhibitor could be used to inhibit the ERα

degradation, enriching ERα in the cell. Furthermore studies on mitotically arrested cells show that drugs being used in order to perturb the spindle assembly such as taxanes, vinca alkaloids, and epothilones are not the best options for ER positive tumors [524]. The reason is that the mechanism of action of these drugs is to perturb the spindle assembly and as a result induce apoptosis. Cancer cells resist apoptosis by a premature exit from the M phase before the onset of apoptosis, thereby avoiding the effects of spindle targeting agents [525]. Spindle Assembly Checkpoint (SAC) inhibition is not a good option to arrest the cells at mitosis, however perturbation of the growth of cancer cells could be accomplished downstream of the M phase checkpoint [525].

It is exciting having discovered that APC targets ERα to the proteasome and it also has precedence in literature as a therapeutic strategy. Huang et. al introduced tert amyl methyl ether (TAME), which is a small molecule that inhibits APC activation directly by preventing Cdc20 binding. A cell-permeable prodrug (proTAME) induces mitotic arrest and cell death [526]. TAME is an alternative for the current inhibitors of M phase, as it arrests the cells at M phase downstream of SAC so does not require checkpoint activity. The bypass of SAC by using TAME makes it possible for tumor cells to go through intrinsic apoptosis or an alternative death pathway when Bcl2 was overexpressed. The use of TAME is supported by our data since it directly inhibits APC (the E3 ligase of ERα) and could be an effective method for enriching ERα expressing cells. However TAME cannot be used in conjunction with taxol, as taxol requires the APC pathway to be intact for its activity [527], so use of these two drugs simultaneously is not an appropriate strategy. The mechanism of action of TAME makes it a good agent to consider as an effective method for mitotic arrest and inturn enrichment of ERα expressing cells.

Based on the data presented in this dissertation, ERα in its unliganded status has an inhibitory effect on the cell cycle progression. Others have also shown that unliganded ERα has a protective role against tumor invasion [507, 523]. This data also implies that *in vivo*, one would need to enrich the cells for ERα by using agents that arrest the cells at M phase, and TAME could serve this purpose. The next step would be to also eliminate estrogen. Elimination of systemic estrogens is a very difficult task but necessary to inhibit cancer progression. In a tissue culture setting elimination of estradiol is easily done by culturing the

cells under estradiol free conditions. However in the whole organism it is hard to eliminate all sources of estradiol. It may be the reason that current anti-estrogen treatments are most effective in post-menopausal women due to the lower level of circulating estradiol [528]. Additionally, the current aromatase inhibitors that are being used are essentially inhibiting the estradiol production by inhibiting the aromatase enzyme in adipose tissues [529]. The largest trial ever completed to date to compare the effects of Tamoxifen alone or aromatase inhibitor alone or the combination of these two together is ATAC trial [528]. In this study, 3000 ER-positive patients were recruited in each arm with a median follow up of 33 months. This trial showed that patients in the anastrozole treatment alone arm had significantly better outcome by reducing the risk of recurrence by a relative 22% (p=0·005) and the risk of developing a second primary cancer by a relative 58% (p=0·007) compared to the other two arms [528]. Therefore, despite their very limited inhibition of estrogen production, aromatase inhibitors have superior effect in terms of disease-free survival (DFS), time to recurrence (TTR), and incidence of contralateral breast cancer (CLBC) compared to antiestrogens [528].

Aromatase inhibitors do not affect the main estradiol production in the body, which is through ovaries and adrenal glands. If there was a drug that could eliminate estrogen from these sources it could revolutionize the treatment of breast cancer and all hormone dependent cancers. In order to keep the estrogen low in the blood circulation of a premenopause patient, chemical oopheroctomy could be performed with GnRH antagonists. GnRH is the hormone, which its secretion is regulated via the hypothalamus. GnRH stimulates FSH and LH synthesis from pituitary glands, which then gives rise to estrogen through the ovaries. GnRH antagonists function by blocking this pathway at pituitary gland by binding to GnRH receptors in the pituitary gland, inhibiting the FSH and LH release, which are the initial step before estrogen production from ovarioes [530, 531]. Currently approved GnRH antagonists include the following: Cetrorelix, Ganirelix, Abarelix, Degarelix [532, 533]. GnRH antagonists have to be combined with either TAME or taxol to enrich for high levels of ERα .Based on the results presented in this dissertation and published work, the most promising treatment could be the combination of an APC inhibitor (TAME) to sustain mitotic arrest and enrichment of the cells for ERα with an aromatase inhibitor or GnRH antoagonists. Anastrozole has been used already in ATAC trial and could provide a good choice for aromatase inhibitor.

A rational clinical trial would be one with three arms to delineate the effects of the combination of drugs that inhibit ER degradation and eliminate estradiol. One arm will have ER-positive patients treated with anastrozole (or GnRH antagonists) and TAME, while the second arm would have patients treated with TAME alone and in the third arm patients would receive anastrozole (or GnRH antagonists), TAME and antiestrogen (Needs to be a SERM rather than SERD). However before the design of this trials get to the clinic a thorough evaluation of these combinations are needed in an *in vitro* settings using cell lines and in preclinical in vivo model systems.

To go back again to the research of Dr. Beatson which was mentioned earlier. He was very wise to treat the patients by eliminating the main source of estradiol and I believe that after more than 100 years, it is recognized again that a drug is needed which eliminates/lower the circulating estradiol in premenopausal women as the best means to treat ER positive breast cancer patients.

Chapter IV: SUPPLEMENTAL DATA

MCF-7 cell growth is not significantly affected within 10 days of E2 treatment.

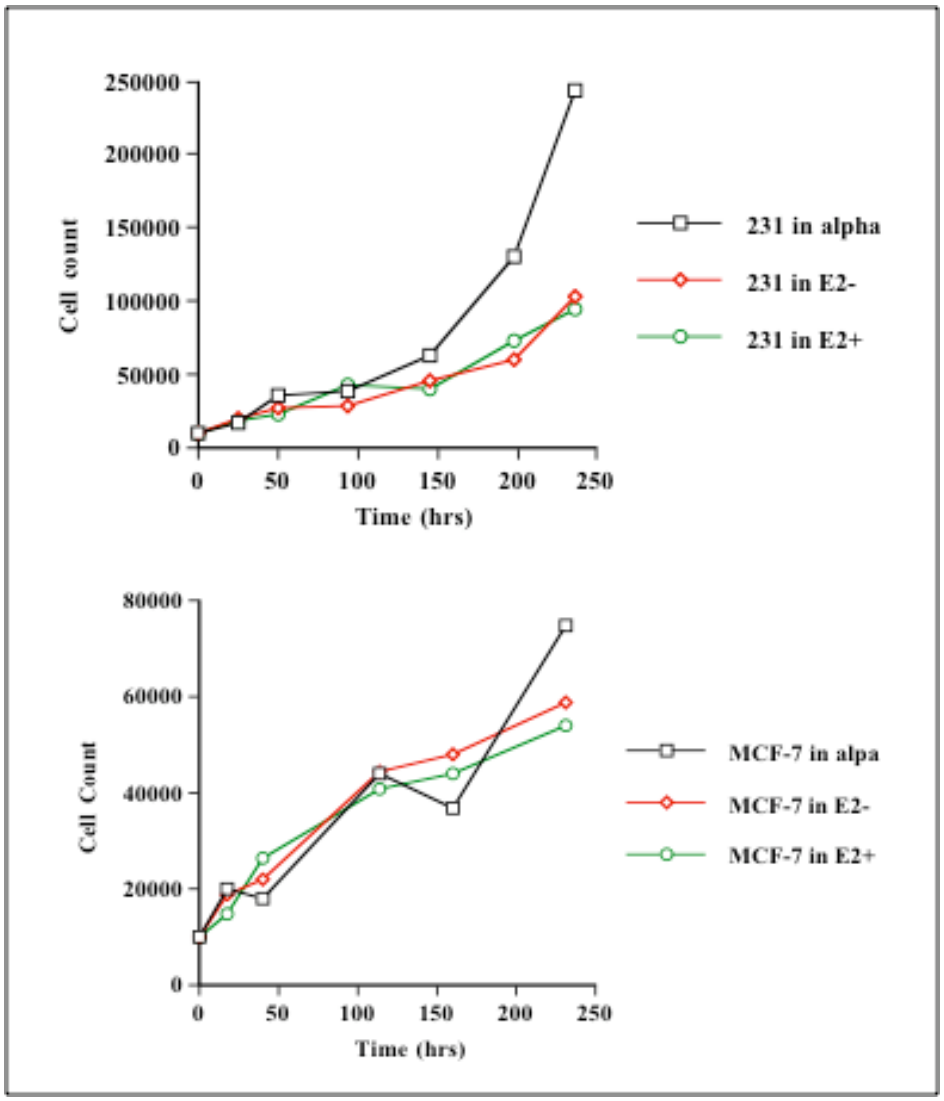
Liganded ERα shortened the S and G2/M phases of the cell cycle in the MCF-7 and MDA-MB231cells (Figures 14 and 24). To determine whether a change in the phases of the cell cycle resulted in a change in proliferation of cells, the doubling time of MCF-7 and MDA-MB231 cells was determined in the presence or absence of E2. Based on preliminary data, increased proliferation and a decreased doubling time was expected in MCF-7 cells in the presence of E2. MCF-7 and MDA-MB231 cells, which had been kept in E2-free media, were divided in to two groups: one to receive E2 and the other group that would remain clear of E2 throughout the experiment. Additionally, a group of cells were cultured in complete alpha medium as a positive control. MDA-MB231 cells are generally rapidly proliferating when cultured in alpha medium, however these cells are ER-negative and therefore were not expected to show a difference in the presence or absence of E2. All cells were plated with the same numbers and under the same conditions in 6 well plates. Cells were harvested and counted and the media was changed on the remaining wells every other day for 10 days. Table 6 shows the doubling time of MCF-7 and MDA-MB231 cells under different conditions. Doubling time was calculated using the cell counts from the exponential growth phase using the following formula:

Doubling time $=[(0.301\ \Delta t)] : [\mathrm{Log}_{10}\ (N/N_0)]$

Doubling Time (hrs)	E2-positive	E2-negative	Alpha-media
MCF-7	35	35.1	40.29
MDA-MB231	59	50	50

Table 6 – Analysis of the doubling time of MCF-7 and MDA-MB231 cells

As supplemental Figure 1 shows, both MDA-MB231 cells and MCF-7 cells had almost the same rates of proliferation despite the presence or absence of E2 (Table 6). These results were unexpected for MCF-7 cells, but could be explained by the possibility that liganded ERα can change the length of S and G2/M phases without changing the overall proliferation of the cell. However, we have also shown that the length of the cell cycle is significantly reduced in MCF-7 cells in the presence of E2. By 10 days, the MCF-7 cells grown under E2-positive conditions did start to show slightly higher growth rates than those in E2-negative conditions. Therefore, it is likely that following the growth of the cells for a longer period of time would show more significant results.



Supplemental Figure 1: Comparison of the proliferation of MCF-7 cells and MDA-MB231 cells under E2-positive and E2-negative conditions.

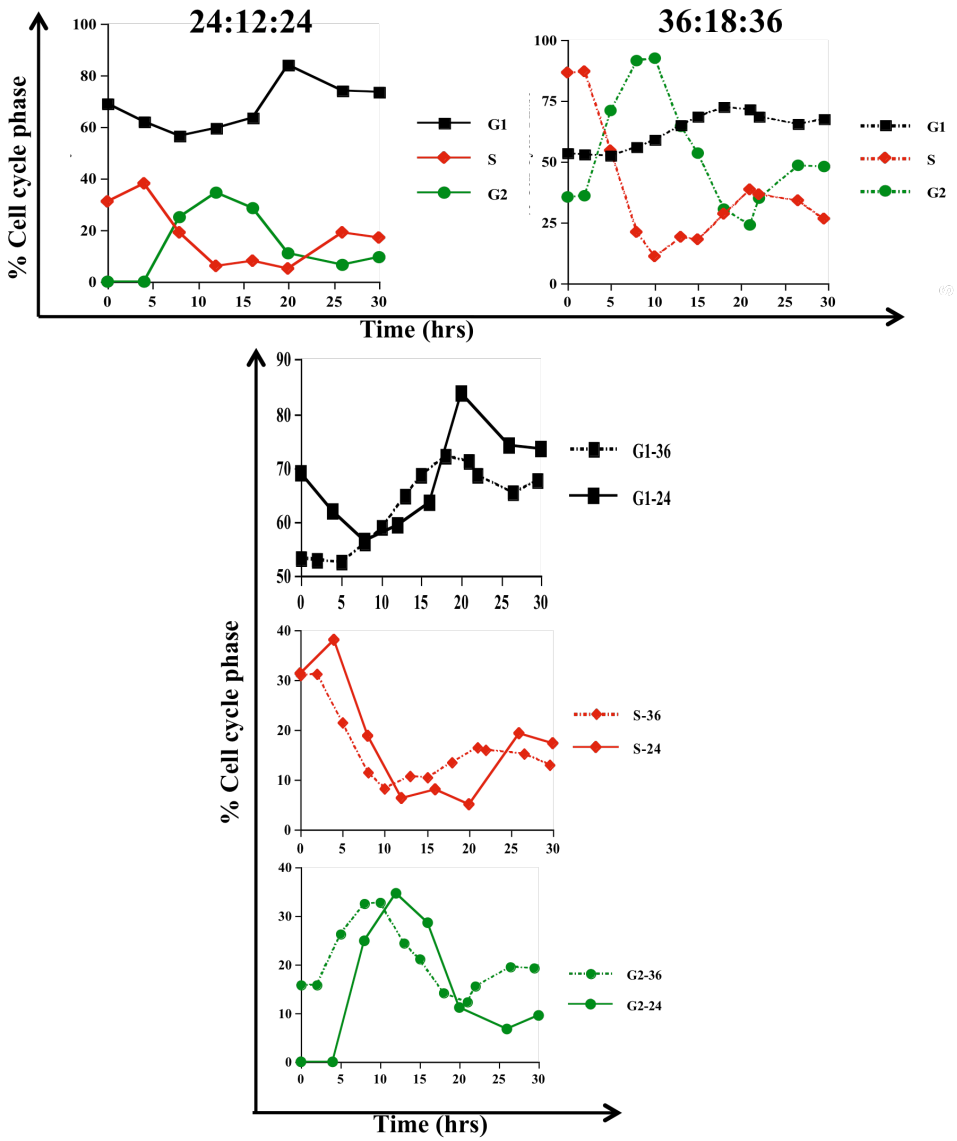
A cell count was taken and graphed of MCF-7 and MDA-MB231 cells every other day for 10 days in the presence or absence of E2.

MCF-7 cells can be synchronized using treatment with double thymidine block.

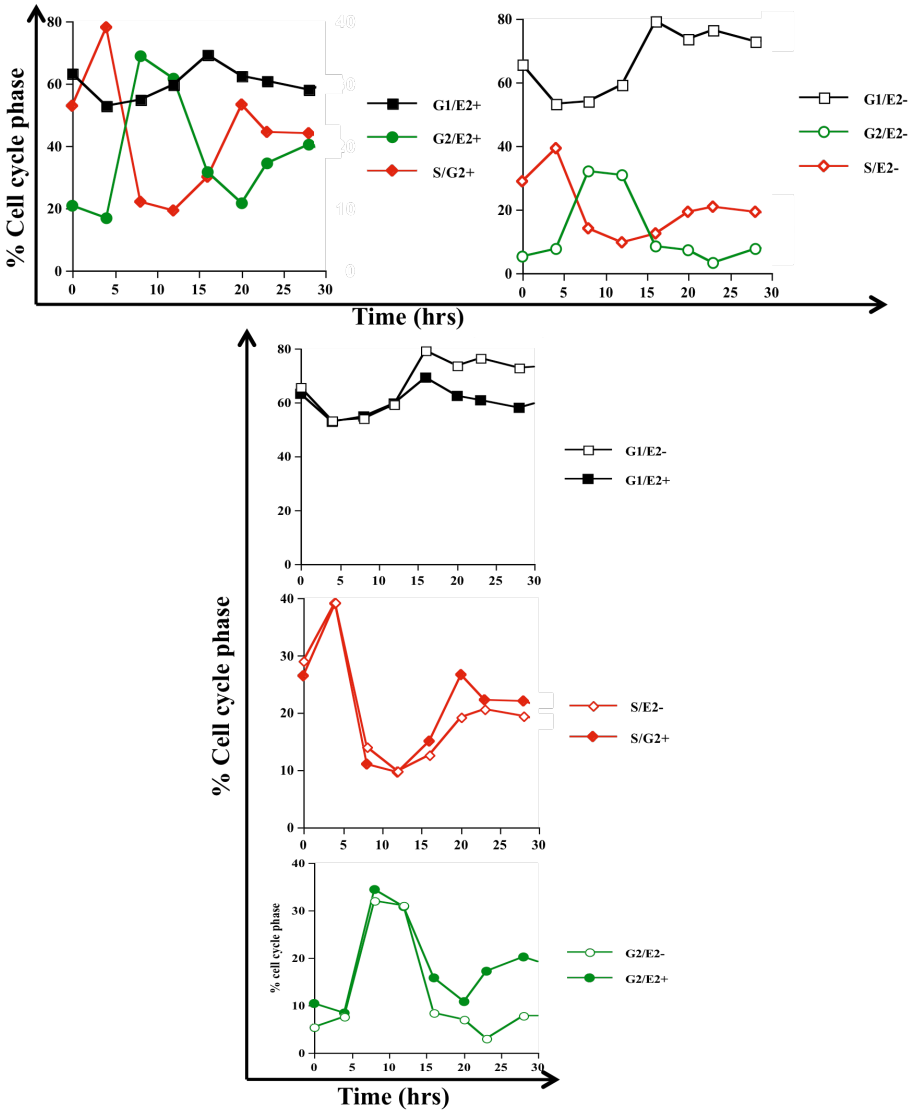
As an alternative method of cell synchronization and to confirm data using lovastatin and aphidicolin as arresting agents, MCF-7 cells were subjected to treatment with double thymidine. Treatment of cells with excess arrests the cells at the border of G1/S transtion as such treatment blocks DNA synthesis.

Supplemental Figure 2 shows that double thymidine block is a suitable method to synchronize the MCF-7 cells at the border of G1 and S. Approximately 75% of the cells arrested at the G1/S border then quickly and synchronously exited G1 and entered into S and G2/M phases of the cell cycle after release in the 24:12:24 schedule compared to about 55% with the 36:18:36 schedule. Therefore the 24:12:24 double thymidine treatment schedule was chosen to study the changes in cell cycle profile due to the presence or absence of E2. Supplemental Figure 3 shows that almost 70% of cells were in G1 phase and 25% in S phase and 5% in G2/M upon release from double thymidine induced arrest. However, FACS analysis showed unremarkable changes in the cell cycle profiles of the cells released under E2-positive versus the E2-negative conditions.

Thymidine arrests cells at the end of G1 phase and as soon as the cells were released from the thymidine arrest they pass quickly through S and G2/M phases. It had been observed previously, when using aphidicolin as an arresting agent, that cells do not express ERα until the second cycle after release (Figure 16). Therefore, it is likely that if a longer time from release would be examined, the expected differences would be observed. By 16 hours after the release, the second S phase begins and a difference between E2-negative versus E2-positive conditions was clearly observed, with cells under E2- positive conditions entering S and G2/M phases earlier than under E2-negative conditions (Supplemental Figure 3). If double thymidine were to be chosen as an arresting agent, cells would need to be collected at time points for an additional 8-10 hours.



Supplemental Figure 2: Double thymidine treatment synchronizes MCF-7 cells at G1 phase. Two different schedules for thymidine treatment were used (24:12:24 or 36:18:36) to arrest MCF-7 cells at G1 phase. Cells were harvest every 4 hours after release and subjected to flow cytometry. The percentage of cells in each cell cycle phase is shown.



Supplemental Figure 3: Effects of estradiol on cell cycle phases do not occur until second cell cycle after arrest with double thymidine.

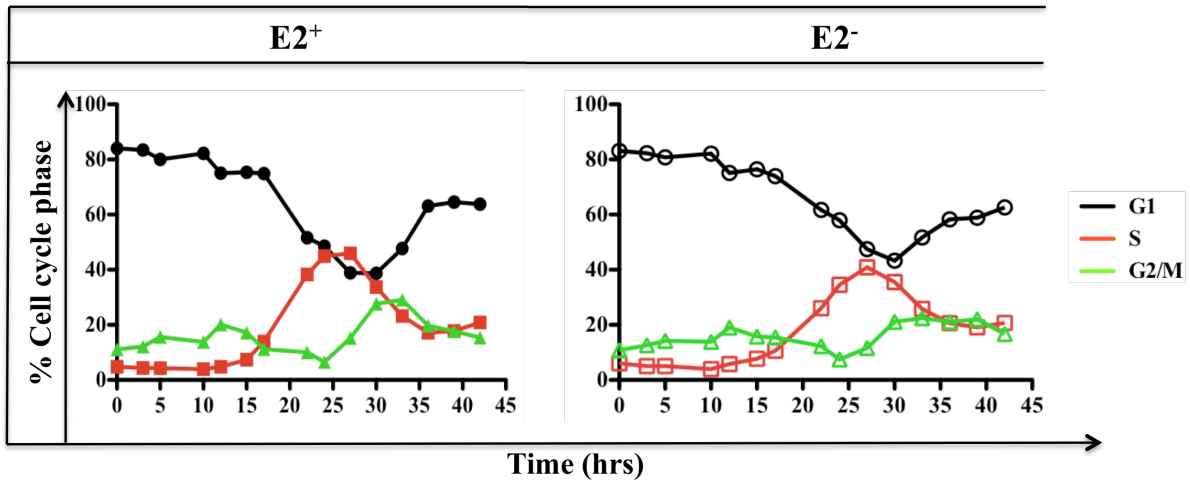
MCF-7 cells were synchronized by double thymidine using the 24:12:24 treatment schedule. Cells were released under E2-positive or E2-negative conditions and cells were collected for FACS analysis and presented as the percentage of cells in each cell cycle phase.

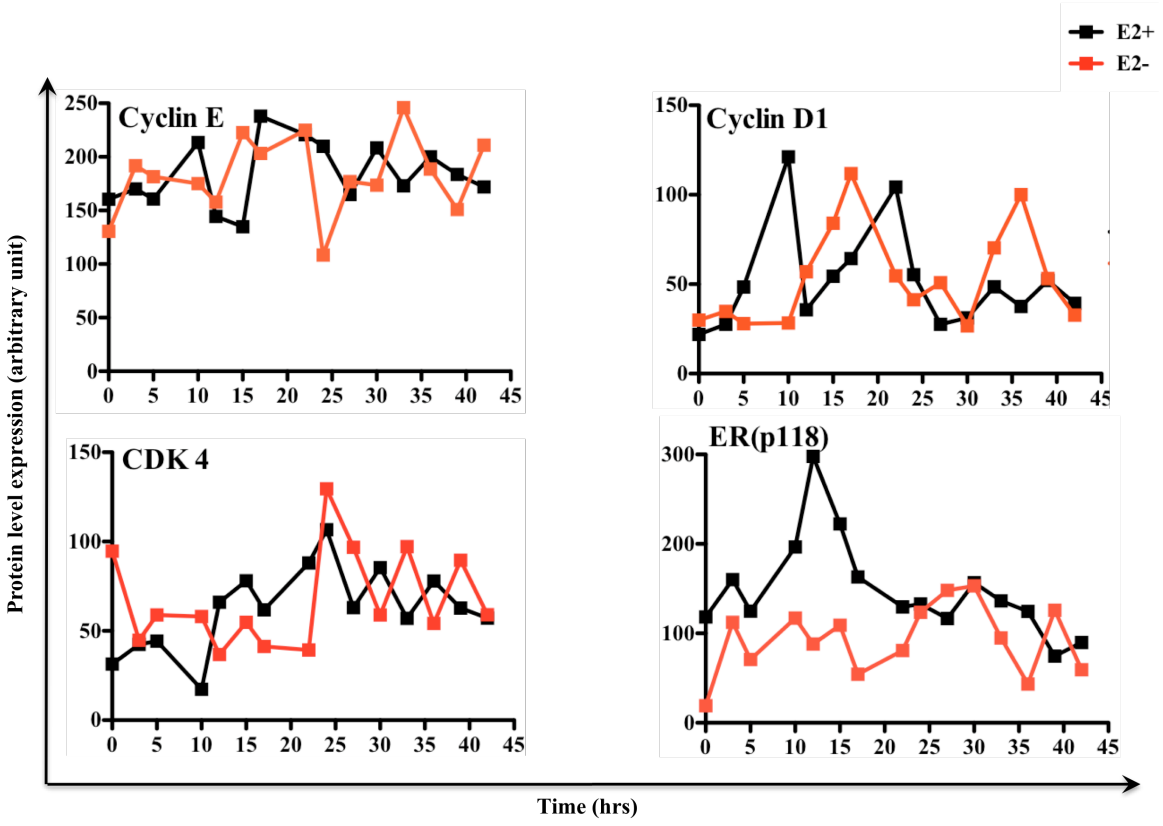
RPPA can be used to screen proteins activated by the ERα pathway.

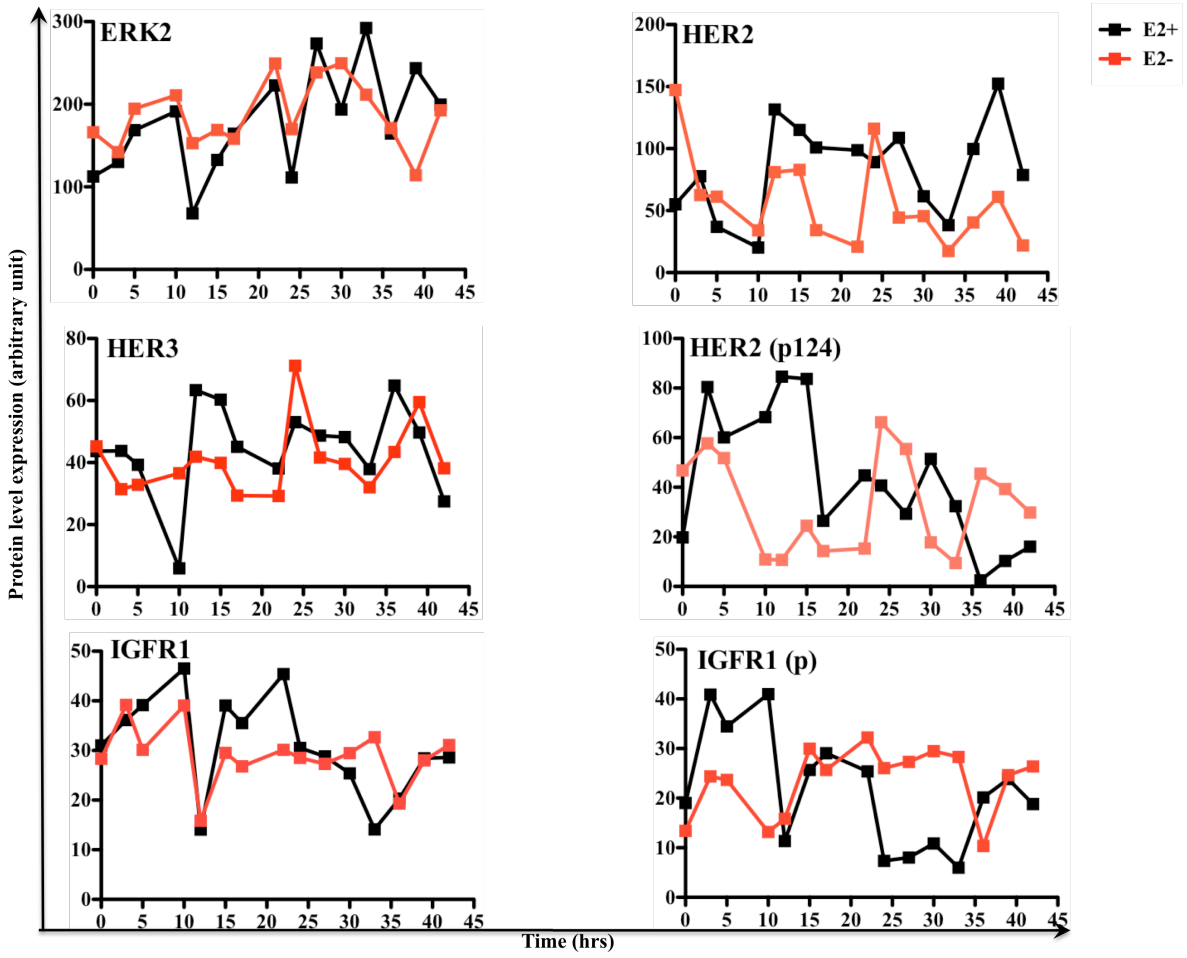
Reverse-Phase Protein Array (RPPA) analysis is an ideal method to examine the expression of a series of proteins in parallel. RPPA is a method to measure the protein expression levels (just like western blot) in a large number of biological samples using high quality antibodies [491]. RPPA is also subject to accurate quantitaton as serial dilutions of the test lysates are printed on each slide alongside with known amounts of protein as controls. RPPA was performed in collaboration with Dr. Gordon Mills' laboratory. The expression pattern of a series of proteins (the list of all proteins examined is in supplemental Figure 4) during the cell cycle was examined in relation to cell cycle phase and ERα expression. Lovastatin synchronized MCF-7 cells, which had been divided in to two groups of E2-treated versus the E2-untreated were harvested and diluted in ppi (protease/phosphatase inhibitor-components listed under western blot analysis in chapter II) and sample buffer to the final concentration of 1μg/μl and subjected to RPPA analysis in the Mills' laboratory.

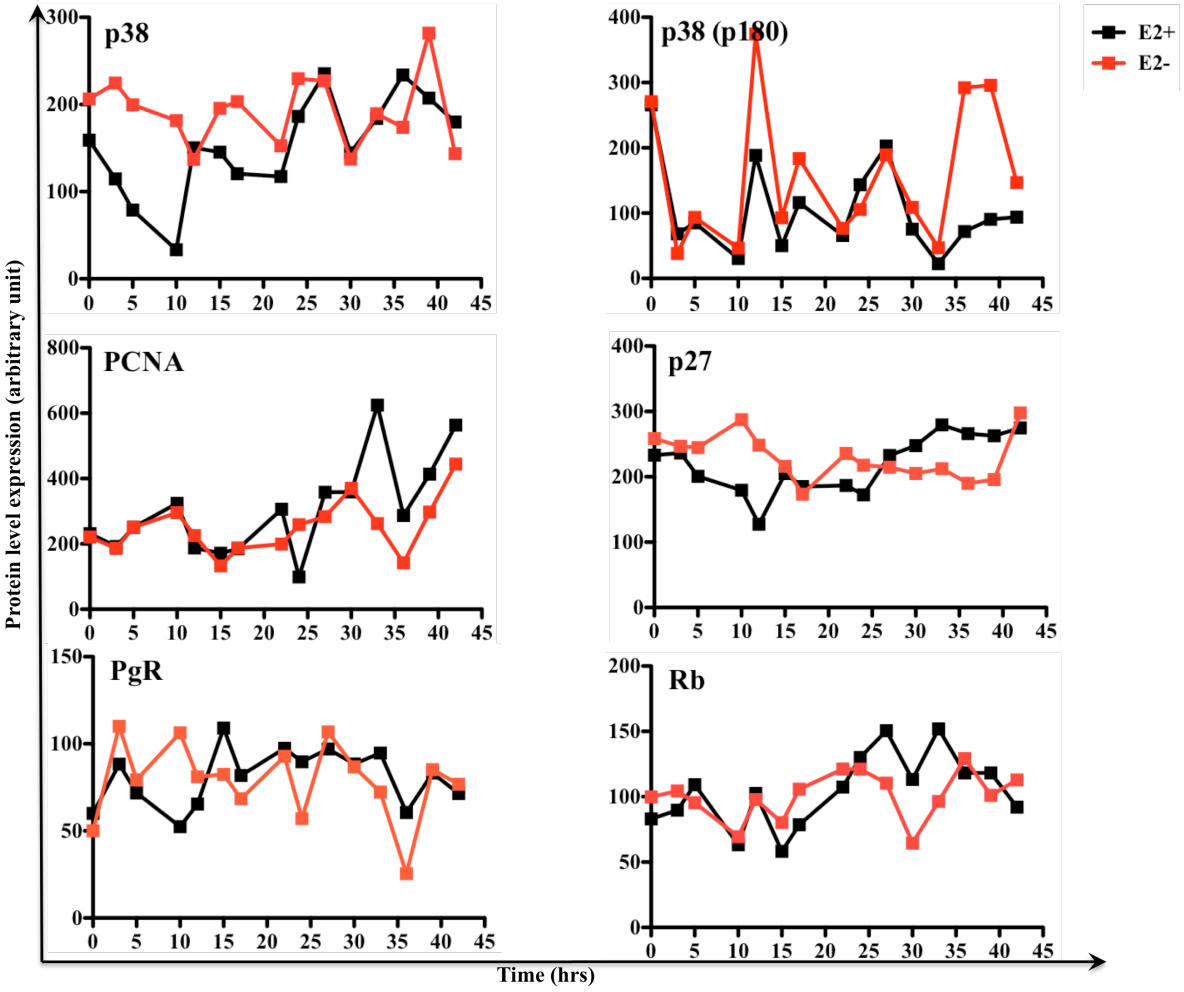
The samples were immobilized (printed) on individual spots on as many microarray slides as there are antibodies. Then each slide was incubated with a single specific antibody to detect expression of the target protein on all the samples simultaneously. Detection was performed using a primary and a secondary labeled antibody. Chemiluminescence was used to detect the expression. The values resulting from intensity of antibody detection were plotted in graphs in (Figures 40 and supplemental Figure 4).

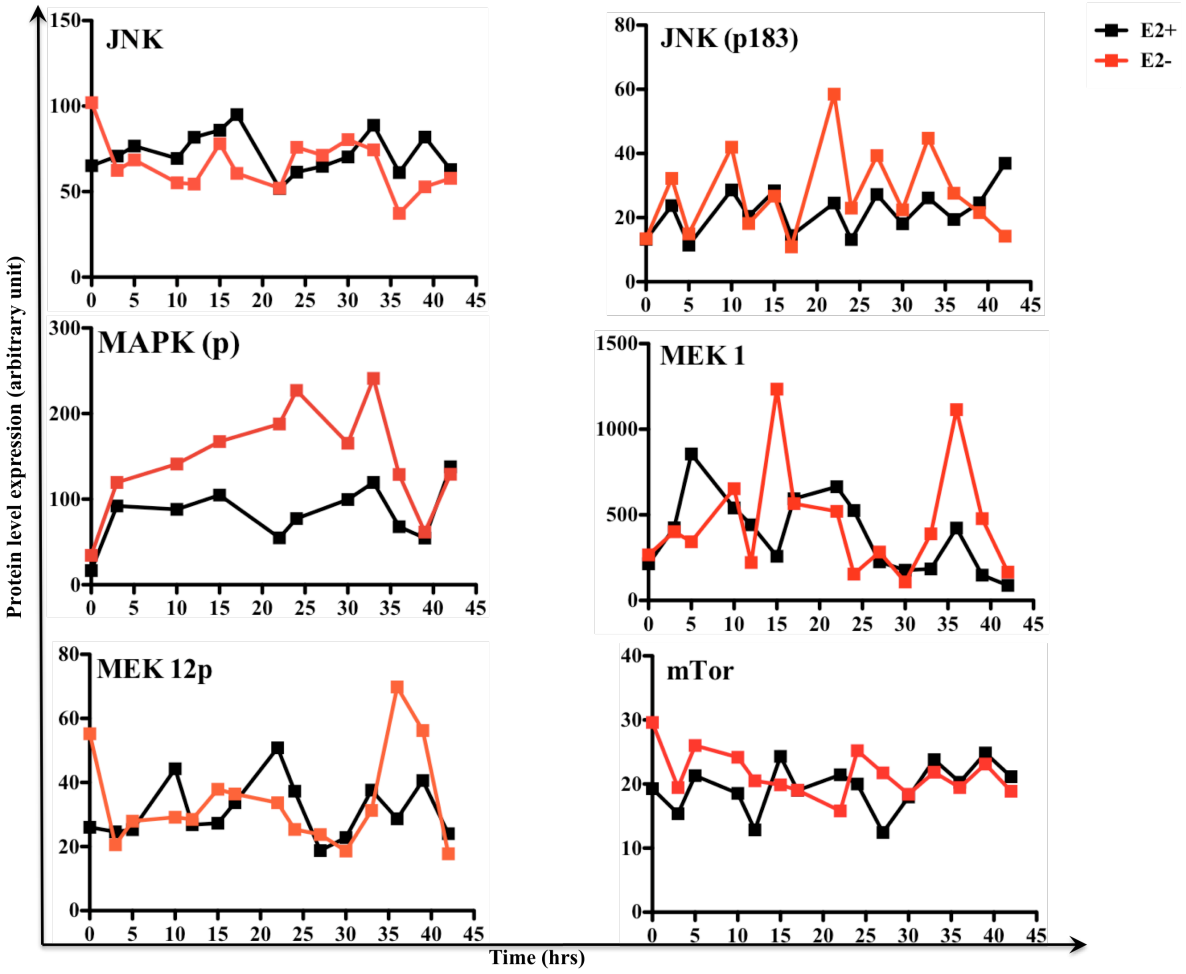
Using this analysis we found that three proteins (ERp118, MAPKp, and AKTp473) had a very different pattern of cell cycle expression depending on whether or not E2 was present (Figure 40). However there was no detectable change in the rest of the examined proteins between E2-positive (black) versus E2-negative (red) (Supplemental Figure 4).

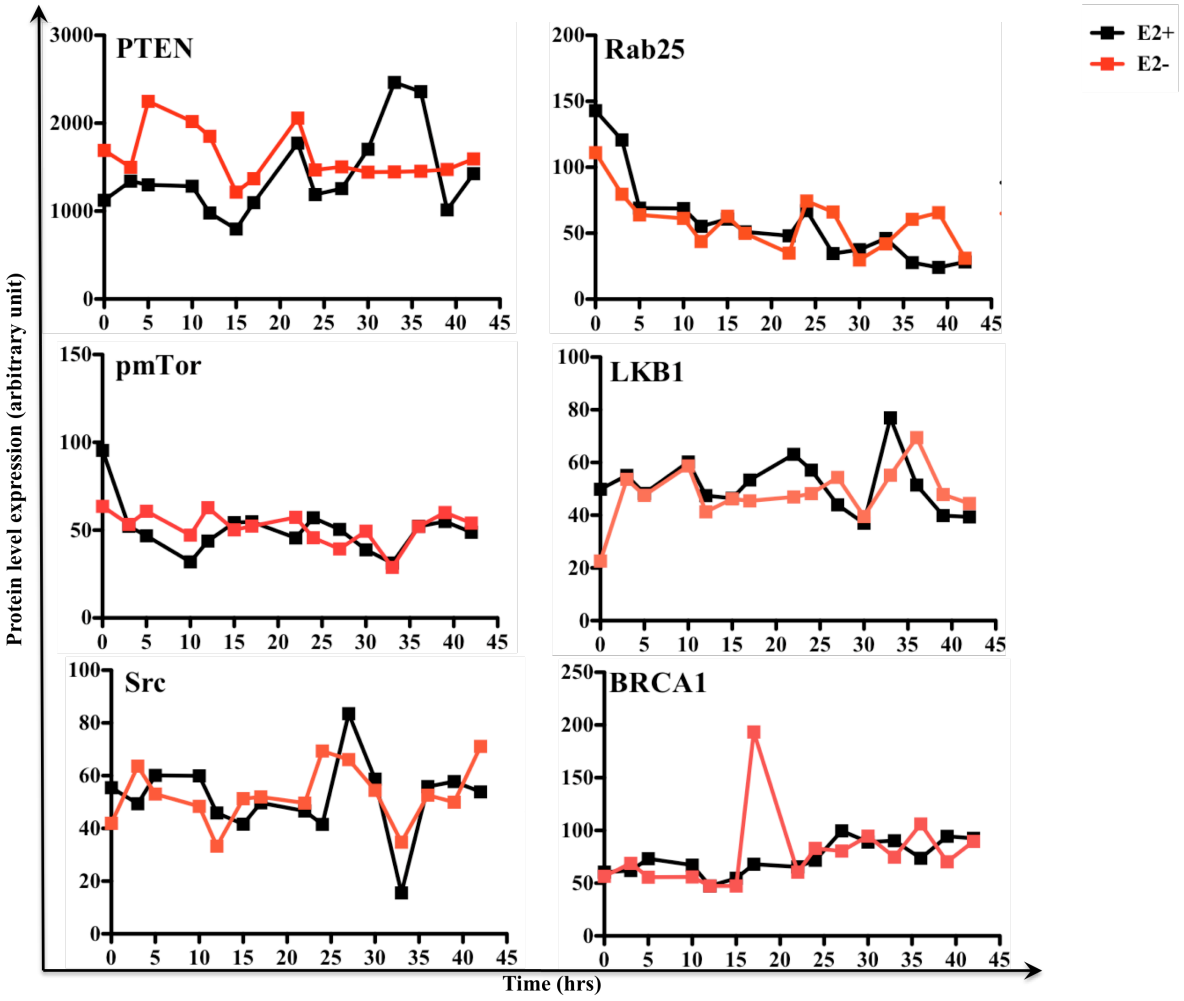

E2+
E2-
% Cell cycle phase
Time (hrs)
G1
S
G2/M

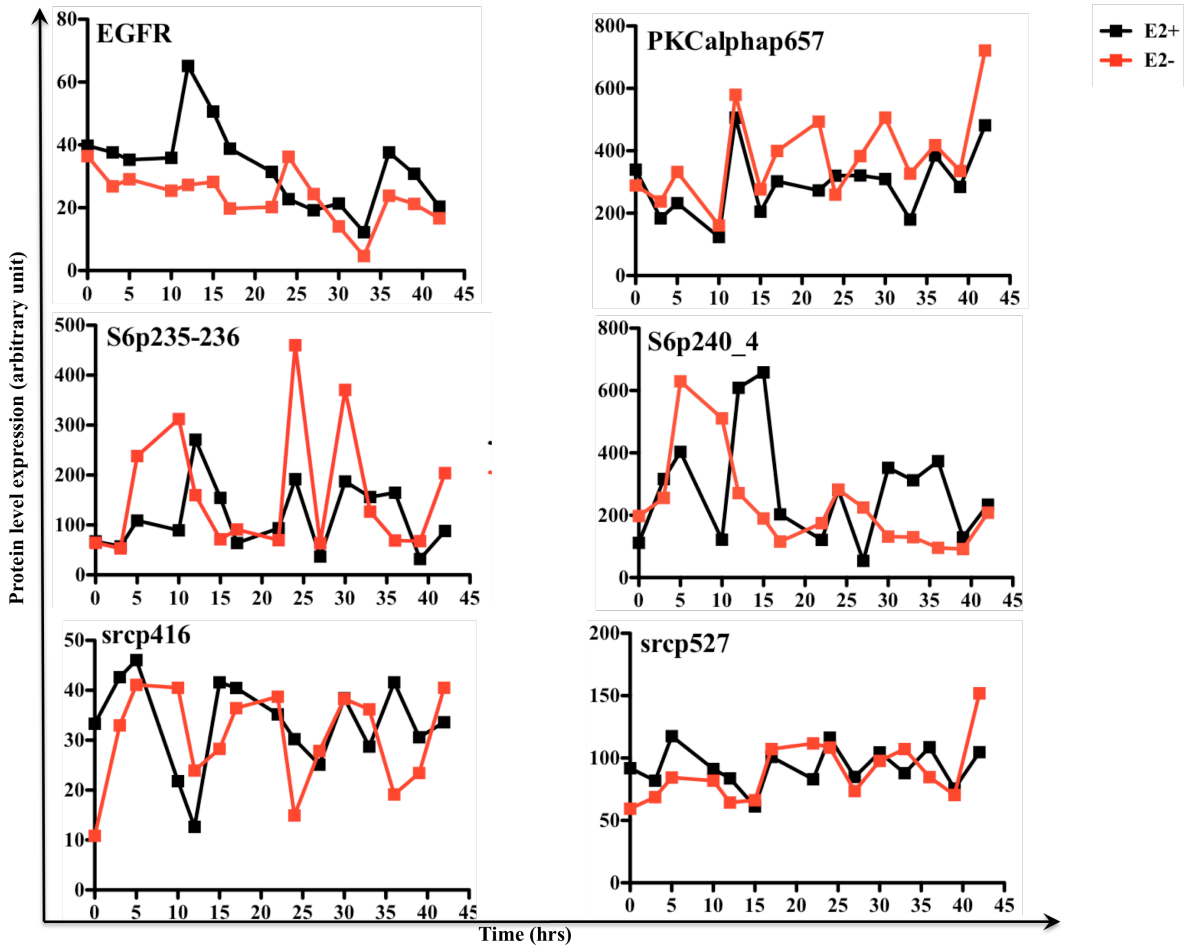

E2+
E2-
Cyclin E
Cyclin D1
CDK 4
ER(p118)
Protein level expression (arbitrary unit)
Time (hrs)

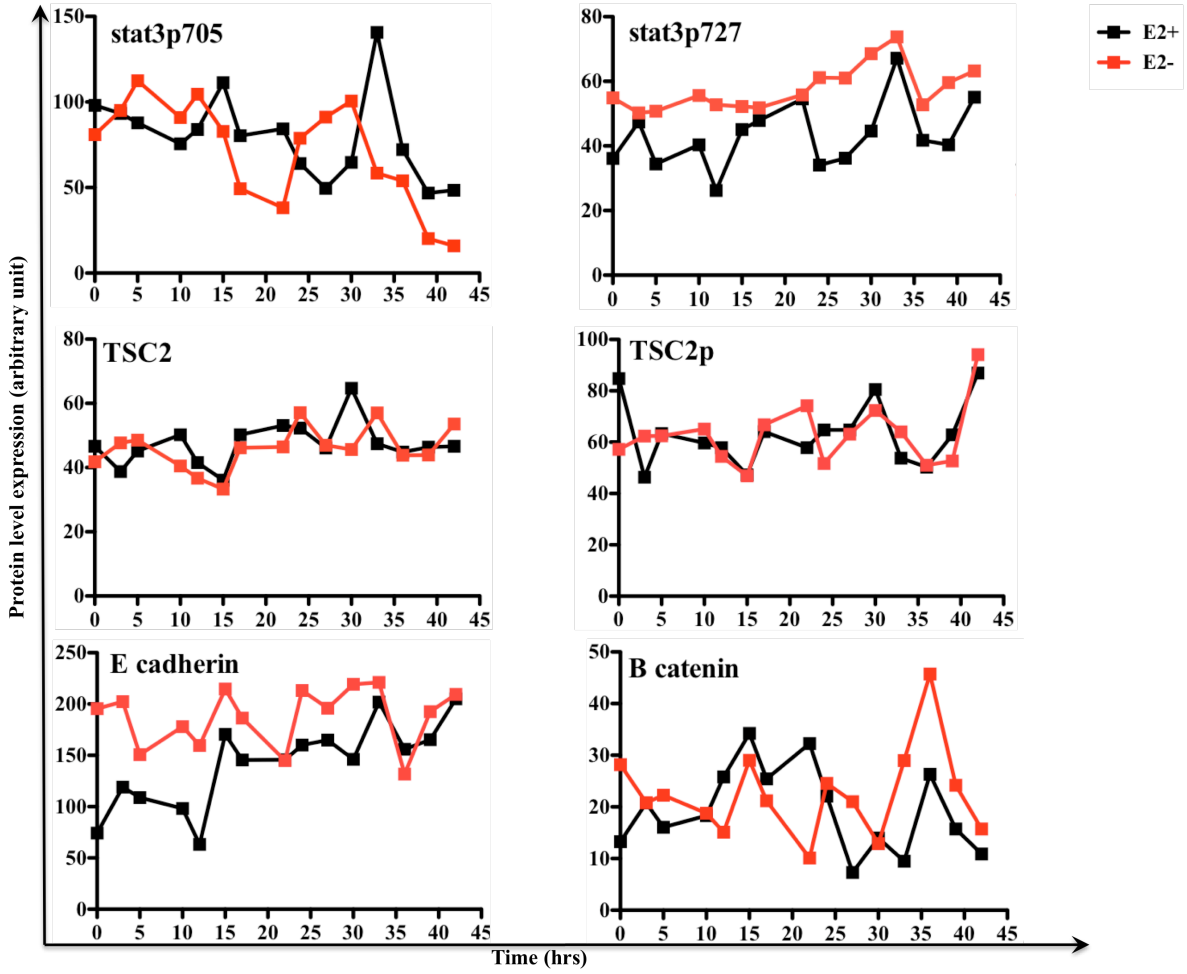

ERK2
HER2
HER3
HER2 (p124)
IGFR1
IGFR1 (p)
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)

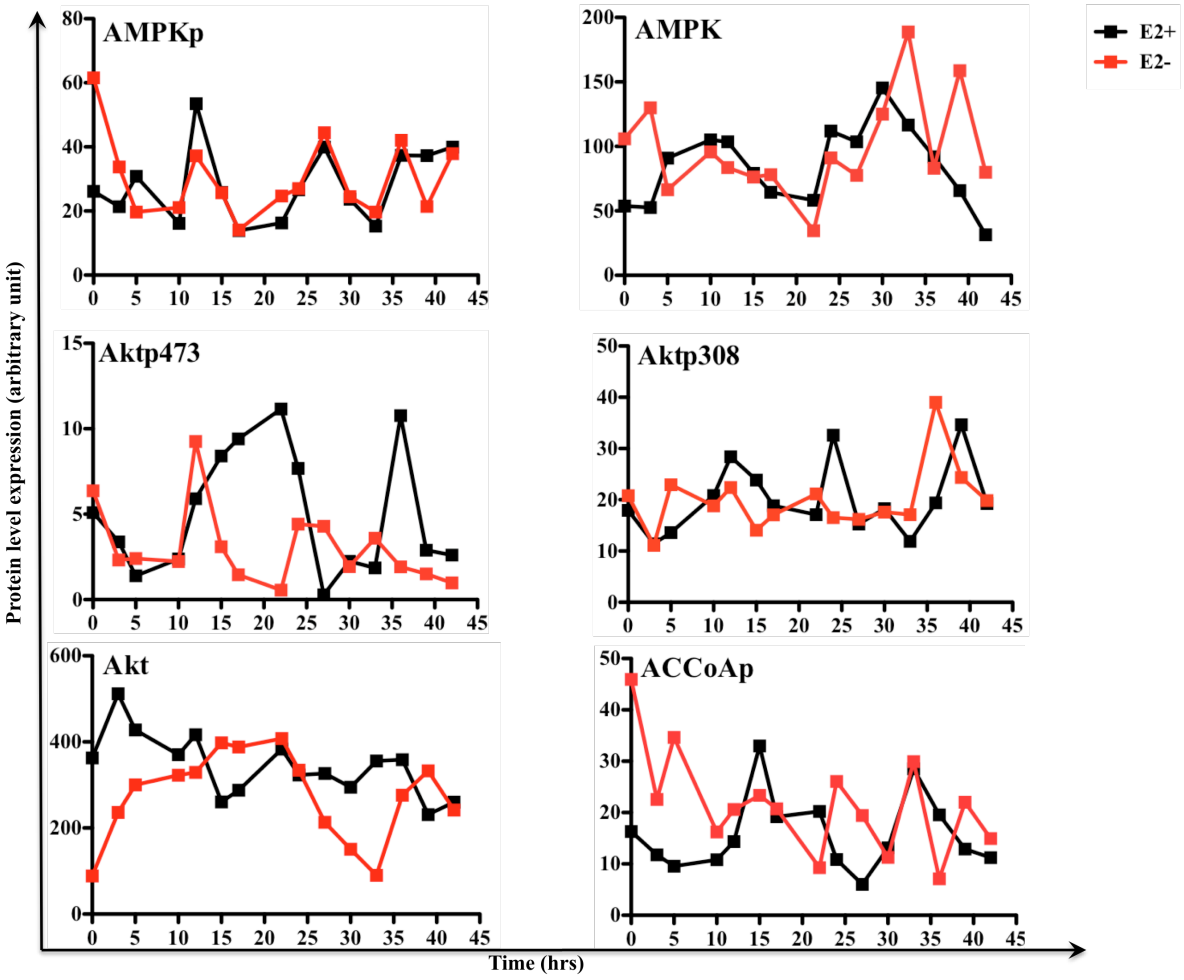

p38
p38 (p180)
PCNA
p27
PgR
Rb
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)

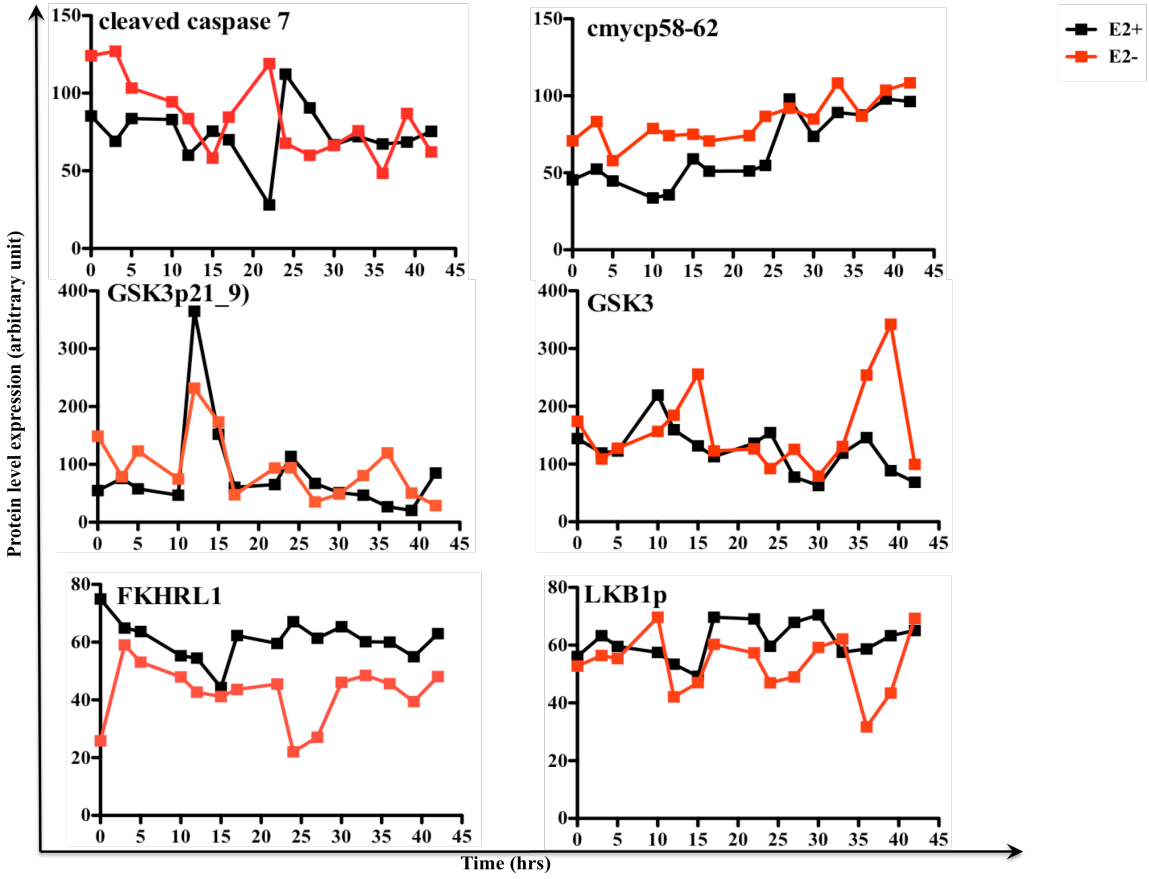

JNK
JNK (p183)
MAPK (p)
MEK 1
MEK 12p
mTor
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)

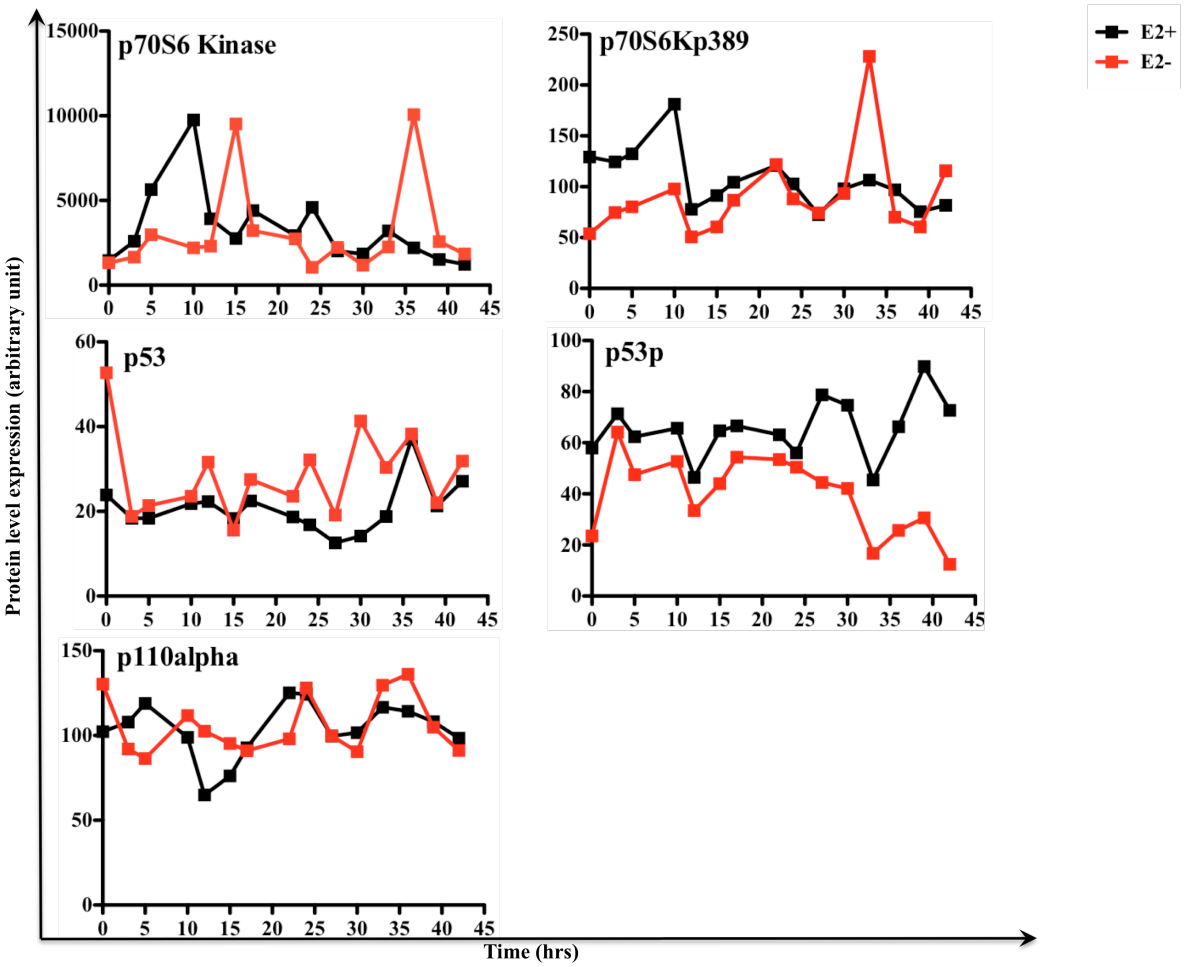

PTEN
Rab25
pmTor
LKB1
Src
BRCA1
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)


EGFR
PKCalphap657
S6p235-236
S6p240_4
srcp416
srcp527
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)


stat3p705
stat3p727
TSC2
TSC2p
E cadherin
B catenin
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)


AMPKp
AMPK
Aktp473
Aktp308
Akt
ACCoAp
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)


cleaved caspase 7
cmycp58-62
GSK3p21_9)
GSK3
FKHRL1
LKB1p
E2+
E2-
Protein level expression (arbitrary unit)
Time (hrs)



Supplemental Figure 4: RPPA detects differential protein expression in key signaling proteins when ERα is liganded to E2 in MCF-7 cells. RPPA analysis was performed on lovastatin synchronized MCF-7 cells in the presence and absence of E2. (A) FACS analysis shows the percentage of cells in each cell cycle phase after release from arrest. (B) Lysates from each time point were subjected to RPPA to detect the expression of an array of protein as marked on top left of each graph in the presence of E2 (black) and absence of E2 (red).

Chapter V: Concluding Remarks & Future Directions

The data presented in this dissertation clearly show that the ligand status of ERα plays a direct and very important role in ERα driven cell cycle effects. The results are supported by using several different assays including FACS and western blot analyses and using multiple cell lines to verify that this is a general effect. Therefore, the conclusions derived from this data can be used to further our understanding of cell proliferation in ER positive compared to ER negative tumors in the presence and absence of estrogen. The following conclusions can be made:

- Endogenous ERα protein in ER-positive cells and exogenous ERα protein in otherwise ER-negative cells regulation, is cell cycle dependent.
- ERα is regulated at the level of protein and not at the transcriptional level.
- Cell cycle regulation of ERα is ligand independent.
- Liganded ERα results in a fast progression through the cell cycle, specifically through S and G2/M phases compared to unliganded ERα.
- Overexpression of ERα either in MCF-7 (ER-positive) and MDA-MB231 cells (ER-negative) cells caused a prolonged S and G2/M phase.
- ERα shows cell cycle specific localization, with ERα residing in the cytoplasm at the end of S phase and moving to the nucleus during G2 and early M phase.
- ERα is in a complex with cyclin B, Cdk1 and Cdc27 during late S, G2 and M
- phase. ERα gets ubiquitinated during S and G2/M phase in order to prepare for its degradation by the proteasome at G2/M phase.
- Liganded ERα increases transcriptional activity at ERE of genes including pS2 and PgR compared to unliganded ERα.

These results support the rationale to treat ER-positive breast cancer patients with a drug that keeps the integrity of the ERα protein intact, while inhibiting the ligand binding to ERα.

Future Directions:

Determine the role of phosphorylated ERα at each phase of the cell cycle.

Future directions include understanding the role of phosphorylation in ERα regulation and whether phosphorylation of ERα is also cell cycle regulated. We will examine whether phosphorylation of ERα results in activation or repression of downstream events and whether phosphorylation depends on ligand. The cyclin A-Cdk2 complex phosphorylates ERα on Serine 104/106, within ER Activation Function-1 domain (AF-1) (22). Cyclin A-Cdk2 kinase activity occurs in the S phase of the cell cycle. Our data showed that S and G2/M phases were the phases of the cell cycle, which were either shortened or elongated by ER in the presence or absence of the ligand, respectively (Figures 14 and 16). Results from RPPA analysis show that ERα that is phosphoryated at amino acid 118 changes in a cell cycle dependent manner, therefore we will expand our focus beyond only cyclin A/Cdk2 as a ERα kinase.

Determine the interaction of ERα with the cytoplasmic pathway(s)

The experiments described throughout this dissertation elucidate the effects of liganded versus unliganded ERα on the cell cycle phases. Initial examination of the proteins involved in the pathways that lead to the cell cycle effects was also performed. For example, ERα co-localized with cyclin B. However, a mechanism for translocation of ERα and cyclin B was not explored. Furthermore, when ERα is cytoplasmic, it could interact with factors of many cytoplasmic pathways, which could ultimately control the proliferation of cells. To this end, a thorough examination of the ERα interactions with both cytoplasmic and nuclear proteins throughout the cell cycle phases should be performed. Mass spectrometry, which is a powerful technique to determine the amino acid sequences of proteins, which can also reveal the structure of the protein, would be a suitable method to pursue this goal. For these experiments, cells will be synchronized in order to enrich cells in different phases. Immunoprecipitation will be performed using anti-ERα and anti phosphorylated-ERα antibodies. A 2-D gel of the immunoprecipiates will be silver stained and subjected to Mass-spectometry. Our expected results would be the identification of binding partners for

liganded versus unliganded ERα during cell cycle phases of the cell cycle that would associate liganded with unliganded ER to the observed biologic effects in these phases of the cell cycle mediated by ERα. Examination of the phosphorylation state of ERα would provide information on whether a kinase is upstream of ERα. For this purpose, we will perform the immunoprecipitation assay using antibodies against other known kinases of ERα such as extracellular signal-regulated kinases1/2 (24), p38 mitogen-activated protein kinase (MAPK), [172, 183], Cdk7 (26), c-Src [168, 534], protein kinase A [405], pp90-rsk1 [184], or AKT [226] to reveal whether these kinases are also involved in phosphorylation of ERα in S and G2/M phase or in the other phases of the cell cycle. Preliminary data also suggested that Cdk1 is a binding partner for ERα. If we reveal the phosphorylation of ERα by any of these kinases, subsequently we will examine the function of the phosphorylated form in the cell cycle. For these set of experiments first we will examine the pattern of phosphorylated ERα during cell cycle phases.

Pursue in depth analysis and examination of the differential nocodazole arrest mechanisms in ER-positive cells compared to ER-negative cells.

As Figure 39 shows, ER-positive cells were unable to exit G2/M upon arrest with nocodazole while ER-negative cells would release from G2/M and enter the G1 phase of the next cell cycle. This was a consistent observation and therefore nocodazole was not used as an agent in to verify the results shown by using lovastatin; instead, aphidicolin, was used as a confirming agent (Figure 16). However, the mechanisms for the differential ability of ER-positive and ER-negative cells to exit from nocodazole arrest could provide more information about the role of ERα in cell cycle regulation. One reason that ER-positive cells did not overcome arrest could be activity of p53. Most of the ER-positive cells have a functional p53 while the ER-negative cells do not [535]. Deregulation of ERα and/or p53 increases the risk of developing mammary preneoplasia [536]. For example, increased ERα expression coupled with p53 heterozygosity is associated with increased levels of phosphorylated AKT and decreased p27 expression.

Aberrations in ERα and p53 also result in a relative increase in Src phosphorylation, leading to cell proliferation [537]. Src and ERα levels were shown to be inversely correlated in primary breast cancers, leading Chu et al. to observation that c-Src phosphorylation

stimulates ERα ubiquitynation and proteasome-dependent degradation [534]. Suppression of Src-induced migration and invasion is one possible pathway that p53 utilizes to inhibit tumor progression and metastasis [538]. Studies have also shown that p53 can directly regulate ERα expression and transcriptional activity [539].

All of these studies suggest an association between ERα and p53 and could help to explain the discrepancy observed in the release, and lack thereof, of ER-negative and ER-positive cells from nocodazole arrest, respectively. Based on the findings by Shirley et al. that p53 regulates ERα transcription [540], it is speculated that accumulation of ERα at G2/M phase due to nocodazole arrest up regulates p53. This feedback mechanism between ERα and p53 and the potential contribution to inhibition of cell cycle progression could be the reason that ERα status is a strong prognostic factor in breast cancer. Hormone-receptor status is also a predictor of response to endocrine therapy in breast cancer. The data presented in this dissertation suggest that ERα expression leads to a favorable outcome in breast cancer both because it provides a target for endocrine therapy and also because the presence of unliganded ERα showing and inhibitory effect on the cell cycle progression.

REFERENCES:

1. Basu A, Rowan BG. Genes related to estrogen action in reproduction and breast cancer. Front Biosci 2005; 10:2346-72.
2. Saji S, Jensen EV, Nilsson S, Rylander T, Warner M, Gustafsson JA. Estrogen receptors alpha and beta in the rodent mammary gland. Proc Natl Acad Sci U S A 2000; 97:337-42.
3. Clarke RB, Howell A, Anderson E. Estrogen sensitivity of normal human breast tissue in vivo and implanted into athymic nude mice: analysis of the relationship between estrogen-induced proliferation and progesterone receptor expression. Breast Cancer Res Treat 1997; 45:121-33.
4. Russo J, Reina D, Frederick J, Russo IH. Expression of phenotypical changes by human breast epithelial cells treated with carcinogens in vitro. Cancer Res 1988; 48:2837-57.
5. Russo J, Calaf G, Russo IH. A critical approach to the malignant transformation of human breast epithelial cells with chemical carcinogens. Crit Rev Oncog 1993; 4:403-17.
6. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ. Comparative study of human and rat mammary tumorigenesis. Lab Invest 1990; 62:244-78.
7. Anderson E, Clarke RB. Steroid receptors and cell cycle in normal mammary epithelium. J Mammary Gland Biol Neoplasia 2004; 9:3-13.
8. Fujimoto N, Jinno N, Kitamura S. Activation of estrogen response element dependent transcription by thyroid hormone with increase in estrogen receptor levels in a rat pituitary cell line, GH3. J Endocrinol 2004; 181:77-83.
9. Allred DC, Brown P, Medina D. The origins of estrogen receptor alpha-positive and estrogen receptor alpha-negative human breast cancer. Breast Cancer Res 2004; 6:240-5.
10. Sims AH, Howell A, Howell SJ, Clarke RB. Origins of breast cancer subtypes and therapeutic implications. Nat Clin Pract Oncol 2007; 4:516-25.
11. Kuukasjarvi T, Kononen J, Helin H, Holli K, Isola J. Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. J Clin Oncol 1996; 14:2584-9.
12. Asselin-Labat ML, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE, Lindeman GJ. Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst 2006; 98:1011-4.

13. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ. Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. J Cell Biol 2007; 176:19-26.
14. Smith RA, Cokkinides V, Brooks D, Saslow D, Brawley OW. Cancer screening in the United States, 2010: a review of current American Cancer Society guidelines and issues in cancer screening. CA Cancer J Clin; 60:99-119.
15. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. CA Cancer J Clin.
16. Patel JD. Lung cancer: a biologically different disease in women? Womens Health (Lond Engl) 2009; 5:685-91.
17. Berg WA. Benefits of screening mammography. JAMA; 303:168-9.
18. Ruoslahti E. How cancer spreads. Sci Am 1996; 275:72-7.
19. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. Nat Rev Cancer 2007; 7:659-72.
20. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. Nature 2000; 406:747-52.
21. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98:10869-74.
22. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lonning PE, Brown PO, Borresen-Dale AL, Botstein D. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003; 100:8418-23.
23. Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L, Nobel A, Parker J, Ewend MG, Sawyer LR, Wu J, Liu Y, Nanda R, Tretiakova M, Ruiz Orrico A, Dreher D, Palazzo JP, Perreard L, Nelson E, Mone M, Hansen H, Mullins M, Quackenbush JF, Ellis MJ, Olopade OI, Bernard PS, Perou CM. The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 2006; 7:96.

24. Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G. Breast cancer. Lancet 2005; 365:1727-41.
25. Maartense S, Hermans J, Leer JW. Radiation therapy in localized prostate cancer: long-term results and late toxicity. Clin Oncol (R Coll Radiol) 2000; 12:222-8.
26. Valerie K, Dritschilo A, McKenna G, Schmidt-Ullrich RK. Novel molecular targets for tumor radiosensitization: Molecular Radiation Biology and Oncology Workshop: translation of molecular mechanisms into clinical radiotherapy. Int J Cancer 2000; 90:51-8.
27. Chabner BA, Roberts TG, Jr. Timeline: Chemotherapy and the war on cancer. Nat Rev Cancer 2005; 5:65-72.
28. Jonat W, Pritchard KI, Sainsbury R, Klijn JG. Trends in endocrine therapy and chemotherapy for early breast cancer: a focus on the premenopausal patient. J Cancer Res Clin Oncol 2006; 132:275-86.
29. Chris H. Takimoto M, PhD, and Emiliano Calvo, MD, PhD. Principles of Oncologic Pharmacotherapy. Cancer Management Handbook- Principles of Oncologic Pharmacotherapy 2009; 11th Edition:1-4.
30. Maughan KL, Lutterbie MA, Ham PS. Treatment of breast cancer. Am Fam Physician; 81:1339-46.
31. Untch M. Targeted Therapy for Early and Locally Advanced Breast Cancer. Breast Care (Basel); 5:144-52.
32. Takada M, Toi M. [Primary systemic chemotherapy for breast cancer]. Gan To Kagaku Ryoho 2007; 34:1730-4.
33. Walker RA. Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment I. Histopathology 2006; 49:406-10.
34. Azizun N, Bhurgri Y, Raza F, Kayani N. Comparison of ER, PR and HER-2/neu (C-erb B 2) reactivity pattern with histologic grade, tumor size and lymph node status in breast cancer. Asian Pac J Cancer Prev 2008; 9:553-6.
35. Qureshi A, Pervez S. Allred scoring for ER reporting and it's impact in clearly distinguishing ER negative from ER positive breast cancers. J Pak Med Assoc; 60:350-3.
36. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999; 17:1474-81.

37. Horwitz KB, McGuire WL. Predicting response to endocrine therapy in human breast cancer: a hypothesis. Science 1975; 189:726-7.
38. Degenshein GA, Bloom N, Tobin E. The value of progesterone receptor assays in the management of advanced breast cancer. Cancer 1980; 46:2789-93.
39. Mohsin SK, Weiss H, Havighurst T, Clark GM, Berardo M, Roanh le D, To TV, Qian Z, Love RR, Allred DC. Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. Mod Pathol 2004; 17:1545-54.
40. Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma. Suggestions for a new method of treatment with illustrative cases. Lancet 1896; 2:104–7.
41. Lonning PE. Additive endocrine therapy for advanced breast cancer - back to the future. Acta Oncol 2009; 48:1092-101.
42. Moursi GE, Abdel-Daim MH, Kelada NL, Abdel-Tawab GA, Girgis LH. The influence of sex, age, synthetic oestrogens, progestogens and oral contraceptives on the excretion of urinary tryptophan metabolites. Bull World Health Organ 1970; 43:651-61.
43. Dao TL, Huggins C. Bilateral adrenalectomy in the treatment of cancer of the breast. AMA Arch Surg 1955; 71:645-57.
44. Nevinny-Stickel HB, Dederick MM, Haines CR, Hall TC. Comparative Study of 6-Dehydro-17alpha-Methyltestosterone and Testosterone Propionate in Human Breast Cancer. Cancer 1964; 17:95-9.
45. Lemon HM. Prednisone therapy of advanced mammary cancer. Cancer 1959; 12:93-107.
46. Lundgren S. Progestins in breast cancer treatment. A review. Acta Oncol 1992; 31:709-22.
47. Lerner LJ, Holthaus FJ, Jr., Thompson CR. A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 1958; 63:295-318.
48. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967; 13:101-19.
49. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 1971; 25:270-5.

50. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 1976; 12:419-24.
51. Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 randomized trial. J Natl Cancer Inst 2001; 93:684-90.
52. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987; 10:31-5.
53. Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326:852-6.
54. Horwitz KB. When tamoxifen turns bad. Endocrinology 1995; 136:821-3.
55. Kansra S, Yamagata S, Sneade L, Foster L, Ben-Jonathan N. Differential effects of estrogen receptor antagonists on pituitary lactotroph proliferation and prolactin release. Mol Cell Endocrinol 2005; 239:27-36.
56. Addo S, Yates RA, Laight A. A phase I trial to assess the pharmacology of the new oestrogen receptor antagonist fulvestrant on the endometrium in healthy postmenopausal volunteers. Br J Cancer 2002; 87:1354-9.
57. Bergman L, Beelen ML, Gallee MP, Hollema H, Benraadt J, van Leeuwen FE. Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. Lancet 2000; 356:881-7.
58. Fawell SE, White R, Hoare S, Sydenham M, Page M, Parker MG. Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. Proc Natl Acad Sci U S A 1990; 87:6883-7.
59. Dauvois S, White R, Parker MG. The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. J Cell Sci 1993; 106 (Pt 4):1377-88.
60. Wakeling AE. The future of new pure antiestrogens in clinical breast cancer. Breast Cancer Res Treat 1993; 25:1-9.

61. Dauvois S, Danielian PS, White R, Parker MG. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. Proc Natl Acad Sci U S A 1992; 89:4037-41.
62. Valachis A, Mauri D, Polyzos NP, Mavroudis D, Georgoulias V, Casazza G. Fulvestrant in the treatment of advanced breast cancer: a systematic review and meta-analysis of randomized controlled trials. Crit Rev Oncol Hematol; 73:220-7.
63. Howell A, Robertson JF, Abram P, Lichinitser MR, Elledge R, Bajetta E, Watanabe T, Morris C, Webster A, Dimery I, Osborne CK. Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: a multinational, double-blind, randomized trial. J Clin Oncol 2004; 22:1605-13.
64. Bonneterre J, Thurlimann B, Robertson JF, Krzakowski M, Mauriac L, Koralewski P, Vergote I, Webster A, Steinberg M, von Euler M. Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomized Group Efficacy and Tolerability study. J Clin Oncol 2000; 18:3748-57.
65. Howell SJ, Johnston SR, Howell A. The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. Best Pract Res Clin Endocrinol Metab 2004; 18:47-66.
66. Miller WR. Aromatase inhibitors: mechanism of action and role in the treatment of breast cancer. Semin Oncol 2003; 30:3-11.
67. Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, Jones SE, Alvarez I, Bertelli G, Ortmann O, Coates AS, Bajetta E, Dodwell D, Coleman RE, Fallowfield LJ, Mickiewicz E, Andersen J, Lonning PE, Cocconi G, Stewart A, Stuart N, Snowdon CF, Carpentieri M, Massimini G, Bliss JM, van de Velde C. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 2004; 350:1081-92.
68. Santen RJ, Samojlik E, Lipton A, Harvey H, Ruby EB, Wells SA, Kendall J. Kinetic, hormonal and clinical studies with aminoglutethimide in breast cancer. Cancer 1977; 39:2948-58.

69. Petru E, Schmahl D. On the role of additive hormone monotherapy with tamoxifen, medroxyprogesterone acetate and aminoglutethimide, in advanced breast cancer. Klin Wochenschr 1987; 65:959-66.
70. Hoffken K. Experience with aromatase inhibitors in the treatment of advanced breast cancer. Cancer Treat Rev 1993; 19 Suppl B:37-44.
71. Santen RJ, Manni A, Harvey H, Redmond C. Endocrine treatment of breast cancer in women. Endocr Rev 1990; 11:221-65.
72. Raats JI, Falkson G, Falkson HC. A study of fadrozole, a new aromatase inhibitor, in postmenopausal women with advanced metastatic breast cancer. J Clin Oncol 1992; 10:111-6.
73. Perez Carrion R, Alberola Candel V, Calabresi F, Michel RT, Santos R, Delozier T, Goss P, Mauriac L, Feuilhade F, Freue M, et al. Comparison of the selective aromatase inhibitor formestane with tamoxifen as first-line hormonal therapy in postmenopausal women with advanced breast cancer. Ann Oncol 1994; 5 Suppl 7:S19-24.
74. Campos SM. Aromatase inhibitors for breast cancer in postmenopausal women. Oncologist 2004; 9:126-36.
75. Coombes RC, Kilburn LS, Snowdon CF, Paridaens R, Coleman RE, Jones SE, Jassem J, Van de Velde CJ, Delozier T, Alvarez I, Del Mastro L, Ortmann O, Diedrich K, Coates AS, Bajetta E, Holmberg SB, Dodwell D, Mickiewicz E, Andersen J, Lonning PE, Cocconi G, Forbes J, Castiglione M, Stuart N, Stewart A, Fallowfield LJ, Bertelli G, Hall E, Bogle RG, Carpentieri M, Colajori E, Subar M, Ireland E, Bliss JM. Survival and safety of exemestane versus tamoxifen after 2-3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. Lancet 2007; 369:559-70.
76. Mouridsen H, Giobbie-Hurder A, Goldhirsch A, Thurlimann B, Paridaens R, Smith I, Mauriac L, Forbes JF, Price KN, Regan MM, Gelber RD, Coates AS. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. N Engl J Med 2009; 361:766-76.
77. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001; 1:222-31.
78. Smith JA, Martin L. Do cells cycle? Proc Natl Acad Sci U S A 1973; 70:1263-7.

79. Nigg EA. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. Bioessays 1995; 17:471-80.
80. Schafer KA. The cell cycle: a review. Vet Pathol 1998; 35:461-78.
81. Bloom J, Cross FR. Multiple levels of cyclin specificity in cell-cycle control. Nat Rev Mol Cell Biol 2007; 8:149-60.
82. Resnitzky D, Reed SI. Different roles for cyclins D1 and E in regulation of the G1-to-S transition. Mol Cell Biol 1995; 15:3463-9.
83. Ito M. Factors controlling cyclin B expression. Plant Mol Biol 2000; 43:677-90.
84. Kurzawa L, Morris MC. Cell-cycle markers and biosensors. Chembiochem; 11:1037-47.
85. Kimura K, Hirano M, Kobayashi R, Hirano T. Phosphorylation and activation of 13S condensin by Cdc2 in vitro. Science 1998; 282:487-90.
86. Heald R, McKeon F. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. Cell 1990; 61:579-89.
87. Igarashi M, Nagata A, Jinno S, Suto K, Okayama H. Wee1(+)-like gene in human cells. Nature 1991; 353:80-3.
88. Berry LD, Gould KL. Regulation of Cdc2 activity by phosphorylation at T14/Y15. Prog Cell Cycle Res 1996; 2:99-105.
89. van de Weerdt BC, Medema RH. Polo-like kinases: a team in control of the division. Cell Cycle 2006; 5:853-64.
90. Hagting A, Jackman M, Simpson K, Pines J. Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Curr Biol 1999; 9:680-9.
91. Yang J, Song H, Walsh S, Bardes ES, Kornbluth S. Combinatorial control of cyclin B1 nuclear trafficking through phosphorylation at multiple sites. J Biol Chem 2001; 276:3604-9.
92. Peters JM. The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat Rev Mol Cell Biol 2006; 7:644-56.
93. Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 1997; 13:261-91.

94. Norbury C, Blow J, Nurse P. Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates. EMBO J 1991; 10:3321-9.
95. Ekholm SV, Reed SI. Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 2000; 12:676-84.
96. Takizawa CG, Morgan DO. Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. Curr Opin Cell Biol 2000; 12:658-65.
97. Tannoch VJ, Hinds PW, Tsai LH. Cell cycle control. Adv Exp Med Biol 2000; 465:127-40.
98. Eward KL, Van Ert MN, Thornton M, Helmstetter CE. Cyclin mRNA stability does not vary during the cell cycle. Cell Cycle 2004; 3:1057-61.
99. Koepp DM, Harper JW, Elledge SJ. How the cyclin became a cyclin: regulated proteolysis in the cell cycle. Cell 1999; 97:431-4.
100. Elledge SJ, Harper JW. The role of protein stability in the cell cycle and cancer. Biochim Biophys Acta 1998; 1377:M61-70.
101. Yanagida M. Fission yeast cut mutations revisited: control of anaphase. Trends Cell Biol 1998; 8:144-9.
102. Sigrist S, Jacobs H, Stratmann R, Lehner CF. Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. EMBO J 1995; 14:4827-38.
103. Diehl JA, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev 1997; 11:957-72.
104. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 1998; 12:3499-511.
105. Scheffner M, Nuber U, Huibregtse JM. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature 1995; 373:81-3.
106. Paterlini P, Suberville AM, Zindy F, Melle J, Sonnier M, Marie JP, Dreyfus F, Brechot C. Cyclin A expression in human hematological malignancies: a new marker of cell proliferation. Cancer Res 1993; 53:235-8.
107. Donnellan R, Chetty R. Cyclin E in human cancers. FASEB J 1999; 13:773-80.

108. Pruneri G, Mazzarol G, Fabris S, Del Curto B, Bertolini F, Neri A, Viale G. Cyclin D3 immunoreactivity in gastrointestinal stromal tumors is independent of cyclin D3 gene amplification and is associated with nuclear p27 accumulation. Mod Pathol 2003; 16:886-92.
109. Pontano LL, Aggarwal P, Barbash O, Brown EJ, Bassing CH, Diehl JA. Genotoxic stress-induced cyclin D1 phosphorylation and proteolysis are required for genomic stability. Mol Cell Biol 2008; 28:7245-58.
110. Strohmaier H, Spruck CH, Kaiser P, Won KA, Sangfelt O, Reed SI. Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. Nature 2001; 413:316-22.
111. Patton EE, Willems AR, Tyers M. Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. Trends Genet 1998; 14:236-43.
112. Fu M, Wang C, Li Z, Sakamaki T, Pestell RG. Minireview: Cyclin D1: normal and abnormal functions. Endocrinology 2004; 145:5439-47.
113. Yang K, Hitomi M, Stacey DW. Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell. Cell Div 2006; 1:32.
114. Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev 1993; 7:812-21.
115. Cross DA, Alessi DR, Vandenheede JR, McDowell HE, Hundal HS, Cohen P. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. Biochem J 1994; 303 (Pt 1):21-6.
116. Saito Y, Vandenheede JR, Cohen P. The mechanism by which epidermal growth factor inhibits glycogen synthase kinase 3 in A431 cells. Biochem J 1994; 303 (Pt 1):27-31.
117. Keenan SM, Lents NH, Baldassare JJ. Expression of cyclin E renders cyclin D-CDK4 dispensable for inactivation of the retinoblastoma tumor suppressor protein, activation of E2F, and G1-S phase progression. J Biol Chem 2004; 279:5387-96.
118. Chen Z, Duan RS, Zhu Y, Folkesson R, Albanese C, Winblad B, Zhu J. Increased cyclin E expression may obviate the role of cyclin D1 during brain development in cyclin D1 knockout mice. J Neurochem 2005; 92:1281-4.

119. Bowe DB, Kenney NJ, Adereth Y, Maroulakou IG. Suppression of Neu-induced mammary tumor growth in cyclin D1 deficient mice is compensated for by cyclin E. Oncogene 2002; 21:291-8.
120. Phillips-Mason PJ, Raben DM, Baldassare JJ. Phosphatidylinositol 3-kinase activity regulates alpha -thrombin-stimulated G1 progression by its effect on cyclin D1 expression and cyclin-dependent kinase 4 activity. J Biol Chem 2000; 275:18046-53.
121. den Elzen N, Pines J. Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. J Cell Biol 2001; 153:121-36.
122. Geley S, Kramer E, Gieffers C, Gannon J, Peters JM, Hunt T. Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. J Cell Biol 2001; 153:137-48.
123. Glotzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. Nature 1991; 349:132-8.
124. Hershko A, Ganoth D, Pehrson J, Palazzo RE, Cohen LH. Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. J Biol Chem 1991; 266:16376-9.
125. Yamano H, Gannon J, Mahbubani H, Hunt T. Cell cycle-regulated recognition of the destruction box of cyclin B by the APC/C in Xenopus egg extracts. Mol Cell 2004; 13:137-47.
126. Suzumori N, Burns KH, Yan W, Matzuk MM. RFPL4 interacts with oocyte proteins of the ubiquitin-proteasome degradation pathway. Proc Natl Acad Sci U S A 2003; 100:550-5.
127. Kobayashi H, Stewart E, Poon R, Adamczewski JP, Gannon J, Hunt T. Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits. Mol Biol Cell 1992; 3:1279-94.
128. Lorca T, Devault A, Colas P, Van Loon A, Fesquet D, Lazaro JB, Doree M. Cyclin A-Cys41 does not undergo cell cycle-dependent degradation in Xenopus extracts. FEBS Lett 1992; 306:90-3.
129. Luca FC, Shibuya EK, Dohrmann CE, Ruderman JV. Both cyclin A delta 60 and B delta 97 are stable and arrest cells in M-phase, but only cyclin B delta 97 turns on cyclin destruction. EMBO J 1991; 10:4311-20.

130. Kaspar M, Dienemann A, Schulze C, Sprenger F. Mitotic degradation of cyclin A is mediated by multiple and novel destruction signals. Curr Biol 2001; 11:685-90.
131. Hershko A. Mechanisms and regulation of the degradation of cyclin B. Philos Trans R Soc Lond B Biol Sci 1999; 354:1571-5; discussion 5-6.
132. Zachariae W, Nasmyth K. Whose end is destruction: cell division and the anaphase-promoting complex. Genes Dev 1999; 13:2039-58.
133. Kirkpatrick DS, Hathaway NA, Hanna J, Elsasser S, Rush J, Finley D, King RW, Gygi SP. Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nat Cell Biol 2006; 8:700-10.
134. Cox CJ, Dutta K, Petri ET, Hwang WC, Lin Y, Pascal SM, Basavappa R. The regions of securin and cyclin B proteins recognized by the ubiquitination machinery are natively unfolded. FEBS Lett 2002; 527:303-8.
135. Pines J, Hunter T. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J Cell Biol 1991; 115:1-17.
136. Golan A, Yudkovsky Y, Hershko A. The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk. J Biol Chem 2002; 277:15552-7.
137. Viallard JF, Lacombe F, Belloc F, Pellegrin JL, Reiffers J. [Molecular mechanisms controlling the cell cycle: fundamental aspects and implications for oncology]. Cancer Radiother 2001; 5:109-29.
138. Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science 1996; 274:1664-72.
139. Pardee AB. A restriction point for control of normal animal cell proliferation. Proc Natl Acad Sci U S A 1974; 71:1286-90.
140. Das SK, Hashimoto T, Shimizu K, Yoshida T, Sakai T, Sowa Y, Komoto A, Kanazawa K. Fucoxanthin induces cell cycle arrest at G0/G1 phase in human colon carcinoma cells through up-regulation of p21WAF1/Cip1. Biochim Biophys Acta 2005; 1726:328-35.
141. Wu CL, Zukerberg LR, Ngwu C, Harlow E, Lees JA. In vivo association of E2F and DP family proteins. Mol Cell Biol 1995; 15:2536-46.

142. De Veylder L, Joubes J, Inze D. Plant cell cycle transitions. Curr Opin Plant Biol 2003; 6:536-43.
143. Luo RX, Postigo AA, Dean DC. Rb interacts with histone deacetylase to repress transcription. Cell 1998; 92:463-73.
144. de Jager SM, Maughan S, Dewitte W, Scofield S, Murray JA. The developmental context of cell-cycle control in plants. Semin Cell Dev Biol 2005; 16:385-96.
145. Sinal SH, Woods CR. Human papillomavirus infections of the genital and respiratory tracts in young children. Semin Pediatr Infect Dis 2005; 16:306-16.
146. Vietri M, Bianchi M, Ludlow JW, Mittnacht S, Villa-Moruzzi E. Direct interaction between the catalytic subunit of Protein Phosphatase 1 and pRb. Cancer Cell Int 2006; 6:3.
147. Munger K, Howley PM. Human papillomavirus immortalization and transformation functions. Virus Res 2002; 89:213-28.
148. Zheng N, Fraenkel E, Pabo CO, Pavletich NP. Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DP. Genes Dev 1999; 13:666-74.
149. Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. Genes Dev 1997; 11:2090-100.
150. Nakayama K. Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. Bioessays 1998; 20:1020-9.
151. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. Dev Cell 2008; 14:159-69.
152. Russo AA, Jeffrey PD, Patten AK, Massague J, Pavletich NP. Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. Nature 1996; 382:325-31.
153. Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde KH, Beach D. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 1995; 269:1281-4.
154. Liggett WH, Jr., Sidransky D. Role of the p16 tumor suppressor gene in cancer. J Clin Oncol 1998; 16:1197-206.

155. Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J 1998; 17:5001-14.
156. Vidal A, Koff A. Cell-cycle inhibitors: three families united by a common cause. Gene 2000; 247:1-15.
157. Giacinti C, Giordano A. RB and cell cycle progression. Oncogene 2006; 25:5220-7.
158. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell 1995; 81:323-30.
159. Hunter T, Pines J. Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell 1994; 79:573-82.
160. Keyomarsi K, Conte D, Jr., Toyofuku W, Fox MP. Deregulation of cyclin E in breast cancer. Oncogene 1995; 11:941-50.
161. Cinti C, Leoncini L, Nyongo A, Ferrari F, Lazzi S, Bellan C, Vatti R, Zamparelli A, Cevenini G, Tosi GM, Claudio PP, Maraldi NM, Tosi P, Giordano A. Genetic alterations of the retinoblastoma-related gene RB2/p130 identify different pathogenetic mechanisms in and among Burkitt's lymphoma subtypes. Am J Pathol 2000; 156:751-60.
162. De Falco G, Bellan C, Lazzi S, Claudio P, La Sala D, Cinti C, Tosi P, Giordano A, Leoncini L. Interaction between HIV-1 Tat and pRb2/p130: a possible mechanism in the pathogenesis of AIDS-related neoplasms. Oncogene 2003; 22:6214-9.
163. Lazzi S, Bellan C, De Falco G, Cinti C, Ferrari F, Nyongo A, Claudio PP, Tosi GM, Vatti R, Gloghini A, Carbone A, Giordano A, Leoncini L, Tosi P. Expression of RB2/p130 tumor-suppressor gene in AIDS-related non-Hodgkin's lymphomas: implications for disease pathogenesis. Hum Pathol 2002; 33:723-31.
164. Sherr CJ. Cancer cell cycles. Science 1996; 274:1672-7.
165. Hinds PW, Dowdy SF, Eaton EN, Arnold A, Weinberg RA. Function of a human cyclin gene as an oncogene. Proc Natl Acad Sci U S A 1994; 91:709-13.
166. MacGregor JI, Jordan VC. Basic guide to the mechanisms of antiestrogen action. Pharmacol Rev 1998; 50:151-96.
167. Leo C, Li H, Chen JD. Differential mechanisms of nuclear receptor regulation by receptor-associated coactivator 3. J Biol Chem 2000; 275:5976-82.

168. Shah YM, Rowan BG. The Src kinase pathway promotes tamoxifen agonist action in Ishikawa endometrial cells through phosphorylation-dependent stabilization of estrogen receptor (alpha) promoter interaction and elevated steroid receptor coactivator 1 activity. Mol Endocrinol 2005; 19:732-48.
169. Dutertre M, Smith CL. Ligand-independent interactions of p160/steroid receptor coactivators and CREB-binding protein (CBP) with estrogen receptor-alpha: regulation by phosphorylation sites in the A/B region depends on other receptor domains. Mol Endocrinol 2003; 17:1296-314.
170. Williams CC, Basu A, El-Gharbawy A, Carrier LM, Smith CL, Rowan BG. Identification of four novel phosphorylation sites in estrogen receptor alpha: impact on receptor-dependent gene expression and phosphorylation by protein kinase CK2. BMC Biochem 2009; 10:36.
171. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. Physiol Rev 2001; 81:1535-65.
172. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 1995; 270:1491-4.
173. Bourguet W, Germain P, Gronemeyer H. Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. Trends Pharmacol Sci 2000; 21:381-8.
174. Gougelet A, Bouclier C, Marsaud V, Maillard S, Mueller SO, Korach KS, Renoir JM. Estrogen receptor alpha and beta subtype expression and transactivation capacity are differentially affected by receptor-, hsp90- and immunophilin-ligands in human breast cancer cells. J Steroid Biochem Mol Biol 2005; 94:71-81.
175. Picard D. Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 2002; 59:1640-8.
176. Mayer MP. Gymnastics of molecular chaperones. Mol Cell; 39:321-31.
177. Kee BL, Arias J, Montminy MR. Adaptor-mediated recruitment of RNA polymerase II to a signal-dependent activator. J Biol Chem 1996; 271:2373-5.

178. Savkur RS, Burris TP. The coactivator LXXLL nuclear receptor recognition motif. J Pept Res 2004; 63:207-12.
179. Danielian PS, White R, Lees JA, Parker MG. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J 1992; 11:1025-33.
180. Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 1997; 387:733-6.
181. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 1997; 387:677-84.
182. Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ, West BL. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science 1998; 280:1747-9.
183. Bunone G, Briand PA, Miksicek RJ, Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 1996; 15:2174-83.
184. Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J, Lannigan DA. pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol Cell Biol 1998; 18:1978-84.
185. Arnold SF, Obourn JD, Jaffe H, Notides AC. Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. Mol Endocrinol 1994; 8:1208-14.
186. Barnes CJ, Vadlamudi RK, Kumar R. Novel estrogen receptor coregulators and signaling molecules in human diseases. Cell Mol Life Sci 2004; 61:281-91.
187. Landers JP, Spelsberg TC. New concepts in steroid hormone action: transcription factors, proto-oncogenes, and the cascade model for steroid regulation of gene expression. Crit Rev Eukaryot Gene Expr 1992; 2:19-63.
188. Gaub MP, Bellard M, Scheuer I, Chambon P, Sassone-Corsi P. Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex. Cell 1990; 63:1267-76.

189. Tora L, Gaub MP, Mader S, Dierich A, Bellard M, Chambon P. Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter. EMBO J 1988; 7:3771-8.
190. Philips A, Chalbos D, Rochefort H. Estradiol increases and anti-estrogens antagonize the growth factor-induced activator protein-1 activity in MCF7 breast cancer cells without affecting c-fos and c-jun synthesis. J Biol Chem 1993; 268:14103-8.
191. Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y, Kamada T. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem 1994; 269:16433-42.
192. Tzukerman M, Zhang XK, Pfahl M. Inhibition of estrogen receptor activity by the tumor promoter 12-O-tetradeconylphorbol-13-acetate: a molecular analysis. Mol Endocrinol 1991; 5:1983-92.
193. Saatcioglu F, Lopez G, West BL, Zandi E, Feng W, Lu H, Esmaili A, Apriletti JW, Kushner PJ, Baxter JD, Karin M. Mutations in the conserved C-terminal sequence in thyroid hormone receptor dissociate hormone-dependent activation from interference with AP-1 activity. Mol Cell Biol 1997; 17:4687-95.
194. Webb P, Lopez GN, Uht RM, Kushner PJ. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 1995; 9:443-56.
195. Kushner PJ, Agard D, Feng WJ, Lopez G, Schiau A, Uht R, Webb P, Greene G. Oestrogen receptor function at classical and alternative response elements. Novartis Found Symp 2000; 230:20-6; discussion 7-40.
196. Gururaj AE, Rayala SK, Vadlamudi RK, Kumar R. Novel mechanisms of resistance to endocrine therapy: genomic and nongenomic considerations. Clin Cancer Res 2006; 12:1001s-7s.
197. Levin ER. Integration of the extranuclear and nuclear actions of estrogen. Mol Endocrinol 2005; 19:1951-9.
198. Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 2005; 19:833-42.

199. Doolan CM, Harvey BJ. A Galphas protein-coupled membrane receptor, distinct from the classical oestrogen receptor, transduces rapid effects of oestradiol on [Ca2+]i in female rat distal colon. Mol Cell Endocrinol 2003; 199:87-103.
200. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 2000; 407:538-41.
201. Xu W, Liu LZ, Loizidou M, Ahmed M, Charles IG. The role of nitric oxide in cancer. Cell Res 2002; 12:311-20.
202. Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, Aswad DW, Stallcup MR. Regulation of transcription by a protein methyltransferase. Science 1999; 284:2174-7.
203. Losel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K, Wehling M. Nongenomic steroid action: controversies, questions, and answers. Physiol Rev 2003; 83:965-1016.
204. Cato AC, Nestl A, Mink S. Rapid actions of steroid receptors in cellular signaling pathways. Sci STKE 2002; 2002:re9.
205. Chiang CH, Cheng KW, Igarashi S, Nathwani PS, Leung PC. Hormonal regulation of estrogen receptor alpha and beta gene expression in human granulosa-luteal cells in vitro. J Clin Endocrinol Metab 2000; 85:3828-39.
206. Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP. Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci U S A 1999; 96:4686-91.
207. 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; 100:4807-12.
208. Figtree GA, McDonald D, Watkins H, Channon KM. Truncated estrogen receptor alpha 46-kDa isoform in human endothelial cells: relationship to acute activation of nitric oxide synthase. Circulation 2003; 107:120-6.
209. Norfleet AM, Thomas ML, Gametchu B, Watson CS. Estrogen receptor-alpha detected on the plasma membrane of aldehyde-fixed GH3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry. Endocrinology 1999; 140:3805-14.

210. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest 1999; 103:401-6.
211. Watson CS, Norfleet AM, Pappas TC, Gametchu B. Rapid actions of estrogens in GH3/B6 pituitary tumor cells via a plasma membrane version of estrogen receptor-alpha. Steroids 1999; 64:5-13.
212. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 1999; 20:358-417.
213. Levin ER. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol 2003; 17:309-17.
214. Alroy I, Yarden Y. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. FEBS Lett 1997; 410:83-6.
215. 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; 275:18447-53.
216. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Bottero D, Varricchio L, Nanayakkara M, Rotondi A, Auricchio F. Sex steroid hormones act as growth factors. J Steroid Biochem Mol Biol 2002; 83:31-5.
217. Chung YL, Sheu ML, Yang SC, Lin CH, Yen SH. Resistance to tamoxifen-induced apoptosis is associated with direct interaction between Her2/neu and cell membrane estrogen receptor in breast cancer. Int J Cancer 2002; 97:306-12.
218. Filardo EJ. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol 2002; 80:231-8.
219. Simoncini T. Mechanisms of action of estrogen receptors in vascular cells: relevance for menopause and aging. Climacteric 2009; 12 Suppl 1:6-11.
220. Anderson RG. The caveolae membrane system. Annu Rev Biochem 1998; 67:199-225.
221. Azarnia R, Reddy S, Kmiecik TE, Shalloway D, Loewenstein WR. The cellular src gene product regulates junctional cell-to-cell communication. Science 1988; 239:398-401.

222. Kavanaugh WM, Williams LT. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science 1994; 266:1862-5.
223. Razandi M, Pedram A, Park ST, Levin ER. Proximal events in signaling by plasma membrane estrogen receptors. J Biol Chem 2003; 278:2701-12.
224. Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. Proc Natl Acad Sci U S A 2002; 99:14783-8.
225. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ. Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. Mol Endocrinol 2002; 16:116-27.
226. Sun M, Paciga JE, Feldman RI, Yuan Z, Coppola D, Lu YY, Shelley SA, Nicosia SV, Cheng JQ. Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. Cancer Res 2001; 61:5985-91.
227. Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995; 270:1354-7.
228. Shibata H, Spencer TE, Onate SA, Jenster G, Tsai SY, Tsai MJ, O'Malley BW. Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog Horm Res 1997; 52:141-64; discussion 64-5.
229. Edwards DP. The role of coactivators and corepressors in the biology and mechanism of action of steroid hormone receptors. J Mammary Gland Biol Neoplasia 2000; 5:307-24.
230. Gao X, Loggie BW, Nawaz Z. The roles of sex steroid receptor coregulators in cancer. Mol Cancer 2002; 1:7.
231. O'Malley BW. A life-long search for the molecular pathways of steroid hormone action. Mol Endocrinol 2005; 19:1402-11.
232. Gill G, Ptashne M. Negative effect of the transcriptional activator GAL4. Nature 1988; 334:721-4.
233. Blanco JC, Minucci S, Lu J, Yang XJ, Walker KK, Chen H, Evans RM, Nakatani Y, Ozato K. The histone acetylase PCAF is a nuclear receptor coactivator. Genes Dev 1998; 12:1638-51.

234. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 1997; 90:569-80.
235. Henttu PM, Kalkhoven E, Parker MG. AF-2 activity and recruitment of steroid receptor coactivator 1 to the estrogen receptor depend on a lysine residue conserved in nuclear receptors. Mol Cell Biol 1997; 17:1832-9.
236. Webb P, Nguyen P, Shinsako J, Anderson C, Feng W, Nguyen MP, Chen D, Huang SM, Subramanian S, McKinerney E, Katzenellenbogen BS, Stallcup MR, Kushner PJ. Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Mol Endocrinol 1998; 12:1605-18.
237. Yamamoto Y, Wada O, Suzawa M, Yogiashi Y, Yano T, Kato S, Yanagisawa J. The tamoxifen-responsive estrogen receptor alpha mutant D351Y shows reduced tamoxifen-dependent interaction with corepressor complexes. J Biol Chem 2001; 276:42684-91.
238. Montano MM, Ekena K, Krueger KD, Keller AL, Katzenellenbogen BS. Human estrogen receptor ligand activity inversion mutants: receptors that interpret antiestrogens as estrogens and estrogens as antiestrogens and discriminate among different antiestrogens. Mol Endocrinol 1996; 10:230-42.
239. Montano MM, Ekena K, Delage-Mourroux R, Chang W, Martini P, Katzenellenbogen BS. An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. Proc Natl Acad Sci U S A 1999; 96:6947-52.
240. Lawrence Zipursky; Arnold Berk; Monty Krieger; Darnell JEL, Harvey F.; Kaiser, Chris; Matthew P Scott; Matsudaira, Paul T.. McGill Lodish.
241. Hong H, Kohli K, Garabedian MJ, Stallcup MR. GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol 1997; 17:2735-44.
242. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 1997; 277:965-8.

243. Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 1996; 85:403-14.
244. Torchia J, Glass C, Rosenfeld MG. Co-activators and co-repressors in the integration of transcriptional responses. Curr Opin Cell Biol 1998; 10:373-83.
245. Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature 1996; 382:319-24.
246. Cheng AS, Jin VX, Fan M, Smith LT, Liyanarachchi S, Yan PS, Leu YW, Chan MW, Plass C, Nephew KP, Davuluri RV, Huang TH. Combinatorial analysis of transcription factor partners reveals recruitment of c-MYC to estrogen receptor-alpha responsive promoters. Mol Cell 2006; 21:393-404.
247. Martinez-Balbas MA, Bannister AJ, Martin K, Haus-Seuffert P, Meisterernst M, Kouzarides T. The acetyltransferase activity of CBP stimulates transcription. EMBO J 1998; 17:2886-93.
248. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 1997; 389:194-8.
249. Demarest SJ, Martinez-Yamout M, Chung J, Chen H, Xu W, Dyson HJ, Evans RM, Wright PE. Mutual synergistic folding in recruitment of CBP/p300 by p160 nuclear receptor coactivators. Nature 2002; 415:549-53.
250. Kobayashi Y, Kitamoto T, Masuhiro Y, Watanabe M, Kase T, Metzger D, Yanagisawa J, Kato S. p300 mediates functional synergism between AF-1 and AF-2 of estrogen receptor alpha and beta by interacting directly with the N-terminal A/B domains. J Biol Chem 2000; 275:15645-51.
251. Kim MY, Hsiao SJ, Kraus WL. A role for coactivators and histone acetylation in estrogen receptor alpha-mediated transcription initiation. EMBO J 2001; 20:6084-94.
252. Sewack GF, Ellis TW, Hansen U. Binding of TATA binding protein to a naturally positioned nucleosome is facilitated by histone acetylation. Mol Cell Biol 2001; 21:1404-15.

253. Heemers HV, Sebo TJ, Debes JD, Regan KM, Raclaw KA, Murphy LM, Hobisch A, Culig Z, Tindall DJ. Androgen deprivation increases p300 expression in prostate cancer cells. Cancer Res 2007; 67:3422-30.
254. Wang Q, Carroll JS, Brown M. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 2005; 19:631-42.
255. Hudelist G, Czerwenka K, Kubista E, Marton E, Pischinger K, Singer CF. Expression of sex steroid receptors and their co-factors in normal and malignant breast tissue: AIB1 is a carcinoma-specific co-activator. Breast Cancer Res Treat 2003; 78:193-204.
256. Fu M, Wang C, Reutens AT, Wang J, Angeletti RH, Siconolfi-Baez L, Ogryzko V, Avantaggiati ML, Pestell RG. p300 and p300/cAMP-response element-binding protein-associated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation. J Biol Chem 2000; 275:20853-60.
257. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, Schiff R. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004; 96:926-35.
258. Debes JD, Sebo TJ, Lohse CM, Murphy LM, Haugen DA, Tindall DJ. p300 in prostate cancer proliferation and progression. Cancer Res 2003; 63:7638-40.
259. Cascio S, Bartella V, Garofalo C, Russo A, Giordano A, Surmacz E. Insulin-like growth factor 1 differentially regulates estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells. J Biol Chem 2007; 282:3498-506.
260. Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 2003; 115:751-63.
261. Qi C, Chang J, Zhu Y, Yeldandi AV, Rao SM, Zhu YJ. Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. J Biol Chem 2002; 277:28624-30.
262. Bedford MT, Richard S. Arginine methylation an emerging regulator of protein function. Mol Cell 2005; 18:263-72.
263. Strahl BD, Briggs SD, Brame CJ, Caldwell JA, Koh SS, Ma H, Cook RG, Shabanowitz J, Hunt DF, Stallcup MR, Allis CD. Methylation of histone H4 at arginine 3

occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. Curr Biol 2001; 11:996-1000.

264. Wang H, Huang ZQ, Xia L, Feng Q, Erdjument-Bromage H, Strahl BD, Briggs SD, Allis CD, Wong J, Tempst P, Zhang Y. Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. Science 2001; 293:853-7.

265. Chen D, Huang SM, Stallcup MR. Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300. J Biol Chem 2000; 275:40810-6.

266. Teyssier C, Chen D, Stallcup MR. Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. J Biol Chem 2002; 277:46066-72.

267. Klinge CM, Jernigan SC, Mattingly KA, Risinger KE, Zhang J. Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors alpha and beta by coactivators and corepressors. J Mol Endocrinol 2004; 33:387-410.

268. Daujat S, Bauer UM, Shah V, Turner B, Berger S, Kouzarides T. Crosstalk between CARM1 methylation and CBP acetylation on histone H3. Curr Biol 2002; 12:2090-7.

269. Zou Y, Tsai WB, Cheng CJ, Hsu C, Chung YM, Li PC, Lin SH, Hu MC. Forkhead box transcription factor FOXO3a suppresses estrogen-dependent breast cancer cell proliferation and tumorigenesis. Breast Cancer Res 2008; 10:R21.

270. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 2009; 325:834-40.

271. Keeton EK, Brown M. Cell cycle progression stimulated by tamoxifen-bound estrogen receptor-alpha and promoter-specific effects in breast cancer cells deficient in N-CoR and SMRT. Mol Endocrinol 2005; 19:1543-54.

272. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000; 103:843-52.

273. Vegeto E, Allan GF, Schrader WT, Tsai MJ, McDonnell DP, O'Malley BW. The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. Cell 1992; 69:703-13.

274. Baniahmad A, Tsai SY, O'Malley BW, Tsai MJ. Kindred S thyroid hormone receptor is an active and constitutive silencer and a repressor for thyroid hormone and retinoic acid responses. Proc Natl Acad Sci U S A 1992; 89:10633-7.
275. Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Natl Acad Sci U S A 1998; 95:2920-5.
276. Carroll JS, Lynch DK, Swarbrick A, Renoir JM, Sarcevic B, Daly RJ, Musgrove EA, Sutherland RL. p27(Kip1) induces quiescence and growth factor insensitivity in tamoxifen-treated breast cancer cells. Cancer Res 2003; 63:4322-6.
277. Perissi V, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG. A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. Cell 2004; 116:511-26.
278. Leung YK, Mak P, Hassan S, Ho SM. Estrogen receptor (ER)-beta isoforms: a key to understanding ER-beta signaling. Proc Natl Acad Sci U S A 2006; 103:13162-7.
279. Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci U S A 2000; 97:10751-6.
280. Flouriot G, Brand H, Denger S, Metivier R, Kos M, Reid G, Sonntag-Buck V, Gannon F. Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. EMBO J 2000; 19:4688-700.
281. Lin CY, Strom A, Li Kong S, Kietz S, Thomsen JS, Tee JB, Vega VB, Miller LD, Smeds J, Bergh J, Gustafsson JA, Liu ET. Inhibitory effects of estrogen receptor beta on specific hormone-responsive gene expression and association with disease outcome in primary breast cancer. Breast Cancer Res 2007; 9:R25.
282. Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci U S A 2004; 101:1566-71.

283. Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH, Jr., Pestell RG, Kushner PJ. Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 2002; 277:24353-60.
284. Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C. Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. Mol Endocrinol 2003; 17:203-8.
285. Faulds MH, Olsen H, Helguero LA, Gustafsson JA, Haldosen LA. Estrogen receptor functional activity changes during differentiation of mammary epithelial cells. Mol Endocrinol 2004; 18:412-21.
286. Matthews J, Wihlen B, Tujague M, Wan J, Strom A, Gustafsson JA. Estrogen receptor (ER) beta modulates ERalpha-mediated transcriptional activation by altering the recruitment of c-Fos and c-Jun to estrogen-responsive promoters. Mol Endocrinol 2006; 20:534-43.
287. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 1998; 95:15677-82.
288. Vidal O, Lindberg MK, Hollberg K, Baylink DJ, Andersson G, Lubahn DB, Mohan S, Gustafsson JA, Ohlsson C. Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. Proc Natl Acad Sci U S A 2000; 97:5474-9.
289. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138:863-70.
290. Palmieri C, Cheng GJ, Saji S, Zelada-Hedman M, Warri A, Weihua Z, Van Noorden S, Wahlstrom T, Coombes RC, Warner M, Gustafsson JA. Estrogen receptor beta in breast cancer. Endocr Relat Cancer 2002; 9:1-13.
291. Palmieri C, Saji S, Sakaguchi H, Cheng G, Sunters A, O'Hare MJ, Warner M, Gustafsson JA, Coombes RC, Lam EW. The expression of oestrogen receptor (ER)-beta and its variants, but not ERalpha, in adult human mammary fibroblasts. J Mol Endocrinol 2004; 33:35-50.

292. Russo J, Ao X, Grill C, Russo IH. Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast Cancer Res Treat 1999; 53:217-27.
293. Anderson TJ, Ferguson DJ, Raab GM. Cell turnover in the "resting" human breast: influence of parity, contraceptive pill, age and laterality. Br J Cancer 1982; 46:376-82.
294. Battersby S, Robertson BJ, Anderson TJ, King RJ, McPherson K. Influence of menstrual cycle, parity and oral contraceptive use on steroid hormone receptors in normal breast. Br J Cancer 1992; 65:601-7.
295. Ferguson DJ, Anderson TJ. Morphological evaluation of cell turnover in relation to the menstrual cycle in the "resting" human breast. Br J Cancer 1981; 44:177-81.
296. Going JJ, Anderson TJ, Battersby S, MacIntyre CC. Proliferative and secretory activity in human breast during natural and artificial menstrual cycles. Am J Pathol 1988; 130:193-204.
297. Meyer JS. Cell proliferation in normal human breast ducts, fibroadenomas, and other ductal hyperplasias measured by nuclear labeling with tritiated thymidine. Effects of menstrual phase, age, and oral contraceptive hormones. Hum Pathol 1977; 8:67-81.
298. Masters JR, Drife JO, Scarisbrick JJ. Cyclic Variation of DNA synthesis in human breast epithelium. J Natl Cancer Inst 1977; 58:1263-5.
299. Potten CS, Watson RJ, Williams GT, Tickle S, Roberts SA, Harris M, Howell A. The effect of age and menstrual cycle upon proliferative activity of the normal human breast. Br J Cancer 1988; 58:163-70.
300. Williams G, Anderson E, Howell A, Watson R, Coyne J, Roberts SA, Potten CS. Oral contraceptive (OCP) use increases proliferation and decreases oestrogen receptor content of epithelial cells in the normal human breast. Int J Cancer 1991; 48:206-10.
301. Laidlaw IJ, Clarke RB, Howell A, Owen AW, Potten CS, Anderson E. The proliferation of normal human breast tissue implanted into athymic nude mice is stimulated by estrogen but not progesterone. Endocrinology 1995; 136:164-71.
302. Russo IH, Russo J. Role of hormones in mammary cancer initiation and progression. J Mammary Gland Biol Neoplasia 1998; 3:49-61.
303. Clarke RB, Howell A, Potten CS, Anderson E. Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res 1997; 57:4987-91.

304. Russo IH RJ. Role of hormones in cancer initiation and progression J Mammary Gland Biol Neolasia 1998; 3:49-61.
305. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983; 31:13-20.
306. Kodama F, Greene GL, Salmon SE. Relation of estrogen receptor expression to clonal growth and antiestrogen effects on human breast cancer cells. Cancer Res 1985; 45:2720-4.
307. Podhajcer OL BA, Sorin I, Guman N, Cerdeiro R, Mordoh. Determination of DNA synthesis, estrogen Hormone receptors and breast cell proliferation 227 receptors, and carcinoembryonic antigen in isolated cellular subpopulations of human breast cancer. Cancer 1986; 58:720–9.
308. Clarke R, Dickson RB, Lippman ME. Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. Crit Rev Oncol Hematol 1992; 12:1-23.
309. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB. Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell 1987; 48:417-28.
310. Dickson RB, Lippman ME. Control of human breast cancer by estrogen, growth factors, and oncogenes. Cancer Treat Res 1988; 40:119-65.
311. Shyamala G, Schneider W, Schott D. Developmental regulation of murine mammary progesterone receptor gene expression. Endocrinology 1990; 126:2882-9.
312. Dos Santos E, Dieudonne MN, Leneveu MC, Serazin V, Rincheval V, Mignotte B, Chouillard E, De Mazancourt P, Giudicelli Y, Pecquery R. Effects of 17beta-estradiol on preadipocyte proliferation in human adipose tissue: Involvement of IGF1-R signaling. Horm Metab Res; 42:514-20.
313. Seagroves TN, Lydon JP, Hovey RC, Vonderhaar BK, Rosen JM. C/EBPbeta (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. Mol Endocrinol 2000; 14:359-68.
314. Zeps N, Bentel JM, Papadimitriou JM, D'Antuono MF, Dawkins HJ. Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. Differentiation 1998; 62:221-6.

315. Clarke RB. Human breast cell proliferation and its relationship to steroid receptor expression. Climacteric 2004; 7:129-37.
316. Weinstat-Saslow D, Merino MJ, Manrow RE, Lawrence JA, Bluth RF, Wittenbel KD, Simpson JF, Page DL, Steeg PS. Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. Nat Med 1995; 1:1257-60.
317. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature 1994; 369:669-71.
318. Courjal F, Louason G, Speiser P, Katsaros D, Zeillinger R, Theillet C. Cyclin gene amplification and overexpression in breast and ovarian cancers: evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. Int J Cancer 1996; 69:247-53.
319. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994; 54:1812-7.
320. Cicatiello L, Addeo R, Sasso A, Altucci L, Petrizzi VB, Borgo R, Cancemi M, Caporali S, Caristi S, Scafoglio C, Teti D, Bresciani F, Perillo B, Weisz A. Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol Cell Biol 2004; 24:7260-74.
321. Eeckhoute J, Carroll JS, Geistlinger TR, Torres-Arzayus MI, Brown M. A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. Genes Dev 2006; 20:2513-26.
322. Bremner R, Cohen BL, Sopta M, Hamel PA, Ingles CJ, Gallie BL, Phillips RA. Direct transcriptional repression by pRB and its reversal by specific cyclins. Mol Cell Biol 1995; 15:3256-65.
323. Rogatsky I, Trowbridge JM, Garabedian MJ. Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A-CDK2 complex. J Biol Chem 1999; 274:22296-302.
324. Beato M, Klug J. Steroid hormone receptors: an update. Hum Reprod Update 2000; 6:225-36.

325. Foster JS, Wimalasena J. Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells. Mol Endocrinol 1996; 10:488-98.
326. Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ, Kato JY. D-type cyclin-dependent kinase activity in mammalian cells. Mol Cell Biol 1994; 14:2066-76.
327. Malik S, Jiang S, Garee JP, Verdin E, Lee AV, O'Malley BW, Zhang M, Belaguli NS, Oesterreich S. Histone deacetylase 7 and FoxA1 in estrogen-mediated repression of RPRM. Mol Cell Biol; 30:399-412.
328. Ohki R, Nemoto J, Murasawa H, Oda E, Inazawa J, Tanaka N, Taniguchi T. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. J Biol Chem 2000; 275:22627-30.
329. Jin S, Tong T, Fan W, Fan F, Antinore MJ, Zhu X, Mazzacurati L, Li X, Petrik KL, Rajasekaran B, Wu M, Zhan Q. GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. Oncogene 2002; 21:8696-704.
330. Taylor WR, DePrimo SE, Agarwal A, Agarwal ML, Schonthal AH, Katula KS, Stark GR. Mechanisms of G2 arrest in response to overexpression of p53. Mol Biol Cell 1999; 10:3607-22.
331. Winters ZE, Ongkeko WM, Harris AL, Norbury CJ. p53 regulates Cdc2 independently of inhibitory phosphorylation to reinforce radiation-induced G2 arrest in human cells. Oncogene 1998; 17:673-84.
332. Badve S, Turbin D, Thorat MA, Morimiya A, Nielsen TO, Perou CM, Dunn S, Huntsman DG, Nakshatri H. FOXA1 expression in breast cancer--correlation with luminal subtype A and survival. Clin Cancer Res 2007; 13:4415-21.
333. An J, Ribeiro RC, Webb P, Gustafsson JA, Kushner PJ, Baxter JD, Leitman DC. Estradiol repression of tumor necrosis factor-alpha transcription requires estrogen receptor activation function-2 and is enhanced by coactivators. Proc Natl Acad Sci U S A 1999; 96:15161-6.
334. Bretschneider N, Brand H, Miller N, Lowery AJ, Kerin MJ, Gannon F, Denger S. Estrogen induces repression of the breast cancer and salivary gland expression gene in an estrogen receptor alpha-dependent manner. Cancer Res 2008; 68:106-14.

335. Garcia-Arencibia M, Molero S, Davila N, Carranza MC, Calle C. 17beta-Estradiol transcriptionally represses human insulin receptor gene expression causing cellular insulin resistance. Leuk Res 2005; 29:79-87.
336. Gottardis MM, Saceda M, Garcia-Morales P, Fung YK, Solomon H, Sholler PF, Lippman ME, Martin MB. Regulation of retinoblastoma gene expression in hormone-dependent breast cancer. Endocrinology 1995; 136:5659-65.
337. Newman SP, Bates NP, Vernimmen D, Parker MG, Hurst HC. Cofactor competition between the ligand-bound oestrogen receptor and an intron 1 enhancer leads to oestrogen repression of ERBB2 expression in breast cancer. Oncogene 2000; 19:490-7.
338. Perissi V, Menini N, Cottone E, Capello D, Sacco M, Montaldo F, De Bortoli M. AP-2 transcription factors in the regulation of ERBB2 gene transcription by oestrogen. Oncogene 2000; 19:280-8.
339. Ray P, Ghosh SK, Zhang DH, Ray A. Repression of interleukin-6 gene expression by 17 beta-estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-kappa B by the estrogen receptor. FEBS Lett 1997; 409:79-85.
340. Uray IP, Liang Y, Hyder SM. Estradiol down-regulates CD36 expression in human breast cancer cells. Cancer Lett 2004; 207:101-7.
341. T'Ang A, Varley JM, Chakraborty S, Murphree AL, Fung YK. Structural rearrangement of the retinoblastoma gene in human breast carcinoma. Science 1988; 242:263-6.
342. van der Poll T, Keogh CV, Guirao X, Buurman WA, Kopf M, Lowry SF. Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. J Infect Dis 1997; 176:439-44.
343. Tamura K, Kanaoka Y, Jinno S, Nagata A, Ogiso Y, Shimizu K, Hayakawa T, Nojima H, Okayama H. Cyclin G: a new mammalian cyclin with homology to fission yeast Cig1. Oncogene 1993; 8:2113-8.
344. Horne MC, Donaldson KL, Goolsby GL, Tran D, Mulheisen M, Hell JW, Wahl AF. Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. J Biol Chem 1997; 272:12650-61.
345. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights

into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 2003; 144:4562-74.
346. Stossi F, Likhite VS, Katzenellenbogen JA, Katzenellenbogen BS. Estrogen-occupied estrogen receptor represses cyclin G2 gene expression and recruits a repressor complex at the cyclin G2 promoter. J Biol Chem 2006; 281:16272-8.
347. Semlali A, Oliva J, Badia E, Pons M, Duchesne MJ. Immediate early gene X-1 (IEX-1), a hydroxytamoxifen regulated gene with increased stimulation in MCF-7 derived resistant breast cancer cells. J Steroid Biochem Mol Biol 2004; 88:247-59.
348. Poelzl G, Kasai Y, Mochizuki N, Shaul PW, Brown M, Mendelsohn ME. Specific association of estrogen receptor beta with the cell cycle spindle assembly checkpoint protein, MAD2. Proc Natl Acad Sci U S A 2000; 97:2836-9.
349. Gustafsson N, Zhao C, Gustafsson JA, Dahlman-Wright K. RBCK1 drives breast cancer cell proliferation by promoting transcription of estrogen receptor alpha and cyclin B1. Cancer Res; 70:1265-74.
350. Pietras RJ, Szego CM. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 1977; 265:69-72.
351. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 2001; 276:36869-72.
352. 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.
353. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, Auricchio F. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 1996; 15:1292-300.
354. Castoria G, Barone MV, Di Domenico M, Bilancio A, Ametrano D, Migliaccio A, Auricchio F. Non-transcriptional action of oestradiol and progestin triggers DNA synthesis. EMBO J 1999; 18:2500-10.
355. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 2001; 104:719-30.

356. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc Natl Acad Sci U S A 2000; 97:5930-5.
357. Denger S, Reid G, Kos M, Flouriot G, Parsch D, Brand H, Korach KS, Sonntag-Buck V, Gannon F. ERalpha gene expression in human primary osteoblasts: evidence for the expression of two receptor proteins. Mol Endocrinol 2001; 15:2064-77.
358. Berry M, Metzger D, Chambon P. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J 1990; 9:2811-8.
359. Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW, McDonnell DP. Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 1994; 8:21-30.
360. Norris JD, Fan D, Kerner SA, McDonnell DP. Identification of a third autonomous activation domain within the human estrogen receptor. Mol Endocrinol 1997; 11:747-54.
361. Monje P, Zanello S, Holick M, Boland R. Differential cellular localization of estrogen receptor alpha in uterine and mammary cells. Mol Cell Endocrinol 2001; 181:117-29.
362. Ivanova MM, Mazhawidza W, Dougherty SM, Klinge CM. Sex differences in estrogen receptor subcellular location and activity in lung adenocarcinoma cells. Am J Respir Cell Mol Biol; 42:320-30.
363. Patel JD. Lung cancer in women. J Clin Oncol 2005; 23:3212-8.
364. Swaneck GE, Fishman J. Covalent binding of the endogenous estrogen 16 alpha-hydroxyestrone to estradiol receptor in human breast cancer cells: characterization and intranuclear localization. Proc Natl Acad Sci U S A 1988; 85:7831-5.
365. Schneider J, Kinne D, Fracchia A, Pierce V, Anderson KE, Bradlow HL, Fishman J. Abnormal oxidative metabolism of estradiol in women with breast cancer. Proc Natl Acad Sci U S A 1982; 79:3047-51.
366. Fishman J, Schneider J, Hershcope RJ, Bradlow HL. Increased estrogen-16 alpha-hydroxylase activity in women with breast and endometrial cancer. J Steroid Biochem 1984; 20:1077-81.

367. Gushima M, Kawate H, Ohnaka K, Nomura M, Takayanagi R. Raloxifene induces nucleolar translocation of the estrogen receptor. Mol Cell Endocrinol; 319:14-22.
368. Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM, Jr., Valencia T, Brun-Zinkernagel AM, Prokai L, Will Y, Dykens J, Koulen P, Simpkins JW. Mitochondrial localization of estrogen receptor beta. Proc Natl Acad Sci U S A 2004; 101:4130-5.
369. Vasconsuelo A, Milanesi L, Boland R. 17Beta-estradiol abrogates apoptosis in murine skeletal muscle cells through estrogen receptors: role of the phosphatidylinositol 3-kinase/Akt pathway. J Endocrinol 2008; 196:385-97.
370. Milanesi L, Vasconsuelo A, de Boland AR, Boland R. Expression and subcellular distribution of native estrogen receptor beta in murine C2C12 cells and skeletal muscle tissue. Steroids 2009; 74:489-97.
371. Watson GH, Muldoon TG. Specific binding of estrogen and estrogen-receptor complex by microsomes from estrogen-responsive tissues of the rat. Endocrinology 1985; 117:1341-9.
372. Muldoon TG, Watson GH, Evans AC, Jr., Steinsapir J. Microsomal receptor for steroid hormones: functional implications for nuclear activity. J Steroid Biochem 1988; 30:23-31.
373. Li S, Han B, Liu G, Ouellet J, Labrie F, Pelletier G. Immunocytochemical localization of sex steroid hormone receptors in normal human mammary gland. J Histochem Cytochem; 58:509-15.
374. Nelson LR, Bulun SE. Estrogen production and action. J Am Acad Dermatol 2001; 45:S116-24.
375. Chen S, Shively JE, Nakajin S, Shinoda M, Hall PF. Amino terminal sequence analysis of human placenta aromatase. Biochem Biophys Res Commun 1986; 135:713-9.
376. Whitlock JP, Jr. The regulation of cytochrome P-450 gene expression. Annu Rev Pharmacol Toxicol 1986; 26:333-69.
377. Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 1994; 15:342-55.

378. McTernan PG, Anderson LA, Anwar AJ, Eggo MC, Crocker J, Barnett AH, Stewart PM, Kumar S. Glucocorticoid regulation of p450 aromatase activity in human adipose tissue: gender and site differences. J Clin Endocrinol Metab 2002; 87:1327-36.
379. Jacobson HI, Lemanski N, Narendran A, Agarwal A, Bennett JA, Andersen TT. Hormones of pregnancy, alpha-feto protein, and reduction of breast cancer risk. Adv Exp Med Biol 2008; 617:477-84.
380. Ziegler RG, Rossi SC, Fears TR, Bradlow HL, Adlercreutz H, Sepkovic D, Kiuru P, Wahala K, Vaught JB, Donaldson JL, Falk RT, Fillmore CM, Siiteri PK, Hoover RN, Gail MH. Quantifying estrogen metabolism: an evaluation of the reproducibility and validity of enzyme immunoassays for 2-hydroxyestrone and 16alpha-hydroxyestrone in urine. Environ Health Perspect 1997; 105 Suppl 3:607-14.
381. Davis DL, Bradlow HL, Wolff M, Woodruff T, Hoel DG, Anton-Culver H. Medical hypothesis: xenoestrogens as preventable causes of breast cancer. Environ Health Perspect 1993; 101:372-7.
382. Bradlow HL, Hershcopf RJ, Martucci CP, Fishman J. Estradiol 16 alpha-hydroxylation in the mouse correlates with mammary tumor incidence and presence of murine mammary tumor virus: a possible model for the hormonal etiology of breast cancer in humans. Proc Natl Acad Sci U S A 1985; 82:6295-9.
383. Abul-Hajj YJ. Binding of catechol estrogens to the estrogen receptor of dimethylbenz(A) anthracene induced rat mammary tumors. J Steroid Biochem 1980; 13:83-8.
384. Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci U S A 1998; 95:6965-70.
385. Shozu M, Akasofu K, Harada T, Kubota Y. A new cause of female pseudohermaphroditism: placental aromatase deficiency. J Clin Endocrinol Metab 1991; 72:560-6.
386. Ito Y, Fisher CR, Conte FA, Grumbach MM, Simpson ER. Molecular basis of aromatase deficiency in an adult female with sexual infantilism and polycystic ovaries. Proc Natl Acad Sci U S A 1993; 90:11673-7.
387. Conte FA, Grumbach MM, Ito Y, Fisher CR, Simpson ER. A syndrome of female pseudohermaphrodism, hypergonadotropic hypogonadism, and multicystic ovaries associated

with missense mutations in the gene encoding aromatase (P450arom). J Clin Endocrinol Metab 1994; 78:1287-92.
388. Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 1995; 80:3689-98.
389. Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER. Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 1997; 337:91-5.
390. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 1994; 331:1056-61.
391. Master-Hunter T, Heiman DL. Amenorrhea: evaluation and treatment. Am Fam Physician 2006; 73:1374-82.
392. Burke SN, Barnes CA. Neural plasticity in the ageing brain. Nat Rev Neurosci 2006; 7:30-40.
393. Shaywitz SE, Shaywitz BA, Pugh KR, Fulbright RK, Skudlarski P, Mencl WE, Constable RT, Naftolin F, Palter SF, Marchione KE, Katz L, Shankweiler DP, Fletcher JM, Lacadie C, Keltz M, Gore JC. Effect of estrogen on brain activation patterns in postmenopausal women during working memory tasks. JAMA 1999; 281:1197-202.
394. Revill P. The functional significance of cerebral estrogen synthesis. Drug News Perspect 2007; 20:387-94.
395. Soto AM, Justicia H, Wray JW, Sonnenschein C. p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 1991; 92:167-73.
396. Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 1993; 132:2279-86.
397. Singer PL. Occupational oligospermia. JAMA 1949; 140:1249.
398. Fry DM. Sex ratio skew and breeding patterns of gulls: demographic and toxicological considerations. Studies in Avian Biology 1987; 10:26-43.
399. L. J. Guillette TSG, G. R. Masson, J. M. Matter, H. F. Percival and A. R. Woodward. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in

juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect 1994; 102:680-8.
400. Guzelian PS. Comparative toxicology of chlorodecone (kepone) in humans and experimental animals. Annu Rev Pharmocol Toxicol 1982; 22:89-113.
401. C. E. Purdom PAH, V. J. Bye, N. C. Eno, C. R. Tyler and J. P. Sumpter. Estrogenic effects from sewage treatmemt works. Chem Ecol 1994; 8:275-85.
402. J. A. Brotons MFO-S, M. Villalobos and N. Olea. Xenoestrogens released from lacquer coating in food cans. Environ Health Perspect 1994; 103:608-12.
403. Pickart CM. Targeting of substrates to the 26S proteasome. FASEB J 1997; 11:1055-66.
404. Haas AL, Siepmann TJ. Pathways of ubiquitin conjugation. FASEB J 1997; 11:1257-68.
405. Chen ZJ, Parent L, Maniatis T. Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell 1996; 84:853-62.
406. Ciechanover A, Schwartz AL. The ubiquitin-mediated proteolytic pathway: mechanisms of recognition of the proteolytic substrate and involvement in the degradation of native cellular proteins. FASEB J 1994; 8:182-91.
407. Murray EJ, Bentley GV, Grisanti MS, Murray SS. The ubiquitin-proteasome system and cellular proliferation and regulation in osteoblastic cells. Exp Cell Res 1998; 242:460-9.
408. Huibregtse JM, Scheffner M, Beaudenon S, Howley PM. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A 1995; 92:2563-7.
409. van Wijk SJ, Timmers HT. The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. FASEB J; 24:981-93.
410. Confalonieri S, Quarto M, Goisis G, Nuciforo P, Donzelli M, Jodice G, Pelosi G, Viale G, Pece S, Di Fiore PP. Alterations of ubiquitin ligases in human cancer and their association with the natural history of the tumor. Oncogene 2009; 28:2959-68.
411. Sherwood SW, Kung AL, Roitelman J, Simoni RD, Schimke RT. In vivo inhibition of cyclin B degradation and induction of cell-cycle arrest in mammalian cells by the neutral cysteine protease inhibitor N-acetylleucylleucylnorleucinal. Proc Natl Acad Sci U S A 1993; 90:3353-7.

412. Hershko A, Ganoth D, Sudakin V, Dahan A, Cohen LH, Luca FC, Ruderman JV, Eytan E. Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2. J Biol Chem 1994; 269:4940-6.
413. Peters JM, King RW, Hoog C, Kirschner MW. Identification of BIME as a subunit of the anaphase-promoting complex. Science 1996; 274:1199-201.
414. Grossberger R, Gieffers C, Zachariae W, Podtelejnikov AV, Schleiffer A, Nasmyth K, Mann M, Peters JM. Characterization of the DOC1/APC10 subunit of the yeast and the human anaphase-promoting complex. J Biol Chem 1999; 274:14500-7.
415. Kim TK, Maniatis T. Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. Science 1996; 273:1717-9.
416. Lonard DM, Nawaz Z, Smith CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell 2000; 5:939-48.
417. Li C, Wu RC, Amazit L, Tsai SY, Tsai MJ, O'Malley BW. Specific amino acid residues in the basic helix-loop-helix domain of SRC-3 are essential for its nuclear localization and proteasome-dependent turnover. Mol Cell Biol 2007; 27:1296-308.
418. Sarff M, Gorski J. Control of estrogen binding protein concentration under basal conditions and after estrogen administration. Biochemistry 1971; 10:2557-63.
419. Jensen EV, Suzuki T, Numata M, Smith S, DeSombre ER. Estrogen-binding substances of target tissues. Steroids 1969; 13:417-27.
420. Dworniczak B, Mirault ME. Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein. Nucleic Acids Res 1987; 15:5181-97.
421. Hohfeld J, Cyr DM, Patterson C. From the cradle to the grave: molecular chaperones that may choose between folding and degradation. EMBO Rep 2001; 2:885-90.
422. Meacham GC, Patterson C, Zhang W, Younger JM, Cyr DM. The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat Cell Biol 2001; 3:100-5.
423. Murata S, Minami Y, Minami M, Chiba T, Tanaka K. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep 2001; 2:1133-8.
424. Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature 2003; 426:895-9.

425. Mader S, Kumar V, de Verneuil H, Chambon P. Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. Nature 1989; 338:271-4.
426. Tateishi Y, Kawabe Y, Chiba T, Murata S, Ichikawa K, Murayama A, Tanaka K, Baba T, Kato S, Yanagisawa J. Ligand-dependent switching of ubiquitin-proteasome pathways for estrogen receptor. EMBO J 2004; 23:4813-23.
427. Fan M, Nakshatri H, Nephew KP. Inhibiting proteasomal proteolysis sustains estrogen receptor-alpha activation. Mol Endocrinol 2004; 18:2603-15.
428. Grisouard J, Medunjanin S, Hermani A, Shukla A, Mayer D. Glycogen synthase kinase-3 protects estrogen receptor alpha from proteasomal degradation and is required for full transcriptional activity of the receptor. Mol Endocrinol 2007; 21:2427-39.
429. Medunjanin S, Hermani A, De Servi B, Grisouard J, Rincke G, Mayer D. Glycogen synthase kinase-3 interacts with and phosphorylates estrogen receptor alpha and is involved in the regulation of receptor activity. J Biol Chem 2005; 280:33006-14.
430. Cardona-Gomez P, Perez M, Avila J, Garcia-Segura LM, Wandosell F. Estradiol inhibits GSK3 and regulates interaction of estrogen receptors, GSK3, and beta-catenin in the hippocampus. Mol Cell Neurosci 2004; 25:363-73.
431. Mendez P, Garcia-Segura LM. Phosphatidylinositol 3-kinase and glycogen synthase kinase 3 regulate estrogen receptor-mediated transcription in neuronal cells. Endocrinology 2006; 147:3027-39.
432. De Servi B, Hermani A, Medunjanin S, Mayer D. Impact of PKCdelta on estrogen receptor localization and activity in breast cancer cells. Oncogene 2005; 24:4946-55.
433. Kenney NJ, Dickson RB. Growth factor and sex steroid interactions in breast cancer. J Mammary Gland Biol Neoplasia 1996; 1:189-98.
434. Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB. Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. Proc Natl Acad Sci U S A 1997; 94:12786-91.
435. Yue W, Wang JP, Li Y, Fan P, Liu G, Zhang N, Conaway M, Wang H, Korach KS, Bocchinfuso W, Santen R. Effects of estrogen on breast cancer development: Role of estrogen receptor independent mechanisms. Int J Cancer.

436. Zumoff B. Does postmenopausal estrogen administration increase the risk of breast cancer? Contributions of animal, biochemical, and clinical investigative studies to a resolution of the controversy. Proc Soc Exp Biol Med 1998; 217:30-7.
437. Hollingsworth AB, Lerner MR, Lightfoot SA, Wilkerson KB, Hanas JS, McCay PB, Brackett DJ. Prevention of DMBA-induced rat mammary carcinomas comparing leuprolide, oophorectomy, and tamoxifen. Breast Cancer Res Treat 1998; 47:63-70.
438. Breast cancer and hormonal contraceptives: further results. Collaborative Group on Hormonal Factors in Breast Cancer. Contraception 1996; 54:1S-106S.
439. Feinleib M. Breast cancer and artificial menopause: a cohort study. J Natl Cancer Inst 1968; 41:315-29.
440. Trichopoulos D, MacMahon B, Cole P. Menopause and breast cancer risk. J Natl Cancer Inst 1972; 48:605-13.
441. Clemons M, Goss P. Estrogen and the risk of breast cancer. N Engl J Med 2001; 344:276-85.
442. Chen WY, Manson JE, Hankinson SE, Rosner B, Holmes MD, Willett WC, Colditz GA. Unopposed estrogen therapy and the risk of invasive breast cancer. Arch Intern Med 2006; 166:1027-32.
443. Hulka BS. Epidemiologic analysis of breast and gynecologic cancers. Prog Clin Biol Res 1997; 396:17-29.
444. Kaaks R, Rinaldi S, Key TJ, Berrino F, Peeters PH, Biessy C, Dossus L, Lukanova A, Bingham S, Khaw KT, Allen NE, Bueno-de-Mesquita HB, van Gils CH, Grobbee D, Boeing H, Lahmann PH, Nagel G, Chang-Claude J, Clavel-Chapelon F, Fournier A, Thiebaut A, Gonzalez CA, Quiros JR, Tormo MJ, Ardanaz E, Amiano P, Krogh V, Palli D, Panico S, Tumino R, Vineis P, Trichopoulou A, Kalapothaki V, Trichopoulos D, Ferrari P, Norat T, Saracci R, Riboli E. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European prospective investigation into cancer and nutrition. Endocr Relat Cancer 2005; 12:1071-82.
445. Santen RJ. To block estrogen's synthesis or action: that is the question. J Clin Endocrinol Metab 2002; 87:3007-12.
446. Liehr JG. Is estradiol a genotoxic mutagenic carcinogen? Endocr Rev 2000; 21:40-54.

447. Hahn WC, Weinberg RA. Rules for making human tumor cells. N Engl J Med 2002; 347:1593-603.
448. Gupta PB, Proia D, Cingoz O, Weremowicz J, Naber SP, Weinberg RA, Kuperwasser C. Systemic stromal effects of estrogen promote the growth of estrogen receptor-negative cancers. Cancer Res 2007; 67:2062-71.
449. Villaseca P, Campino C, Oestreicher E, Mayerson D, Seron-Ferre M, Arteaga E. Bilateral oophorectomy in a pregnant woman: hormonal profile from late gestation to post-partum: case report. Hum Reprod 2005; 20:397-401.
450. Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Popescu NC, Hahn WC, Weinberg RA. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev 2001; 15:50-65.
451. Prossnitz ER, Barton M. Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER. Prostaglandins Other Lipid Mediat 2009; 89:89-97.
452. Smith HO, Arias-Pulido H, Kuo DY, Howard T, Qualls CR, Lee SJ, Verschraegen CF, Hathaway HJ, Joste NE, Prossnitz ER. GPR30 predicts poor survival for ovarian cancer. Gynecol Oncol 2009; 114:465-71.
453. Kuo WH, Chang LY, Liu DL, Hwa HL, Lin JJ, Lee PH, Chen CN, Lien HC, Yuan RH, Shun CT, Chang KJ, Hsieh FJ. The interactions between GPR30 and the major biomarkers in infiltrating ductal carcinoma of the breast in an Asian population. Taiwan J Obstet Gynecol 2007; 46:135-45.
454. Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, Steinhoff MM, Sabo E. Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. Clin Cancer Res 2006; 12:6359-66.
455. Vivacqua A, Bonofiglio D, Albanito L, Madeo A, Rago V, Carpino A, Musti AM, Picard D, Ando S, Maggiolini M. 17beta-estradiol, genistein, and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the g protein-coupled receptor GPR30. Mol Pharmacol 2006; 70:1414-23.
456. Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Ando S, Maggiolini M. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by

17beta-estradiol and hydroxytamoxifen in endometrial cancer cells. Mol Endocrinol 2006; 20:631-46.

457. Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, Oprea TI, Prossnitz ER, Musti AM, Ando S, Maggiolini M. 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; 67:1859-66.

458. Albanito L, Sisci D, Aquila S, Brunelli E, Vivacqua A, Madeo A, Lappano R, Pandey DP, Picard D, Mauro L, Ando S, Maggiolini M. Epidermal growth factor induces G protein-coupled receptor 30 expression in estrogen receptor-negative breast cancer cells. Endocrinology 2008; 149:3799-808.

459. Ahola TM, Manninen T, Alkio N, Ylikomi T. G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. Endocrinology 2002; 143:3376-84.

460. 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; 103:9063-8.

461. Kang L, Zhang X, Xie Y, Tu Y, Wang D, Liu Z, Wang ZY. Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. Mol Endocrinol; 24:709-21.

462. 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; 83:2496-500.

463. Keyomarsi K, Sandoval L, Band V, Pardee AB. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res 1991; 51:3602-9.

464. Hyland PL, Keegan AL, Curran MD, Middleton D, McKenna PG, Barnett YA. Effect of a dCTP:dTTP pool imbalance on DNA replication fidelity in Friend murine erythroleukemia cells. Environ Mol Mutagen 2000; 36:87-96.

465. Harper JV. Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. Methods Mol Biol 2005; 296:157-66.

466. Pedrali-Noy G, Spadari S. Mechanism of inhibition of herpes simplex virus and vaccinia virus DNA polymerases by aphidicolin, a highly specific inhibitor of DNA replication in eucaryotes. J Virol 1980; 36:457-64.
467. Jordan MA, Thrower D, Wilson L. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. J Cell Sci 1992; 102 (Pt 3):401-16.
468. Vasquez RJ, Howell B, Yvon AM, Wadsworth P, Cassimeris L. Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. Mol Biol Cell 1997; 8:973-85.
469. Remy-Heintz N, Duperray C, Bali JP. [Study of S phase synchronization of the human gastric cancer line HGT-1]. C R Seances Soc Biol Fil 1993; 187:678-88.
470. Javanmoghadam-Kamrani S, Keyomarsi K. Synchronization of the cell cycle using lovastatin. Cell Cycle 2008; 7:2434-40.
471. Ikegami S, Amemiya S, Oguro M, Nagano H, Mano Y. Inhibition by aphidicolin of cell cycle progression and DNA replication in sea urchin embryos. J Cell Physiol 1979; 100:439-44.
472. Kohrmann A, Kammerer U, Kapp M, Dietl J, Anacker J. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer 2009; 9:188.
473. Hansen RK, Bissell MJ. Tissue architecture and breast cancer: the role of extracellular matrix and steroid hormones. Endocr Relat Cancer 2000; 7:95-113.
474. Akli S, Keyomarsi K. Cyclin E and its low molecular weight forms in human cancer and as targets for cancer therapy. Cancer Biol Ther 2003; 2:S38-47.
475. Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 2006; 6:181-202.
476. Ali SH, O'Donnell AL, Balu D, Pohl MB, Seyler MJ, Mohamed S, Mousa S, Dandona P. Estrogen receptor-alpha in the inhibition of cancer growth and angiogenesis. Cancer Res 2000; 60:7094-8.
477. Reis-Filho JS, Lakhani SR. Breast cancer special types: why bother? J Pathol 2008; 216:394-8.

478. Lim HS, Archer CT, Kodadek T. Identification of a peptoid inhibitor of the proteasome 19S regulatory particle. J Am Chem Soc 2007; 129:7750-1.
479. Voorhees PM, Orlowski RZ. The proteasome and proteasome inhibitors in cancer therapy. Annu Rev Pharmacol Toxicol 2006; 46:189-213.
480. Teicher BA, Ara G, Herbst R, Palombella VJ, Adams J. The proteasome inhibitor PS-341 in cancer therapy. Clin Cancer Res 1999; 5:2638-45.
481. Long X, Nephew KP. Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-alpha. J Biol Chem 2006; 281:9607-15.
482. Reid G, Hubner MR, Metivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F. Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. Mol Cell 2003; 11:695-707.
483. Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW. Proteasome-dependent degradation of the human estrogen receptor. Proc Natl Acad Sci U S A 1999; 96:1858-62.
484. Balleine RL, Hunt SM, Clarke CL. Coexpression of alternatively spliced estrogen and progesterone receptor transcripts in human breast cancer. J Clin Endocrinol Metab 1999; 84:1370-7.
485. Jeltsch JM, Roberts M, Schatz C, Garnier JM, Brown AM, Chambon P. Structure of the human oestrogen-responsive gene pS2. Nucleic Acids Res 1987; 15:1401-14.
486. Zhu Y, Qi C, Jain S, Le Beau MM, Espinosa R, 3rd, Atkins GB, Lazar MA, Yeldandi AV, Rao MS, Reddy JK. Amplification and overexpression of peroxisome proliferator-activated receptor binding protein (PBP/PPARBP) gene in breast cancer. Proc Natl Acad Sci U S A 1999; 96:10848-53.
487. Hanahan D. Studies on transformation of Escherichia coli with plasmids. J Mol Biol 1983; 166:557-80.
488. Narayanan R, Edwards DP, Weigel NL. Human progesterone receptor displays cell cycle-dependent changes in transcriptional activity. Mol Cell Biol 2005; 25:2885-98.
489. Morgan DO. The Cell Cycle: Principles of Control. London: New Science Press.
490. Schwartz JA, Zhong L, Deighton-Collins S, Zhao C, Skafar DF. Mutations targeted to a predicted helix in the extreme carboxyl-terminal region of the human estrogen receptor-

alpha alter its response to estradiol and 4-hydroxytamoxifen. J Biol Chem 2002; 277:13202-9.

491. Spurrier B, Ramalingam S, Nishizuka S. Reverse-phase protein lysate microarrays for cell signaling analysis. Nat Protoc 2008; 3:1796-808.

492. Licznar A, Caporali S, Lucas A, Weisz A, Vignon F, Lazennec G. Identification of genes involved in growth inhibition of breast cancer cells transduced with estrogen receptor. FEBS Lett 2003; 553:445-50.

493. Charpentier AH, Bednarek AK, Daniel RL, Hawkins KA, Laflin KJ, Gaddis S, MacLeod MC, Aldaz CM. Effects of estrogen on global gene expression: identification of novel targets of estrogen action. Cancer Res 2000; 60:5977-83.

494. Hodges LC, Cook JD, Lobenhofer EK, Li L, Bennett L, Bushel PR, Aldaz CM, Afshari CA, Walker CL. Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. Mol Cancer Res 2003; 1:300-11.

495. Bates S, Rowan S, Vousden KH. Characterisation of human cyclin G1 and G2: DNA damage inducible genes. Oncogene 1996; 13:1103-9.

496. Horne MC, Goolsby GL, Donaldson KL, Tran D, Neubauer M, Wahl AF. Cyclin G1 and cyclin G2 comprise a new family of cyclins with contrasting tissue-specific and cell cycle-regulated expression. J Biol Chem 1996; 271:6050-61.

497. Suzuki M. SPXX, a frequent sequence motif in gene regulatory proteins. J Mol Biol 1989; 207:61-84.

498. Platet N, Cunat S, Chalbos D, Rochefort H, Garcia M. Unliganded and liganded estrogen receptors protect against cancer invasion via different mechanisms. Mol Endocrinol 2000; 14:999-1009.

499. Spelsberg TC, Rories C, Rejman JJ, Goldberger A, Fink K, Lau CK, Colvard DS, Wiseman G. Steroid action on gene expression: possible roles of regulatory genes and nuclear acceptor sites. Biol Reprod 1989; 40:54-69.

500. Narayanan R, Adigun AA, Edwards DP, Weigel NL. Cyclin-dependent kinase activity is required for progesterone receptor function: novel role for cyclin A/Cdk2 as a progesterone receptor coactivator. Mol Cell Biol 2005; 25:264-77.

501. Musgrove EA, Lee CS, Sutherland RL. Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor

alpha, epidermal growth factor receptor, c-fos, and c-myc genes. Mol Cell Biol 1991; 11:5032-43.

502. Randy Poon TKF. Cyclin A2. 2007.

503. Chan QK, Lam HM, Ng CF, Lee AY, Chan ES, Ng HK, Ho SM, Lau KM. Activation of GPR30 inhibits the growth of prostate cancer cells through sustained activation of Erk1/2, c-jun/c-fos-dependent upregulation of p21, and induction of G(2) cell-cycle arrest. Cell Death Differ; 17:1511-23.

504. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998; 396:580-4.

505. Rosa J, Canovas P, Islam A, Altieri DC, Doxsey SJ. Survivin modulates microtubule dynamics and nucleation throughout the cell cycle. Mol Biol Cell 2006; 17:1483-93.

506. Schreihofer DA, Resnick EM, Lin VY, Shupnik MA. Ligand-independent activation of pituitary ER: dependence on PKA-stimulated pathways. Endocrinology 2001; 142:3361-8.

507. Maynadier M, Ramirez JM, Cathiard AM, Platet N, Gras D, Gleizes M, Sheikh MS, Nirde P, Garcia M. Unliganded estrogen receptor alpha inhibits breast cancer cell growth through interaction with a cyclin-dependent kinase inhibitor (p21(WAF1)). FASEB J 2008; 22:671-81.

508. Chen X, Danes C, Lowe M, Herliczek TW, Keyomarsi K. Activation of the estrogen-signaling pathway by p21(WAF1/CIP1) in estrogen receptor-negative breast cancer cells. J Natl Cancer Inst 2000; 92:1403-13.

509. Li J, Xu M, Zhou H, Ma J, Potter H. Alzheimer presenilins in the nuclear membrane, interphase kinetochores, and centrosomes suggest a role in chromosome segregation. Cell 1997; 90:917-27.

510. Lengyel F, Vertes Z, Kovacs KA, Kornyei JL, Sumegi B, Vertes M. Effect of estrogen and inhibition of phosphatidylinositol-3 kinase on Akt and FOXO1 in rat uterus. Steroids 2007; 72:422-8.

511. Apostolakis EM, Lanz R, O'Malley BW. Pituitary adenylate cyclase-activating peptide: a pivotal modulator of steroid-induced reproductive behavior in female rodents. Mol Endocrinol 2004; 18:173-83.

512. Lange CA. Making sense of cross-talk between steroid hormone receptors and intracellular signaling pathways: who will have the last word? Mol Endocrinol 2004; 18:269-78.
513. Knudsen KE, Cavenee WK, Arden KC. D-type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability. Cancer Res 1999; 59:2297-301.
514. Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown M, Ewen ME. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. Mol Cell Biol 1997; 17:5338-47.
515. Yamamoto A, Hashimoto Y, Kohri K, Ogata E, Kato S, Ikeda K, Nakanishi M. Cyclin E as a coactivator of the androgen receptor. J Cell Biol 2000; 150:873-80.
516. Peters GA, Khan SA. Estrogen receptor domains E and F: role in dimerization and interaction with coactivator RIP-140. Mol Endocrinol 1999; 13:286-96.
517. Thornton BR, Toczyski DP. Securin and B-cyclin/CDK are the only essential targets of the APC. Nat Cell Biol 2003; 5:1090-4.
518. Stark GR, Taylor WR. Control of the G2/M transition. Mol Biotechnol 2006; 32:227-48.
519. Ho KK, Myatt SS, Lam EW. Many forks in the path: cycling with FoxO. Oncogene 2008; 27:2300-11.
520. Chua BT, Gallego-Ortega D, Ramirez de Molina A, Ullrich A, Lacal JC, Downward J. Regulation of Akt(ser473) phosphorylation by choline kinase in breast carcinoma cells. Mol Cancer 2009; 8:131.
521. Koga M, Hirano K, Hirano M, Nishimura J, Nakano H, Kanaide H. Akt plays a central role in the anti-apoptotic effect of estrogen in endothelial cells. Biochem Biophys Res Commun 2004; 324:321-5.
522. Khor TO, Gul YA, Ithnin H, Seow HF. Positive correlation between overexpression of phospho-BAD with phosphorylated Akt at serine 473 but not threonine 308 in colorectal carcinoma. Cancer Lett 2004; 210:139-50.
523. Sisci D, Aquila S, Middea E, Gentile M, Maggiolini M, Mastroianni F, Montanaro D, Ando S. Fibronectin and type IV collagen activate ERalpha AF-1 by c-Src pathway: effect on breast cancer cell motility. Oncogene 2004; 23:8920-30.

524. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004; 4:253-65.
525. Huang HC, Shi J, Orth JD, Mitchison TJ. Evidence that mitotic exit is a better cancer therapeutic target than spindle assembly. Cancer Cell 2009; 16:347-58.
526. Zeng X, Sigoillot F, Gaur S, Choi S, Pfaff KL, Oh DC, Hathaway N, Dimova N, Cuny GD, King RW. Pharmacologic inhibition of the anaphase-promoting complex induces a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. Cancer Cell; 18:382-95.
527. Radulescu S, Ridgway RA, Appleton P, Kroboth K, Patel S, Woodgett J, Taylor S, Nathke IS, Sansom OJ. Defining the role of APC in the mitotic spindle checkpoint in vivo: APC-deficient cells are resistant to Taxol. Oncogene; 29:6418-27.
528. Baum M, Budzar AU, Cuzick J, Forbes J, Houghton JH, Klijn JG, Sahmoud T. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet 2002; 359:2131-9.
529. Esteva FJ, Hortobagyi GN. Comparative assessment of lipid effects of endocrine therapy for breast cancer: implications for cardiovascular disease prevention in postmenopausal women. Breast 2006; 15:301-12.
530. Broqua P, Riviere PJ, Conn PM, Rivier JE, Aubert ML, Junien JL. Pharmacological profile of a new, potent, and long-acting gonadotropin-releasing hormone antagonist: degarelix. J Pharmacol Exp Ther 2002; 301:95-102.
531. Engel JB, Schally AV. Drug Insight: clinical use of agonists and antagonists of luteinizing-hormone-releasing hormone. Nat Clin Pract Endocrinol Metab 2007; 3:157-67.
532. Lin YH, Wen YR, Chang Y, Seow KM, Hsieh BC, Hwang JL, Tzeng CR. Safety and efficacy of mixing cetrorelix with follitropin alfa: a randomized study. Fertil Steril; 94:179-83.
533. Van Poppel H, Tombal B, de la Rosette JJ, Persson BE, Jensen JK, Kold Olesen T. Degarelix: a novel gonadotropin-releasing hormone (GnRH) receptor blocker--results from a 1-yr, multicentre, randomised, phase 2 dosage-finding study in the treatment of prostate cancer. Eur Urol 2008; 54:805-13.

534. Chu I, Arnaout A, Loiseau S, Sun J, Seth A, McMahon C, Chun K, Hennessy B, Mills GB, Nawaz Z, Slingerland JM. Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. J Clin Invest 2007; 117:2205-15.
535. Moriki T, Takahashi T, Kataoka H, Hiroi M, Yamane T, Hara H. Proliferation marker MIB-1 correlates well with proliferative activity evaluated by BrdU in breast cancer: an immunohistochemical study including correlation with PCNA, p53, c-erbB-2 and estrogen receptor status. Pathol Int 1996; 46:953-61.
536. Zagorianakou N, Ioachim E, Mitselou A, Kitsou E, Zagorianakou P, Makrydimas G, Salmas M, Agnantis NJ. Immunohistochemical expression of heat shock protein 27, in normal hyperplastic and neoplastic endometrium: correlation with estrogen and progesterone receptor status, p53, pRb and proliferation associated indices (PCNA, MIB1). Eur J Gynaecol Oncol 2003; 24:299-304.
537. Diaz-Cruz ES, Furth PA. Deregulated estrogen receptor alpha and p53 heterozygosity collaborate in the development of mammary hyperplasia. Cancer Res; 70:3965-74.
538. Mukhopadhyay UK, Eves R, Jia L, Mooney P, Mak AS. p53 suppresses Src-induced podosome and rosette formation and cellular invasiveness through the upregulation of caldesmon. Mol Cell Biol 2009; 29:3088-98.
539. Akaogi K, Nakajima Y, Ito I, Kawasaki S, Oie SH, Murayama A, Kimura K, Yanagisawa J. KLF4 suppresses estrogen-dependent breast cancer growth by inhibiting the transcriptional activity of ERalpha. Oncogene 2009; 28:2894-902.
540. Shirley SH, Rundhaug JE, Tian J, Cullinan-Ammann N, Lambertz I, Conti CJ, Fuchs-Young R. Transcriptional regulation of estrogen receptor-alpha by p53 in human breast cancer cells. Cancer Res 2009; 69:3405-14.

VITA

Sonia Javan Moghaddam was born June 21, 1973 in Urmia, Iran. She grew up in Urmia, the little historic city hidden in the mountains located in the north west of Iran, until age 10 and then moved to Tehran. After graduating (majoring in mathematics) from Farasat high school in Tehran, in 1991, Sonia attended the Azad University in Tehran. She graduated with a B.S. in Microbiology in 1997. Upon finishing her B.S. she was actively involved in research as a research assistant in a genetics laboratory with the mentorship of Dr. Hossein Najmabadi. She moved once more. This time it was a much longer trip in distance and life, to AnnArbor, MI. Sonia married Ali kamrani and went back to school to pursue a Masters degree in Cellular and Molecular Biology under the mentorship of Dr. Daniel Clemans. She started to move toward her dream by engaging in a project on breast cancer at the University of Michigan with the mentorship of Dr. Kevin McDonagh, Upon another move from AnnArbor, MI to Houston, TX, Sonia returned to graduate school to pursue her Ph.D. degree in Cancer biology at the University of Texas- Houston Graduate School of Biomedical Sciences (GSBS) in 2004 under the mentorship of Dr. Khandan Keyomarsi. In Dr. Keyomarsi's lab she was compelled by the amount of research and resources dedicated toward breast cancer research. During these years Sonia and Ali were very fortunate to welcome their first son (Arshya Keanu) in to their lives in 2005, and their second son (Aria Sean) in 2009. The experiences she gained in these years have been beyond imagination.

She will use all the gained knowledge and foundation through these years of graduate school and research careers to pursue her final goal, which is her inner most desire to share them with others.