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Genomic Effects of Hormonal Adjuvant Therapies that Could Support the
Emergence of Drug Resistance in Breast Cancer

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An Abstract of
Genomic Effects of Hormonal Adjuvant Therapies that Could Support the Emergence
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The majority of breast tumors are estrogen receptor α (ER) positive. Hormonal adjuvant
therapy using tamoxifen (OH-Tam) or aromatase inhibitors (AIs) are the mainstay in the
treatment of ER+ breast cancer. However, resistance mechanisms develop during either
hormone depletion or OH-Tam adjuvant therapy. The mechanistic events underlying the
progression to emergence of drug resistance are poorly understood.

Hormonal adjuvant therapies are overall tumorstatic. In hormone-depleted MCF-7
breast cancer cells, ligand-independent actions of ER supported a basal fraction of S-
phase cells through maintenance of the basal expression of the retinoic acid receptor α1
(RARα1). The apo-ER/apo-RAR α1 axis regulated a set of genes that were both OH-Tam
insensitive and all-trans retinoic acid (ATRA)-insensitive. The target genes that were
activated by this axis predominantly promote cell cycle progression and mitosis whereas
the target genes repressed through the axis are predominantly negative regulators of the
cell cycle and mitosis. RARα1 was the only RARα isoform expressed in MCF-7 cells and most breast tumors and since this RAR subtype is known to be genetically redundant; we therefore propose that targeted inactivation or downregulation of RARα1 may improve hormonal adjuvant therapies by significantly reducing the ability of hormone-sensitive breast cancer cells to maintain a basal level of proliferation under conditions of hormone-depletion or OH-Tam treatment with minimal side effects on non-target tissues.

Another aspect of ER action that has been remarkably under-investigated is gene repression. E$_2$ directly and indirectly represses more genes than it activates and in the majority of those genes, hormonal adjuvants partially or fully block the repression. Using the folate receptor α gene promoter as a model, we established a novel non-classical mechanism for direct gene repression by E$_2$ in which ER forms a TAFII30-associated co-repressor complex; OH-Tam prevents this repression by simply dissociating the complex. Comprehensive ontology analysis of the subset of OH-Tam sensitive genes repressed by E$_2$ revealed predominant associations with physiological functions that support an aggressive tumor phenotype correlated with poor prognosis. Hormonal adjuvant treatments may prevent this repression and encourage aggressive phenotypes in tumors cells that have acquired hormone-insensitivity for growth.
I dedicate this Ph.D. thesis to my aunt Márcia Salazar-Valentine and Craig Valentine for their love and support throughout this journey.
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CHAPTER 1

LITERATURE REVIEW

Breast cancer etiology and epidemiology

Breast cancer is the most common type of cancer diagnosed and the second leading cause of death in American women, affecting 1 in every 8 women, according to the U.S. Cancer Statistics Working Group and the Center for Disease Control and Prevention (www.cdc.gov/uscs). Most breast cancer malignancies are lesions occurring in the epithelial lining of the ductal-lobular system involved in breast milk production (Yoder, Wilkinson et al. 2007). Ductal carcinomas account for approximately 75-80% of all cases of breast carcinomas and are divided into two subtypes: ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). Ductal carcinoma in situ or intraductal carcinoma usually characterizes early stages of the disease, when tumor cells are not invasive and proliferation is still confined to the mammary ducts (Tamimi, Baer et al. 2008); however, patients with DCIS are at a greater risk of developing IDC, characterized by an aggressive metastatic phenotype and poorer prognosis (Lagios 1995).

Unlike DCIS, the contribution of lobular carcinoma in situ lesions (LCIS), also known as lobular neoplasia to the invasive phenotype (Carolin, Tekyi-Mensah et al. 2002), as well as the incidence of LCIS in the general population is still unclear (Simpson, Gale et al. 2003). Invasive lobular carcinomas (ILC) arise in the milk producing glands at the terminal ends of the ducts, representing around 10% of all breast cancer cases.
Importantly, ILC and IDC have distinct metastatic patterns; metastasis to the peritoneum, ovary and gastrointestinal systems are associated with ILC whereas IDC are more likely to metastasize to the lungs and CNS (Arpino, Bardou et al. 2004). Other types of breast cancer, including tubular, invasive cribriform, invasive solid papillary, pure mucinous and apocrine carcinomas are rare (Yerushalmi, Hayes et al. 2009).

Even though breast cancer etiology is poorly understood, several risk factors have been identified and altogether, are important to help to prevent and reduce breast cancer incidence in women at high risk. Family history, hormonal and reproductive factors, as well as exposure to environmental carcinogens have been shown to greatly increase the risk of developing breast cancer (DeBruin and Josephy 2002; Hankinson, Colditz et al. 2004). Among women with a first degree relative who had breast cancer, inheritance of mutant BRCA1 or BRCA2 genes at least doubles the risk of developing sporadic breast tumors when compared with the general population (Colditz, Willett et al. 1993; Hedenfalk, Duggan et al. 2001); however the prognostic value of family history for screening purposes remains controversial (Phillips, Andruulis et al. 1999; Thalib, Wedren et al. 2004) and genetic predisposition only accounts for up to 10% of all cases of breast cancer (McPherson, Steel et al. 2000).

Regardless of family history of breast cancer and genetic predisposition, breast cancer incidence in humans increases with aging (Benz and Yau 2008). While hormonal replacement therapy (HRT) attenuates menopausal symptoms and helps to prevent osteoporosis and other chronic conditions in women (Nelson, Humphrey et al. 2002),
studies have demonstrated a direct association between HRT using estrogen-progestin regimen with increased breast cancer risk during the course of treatment (Schairer, Lubin et al. 2000; Chlebowski, Kuller et al. 2009). Further, postmenopausal women undergoing HRT using estrogen plus progestin that developed breast cancer were more likely to be diagnosed with advanced stage invasive breast cancer than the placebo group (Chlebowski, Hendrix et al. 2003).

Acquired mutations due to occupational exposure to hazardous agents, chemical pollutants and lifestyle factors like smoking and diet are known to represent potential risk factors for development of cancer in general, but a direct relationship between exposure to environmental xenoestrogens or synthetic chemicals and risk of developing breast cancer may be difficult to establish due to the lack of proper controlled experimental populations (Weyandt, Ellsworth et al. 2008).

The biology of Estrogen

Estrogens are steroid hormones involved in reproductive and non-reproductive processes in mammals. Synthesized and released mainly by the ovaries, estrogens are essential for the progression of the ovarian cycle, bone homeostasis, protection of the cardiovascular system and cognitive networks in the brain (Riggs, Khosla et al. 2002; Huang and Kaley 2004; Maggi, Ciana et al. 2004; Hewitt, Harrell et al. 2005). Estradiol (17-β estradiol) (E$_2$) is the major circulating form of estrogen and the most potent estrogen steroid. Due to its hydrophobic properties, E$_2$ easily diffuses across the cell membrane, binding to intracellular estrogen receptors (ER) with a high affinity. ER types
-α and β are members of the steroid/thyroid hormone superfamily of nuclear transcription factors, modulating gene expression either by direct interaction with the DNA or through tethering mechanisms on the target gene. In spite of the physiological regulatory roles of E₂ in maintaining homeostasis, there is a strong correlation between exposure to high levels of E₂ and development of breast cancer (Nilsson, Makela et al. 2001).

Estrogen and its presence in the ovaries was first described in 1923 (Allen and Doisy 1923), but the carcinogenic action of estrogen on breast cancer was only recognized in the following decade (Lacassagne 1936). However, during the late nineteenth century, Beaston’s work was the first to establish a link between oophorectomy and regression of metastatic breast cancer (Beatson 1896). After conducting oophorectomy surgery in two premenopausal women with breast cancer, he observed an initial tumor regression, which represented a turning point in the field of breast cancer research (Jordan 2009).

In both pre- and postmenopausal women, E₂ stimulates breast cancer cell proliferation by supporting entry into S phase during cell cycle progression (Prall, Sarcevic et al. 1997; Foster, Henley et al. 2001); while in pre menopausal women E₂ is mostly produced in the ovaries, in post menopausal women estrogens are mainly synthesized from steroid hormone precursors in peripheral tissues. During the development and progression of breast cancer, high levels of E₂ are also synthesized within the intratumoral microenvironment, while E₂ levels are several folds higher than the circulating plasma levels (Castagnetta, Lo Casto et al. 1996; Pasqualini, Chetrite et al. 1996; Ali and Coombes 2002). Studies in vitro using MCF-7 cells have also indicated that upon
binding to ER, E₂ up-regulates the anti-apoptotic protein Bcl-2 by directly binding to two estrogen response elements (ERE) present within the Bcl-2 gene coding region, thus protecting breast cancer cells against cell death by apoptosis (Perillo, Sasso et al. 2000).

In normal breast tissue, estrogen signaling is essential for mammary gland development and proper ductal elongation; though ERα is expressed at low to intermediate levels mostly in non-proliferating luminal epithelial cells, estrogen-induced proliferation and ductal morphogenesis results from estrogen-mediated secretion of paracrine factors in the breast stroma, promoting ductal outgrowth of adjacent neighboring cells (LaMarca and Rosen 2007). In contrast, the majority of breast tumors express high levels of ERα in proliferating breast cancer cells, leading to the activation of signaling pathways involved in tumor progression (Fowler and Alarid 2007). Although the role of ERα in stimulating breast cancer growth and progression is clear, the role of ERβ is less understood (Ali and Coombes 2000; Fox, Davis et al. 2008).

**The Current Clinical Status of OH-Tam**

OH-Tam is a Selective Estrogen Receptor Modulator (SERM) widely used in adjuvant therapy following surgery and chemotherapy/radiotherapy for the treatment of ERα + breast tumors. In estrogen-dependent breast cancer cells, OH-Tam opposes estrogen-stimulated growth and induces apoptosis, inhibiting overall tumor growth (Jordan 2004). OH-Tam inhibits breast tumor growth is by promoting the preferential recruitment of co-repressor complexes associated with histone deacetylases (HDAC) to E₂ responsive genes, creating a repressive chromatin conformation and silencing
transcription of E₂ target genes (Shiau, Barstad et al. 1998; Liu and Bagchi 2004). Alternatively, OH-Tam has been shown to stimulate apoptosis in breast cancer cells through downregulation of anti-apoptotic proteins of the Bcl-2 family, by stimulating mitochondrial nitric oxide synthase (mtNOS) activity, inducing mitochondrial oxidative stress and by up-regulating c-Myc expression (Zhang, Kimijima et al. 1999; Mandlekar and Kong 2001; Nazarewicz, Zenebe et al. 2007).

As a chemopreventative agent, OH-Tam is equally effective in reducing breast cancer incidence in both pre- and postmenopausal woman at high risk. Treatment with OH-Tam is usually successful over a period of 5 years; and the benefits of OH-Tam actions persist for an additional 5 years after cessation of treatment (Parton and Smith 2008). However, OH-Tam treatment for more than 3 years accounts for a reduction in the incidence of invasive breast cancer of only about 50% in woman at high risk, leaving considerable room for improvement (Dunn and Ford 2001; Cuzick, Powles et al. 2003). Case control studies have shown that the incidence of endometrial cancer increases with the duration of OH-Tam treatment and that the risk was not reduced up to at least 5 years after cessation of the treatment (Cuzick, Powles et al. 2003; Swerdlow and Jones 2005).

Around one third of the ER+ breast cancer cells are intrinsically resistant to OH-Tam and fail to respond to initial treatment. On the other hand, acquired mechanisms of OH-Tam resistance usually take place after long term treatment, resulting in disease recurrence. Eventually, the tumor cells could even become dependent on OH-Tam for growth (Osborne 1998; Fisher, Jeong et al. 2001; Ali and Coombes 2002; Lewis and
Jordan 2005; Hayashi and Yamaguchi 2006; Sengupta and Jordan 2008). The potential tumorigenic actions of OH-Tam were further confirmed in vivo using xenograft models of OH-Tam-dependent human endometrial cancer (O'Regan, Cisneros et al. 1998; O'Regan, Gajdos et al. 2002) and breast cancer (Osborne, Hobbs et al. 1985; Osborne, Coronado et al. 1987; Gottardis and Jordan 1988; Wolf and Jordan 1994).

The mechanisms underlying OH-Tam resistance are poorly understood. It is possible that during tumorigenesis, de novo ER+ tumors counteract OH-Tam-mediated growth inhibition by facilitating promiscuous interactions of ER with co-regulatory proteins and/or DNA, leading to activation of alternative signaling pathways involved with cellular proliferation and survival. The mechanisms contributing to this phenomenon remains to be fully characterized (Schafer, Lee et al. 2001).

Several mechanisms involved in drug uptake and retention or drug metabolism, overexpression of proto-oncogenes and increased tumor angiogenesis certainly play a role in the development of resistance to hormone therapy (MacGregor and Jordan 1998). Genetic polymorphisms associated with the cytochrome P450 2D6 (CYP2D6) enzyme, important to metabolize OH-Tam into active and more potent antagonists like endoxifen, can predict OH-Tam response. To date, there are over 80 different single nucleotide polymorphisms associated with the CYP2D6 gene identified. Individuals carrying non-functional or dysfunctional alleles are considered to be poor or intermediate metabolizers, which compromises the overall OH-Tam response when compared with individuals carrying CYP2D6 functional alleles (Beverage, Sissung et al. 2007).
Increase in the availability of co-activators (eg. SRC-3/NCOA3/AIB1) (Anzick, Kononen et al. 1997; Osborne, Bardou et al. 2003) or a reduction in the availability of co-repressors (eg. NCoR) contributes to the development of OH-Tam resistance and dependence for growth in breast and endometrial cancer cells. Amplified in breast cancer 1 (AIB1) is a member of the p160/steroid receptor coactivator and considered to be an important oncogene in breast cancer. In breast cancer cells, AIB1 competes with PAX2, a transcriptional co-repressor for binding to the cis-regulatory elements of the erbB2 (epidermal growth factor EGF-receptor related protein tyrosine kinase B2) gene, modulating mRNA expression by tethering to liganded ER (Hurtado, Holmes et al. 2008). Amplification/overexpression of erbB2 is directly correlated with resistance to hormonal adjuvant therapy and the ratio between AIB1 versus PAX2 expression levels determines whether transcription of the erbB2 gene is repressed or activated (Hurtado, Holmes et al. 2008). Even though it is controversial whether or not overexpression of AIB1 alone in patients with breast cancer is correlated with a worse outcome, high levels of AIB1 in hormone-responsive tumors overexpressing the human epidermal growth factor receptor 2 (HER-2, also known as erbB2) is associated with OH-Tam resistance and shorter disease free survival (Shou, Massarweh et al. 2004; Su, Hu et al. 2008; Lahusen, Henke et al. 2009). Knocking down AIB1 in vitro in MCF-7 cells reduced estrogen-stimulated cell proliferation by inducing S/G2-M cell cycle arrest and increased the rate of apoptosis, further confirming the regulatory role of AIB1 in estrogen-mediated cell growth (Karmakar, Foster et al. 2009). In addition to AIB1 and HER-2, which have been shown to mediate estrogen-like activity of OH-Tam in breast cancer cells, OH-Tam
can also act as a SRC-1-dependent $E_2$ agonist in Ishikawa cells, supporting endometrial cancer cell growth and contributing to resistance (Shang and Brown 2002; Shou, Massarweh et al. 2004).

Amplification of HER-2 occurs in approximately 30% of breast cancers (Slamon, Clark et al. 1987; Slamon, Godolphin et al. 1989). HER-2 is a ligandless receptor member of the tyrosine kinase family. In mammary carcinomas, HER-2 amplification and overexpression is an independent predictor of early metastasis, shorter disease free survival and OH-Tam resistance (King, Kraus et al. 1985; Semba, Kamata et al. 1985; Slamon, Clark et al. 1987; Tiwari, Borgen et al. 1992; Borg, Baldetorp et al. 1994). *In vitro*, overexpression of HER-2 proto-oncogene in MCF-7 cells leads to acquired OH-Tam resistance due to phosphorylation of ER and activation of multiple ligand-independent signaling pathways including AKT and ERK1,2 mitogen-activated protein kinase (MAPK) (Shou, Massarweh et al. 2004). In pre-clinical and in early-stage breast cancer patients, overexpression and/or amplification of HER-2 confers intrinsic resistance to OH-Tam therapy (Benz, Scott et al. 1992; Carломagno, Perrone et al. 1996).

Alternative mechanisms of resistance involve phosphorylation of ER through the RAS-RAF-ERK pathway, the PI3/AKT pathway and G-protein-coupled receptors, as well as other kinases, conferring ligand-independent transcriptional activity (Ali and Coombes 2002; Lewis and Jordan 2005). On the other hand, protein Kinase A (PKA) or Src-mediated phosphorylation of specific ER residues may also increase estrogen binding
affinity up to 7-fold, possibly contributing to E$_2$-hypersensitivity (Weigel and Moore 2007).

So far, the mechanisms leading to OH-Tam resistance that were previously suggested only partially address the clinical problem. Importantly, acquired resistance to OH-Tam is not usually correlated with ER down-regulation, and most of the breast cancer cells remain sensitive to agents that down-regulate ER (Howell, DeFriend et al. 1996). Mutations in the ER coding region are unusual, accounting for only 1% of primary breast tumors and rarely affecting its function (Ali and Coombes 2002; Pearce and Jordan 2004). In the vast majority of the cases, OH-Tam resistance must occur through altered ER signaling events. Besides the development of new therapeutic alternatives to OH-Tam for the treatment of early and advanced stages of breast cancer in postmenopausal women, pre-menopausal women still rely on OH-Tam as the drug of choice. Thus, OH-Tam is a mainstay in the prevention and treatment of breast cancer.

**Aromatase inhibitors**

Postmenopausal women with ER+ breast cancer can also benefit from adjuvant therapy using aromatase inhibitors (AI). Aromatase is an estrogen synthetase, which exerts its mechanisms of action by catalyzing E$_2$ synthesis from steroid precursors in the ovaries and in peripheral tissues including bone, brain, breast and adipose tissue. In the presence of high affinity AI, whole-body aromatase activity is inhibited in a dose-dependent manner and with relatively high selectivity. Type I aromatase inhibitors are steroidal inhibitors such as exemestane, which irreversibly bind to the catalytic site of the
enzyme, leading to long term inhibition of aromatase activity by a mechanism called “suicide inhibition” (Miller 2003). Recent studies using MCF-7 cells stably transfected with the aromatase gene (MCF-7 arco cells) have suggested that exemestane also induces proteasomal degradation of aromatase (Wang and Chen 2006). In this case, the duration of inhibition of E₂ biosynthesis is directly dependent upon the rate of de novo synthesis of the aromatase. In contrast, type II AI inhibitors are non-steroidal compounds such as anastrozole and letrozole, which function as pseudo-substrates causing short term inhibition of aromatase activity in a reversible manner by competing with androstenedione substrates; as soon as the treatment is discontinued, estrogen biosynthesis is restricted to normal levels (Johnston and Dowsett 2003; Campos 2004).

During the development and progression of breast cancer in postmenopausal women, aromatase activity persists in non-ovarian tissues and E₂ biosynthesis mediated by aromatase is responsible for driving E₂-mediated tumor cell proliferation. Upon treatment with AI, intra-tumoral and circulating E₂ levels are reduced to virtually being absent, blocking tumor growth (Santen, Santner et al. 1997; Goss and von Eichel 2007). In addition to inhibiting E₂-stimulated tumor growth by depleting E₂, AI also cause tumor regression by activating pro-apoptotic signaling pathways. Even though polymorphisms associated with the aromatase gene CYP19 were previously identified, to date, no correlation with increased breast cancer risk or aromatase activity have been described (Healey, Dunning et al. 2000).
Treatment of breast cancer cells in vitro using AI induced cell death by up-regulating the levels of pro-apoptotic proteins such as P53, P21 and Bax, activating caspases 6, -7 and -9, along with downregulating cyclin D1 and c-Myc (Thiantanawat, Long et al. 2003). Alternatively, treatment of estrogen-dependent MCF-7 cells stably transfected with the aromatase gene with AI also induced vacuolization of the cytoplasm, features related to cell death by autophagy (Cepa, Correia-da-Silva et al. 2008). So far, the use of AI is restricted to postmenopausal women due to feedback loop responses to AI in the hypothalamus-pituitary axis in premenopausal women, leading to an increase in gonadotropin secretion and ultimately, estrogen synthesis (Freedman, Verma et al. 2006).

Several multicentre, double-blind clinical trials have demonstrated the superiority of third generation AI (anastrozole, letrozole and exemestane) over OH-Tam in the adjuvant setting for treatment of early and late stage ER+ breast cancer (Brodie 2002; Dixon, Jackson et al. 2003; Howell, Cuzick et al. 2005; Mauri, Pavlidis et al. 2006; Dowsett, Allred et al. 2008). Breast cancer clinical trials are underway to investigate the chemopreventive effects of AI versus OH-Tam. Preliminary results suggest that treatment with AI confers superior protection against contra-lateral breast tumors in high risk postmenopausal women undergoing adjuvant therapy (Cuzick 2008). Results of the ATAC trial (Arimidex, OH-Tam, alone or in combination) have shown that 5 years of adjuvant therapy using anastrazole is accompanied by enhanced tolerability, fewer side-effects and prolonged disease free survival when compared with 5 years of treatment with OH-Tam (Howell, Cuzick et al. 2005). Interestingly, administration of 3 years of adjuvant therapy using letrozole after initial treatment with OH-Tam for 2 years or vice-
versa did not improve disease free survival when compared with monotherapy using letrozole for 5 years (Mouridsen, Giobbie-Hurder et al. 2009).

Currently, anastrozole and letrozole are approved by the Food and Drug Administration (FDA) as first and second line hormonal adjuvant therapy for the treatment of early, advanced or metastatic ER+ breast cancer in postmenopausal women, including patients who have progressed after failing OH-Tam treatment. Exemestane is FDA approved for the treatment of ER+ breast cancer in postmenopausal women who have received 2-3 years of prior OH-Tam prior treatment, in order to complete a total of 5 years of hormonal adjuvant therapy (www.cancer.gov/cancertopics/druginfo). In spite of the positive clinical responses initially observed using AI in breast cancer clinical trials, generally only around 20% to 50% of the patients respond to third generation AI therapy (Chen, Masri et al. 2007); more aggressive tumor phenotypes are usually associated with de novo and/or acquired resistance to AI (Dowsett, Martin et al. 2005).

The mechanisms underlying intrinsic resistance to AI therapy are poorly understood. Previous studies have shown that overexpression of aromatase by the tumor cells or surrounding stromal cells leads to an increase in intra-tumoral estrogen biosynthesis, conferring estrogen-hypersensitivity and supporting estrogen-dependent breast cancer cellular proliferation; however, the true contribution of estrogen-hypersensitivity to the resistant phenotype is still questionable (Chen, Masri et al. 2006; Miller 2008). On the other hand, in vivo treatment of letrozole refractory tumors overexpressing HER-2 using trastuzumab (HER-2 targeted antibody) plus letrozole reversed letrozole resistance and
restored estrogen sensitivity, further confirming the role of HER-2 in ER+ breast tumors as a strong predictor of poor response to endocrine therapy (Sabnis, Schayowitz et al. 2009). In contrast with de novo resistance, acquired resistance to AI develops during the course of treatment after initial response, probably resulting from a selection process involving activation of additional signaling molecules engaged in cross-talk between ER and growth factor signaling pathways (Dowsett, Martin et al. 2005).

**Selective Estrogen Receptor Down-regulators (SERD)**

Regardless of the different mechanisms known to play a role in development of resistance to hormonal adjuvant therapy in ER+ breast cancer cells, in most cases, tumor cells become hormone-insensitive to treatment while retaining ER expression. Activation of multiple ligand-independent ER signaling pathways in hormone refractory breast cancer cells is believed to represent one of the key mechanisms responsible for driving tumor growth and promoting an aggressive phenotype (Kuske, Naughton et al. 2006; Musgrove and Sutherland 2009). As SERDs compete with E$_2$ for binding to ER, they reduce ER availability in a dose-dependent manner by inducing ER degradation, impairment of ER dimerization and disruption of nuclear localization of ER (Dauvois, White et al. 1993; Parker 1993; Pink and Jordan 1996; Osborne, Wakeling et al. 2004).

Pure anti-estrogens (eg. Fulvestrant) are SERDs with no agonist activity, currently FDA approved only for the treatment of metastatic/locally advanced breast cancer as second or third line of therapy in postmenopausal patients who have failed to respond to standard OH-Tam therapy (Bross, Cohen et al. 2002). Clinical studies have demonstrated
that in postmenopausal woman with metastatic/locally advanced breast cancer previously untreated with hormonal adjuvant, clinical response to treatment with fulvestrant was similar to OH-Tam (Howell, Robertson et al. 2004). However, in patients with endocrine resistant tumors, treatment with fulvestrant caused tumor regression mainly through inhibition of constitutive activated ligand-independent ER signaling pathways (Johnston 2004; Nicholson, Hutcheson et al. 2005). As a second line therapy, treatment with fulvestrant was as effective as anastrozole in delaying time to progression (TTP) in patients with advanced breast cancer disease whose cancer had progressed on OH-Tam (TTP: 5.5 months for fulvestrant and 5.1 months for anastrozole); however, there was no statistically significant difference in the overall clinical benefit between the two drugs (Howell, Robertson et al. 2002; Morris and Wakeling 2002).

Resistance to pure-antiestrogens is associated with permanent loss of ER, where breast tumors acquire an ER negative breast cancer phenotype (Sommer, Hoffmann et al. 2003; Massarweh, Osborne et al. 2006). In vitro, prolonged exposure of MCF-7 and T47-D breast cancer cells to fulvestrant was accompanied by activation of src, AKT and ERK1/2 and selection for an aggressive resistant phenotype, characterized by highly invasive cells with enhanced migratory potential (Hiscox, Jordan et al. 2006). Additional mechanisms of resistance to fulvestrant include further activation of HER2/HER3 and EGFR signaling pathways in vitro and in pre-clinical models of OH-Tam resistant breast cancer (Osipo, Meeke et al. 2007; Frogne, Benjaminsen et al. 2009).
The estrogen receptor and its interactions with target genes and co-regulators

Estrogen receptor (ER) is a transcription factor that belongs to the class I steroid hormone receptor superfamily of nuclear receptors, sharing typical domain structures with other steroid receptors (Mangelsdorf, Thummel et al. 1995; Hall, Couse et al. 2001; Nilsson, Makela et al. 2001; McDonnell 2005). The ER structure is composed of a NH$_2$-terminal domain or A/B domain, which encodes a constitutive active ligand-independent activation function (AF1); a DNA binding domain (DBD) (C); a hinge region, important for synergy between the AF1 and COOH-terminal domain; and a COOH-terminal domain or E/F, which harbors a ligand-dependent binding domain (AF2) (Figure 1) (Zwart, de Leeuw et al.; Lees, Fawell et al. 1989). Estrogen is an agonist of AF1 and AF2, inducing the activation of the full-length receptor. In contrast, OH-Tam act as a partial agonist of AF1 and as an antagonist of AF2 (Berry, Metzger et al. 1990). The AF1 domain is relatively weak in mediating ligand-independent protein-protein interactions and it usually synergizes with AF2, but depending of the cellular and promoter contexts, AF1 could be dominant and act independently (Tora, White et al. 1989; Berry, Metzger et al. 1990; Tzukerman, Esty et al. 1994). The DBD and the AF-2 domains associate with co-activators or co-repressors in a ligand-dependent manner (Rosenfeld and Glass 2001; McKenna and O'Malley 2002; McKenna and O'Malley 2002) and even though AF-1 and AF-2 complexes share similarities, it may also bind to distinct co-activators (Endoh, Maruyama et al. 1999).
When ER is bound to agonists like E₂, it alters ER conformation favoring the recruitment and interaction with co-activators instead of co-repressors, resulting in transactivation of the target promoter. However, upon binding to antagonists of E₂ action (eg., OH-Tam), ER preferentially induces the recruitment of co-repressors, silencing gene expression (Glass and Rosenfeld 2000; Smith and O'Malley 2004). The classical mechanism of transcriptional regulation of target genes by ER is characterized by direct binding of liganded ER complexes to estrogen responsive elements (ERE) at the promoter region, activating or repressing gene expression (Osborne and Schiff 2005). Alternatively, non-classical mechanisms of transcriptional regulation by ER involve ER tethering to DNA through interactions with SP1 or AP-1 transcription factors at the promoter region of target genes that do not contain ERE (Safe and Kim 2008). According to the classical mechanisms of ER-mediated gene regulation, the p160 family of proteins (SRC1, SRC2/TIF2/GRIP-1, SRC3/ AIB1), TIF-1, p300/cbp etc. interact with ER in response to bound agonist, while major co-repressors SMRT and NCoR are preferentially recruited in response to antagonist binding (Glass and Rosenfeld 2000; Robyr, Wolffe et al. 2000; Green and Carroll 2007; Lonard and O'Malley B 2007).
contrast, non-classical mechanisms of E₂ signaling have shown that when overexpressed, SMRT together with SRC-3 can act as a co-activator instead of a co-repressor, regulating ER-dependent gene expression of cyclin D1 and progesterone receptor, possibly playing an important role in breast cancer progression (Karmakar, Gao et al. 2010).

In the absence of a classical estrogen response element (ERE), ER may target genes for activation by tethering to the target promoters through DNA-bound transcription factors such as Sp1 (Safe and Kim 2004), AP-1 (DeNardo, Kim et al. 2005), C/EBP and Oct (Carroll, Meyer et al. 2006). OH-Tam may act as an agonist in Ishikawa endometrial cancer cells by activating promoters that contains AP-1 tethered ER (Webb, Lopez et al. 1995). Binding of ER to chromatin may also be facilitated by FoxA1 (Carroll, Liu et al. 2005; Laganiere, Deblois et al. 2005; Carroll, Meyer et al. 2006).

ER is known to interact with the core transcription initiation complex through TBP, TFIIB and TFIID (Ing, Beekman et al. 1992; Jacq, Brou et al. 1994; Sadovsky, Webb et al. 1995). The recruitment of co-regulators by ER is believed to happen in a sequential order, with each co-regulator serving as an anchor for the recruitment of, or replacement by others (Dilworth and Chambon 2001; Urnov and Wolffe 2001). Transcriptional regulation of ER targeted genes requires ER interaction with ATP-dependent chromatin remodeling SWI/SNF complexes followed by five basic distinct regulatory mechanisms: (i) acetylation of histones and transcription factors (Sterner and Berger 2000); (ii) methylation of histones and transcription factors (Zhang and Reinberg 2001); (iii) platform or scaffolding function to recruit histone modifying enzymes (Rosenfeld and
Glass 2001); (iv) direct interaction with the basal transcription machinery to modulate recruitment of RNA polymerase II (Rosenfeld and Glass 2001); (v) ubiquitination and proteolytic activity (Zhang, Guenther et al. 1998; Lonard, Nawaz et al. 2000). Transcription regulation by ER ligands might ultimately result from the combination of cell-specific and promoter-specific co-regulator associations available.

ER activation of non-genomic signaling pathways is rapid and transient. Usually attributed to membrane action, it results in the activation of kinases and downstream signaling pathways, like PKC and PKA, as well as PI3K (Kousteni, Bellido et al. 2001; Sanchez, Nguyen et al. 2002). However, posttranslational modifications of nuclear ER through activation of multiple protein kinases including MAPK and AKT, particularly by phosphorylation, also modulate ER actions in a ligand-dependent and independent manner and are associated with resistance to OH-Tam (Campbell, Bhat-Nakshatri et al. 2001; Hayashi and Yamaguchi 2008). Phosphorylation of multiple ER sites situated at the N-terminal and C-terminal domains were detected using antibodies against phospho-specific ER epitopes or identified by mass spectometry in breast cancer cell lines and human breast carcinomas (Al-Dhaheri and Rowan 2006; Atsriku, Britton et al. 2009; Skliris, Rowan et al. 2009). Phosphorylation of at least one ER site was observed in 69% of invasive ductal breast carcinomas by immunohistochemistry, indicating that ER phosphorylation is major event modulating ER signaling pathways (Skliris, Rowan et al. 2009).
Throughout tumor progression, a vast majority of the ERα positive breast tumors develop resistance to endocrine therapy, in spite of the high intracellular levels of the ER protein. At advanced stages, ER most likely supports breast cancer cell growth through alternative mechanisms.

**Genomic associations of ER**

According to the global chromatin associations studies of ER using ChIP-chip or ChIP cloning methodologies (Carroll, Meyer et al. 2006; Lin, Reierstad et al. 2007; Levy, Tatomer et al. 2008), ER was found to be mainly associated at distal regions (>10 kb) from the putative target promoters. Recently, ChIP-chip studies have suggested that optimal ER-mediated transcriptional regulation and binding at distal regions of the chromatin requires the presence of FOXA1, a transcription factor that belongs to the forkhead family and is necessary for promoting the recruitment of additional transcription factors to the chromatin region and inducing localized chromatin remodeling (Carroll, Liu et al. 2005; Laganiere, Deblois et al. 2005). FOXA1 expression is directly associated with ER expression and an independent marker of favorable prognosis in breast cancer patients (Badve, Turbin et al. 2007; Wolf, Bose et al. 2007). Knock down of FOXA1 in breast cancer cells prevented E₂-mediated cell cycle progression and inhibited E₂-stimulated growth (Laganiere, Deblois et al. 2005; Yamaguchi, Ito et al. 2008).

However, previous studies have shown important examples of ER association either by DNA binding or by tethering at promoter proximal regions. Kwon and colleagues, using ChIP-DSL, which detects ER binding sites with higher sensitivity, identified a large
number of ER binding sites at promoter proximal and core promoter regions. In fact, the authors observed that in MCF-7 cells, over 3% of all genes are bound to ER in promoter proximal regions (Kwon, Garcia-Bassets et al. 2007). On the other hand, only a small portion of the unique ER binding sites were mapped at promoter proximal regions compared to unique RNA polII sites by ChIP-chip (Carroll, Meyer et al. 2006). Altogether, it is clear that ER binds at a high frequency both at proximal and distal sites to the target promoters.

**Non-genomic actions of estrogen**

Recently, the orphan receptor GPR30, a heterotrimeric G protein with 7 transmembrane spanning receptors, has been shown to mediate rapid actions of E₂ through stimulation of adenylyl cyclase activity and cAMP activation as well as heparan-bound epidermal growth factor release, supporting breast cancer cell proliferation (Filardo and Thomas 2005). Similar levels of GPR30 expression were detected by immunohistochemistry analysis in both normal breast and breast tumor samples; GPR30 expression appeared to be independent of ER since approximately 50% of the ER- tumor samples retained GPR30 expression, while rapid E₂ responses were also observed in ER-MB-MDA-231 breast cancer cells after transfection with a GPR30 expression vector (Filardo, Quinn et al. 2002; Filardo, Graeber et al. 2006). The role of GPR30 in breast cancer pathophysiology is not clear; however, activation of GPR30 signaling pathways in ER- breast cancer cells *in vitro* by E₂ or OH-Tam, an ER partial antagonist but a GPR30 agonist, stimulated cell proliferation and migration (Pandey, Lappano et al. 2009).
Even though the non-genomic actions of GPR30 are independent of ER, activating distinct biological responses, convergence of non-genomic and genomic actions triggered by E₂ ultimately leads to transcriptional transactivation of downstream genes responsive to ER, which is sensitive to conformational changes in the receptor induced by different SERMs (Pedram, Razandi et al. 2002).

**Development of new SERMs to address the problem of OH-Tam resistance**

Classification of new SERMs has largely relied on empirical observations. Attempts to classify new SERMs based on global gene expression patterns has failed to generate reliable “gene signatures” profiles partly due to the close similarities of such signatures even among distinct SERMs and the technical difficulties of screening and comparing a large body of compounds (Levenson, Kliakhandler et al. 2002). Considering the limitations of OH-Tam in reducing the incidence and suppressing breast cancer growth, it is necessary to develop more sophisticated systems to systematically classify SERMs to identify those with superior properties. Ideally, a new SERM should have a decreased frequency of resistance to it compared to OH-Tam. It is also desirable for the utility of the SERM not to be limited by the menopausal status, in contrast to pure anti-estrogens and aromatase inhibitors. A mechanism-based classification of ER antagonists would present a distinctive and more practical way to search systematically through large libraries of SERMs for the best lead drug.
Gene repression by E\textsubscript{2} and the effect of OH-Tam

Recent studies have shown that E\textsubscript{2} directly or indirectly inhibited as many genes as it activated (Frasor, Danes et al. 2003; Hayashi 2004; Carroll, Meyer et al. 2006). In MCF-7 breast cancer cells, E\textsubscript{2} was shown to inhibit twice as many genes as it activated, and OH-Tam completely prevented E\textsubscript{2}-mediated gene repression in about a fourth of those genes and partially in close to half of them (Frasor, Stossi et al. 2004). The mechanisms by which E\textsubscript{2} represses genes have not been adequately elucidated. It has been suggested that one possible mechanism by which E\textsubscript{2} represses genes is through physiological “squelching”, which limits the availability of transcription factors by ER (Kalaitzidis and Gilmore 2005). However, physiological “squelching” appears to be a common event at early time points (<3h) of E\textsubscript{2} treatment (Carroll, Meyer et al. 2006). Gene repression by ER is also facilitated by interactions with the induced co-repressor NRIP1 (Teyssier, Belguise et al. 2003; Carroll, Meyer et al. 2006). Studies have shown that direct gene repression by E\textsubscript{2}/ER occurs at target genes that lack an ERE. In this case, repressive actions of ER is mediated by direct interaction with other transcription factors such as GATA-1 (Blobel, Sieff et al. 1995), Sp3/Sp1 (Stoner, Wang et al. 2000; Varshochi, Halim et al. 2005; Stossi, Likhite et al. 2006; Higgins, Liu et al. 2008), AP-1 (Schmitt, Bausero et al. 1995), NFκB and C/EBPβ (Stein and Yang 1995; Cvoro, Tzagarakis-Foster et al. 2006).

Abrogation of E\textsubscript{2}-mediated repression by OH-Tam in ER\textalpha+ breast cancer is known to occur in specific genes; eg., the homeobox B13 gene, a negative prognosticator of breast cancer overexpressed in breast cancer cells of patients treated with OH-Tam (Rodriguez,
Cheng et al. 2008) and fibronectin gene, a positive prognosticator of breast cancer (Horii, Takei et al. 2006). E2-mediated repression of the erbB2 gene, a proto-oncogene expressed in OH-Tam-resistant breast cancer cells, occurs through ER tethering to DNA binding sites and is dependent of intracellular availability of co-regulators, such as PAX2 or AIB1 (Bates and Hurst 1997; Hurtado, Holmes et al. 2008). In the presence of liganded-ER, PAX2 acts as a co-repressor inhibiting erbB2 gene expression, while AIB1 competes for binding to ER with PAX2 and overexpression of AIB1 leads to up-regulation of erbB2 gene expression (Hurtado, Holmes et al. 2008). It is controversial whether OH-Tam treatment represses or up-regulates erbB2 gene expression in breast cancer cells. Data obtained from primary invasive breast tumors suggested that development of OH-Tam resistance occurs following OH-Tam-mediated up-regulation of erbB2 gene expression during the course of hormonal adjuvant therapy (Borg, Baledort et al. 1994). In contrast, in vitro studies using MCF-7 have shown that OH-Tam-ER complex directly represses erbB2 expression (Hurtado, Holmes et al. 2008).

FRα is a negative prognosticator of breast cancer (Rochman, Selhub et al. 1985). Recent studies published by this laboratory on the folate receptor α (FRα) gene and various chimeric promoters (Kelley, Rowan et al. 2003; Hao, d'Alincourt-Salazar et al. 2007) provided for the first time, detailed mechanistic information on OH-Tam-sensitive gene repression by E2. In the FRα model, E2 induces recruitment of ER to the core promoter together with the co-repressors SMRT and NCoR with the help of TAFII30; OH-Tam passively de-represses the gene by disrupting the complex (Figure 2).
**Figure 2.** A non-classical model of the effect of E$_2$ and OH-Tam on core promoter repression by ER.

**Retinoic acid receptors and retinoids in breast cancer therapy**

Retinoic acid receptors (RAR) belongs to the class II family of nuclear hormone receptors, characterized by forming heterodimers with retinoid X receptors (RXR), modulating the expression of target genes by binding to direct DNA repeats within the promoter region (Mangelsdorf, Thummel et al. 1995). In the absence of ligand, the RAR/RXR heterodimer is associated with co-repressors and histone deacetylase complexes (HDACs) at the promoter region of target genes, rendering the chromatin inaccessible to the transcription machinery (Chambon 1996).

The RAR subfamily of nuclear hormone receptors consists of RAR isotypes $\alpha$, $\beta$ and $\gamma$, which are encoded by different genes and highly conserved among metazoans. On the other hand, RAR isoforms results from alternate promoter usage or alternative splicing
Knockdown of any one of the RAR isotypes in vivo results in viable embryos with mild congenital malformations; however, combined knockdown of any two isotypes is embryonically lethal, indicating some degree of functional overlap between the RAR isotypes (Mark, Ghyselinck et al. 2009).

Retinoids are natural or synthetic derivatives of vitamin A, which is essential for normal organogenesis, differentiation and bilateral symmetry during embryogenesis (Mark, Ghyselinck et al. 2009). Retinoid signaling is also critical for maintaining homeostasis and vitamin A precursors must be ingested in the diet. Retinoic acid (RA), the active form of vitamin A, is known to bind RARs with high affinity to modulate expression of RAR target genes. Ligand-induced conformational changes in the LBD of the RAR/RXR heterodimer leads to co-repressor dissociation and recruitment of co-activators with histone acetyltransferase (HAT) activity, relaxing the chromatin structure and activating gene transcription (Figure 3) (Bastien and Rochette-Egly 2004).

The use of retinoids in breast cancer treatment and prevention has been extensively studied, partly due to the success of retinoid-induced differentiation therapy in the treatment of acute promyelocytic leukemia (Soprano, Qin et al. 2004). Early studies have also suggested that there is an inverse relationship between vitamin A serum levels and incidence of pre-malignant epithelial lesions (Sporn 1976). Even though retinoid treatment prevented malignant transformation of human bronchial epithelial cells exposed to carcinogens in vitro (Langenfeld, Lonardo et al. 1996), clinical trials of cancer
prevention using retinoids such as fenretinide only marginally protected women at high risk of developing breast cancer (Zanardi, Serrano et al. 2006).

**Figure 3.** ATRA activation of RAR target genes.

Retinoic acid signaling is necessary for maintaining epithelial differentiation, lumen morphogenesis and mammary gland involution after weaning in normal breast epithelial cells (Montesano and Soulie 2002; Zaragoza, Gimeno et al. 2007). In ER+ breast cancer cells, treatment with supraphysiological concentrations of *all*- trans RA (ATRA) prevented S-phase entry into the cell cycle and induced apoptosis independent of p53 status (Seewaldt, Dietze et al. 1999). The anti-tumor effects of ATRA are mediated by several mechanisms including transrepression of AP-1 (Yang, Kim et al. 1997), inhibition
of IGF-I signaling (Adamo, Shao et al. 1992) and inhibition of G1 cycling proteins in breast cancer cells (Teixeira and Pratt 1997). Pre-clinical studies have also reported synergism between ATRA and other growth inhibitory agents, including OH-Tam (Wang, He et al. 2007), troglitazone (Elstner, Muller et al. 1998), lutein (Sumantran, Zhang et al. 2000) and melatonin (Eck, Yuan et al. 1998) in inducing cell cycle arrest and apoptosis in breast cancer cells. However, clinical trials have failed to demonstrate the growth inhibitory effects of ATRA in patients with advanced breast cancer and additive anti-tumor activity of ATRA in combination with OH-Tam (Sutton, Warmuth et al. 1997; Toma, Raffo et al. 2000).

**Breast cancer treatment options: overview**

Newly diagnosed ER+ breast tumors are usually removed by surgery, followed by brief chemotherapy/radiotherapy. Once the breast cancer patient goes into remission, hormonal adjuvant therapy is given for up to 5 years. However, around 50% of the patients experience relapse during the course of hormone adjuvant therapy due to the emergence of intrinsic or *de novo* resistance mechanisms. Postmenopausal women with metastatic breast cancer can further benefit from therapy using faslodex (SERD) to extend the disease free survival period by an average of 6 months. On the other hand, at advanced stages, chemotherapy/radiotherapy is the only treatment of choice for premenopausal woman with hormone-refractory breast cancer.

Resistance mechanisms are typically investigated with the goal of identifying alternate therapies that overcome the resistance. Even though it is important to comprehend the
molecular basis of various mechanisms of drug resistance, a better understanding of the early events in breast cancer driving the transition from hormone-sensitivity towards hormone-refractoriness should enable the development of strategies to decrease the incidence of drug resistance or delay it onset.

CHAPTER 2

Under Conditions of Hormonal Adjuvant Treatment the Estrogen Receptor
Apoprotein Supports a Basal Level of Breast Cancer Cell Cycling through the RARα1 Apoprotein°

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ABSTRACT

Development of resistance to hormonal adjuvants in breast cancer requires a basal level of cell cycling during the treatment for which the underlying causes are unclear. Basal proliferation persisted in estrogen-sensitive MCF-7 breast cancer cells grown in hormone depleted conditioned media or with 4-hydroxytamoxifen (OH-Tam). Downregulating ER using either siRNA or fulvestrant inhibited basal proliferation by promoting cell cycle arrest, without enrichment for ErbB2/3+ overexpressing cells. The basal expression of retinoic acid receptor α1 (RARα1), the only RARα isoform that was expressed in MCF-7 cells and in most breast tumors, was supported by apo-ER but was unaffected by OH-Tam; RAR -β and -γ were not regulated by apo-ER. Depleting basal RARα1 reproduced the antiproliferative effect of depleting ER whereas its restoration in the ER depleted cells rescued basal cycling. The overlapping tamoxifen-insensitive gene regulation by apo-ER and apo-RARα1 comprised activation of mainly genes promoting cell cycle and mitosis and suppression of genes involved in growth inhibition; these target genes were generally insensitive to all-trans-retinoic acid (ATRA) but were enriched in RAR binding sites in associated chromatin regions. Thus, in hormone-sensitive breast cancer, ER can support a basal fraction of S-phase cells: (i) without
obvious association with ErbB2/3 expression, (ii) by mechanisms unaffected by hormone depletion or OH-Tam and (iii) through maintenance of the basal expression of apo-RARα1 to regulate a set of ATRA-insensitive genes. ER may thus facilitate the development of resistance to hormonal adjuvants. Since isoform 1 of RARα is genetically redundant, its targeted inactivation or downregulation may improve hormonal adjuvant therapies with minimal side effects.

INTRODUCTION

Most breast tumors in both premenopausal and postmenopausal women express estrogen receptor type alpha (ER). Tamoxifen is a selective estrogen receptor modulator (SERM) widely used for adjuvant therapy in the treatment of ER+ breast cancer. In the hormone-sensitive tumors, tamoxifen acts as a partial antagonist, impairing ER function by competing with estrogen for binding to the receptor [1]; however, > 3 years of tamoxifen treatment only results in approx. 50% reduction in the incidence of invasive breast cancer in women at high risk, whereas about a third of ER+ breast tumors are intrinsically resistant to tamoxifen [2, 3].

Third generation aromatase inhibitors (AI) present a valuable alternative to tamoxifen adjuvant therapy in postmenopausal women with ER+ breast cancer [4-6]. Aromatase activity is essential for catalyzing the conversion to estrogen of steroid precursors in peripheral tissues, the major source of estrogen production in postmenopausal women. Upon treatment with AI, aromatase activity is reduced by at least 96% and circulating
estrogen is virtually absent, inhibiting hormone-dependent tumor growth [7]. In spite of the sensitivity of tamoxifen-resistant tumors to AI, breast the tumors also acquire resistance to AI after long term treatment, resulting in disease recurrence and aggressive tumor growth [8, 9]. Clinical trials are underway to assess the possibility of delaying the onset of resistance by administering AI for 2-3 years following 2-3 years of tamoxifen treatment [10, 11]. The mechanistic basis underlying breast tumor resistance to either hormone depletion or to tamoxifen is still inadequately understood. In the vast majority of the cases, resistance must occur through hormone-independent ER signaling events [12, 13]. Accordingly, selective estrogen receptor downregulators (SERDs, eg., Faslodex) have been found to be effective inhibitors of ER+ breast tumor growth but their utility is limited to their use as second or third line therapeutics in postmenopausal women with metastatic disease due to their broader impact on physiological ER signaling pathways in normal tissues [14, 15]. Therefore, it is imperative to continue to identify critical downstream events of ER signaling in breast cancer.

Breast cancer therapy trials have also been designed to explore the effect of retinoid compounds either alone or in combination with tamoxifen [16]. In in vitro and preclinical models of breast cancer using MCF-7 cell xenografts, all-trans- retinoic acid (ATRA) alone or in combination with tamoxifen induced cell cycle arrest and apoptosis, leading to tumor regression through activation of multiple signal transduction pathways [17-19]. Synergistic anti-tumor effects have been noted in vitro for the combination of retinoid and tamoxifen and multiple molecular mechanisms for the ligand effects have been reported [20, 21]. However, toxicity issues due to ATRA treatment was a challenge
in patients with advanced breast cancer during phase I/II clinical trials [22]. Fenretinide, a synthetic amide of retinoic acid, has a better toxicological profile acting on both ER+ and ER- breast tumors principally by inducing apoptosis by both retinoic acid receptor (RAR) -dependent and -independent mechanisms; this drug showed a modest chemopreventive effect only in younger premenopausal women [23].

Hormonal adjuvant therapy of breast cancer is overall tumoristatic [24]. From a fundamental mechanistic standpoint, for resistance to develop in the long term during either hormone depletion or tamoxifen adjuvant therapy, the latent tumors must sustain a basal level of cell cycling to enable the generation and/or progression of genetic or epigenetic changes [25] leading to resistance. It is the premise of this study that understanding the mechanisms that support the persistence of a small fraction of cells in S-phase throughout the course of hormonal adjuvant therapy in breast cancer will shed light on this critical precondition for the eventual development of resistance to the treatments. Since estrogen-independent ER signaling has been implicated in the development of resistance to adjuvant therapy, it was the goal of this study to examine the relationship between hormone-independent actions of ER and the basal cycling state of estrogen deprived breast cancer cells. Further, the ER-retinoic acid receptor (RAR) axis has only been investigated in the context of ligand-dependent effects [26]; it was therefore of additional interest to explore a possible interplay between the apo- forms of ER and RAR and its impact on basal proliferation i.e., under conditions of hormone depletion or tamoxifen antagonism.
MCF-7 cells have proven to be an exceptionally reliable predictive model both *in vitro* and *in vivo* for clinical drug response and the development of clinical drug resistance in breast cancer [27-30]. We have observed that the expected basal proliferating state of hormone-depleted or tamoxifen treated breast cancer cells may be reproduced *in vitro* in MCF-7 cells for indefinite by avoiding the common practice of intermittently replenishing the culture media, thus avoiding depletion of autocrine growth factors. We therefore used this *in vitro* model to investigate the potential impact of hormone-independent actions of ER on the survival or proliferation of hormone-sensitive breast cancer cells *in vitro* and the related mechanisms under conditions that mimic hormonal adjuvant therapy, i.e., estrogen-depletion and tamoxifen treatment.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Dulbecco’s minimum essential medium (DMEM), glutamine and penicillin/streptomycin/glutamine stock mix were purchased from Life Technologies, Inc. (Carlsbad, CA, USA). Fetal bovine serum (FBS) and charcoal-stripped FBS were from Invitrogen (Carlsbad, CA, USA). Fugene 6 and Dharmafect 1 were from Roche Diagnostics (Indianapolis, IN, USA) and Dharmacon (Thermo Scientific Dharmacon, Inc., Lafayette, CO, USA), respectively. ERα (J-003401-12), RARα (J-003437-07) and control (D-001810-02) small interfering RNA (siRNA) were purchased from Dharmacon (Thermo Scientific Dharmacon, Inc., Lafayette, CO, USA). Affinity purified rabbit antibodies to human ERα (sc-543), RARα (sc-551), RARβ (sc-552), RARγ (sc-550) and glyceraldehyde-3-phosphate dehydrogenase (sc-47724) were from Santa Cruz
Biotechnologies (Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibody was from Vector Laboratories (Burlingame, CA, USA). For standard PCR, HotStart Taq Plus DNA Polymerase was used (Qiagen, Maryland, USA). Reagents for real time PCR, primers and TaqMan probes were purchased from Applied Biosystems (Branchburg, NJ, USA). PI/RNase staining buffer was from BD Pharmigen (BD Pharmigen, San Diego, CA, USA). The Guava Nexin Reagent was purchased from Guava Technologies (Guava Technologies, Inc., Hayward, CA, USA). The protease inhibitor cocktail kit was obtained from Pierce Biotechnology (Thermo Scientific, Rockford, IL, USA). 17β-estradiol (E2), 4-hydroxytamoxifen (OH-Tam) and fulvestrant were purchased from Sigma Aldrich (Saint Louis, MO). First strand cDNA from human peripheral blood leukocytes (PBL), thymus and spleen were obtained from Biochain Institute (Biochain Institute Inc., Hayward, CA). Total RNA from normal human breast and human breast tumors were obtained from Biochain Institute Institute (Biochain Institute Inc., Hayward, CA) and Clonetech (Clonetech Laboratories, Inc., Montain View, CA).

**Cell culture.** MCF-7 (American Type Culture Collection) cells were cultured in DMEM supplemented with FBS (10%), penicillin (100unit/ml), streptomycin (100μg/ml) and L-glutamine (2mM). Hormone depleted cells were grown in low glucose phenol-red free media supplemented with 5% charcoal-stripped FBS (v/v) and L-glutamine (2mM) for 48 hours before the experiments.

**Transfection and gene silencing.** MCF-7 cells were plated at 20% confluence in low glucose phenol red free medium supplemented with 5% charcoal stripped FBS and
glutamine 24h prior to transfection. Treatment with vehicle, E₂ or OH-Tam was begun an additional 24h later. Cells were transfected with control siRNA, ERα siRNA or RARα siRNA in 24 well microplates (2.5pmol/well) or 25cm² flasks (31.25pmol per flask) using 2µl and 12.5 µl of Dharmafect 1 (Thermo Scientific Dharmacon), respectively, according to the vendor’s protocol. The cell culture medium was not replenished for the duration of the experiment. In the RARα1 rescue experiments, 2x10⁶ cells were co-transfected with 2µg of either the vector plasmid or RARα1 expression plasmid and with control siRNA or ERα siRNA by nucleofection using the Kit V Amaxa Nucleofection System (Amaxa Biosystems) according to the vendor’s instructions.

**Cell growth assay.** MCF-7 cells were seeded in 24 well microplates at 20% confluence in phenol-red free media supplemented with 5% charcoal-stripped FBS (v/v) and incubated at 37°C with 5% CO₂ for 24h. Cells were transfected with either control siRNA or siRNA targeting ERα using Dharmafect 1. Twenty four hours after transfection the, media was replaced with fresh phenol-red free media supplemented with 5% charcoal-stripped FBS (v/v) and the cells were treated with vehicle (ethanol), E₂ (1nM) or 4-hydroxy-tamoxifen (100nM) for the following 5 days; the culture media was not changed during this period but E₂ and OH-Tam were replenished every 48h. Viable cell counts were monitored using the trypan blue dye exclusion assay at intervals of 24h.

**Cell cycle analysis.** MCF-7 cells were trypsinized and harvested in phenol red free medium supplemented with charcoal stripped FBS. Cells (1x10⁶) were washed and resuspended in 500µl PBS. The cells were fixed by adding 500µl 100% ice cold ethanol,
drop-wise with agitation and incubated on ice for 20 min. The cells were sedimented by brief centrifugation at 200 xg for 5 min and the excess ethanol decanted. After the remaining ethanol was dried off, the cells were resuspended in 500µl of PI/RNase solution. The cells were incubated in the dark at room temperature for 20 min and the cell cycle distribution determined by flow cytometric analysis using a FACSCalibur cell analyzer (BD Biosciences). The data was acquired with BD CellQuest Pro software and analyzed using ModFit LT software.

**Apoptosis assay.** Early stage apoptosis of MCF-7 cells was measured by Guava Nexin analysis using the Guava Nexin Reagent staining kit according to the manufacturer’s instructions. Briefly, 8x10⁴ cells were incubated for 20 minutes at room temperature with the Guava Nexin Reagent and two thousand cells per sample were analyzed using the Guava System.

**Western blot.** Cells were harvested by trypsinization, lysed in a high salt-detergent buffer (400mM NaCl; 10nM Tris, pH 8.0; 1mM EDTA; 1mM EGTA; β-mercaptoethanol; and 0.1% Triton x-100) containing a protease inhibitor cocktail kit and incubated on ice for 30 minutes. Cell lysates were heated to 95°C for 5 min. Protein samples (10-20µg) were resolved by electrophoresis on 8% sodium dedecylsulfate- polyacrylamide gels and electrophoretically transferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA). The blots were probed with the appropriate primary antibody and the appropriate horse radish peroxidase conjugated secondary goat antibody and the protein bands visualized using enhanced chemiluminescence as described [31].
**RNA isolation, reverse transcription PCR and Real time PCR.** Total RNA from MCF-7 cells was isolated using the RNeasy mini kit (Qiagen, Maryland, MD, USA). Reverse transcription PCR reactions were performed using 500ng of total RNA and the high capacity complementary DNA Archive kit (Applied Biosystems, Branchburg, NJ, USA) according to the vendor’s protocol. cDNAs of RARα1 and RARα2 were amplified by competitive PCR. The upstream and downstream primers used for amplification of RARα1 and RARα2 were as follows: RARα1, 5’-GCCAGGCGCTCTGACCACTC-3’ AND 5’-AGCCCTTGTAGCCCTCACAG-3’; RARα2, 5’-ACTCCGCTTTGGAATGGCTCAAAC-3’ and 5’-AGCCCTTGTAGCCCTCACAG-3’. The cDNA for the house keeping gene glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was amplified and the primer sequences used were as follows: 5’-TGGTCACCAGGGCTGCTTTT-3’ and 5’-GGTGAAGACGCCAGTGGACT-3’. The cycling parameters were: 95°C for 15 min; 94°C for 30 sec; 60°C for 30 sec; 72°C for 30 sec and 72°C for 10 min. RARα1 and RARα2 cDNAs were amplified in the same reaction, yielding products of 222bp and 182bp, respectively. PCR products were separated in ethidium bromide-stained 2% agarose gels by electrophoresis. cDNA was also measured by quantitative real time PCR in the 7500 StepOne Plus Real time PCR System (Applied Biosystems). Primers and TaqMan probes for the human ERα, CCNA, CDKN1, ERBB2, ERBB3, MUC20, LYPD1, RARα and GAPDH genes were obtained from the Applied Biosystems inventory. All samples were measured in triplicate and normalized to GAPDH values.
mRNA profiling. The Affymetrix DNA microarray analysis was performed as a full service global gene expression study at the transcriptional profiling core facility of the Cancer Institute of New Jersey. Total RNA samples were used to generate labeled cRNAs, which were hybridized to human U133 Plus2.0 Affymetrix microarrays. The expression data were analysed initially using Affymetrix GeneChip Operating Software to create CEL files. The CEL files were imported into the Bioconductor program affylmGUI [32]. The probe set level intensities were quantified and normalized using robust multiarray averaging and quantile normalization. Differential expression between treatments was determined using the limma linear modeling method, and the significance of differences were ranked by the moderated $t$-statistic.

Statistical analyses. Experimental values are presented as mean ± s.d. The statistical significance of differences ($P$-value) between values being compared was determined using analysis of variance. In all cases, the differences noted in the text are reflected by a $P$-value of <0.001.

RESULTS

In Estrogen-sensitive MCF-7 cells basal proliferation is supported by ER in the absence of hormone. In the following studies, in experiments in which MCF-7 cells were depleted of hormone, the virtual absence of hormone was confirmed in two ways. First, the effect of OH-Tam on the expression of the classical $E_2$ target gene, EGR3 (early growth response 3 gene) was examined as a functional test. The expression of EGR3
mRNA is exquisitely sensitive to upregulation by E₂, in a manner that is completely antagonized by OH-Tam. In the hormone depleted cells, the inability of OH-Tam to further decrease EGR3 mRNA indicated the virtual absence of hormone (Figure 1A). Second, the effect of E₂ on the relative phosphorylation level at ser¹¹⁸ of ER was examined. The binding of E₂ strongly induces phosphorylation of ER at this site [33]. The absence of hormone was further confirmed by the observation of much lower phosphorylation at ser¹¹⁸ of ER in the hormone depleted cells, when compared with control cells treated with E₂ (Figure 1B).

When hormone depleted MCF-7 cells were seeded at a low confluency (< 20 percent) and grown, in the absence of hormone and without replenishing the medium, they continued to proliferate but the proliferation was not inhibited by OH-Tam; we will call this ‘basal’ proliferation. Treatment with E₂ stimulated the cell growth demonstrating that the cells were hormone-sensitive (Figure 1B). The basal proliferation was diminished by knocking down ER (Figure 1C). The expression level of ER mRNA (Figure 1D) progressively decreased with ligand (E₂ or OH-Tam) treatment but the ER protein level (Figure 1E) was stabilized by the ligands during the treatment period; however, ER siRNA specifically and substantially down-regulated ER mRNA (within 24h) and ER protein (within 48h) (Figure 1D and 1E). The results demonstrate a profound role for apo-ER in supporting basal proliferation in hormone-sensitive MCF-7 cells.

**Apo-ER supports MCF-7 cell proliferation by promoting cell cycle progression.** The net proliferation rate of MCF-7 cells under various conditions indicated in Figure 1 may
be determined by changes in the rates of cell cycling as well as the rates of cell death. Figure 2A illustrates that during basal proliferation approx. 20 percent of the cells were in S-phase. Whereas E₂ roughly doubled the proportion of S-phase cells, OH-Tam (100 nM) did not appreciably alter the basal cell cycle distribution (Figure 2A). However, knocking down ER in the hormone-depleted cells decreased the S-phase cells by approx. 50 percent (Figure 2A); the S-phase inhibition was accompanied by an increase in the proportion of cells in G1 phase indicating that the ER knockdown inhibited cell proliferation by inducing cell cycle arrest.

As an alternative method of depleting ER, the hormone depleted MCF-7 cells were treated with fulvestrant, a well established SERD, which causes proteolytic degradation of ER. As expected, fulvestrant treatment resulted in a substantial decrease in ER protein (Figure 3A), without affecting the level of ER mRNA (Figure 3B). Similar to knocking down ER with siRNA, treatment with fulvestrant caused cell cycle arrest (Figure 3C), providing complementary evidence for the role of apo-ER in supporting cell cycling in hormone depleted MCF-7 cells.

In contrast to cell cycle distribution the rate of apoptosis, measured by staining the cells for annexin V, showed only a marginal (up to an additional 1.6 percent) increase due to ligand treatment or knocking down ER compared to hormone depletion (Figure 2B). Fulvestrant treatment also did not significantly impact cell survival and only resulted in a marginal decrease (from 8.3 percent to 7 percent) in annexin V staining (Figure 3D).
Therefore the principal mechanism by which ER supports basal proliferation of hormone-sensitive MCF-7 cells is by promoting cell cycle progression.

**Apo-ER supports basal cycling of MCF-7 cells through regulation of apo-RARα.** Since the actions of antiestrogens and retinoids on breast cancer cells are profoundly regulated by an ER-RAR axis, it was of interest to examine a possible functional relationship between the two receptors in the absence of ligand in the context of basal cycling of ER+ breast cancer cells. In hormone depleted MCF-7 cells, knocking down ER decreased the level of RARα mRNA by about 40 percent and to a greater extent the RARα protein (Figure 4A). However, the basal ER level was unaltered by knocking down RARα (Figure 4A). Knocking down either ER or RARα did not significantly alter the expression of RARs -β and -γ (Figure 4B). The results indicate that apo-RARα is specifically regulated by apo-ER but not vice versa.

Similar to knocking down ER, knocking down RARα in hormone-depleted MCF-7 cells decreased the fraction of S-phase cells (Figure 4C). To test whether the effect of knocking down ER on the basal cycling of MCF-7 cells could be mediated by apo-RARα, the latter protein was introduced ectopically at the time of knocking down ER by co-transfecting an RARα isoform1 expression plasmid (Figure 4D). Restoring RARα at a level approaching the original basal level of the protein partially rescued cell cycling (Figure 4D). Similarly, RARα also rescued basal cell cycling in MCF-7 cells treated with fulvestrant (Figure 4E). Taken together, the above results demonstrate that the ability of
apo-ER to support basal cell proliferation is mediated to a large extent by its ability to support the expression of the basal level of apo-RARα.

**Apo-ER and apo-RARα support cell cycle in MCF-7 cells without obvious or obligatory dependence on ErbB2 and ErbB3 status.** Since the MCF-7 cell line comprises a heterogeneous population of cells, and since ErbB2 and ErbB3 overexpression are associated with resistance to hormonal adjuvants it was of interest to test whether the ErbB2 and ErbB3 status of the cells was related to the dependence of basal cell cycling of MCF-7 cells on apo-ER or apo-RARα. As expected, treating hormone depleted MCF-7 cells with E2 inhibited ErbB2 and ErbB3 mRNA expression (Figure 4E). Following knockdown of either ER or RARα, there was not a significant change in the mRNA levels of ErbB2 and ErbB3 compared to the hormone depleted control cells (Figure 4E). Thus, the regulation of cell cycling of MCF-7 cells by apo-ER or apo-RARα did not show either an obvious or an obligatory selectivity for a subpopulation of cells distinguishable by their ErbB2 or ErbB3 status.

**MCF-7 cells and clinical breast tumors largely exclusively express isoform 1 of RARα.** Given the significant differences in structure and regulation between the two RARα isoforms [34] it was of interest to determine whether only one or both isoforms were relevant in the regulation of RARα by apo-ER in breast cancer cells. cDNA prepared from the total RNA of MCF-7 cells as well as 5 breast tumors and normal human tissue controls (breast, spleen, thymus and peripheral blood leukocytes ) were analyzed for RARα isoform expression by PCR. As seen in Figure 5, whereas both
RARα1 and RARα2 were expressed in spleen, thymus and peripheral blood leukocytes, normal breast and MCF-7 cells as well as 4 of the 5 breast tumors expressed virtually exclusively RARα1 (Figure 5). This observation is consistent with previous evidence [35] for epigenetic silencing of RARα2 expression in MCF-7 cells and, together with the preceding observation that RARα1 rescued basal cell cycling in the ER knockdown cells, indicates that RARα1 is the relevant receptor isoform in the current study of breast tumor cells.

**Apo-RARα1 mediates regulation by apo-ER of tamoxifen-insensitive gene complements principally engaged in the cell division cycle.** The down-regulation of RARα1 upon knocking down ER that occurred in hormone-depleted MCF-7 cells was also insensitive to tamoxifen (Figure 6A); the levels of RARs -β and -γ were also unaffected by tamoxifen treatment (Figure 6A). The gene targets downstream of the apo-ER → apo-RARα1 pathway were identified by mRNA profiling using Affymetrix microarray analysis. Accordingly, data from separate ER knockdown and RARα1 knockdown experiments in hormone depleted MCF-7 cells were used to identify overlapping sets of genes that were either up or down regulated by both ER and RARα1. The Affymetrix microarray analysis was validated for a few target genes known to regulate cell proliferation by real time RT-PCR; they include CDKN1, MUC20 and LYPD1, which are negative regulators of cell proliferation whose basal expression was repressed by apo-ER and apo-RAR as well as CCNA1, a positive regulator of proliferation whose basal expression was supported by apo-ER and apo-RAR (Figure 6B).
Table 1 lists the common target genes of apo-ER and apo-RARα1 whose basal expression was supported by both apo-ER and apo-RARα1 in a tamoxifen-insensitive manner; these genes were identified based on a decrease in basal mRNA level by at least 40 percent due to transfection of cells with siRNA specific for either ER or RARα1. Of the 53 annotated genes that were identified in this manner, gene ontology analysis (DAVID Bioinformatics Resources 2008) [36, 37] revealed genes known to support the cell division cycle and mitosis as the predominant functional category (Figure 6C); the gene sets included additional functional categories that support proliferation (Figure 6C).

Table 2 lists the common target genes of apo-ER and apo-RARα1 whose basal expression was decreased by apo-ER or apo-RARα1 in a tamoxifen-insensitive manner; these genes were identified based on an increase in basal mRNA level by at least 50 percent due to transfection of cells with siRNA specific for either ER or RARα1. Of the 68 annotated genes that were identified in this manner, gene ontology analysis (DAVID Bioinformatics Resources 2008) revealed several categories of genes (Figure 6D); however, among genes functionally related to proliferation, there was enrichment for those that are known to negatively regulate cell proliferation (Figure 6D) in contrast to the genes activated by apo-ER/apo-RARα1 noted above.

The common gene targets of apo-ER and apo-RARα1 are generally insensitive to ATRA but are enriched in RAR binding sites in associated chromatin regions. In the classical mechanism of the transcriptional activity of class II nuclear receptors,
including RAR, the apo-protein is in a repressive association with the target gene and the binding of agonist to the receptor results in gene activation due to a co-regulator switch [38]. However, affymetrix microarray analysis of MCF-7 cells following treatment with ATRA indicated that only a fraction of the genes regulated by apo-ER/apo-RARα1 were regulated by ATRA (Tables 1 and 2). Specifically, among the 53 genes whose basal expression was supported by apo-ER/apo-RARα1, 6 genes were inhibited by ATRA and 1 was activated (Table 1). On the other hand, among the 68 genes whose basal expression was repressed by apo-ER/apo-RARα1, 15 genes were activated by ATRA and 10 genes were inhibited. Notably, none of the ATRA regulated genes in Tables 1 and 2 are functionally associated with the cell division cycle.

Since putative RAR binding sites have been globally mapped in the chromatin of MCF-7 cells [39], we used this information to identify the presence of RAR binding sites associated with the apo-ER/apo-RARα1 target genes listed in Tables 1 and 2. As indicated in the Tables, 18 of the 53 genes in Table 1 and 14 of the 68 genes in Table 2 were associated with RAR binding sites within a distance of 10kb of the transcription start sites. The genes in Table 1 had significant enrichment (Hypergeometric test) for RAR binding sites within 10kb with $P=0.03$. The enrichment for RAR binding sites for the genes in Table 2 had a $P$ value of 0.19. Thus, despite the low frequency with which the apo-ER/apo-RARα1 target genes are regulated by ATRA, there is a significant enrichment among them for associated RAR binding sites, largely among gene targets that are insensitive to ATRA.
DISCUSSION

ER is known to regulate genes in a ligand-independent manner [40, 41]. Hormone-independent actions of ER play an important role in supporting the growth of hormone-refractory breast tumors [12]. On the other hand, studies of gene regulation by ER in estrogen-sensitive breast cancer cells have mostly focused on estrogen-responsive genes that have profound roles in tumor growth and development and the effects of tamoxifen on gene regulation by estrogen [42]. The findings of this study however, highlight a potentially significant mechanism of hormone-independent transcriptional action of ER in hormone-sensitive breast cancer cells. This action of ER is clearly a major contributor to the ability of hormone-sensitive breast cancer cells to maintain a basal level of proliferation under conditions of hormone-depletion. This effect of apo-ER occurred primarily through supporting the cell division cycle. Remarkably, the action of apo-ER was also rather insensitive to tamoxifen at a dose that is clinically relevant to circulating concentrations of the drug that induce all of the surrogate biomarkers of clinical response [43, 44]. Similar to clinical breast tumors, MCF-7 cells are heterogeneous and can yield clonal populations of inherently tamoxifen resistant cells that are variably ER-dependent [45]; nevertheless, the fraction of S-phase cells in hormone-depleted or tamoxifen-treated MCF-7 cells under the in vitro conditions in this study was much higher than the frequency of emergence of aggressively growing colonies in tamoxifen-treated cultures.
Therefore it is likely that the basal level of S-phase cells observed in hormone-depleted or tamoxifen treated cultures represent a substantial proportion of cells in which the cell cycle progression is slowed. In a tumor environment however, this slow proliferation must be offset by cell death, resulting in an overall tumoristatic effect. Since a basal level of cell division is an essential pre-condition for progressive events leading to the eventual development of resistance of breast tumors to hormonal adjuvant therapy, understanding the mechanism of the hormone-independent effects of ER in hormone-sensitive cells is important.

In hormone-sensitive breast cancer cells, the well established ER-RAR axis has been best characterized in the context of ligand effects (estrogen, retinoids, tamoxifen + retinoids) [20, 46]. The results of this study however establish that in hormone-sensitive cells that are depleted of hormone or treated with tamoxifen, a major mechanism by which ER supports the cell cycle is by supporting the basal expression of RARα1. The role of RARα1 in mediating the action of apo-ER is strongly evident from the following observations: (i) In hormone-depleted cells, apo-ER maintained the basal expression level of RARα1 but was not itself regulated by RARα1; (ii) The regulation of RARα1 by apo-ER was insensitive to tamoxifen; (iii) Knocking down RARα1 negatively impacted the basal cell cycle progression and restoring basal apo-RARα1 levels rescued basal level cell division following depletion of ER; (iv) Apo-RARα1 independently regulated a complement of genes in a manner that strongly favored cell division similar to their regulation by apo-ER. This mechanism was remarkable for the following reasons. First, apo-ER regulated the α1 subtype of RAR but not RARs -β or -γ. Second, most of the
common target genes of apo-ER and apo-RARα1 including all of the genes involved in the cell division cycle were insensitive to ATRA. These findings suggest that a major molecular mechanism by which apo-ER supports basal cell division in hormone-sensitive breast cancer cells may not be sensitive to conventional RAR ligands (agonists), but would be predictably opposed by specific inactivators or down-regulators of RARα1.

Similar to primary clinical breast tumors, MCF-7 cells comprise heterogeneous cell types. The cell cycle regulation in MCF-7 cells which occurs through the apo-ER/apo-RARα1 axis could theoretically exclude a subpopulation(s) of cells; such a subpopulation(s) could represent progenitors of tumor cells that are either inherently resistant to hormonal adjuvants or that undergo progressive changes leading to resistance. Whereas the findings in this study do not preclude this possibility, we found no evidence for cells independent of the apo-ER/apo-RARα1 axis that were characterized by ErbB2 or ErbB3 overexpression, common features associated with a resistant phenotype [47, 48].

It is well established that the RARα gene is activated by estrogen; however there is evidence in the literature that ER associates at a basal level with the core promoter of the RARα gene by tethering to DNA bound Sp1 [49]. Apo-ER may thus directly regulate the RARα gene to maintain the basal expression level of RARα1. The observation that the RARα1 protein level decreased more dramatically than its mRNA upon knocking down ER suggests that apo-ER also regulates RARα1 by additional posttranscriptional mechanisms.
The results of this study further indicate that multiple molecular mechanisms must underlie the downstream action of apo-RARα1 on target genes in the context of mediating the effects of apo-ER. The apo-ER/apo-RARα1 axis regulates genes in both a positive and a negative manner to support cell division; both sets of target genes were enriched for associated chromatin sites of RAR binding, suggesting that RARα1 must act on these target genes by direct as well as indirect mechanisms. RAR belongs to the ClassII subfamily of nuclear receptors which typically, in their ligand-free (apoprotein) form, maintain a transcriptionally repressed state of target genes activated by the corresponding agonists [38]. However, only a small fraction of genes regulated by the apo-ER-RARα1 axis appeared to be regulated by this classical mechanism of action of RARα1, since (i) the genes repressed by apo-RARα1 were largely insensitive to ATRA and (ii) most genes activated by apo-RARα1 were ATRA-insensitive. Therefore, apo-RARα1 must act by non-classical mechanisms on most of the target genes, including those with associated RAR binding sites.

RARα is consistently present in the nucleus in breast tumors and its expression levels correlate with that of the proliferation marker, ki-67 [50]. The functional RARα isoform in MCF-7 cells and that identified in most breast tumors was exclusively of type 1, an isoform that is believed to be genetically redundant [51]. The structural divergence of the two RARα isoforms arising from alternative promoter usage and alternative splicing includes differences in functional sub-domains [34] which may enable their differential targeting with pharmacological agents. The mechanistic model elucidated in this study would predict that agents that specifically target the α1 subtype of RAR for
functional inhibition or degradation will enhance current hormonal adjuvant therapies of ER+ breast cancer. This approach may have fewer side effects than SERDs due to a redundancy of RAR subtypes in other tissues. Studies are underway to test this concept in pre-clinical models of hormone-sensitive breast cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.
REFERENCES


**Figure 1-I. Hormone-independent effects of ER on the proliferation of hormone-sensitive MCF-7 cells.** In all the experiments, MCF-7 cells were first cultivated in phenol red free DMEM containing 5% charcoal-stripped FBS for 48 h to deplete hormone. EGR3 mRNA levels in the cells were measured by real time RT-PCR after a brief (8h) treatment with vehicle (No ligand), E2 (1nM) or OH-Tam (100nM) (A). Cells were also treated with vehicle (No ligand) or E2 (10nM) for 30 minutes and ligand-dependent activation of ER was analyzed by western blot using a specific antibody to detect phosphorylation at ser118 residue of ER; GAPDH was probed as a loading control (B). Hormone-depleted MCF-7 cells were transfected with either ER siRNA or control siRNA and maintained in hormone-depleted conditioned media without further replenishment of the media. Twenty four hours after transfection, cells were treated with vehicle (no ligand), E2 (1nM) or OH-Tam (100nM); the treatments were repeated every 48h without changing the media. Following the treatment, viable cells were counted daily for 6 days by the Trypan Blue dye exclusion assay (C). In parallel, cells were harvested on each day of the treatments and total RNA extracted from them; the mRNA for ER was measured by real time RT-PCR and the values were normalized to those for GAPDH (D). In addition, cells were harvested in parallel on each day of the treatments for western blot analysis using antibody to ER; GAPDH was probed in each blot as a loading control (E).
Figure 2-I. Hormone-independent effects of ER on cell cycle phase distribution in hormone-sensitive MCF-7 cells. Hormone-depleted MCF-7 cells were transfected with either ER siRNA or control siRNA and maintained in hormone-depleted conditioned media without further replenishment of the media. Twenty four hours after transfection, cells were treated with vehicle (no ligand), E<sub>2</sub> (1nM) or OH-Tam (100nM); the treatments were repeated every 48h without changing the media. (A) On the indicated days, the cells as treated above were harvested for flow cytometry analysis to determine their cell cycle phase distribution. (B) On day 4 of the above treatment, cells were harvested to measure the proportion of apoptotic cells by Annexin V staining. The figure is a representative example of replicate experiments.
Figure 2-L

A.

![Graph showing distribution in cell cycle for different conditions.]

B.

![Bar graph showing the percentage of Amelio cells across different conditions.]

Day: 0 3 4 5 6

No ligand

E2 (1nM)

OH-Tam (100nM)

No ligand +siER
Figure 3-I. Effect of fulvestrant on cell cycle phase distribution in hormone-depleted MCF-7 cells. Hormone-depleted MCF-7 cells were treated with vehicle control or fulvestrant (100nM) and maintained in hormone-depleted conditioned media without further replenishment of the media. The treatments were repeated every 48h for up to five days. (A) Cells were harvested after 3 and 4 days of treatment as described above and total RNA extracted from them. The mRNA for ER was measured by real time RT-PCR and the values were normalized to those for GAPDH. (B) Cells were harvested after 3 and 4 days of treatment as described above for western blot analysis using antibody to ER. GAPDH was probed as a loading control. (C) The cells treated as above were harvested on days 3 and 4 for flow cytometry analysis to determine their cell cycle phase distribution. (D) On day 4 of the above treatment, cells were harvested to measure the proportion of apoptotic cells by Annexin V staining; the figure is a representative example of replicate experiments.
Figure 3.1.

A. Relative ER mRNA Level

B. No Fulvestrant ligand (100nM)

C. Distribution in Cell Cycle

D. Alkaline Phosphatase (AP) Activity
Figure 4-I. The role of RARα in mediating the hormone-independent effect of ER on basal level cell cycling in MCF-7 cells. (A) Effect of knocking down either ER or RARα on the mRNA levels (left panel) and protein levels (right panel) of ER and RARα. Cells were transfected with control siRNA, ER siRNA or RARα siRNA and 4 days later the cells were harvested to extract total RNA for the measurement of ER and RARα mRNA by real time RT-PCR; the values were normalized those for GAPDH (control). The cells were also harvested 4 days after transfection for western blot analysis using antibody to either ER or RARα; the blots were probed for GAPDH as a loading control. (B) The cells were transfected a described for Panel A and the cell lysates were probed by western blot using antibodies specific for RARβ and RARγ; GAPDH was probed as a loading control. (C) Cells transfected as described in Panel A with control siRNA, ERsiRNA and RARα siRNA were analysed by flow cytometry for cell cycle phase distribution. (D) RARα1 expression plasmid and siRNA against ER were co-transfected into hormone-depleted MCF-7 cells by nucleofection. As controls, cells were co-transfected with either control siRNA or ERsiRNA and the vector plasmid. Cells were harvested 3 days after transfection and the cell cycle phase distribution determined by flow cytometry (left panel). The cells were also harvested at the same time for western blot analysis of the lysates using antibody to ER and RARα (right panel); GAPDH was probed as a loading control. (E) Cells were transfected as described in Panel A with control siRNA, ERsiRNA and RARα and following extraction of total RNA, the mRNAs for ERBB2 and ERBB3 were measured by real time RT-PCR, and normalized to the GAPDH values.
Figure 5-I. Identification of major RARα isoforms in various normal tissues and in breast cancer. Total RNA were extracted from MCF-7 cells, 5 human breast tumors and normal tissue controls (peripheral blood leukocytes, thymus, spleen and breast) and reverse transcribed into cDNA by RT-PCR. The cDNA fragments were amplified by competitive PCR using forward primers specifically against RARα1 and RARα2 and a common reverse primer. The PCR products were identified by electrophoresis on a 2% agarose gel by ethedium bromide staining. The cDNA for GAPDH was amplified in each sample as an internal control.
Figure 5-I.
Figure 6-I. Tamoxifen-insensitivity of basal RAR expression in MCF-7 cells and the functional categories of the target genes of the apo-ER → apo-RARα1 axis. (A) Hormone-depleted MCF-7 cells were treated with OH-Tam (100nM) or vehicle control. 72h later, the cell lysates were prepared and analysed by western blot using antibodies specific for RARα, RARβ or RARγ; GAPDH was probed as a loading control. (B) Real time RT-PCR analysis to confirm the effect of knocking down either ER or RARα on the mRNA levels of representative target genes found by Affymetrix DNA microarray in this study to be regulated by apo-ER and apo-RAR in MCF-7 cells: Cells were transfected with control siRNA, ER siRNA or RARα siRNA and 4 days later the cells were harvested to extract total RNA for the measurement of the relevant mRNAs; the values were normalized those for GAPDH (control). (C) and (D) mRNA profiling was performed to identify common target genes of apo-ER and apo-RARα1 in MCF-7 cells. Apo-ER and apo-RARα1 were knocked down separately in hormone-depleted MCF-7 cells. Seventy two hours after transfection with the appropriate siRNA, total mRNA was extracted and mRNA profiling was carried out using Affymetrix microarray analysis. The overlapping set of up- or down- regulated genes were further filtered to exclude tamoxifen-sensitive genes.
Figure 6-L

A. 

B. 

C. Functional categories
- Support cell cycle/Mitosis
- Transcription/splicing
- Unknown
- Regulation of actin cytoskeleton
- Cell metabolism, enzyme regulation
- Signal transduction (proliferation)
- Signal transduction (other)
- Regulate cell cycle
- Immune response
- Membrane transport
- Protein synthesis
- Ubiquitination

D. Functional categories
- Signal transduction / Cell adhesion / Motility
- Unknown
- Transport and metabolism
- Immune recognition / Immune responses
- Transcription
- Inhibition of cell cycle / Proliferation
- Cytoskeleton
- Regulation of cell cycle
- Protein synthesis
- Differentiation
- Induce apoptosis
Table 1: Tamoxifen-Inensitive genes Supported by the Apo-ER → Apo-RARα Axis

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* RAR binding site identified within 10kb of the transcription start site (Hua et al., 2009).

**++ RAR binding site detected under high stringency (Hua et al., 2009).

+ RAR binding site detected under high stringency (Hua et al., 2009).

↑ Up-regulated by ATRA.

↓ Down-regulated by ATRA.
Table 2: Tamoxifen Insensitive genes Repressed by the Apo-ER → Apo-RARα Axis

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*a RAR binding site identified within 10kb of the transcription start site (Hua et al., 2009).
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+ RAR binding site detected under high stringency (Hua et al., 2009).
↑ Up-regulated by ATRA.
↓ Down-regulated by ATRA.
CHAPTER 3

Estrogen-Induced and TAFII30-Mediated Gene Repression by Direct Recruitment of the Estrogen Receptor and Co-repressors to the Core Promoter and its Reversal by Tamoxifen*

Hong Hao°, Marcela d’Alincourt-Salazar°, Karen M.M. Kelley, Aymen Shatnawi, Sumanta Mukherjee, Yatrik M. Shah and Manohar Ratnam*

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ABSTRACT

Estradiol (E$_2$) acts through the estrogen receptor (ER) to down-regulate many genes and tamoxifen (Tam) largely reverses this repression but the underlying mechanisms are unclear. Repression of the folate receptor (FR)-α P4 core promoter by ER is enhanced by E$_2$ and reversed by Tam. This effect was unaffected by inhibition of new protein synthesis and required the E/F and the DNA binding domains of ER without direct binding of ER to DNA. The repression by E$_2$/ER was not specific for either Sp1 or TATA elements but was loosely selective for the initiator and flanking sequence. Insertion of a response element or a relatively strong Sp1 cluster to recruit ER upstream of the core promoters caused a switch to activation by E$_2$/ER that was inhibited by Tam. In nuclear extracts, association of ER with a biotinylated core promoter fragment was promoted by E$_2$ but Tam blocked this effect. Repression/de-repression of the P4 promoter and endogenous FR-α expression by E$_2$/Tam required SMRT and/or NCoR. ER associated with the chromosomal P4 promoter and SMRT and NCoR associated with it in an ER-dependent manner; these associations were favored by E$_2$ but disrupted by Tam, in the short term, without changes in ER expression. TAFII30 was required for optimal P4 promoter activity and for the repressive association of ER. E$_2$ may thus maintain a low transcriptional status of genes by favoring direct TAFII30-dependent association of ER with the core promoter in a co-repressor complex containing SMRT and/or NCoR; this repression is overridden in target genes containing an upstream element that strongly recruits ER. In addition to suppressing the activation of classical E$_2$ target genes, Tam may up-regulate genes by passively dissociating the ER co-repressor complex.
INTRODUCTION

Within the large superfamily of nuclear receptors, the estrogen receptor (ER) is among the best characterized as a regulator of gene transcription (Hall et al., 2001; McDonnell, 2005). Steroid hormone receptors including ER have been grouped as Class 1 nuclear receptors (Mangelsdorf et al., 1995) that typically form homodimers in response to ligand binding and bind to response elements that occur as inverted repeat half-sites in the target gene. ER agonists such as estradiol (E2) induce preferential recruitment of co-activators vs. co-repressors by the DNA-bound receptor resulting in transactivation of the target promoter. Selective estrogen receptor modulators (SERMs) such as tamoxifen (Tam) may bind to ER and act as antagonists of E2 action by favoring the recruitment of co-repressors (Glass and Rosenfeld, 2000; McDonnell, 2005; Smith and O'Malley, 2004). Depending upon the cellular and promoter context, SERMs may also act as agonists, behaving like E2 (Glass and Rosenfeld, 2000; McDonnell, 2005; Smith and O'Malley, 2004). In contrast to SERMs, pure ER antagonists, such as ICI 182,780 do not display partial agonist activity. ICI 182, 780 acts by decreasing the availability of functional ER by different mechanisms including induction of proteolytic degradation of ER, impairment of ER dimerization and disruption of nuclear localization of ER (Dauvois et al., 1993; Parker, 1993; Pink and Jordan, 1996). Target genes for activation by E2/ER may lack a classical estrogen response element (ERE); in such instances, ER may be tethered to the target promoter by DNA-bound transcription factors such as Sp1 (Safe and Kim, 2004) and AP-1 (DeNardo et al., 2005).
Recent gene expression profiling studies (Frasor et al., 2003; Frasor et al., 2004; Hayashi, 2004; Wang et al., 2004) show that in ER+ cell lines and primary tumors, E₂ inhibits the expression of a large proportion of ER-responsive genes. In MCF-7 breast cancer cells, E₂ inhibited approximately twice as many genes as it activated (Frasor et al., 2003; Frasor et al., 2004). In these cells, Tam completely reversed gene repression by E₂ in 26% of the down-regulated genes and partially reversed the repression in 43% of the genes (Frasor et al., 2004). The remaining 31% of the genes down-regulated by E₂ were also inhibited by Tam (Frasor et al., 2004). There is only a limited amount of mechanistic information on gene repression by E₂/ER and despite the large number of cases in which Tam reverses the repression the mechanism of Tam action in this context is unclear. In the few examples in which direct gene repression by E₂/ER has been investigated, it appears that the target genes lack a ERE and that the repressive action is mediated by direct interaction of ER with other transcription factors such as GATA-1 (Blobel et al., 1995), Sp3/Sp1 (Stoner et al., 2000; Stossi et al., 2006; Varshochi et al., 2005), AP-1 (Schmitt et al., 1995), NF-κB and C/EBPβ (Cvoro et al., 2006; Stein and Yang, 1995). In one instance (Blobel et al., 1995), the repressive effect of E₂/ER is thought to be due to interference with the interaction of a transcription factor (GATA-1) and in two instances (Stossi et al., 2006; Varshochi et al., 2005) the repression involved recruitment of histone deacetylase (HDAC). Further, among these examples, GATA-1-mediated repression is the only case in which Tam has been shown to reverse the repression by E₂ (Blobel et al., 1995).
Understanding the different mechanisms by which E\textsubscript{2}/ER and the prototypical SERM, Tam, regulate model genes is necessary to identify SERMs that are ‘dissociated’ from regulatory characteristics that are undesirable for their use in cancer chemoprevention/therapy. We have previously reported (Kelley et al., 2003) that the TATA-less and Sp1-dependent folate receptor (FR)-\(\alpha\) P4 promoter and endogenous FR-\(\alpha\) expression are repressed in the short term by ER type \(\alpha\) (referred to here as ER) in a manner that is further enhanced by E\textsubscript{2} and that this repression is relieved by Tam and ICI 182,780. ER\(\beta\) repressed the promoter only modestly compared to ER\(\alpha\) but did not interfere with the ER\(\alpha\) effect. We reported a limited initial promoter analysis involving an essential G/C-rich (Sp1 binding) element in the promoter and also observed that when this element was mixed with a nuclear extract, ER very weakly associated with it. In light of the known ability of ER and Sp1 to physically associate with each other, the data was interpreted to suggest that the repressive action of ER occurred through its tethering to the P4 promoter through Sp1 (Kelley et al., 2003). A closer investigation of the mechanism of repression of the FR-\(\alpha\) P4 promoter by ER and its de-repression by Tam reported here, discounts a role for Sp1. This study points instead to the initiator and flanking sequence within the core promoter (that may contain either G/C-rich or TATA box elements) as the principal determinant of the direct and repressive action of a co-repressor complex comprising ER and SMRT and/or NCoR on the basal promoter activity. Evidence is presented to support a model in which de-repression by Tam occurs by its ability to disrupt such a co-repressor complex. The results also show that when either a classical estrogen response element (ERE) or a relatively strong cluster of Sp1 elements that strongly recruits ER is present, it will override the repressive association of
ER with the core promoter. Further, TAFII30 is required for optimal activity of the P4 core promoter and also mediates its repressive association with ER. This model is consistent with the emerging concept (Smale and Kadonaga, 2003) of core promoter diversity that influences the formation and composition of the transcription pre-initiation complex. The ligand-dependence of ER and co-repressor associations with the promoter as well as the ligand effects on the target gene expression are considerably more pronounced in the chromosomal context. The findings contribute to a further mechanistic understanding of the global effects of E2/Tam on gene expression.

MATERIALS AND METHODS

Antibodies and siRNA. Anti-human ER mouse monoclonal antibody D12 and affinity purified rabbit anti-human ER antibody were from Santa Cruz Biotechnologies (Santa Cruz, CA). Affinity purified rabbit anti-human SMRT or NCoR was from Upstate Cell Signaling Solutions (Lake Placid, NY). Affinity purified polyclonal rabbit anti-human TAFII30 antibody was from Aviva Systems Biology (San Diego, CA). Mouse anti-α tubulin clone B-5-1-2 antibody was from Sigma (St. Louis, MO). The small interfering RNAs (siRNA) for SMRT or NCoR were from Dharmacon Research, Inc (Lafayette, CO.). The siRNA for TAFII30 was purchased from Sigma.

DNA constructs and expression plasmids. PCR products or synthetic oligonucleotides were first digested with the appropriate restriction enzymes and inserted into the PGL3-
basic plasmid (Promega) or an appropriate FR-α promoter construct. The construct
SV40(GC)_6-P4InrF, in which the G/C-rich region of the FR-α P4 promoter (-147 to -18)
was replaced by the G/C-rich region (-113 to -43) of the SV40 promoter, was generated
using the upstream primer 5'-GTCAGCATATGTA GTCCCGC CC-3' and the down
stream primer 5'-AAACTTAAGCAGCGATG GGC-3' corresponding to regions in the
SV40 promoter of the pGL3-control plasmid (Promega). The P4/SV40_inrF construct, in
which the FR-α P4 InrF (-28 to +33) was replaced with the SV40 InrF (-48 to +65), was
generated using the upstream primer 5'-ATTCTCCGCGGC ATCGCTGAC-3' and
downstream primer 5'-C ACTGCATACGACGATTCTGTG-3' corresponding to regions
in the SV40 promoter and the luciferase gene of the pGL3-control plasmid (Promega).

The 5' deletion constructs of the FR-α P4 promoter (-272 to +33) and P4 min (-49 to +33)
were constructed by PCR using the appropriate primers and subcloned at MluI (upstream)
and XhoI (downstream) sites in the pGL3 basic plasmid. In the construct TATA-P4InrF,
the G/C-rich sequence (-49 to -35) within the FR-α P4 promoter-luciferase construct was
replaced by a TATA-box element (5'-AATAATTAA-3') using PCR. The ERE-P4 min
and ERE-TATA-P4 InrF constructs were constructed by inserting double stranded
synthetic oligonucleotides corresponding to the sequence (5'-G
GTACCTCAGGTCACA CTGACCTGATCAGGTCACAGTGACCTGATCAGGT
CACAGTGACCTGAGAGCTC-3') into P4 min or TATA-P4 InrF at KpnI (upstream)
and SacI (downstream) sites. In the construct P4/RSV_inrF, the InrF sequence (-35 to +33)
within the FR-α P4 promoter-luciferase construct was replaced by a RSV InrF sequence
(-21 to +22) using PCR. In P4/TK_inrF, the FR InrF (-35 to +33) was replaced by the TK
InrF (-19 to +33). The ER-ABCD construct was generated by PCR using the upstream
primer 5'-AAGCTGgctagcATGGACTACAAGGACGACGATGACAAGAA GGAGAC TCGCTACTGTGCAG-3' containing an Nhe I synthetic restriction site, a 24 bp flag-tag sequence, and a region of the ER-α mRNA sequence encompassing the ATG site. The downstream primer 5'-CGGGCCC tetagaCTACTTAGAGCGTTGATCATGAG CGG-3’ corresponds to the D-domain of the ER-α mRNA sequence and contains a flanking Xba I restriction site. In a similar way, the ER-CDEF construct was generated using the upstream primer 5'-AAGCTGgctagcATGGACTACAAGGACGACGATGACAAGAA GGAGAC GACTCGCTACTGTGCAG -3' containing a synthetic Nhe I restriction site, a synthetic ATG site, a 24 bp flag-tag sequence, and the beginning of the C-domain of the ER-α mRNA sequence. The downstream primer 5'-GCCAGGagatctTCAGACTGTGGCAGG GAAACCCTC -3' contains a Bgl II synthetic restriction site and sequence corresponding to the C-terminus of the ER-α mRNA sequence. Each ER construct was then inserted into the polylinker of the PCR3.1 plasmid using the restriction sites noted. The recombinant plasmids were amplified in E. coli XL1Blue and purified using the Qiagen plasmid kit (Qiagen, Chatsworth, CA). The cloned DNA sequence was verified by sequencing.

Construction and packaging of recombinant lentiviruses and infection of cells. The FR-α P4 promoter (-175 to +33 ) was amplified from the FR-α promoter luciferase construct in pGL3 Basic vector with the upstream primer 5’-ACAATGGGGCCCGTGAC CACCTGGAGAAGG-3’ and downstream primer 5’-ACTGCTCGAGAGAAAGCT GTGGTCAGTGGCACC-3’. The ERE-TK promoter was amplified from an ERE-TK luciferase construct (plasmid XETL) with the upstream primer 5’-ATATGGGCCCCAA
TTAACCTCACTAAAGGG-3’ and down stream primer 5’-ATATCTCGAGATCTGCGGCACGCTGTTGACG-3’. Both PCR products were cut at *ApaI* and *XhoI* restriction sites introduced through the upstream and downstream primers and cloned into the parental lentiviral construct pLL3.7 in front of a luciferase and GFP gene. The lentiviruses were packaged in 293 FT cells. The 293 FT cells were transfected with the lentivirus and plasmids pMD2G, pMDLg/pRRE and pRSV-Rev by LipofectAMINE 2000 according to the vendor’s instructions. Viruses were harvested at 48h and 72h and concentrated by ultracentrifugation at 25000rpm for 90min at 4°C. T47D-B cells were infected with lentivirus at 30% confluence in the presence of polybrene (8 µg/ml) and then treated with vehicle, E2 or E2 plus Tam. RNA was isolated 48 h after treatment and the luciferase mRNA levels were measured by real-time RT-PCR.

**Cell culture and transfection.** T47D-B cells were provided by Dr. Katherine Horwitz. 293 FT cells were provided by Dr. Kam Yeung. T47D-B cells were cultured in phenol red-free MEM supplemented with FBS (5%), penicillin (100 units/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), 1x MEM non-essential amino acid and G418 (200 µg/ml). HeLa (American Type Culture Collection) cells were cultured in phenol red-free MEM supplemented with FBS (10%), penicillin (100 units/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM). 293 FT cells were cultured in DMEM supplemented with heat-inactivated FBS (10%), penicillin (100 units/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), 1x MEM non-essential amino acid and G418 (0.5mg/ml). Three days before transfection and after transfection, HeLa cells were grown in phenol red-free media supplemented with charcoal-stripped FBS (5% v/v), L-glutamine (2 mM), insulin (2
µg/ml), and transferrin (40 µg/ml). E₂ or Tam was used where indicated at the concentrations specified. HeLa cells were transfected with DNA constructs in 6-well plates (Corning, New York, NY) using FuGENE 6 (Roche Diagnostics), according to the manufacturer’s protocol. The β-galactosidase expression plasmid, pSV-β-gal (Promega) was co-transfected to monitor uniformity of transfection. β-Galactosidase activity was measured by the colorimetric assay system from Promega.

**Stable transfection.** HeLa cells were cotransfected in 100-mm plates at 60% confluence with 9 µg each of the appropriate promoter-luciferase construct and 3 µg pcDNA1/Neo plasmid (Invitrogen, Carlsbad, CA) using Fugene (Roche) according to the vendor's protocol. Twenty four hours after transfection, the cells were transferred into DMEM containing G418 (0.5 mg/mL; Gibco-BRL). The cells were selected for G418 resistance for 3 weeks, pooled, and seeded in 6-well plates for the nucleotransfection of ER using the Amaza Nucleofection System (Amaza Biosystems) according to the vendor’s instruction. The cells were treated with vehicle, E₂ or E₂ plus Tam for 48h, and then the cell lysates were assayed for luciferase activity.

**Luciferase assay.** Cells lysates were prepared and assayed for luciferase activity using luciferase substrate (Promega) in a luminometer (Lumat LB 9501; Berthold) according to the vendor’s protocol.

**DNA pull-down assay.** HeLa cells were transfected with ER expression plasmid or plasmid vector (negative control). Forty eight hours after transfection, cells were treated
with vehicle, E_2 (1 nM) or E_2 (1 nM) plus tamoxifen (1 μM) for 60 min, washed twice with PBS and harvested in PBS. Cell pellets were lysed with lysis buffer (400 mM NaCl; 10 mM Tris, pH 8.0; 1mM EDTA; 1 mM EGTA; 0.1% Triton X-100; 1 mM PMSF; and 5 μg/mL each of aprotinin, leupeptin, and pepstatin A) supplemented with vehicle, E_2 (10nM), or E_2 (10nM) plus tamoxifen (1μM). The lysates were centrifuged at 16,000g for 10 min and the resultant supernatants were diluted 1:4 with dilution buffer (10 mM Tris-HCl, pH 8.0; 0.5 mM EGTA; 10% glycerol; 0.25% nonidet P-40) in the presence of ligand as indicated. Then 300μg of lysate was incubated with 1 μg of the appropriate biotinylated DNA probe and 10μg poly (dI-dC) at 4°C on a rotary shaker for 1 h, and then incubated with 30 μl of 50% (v/v) streptavidin-sepharose beads overnight. The samples were centrifuged at 600g for 5 minutes, and the pellets were washed four times with washing buffer (10 mM Tris-HCl, pH 8.0; 1mM EDTA; 0.5 mM EGTA; 100mM NaCl; 10% glycerol; 0.25% nonidet P-40) in the presence of ligand for 5 minutes with rotation. The proteins were released by boiling in 100 μL SDS loading buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% 2-mercaptoethanol; and 0.00125% bromophenol blue) and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and western blots were probed for ER. The 5’ biotin-labeled probes were: TATA-P4 InrF (5’-biotin-CTTACGCCTAAATATGGAGCCCTGCACTAAGGACCCCTGACGATTAAGGACCCCCACCTTCGTCATTTGTTTCTTCCAGGGA-3’), P4InrF (5’-biotin-TGGAGCCCTGCACACACACTTAAGGCCCCACCTCCAGCATTCTTGTGGTCCACTGACCACAGCTCTTTTCAGGGA-3’) and ERE (5’-biotin-GTCCA AAG TCAGGTACAGTGACCTGATCAAAGTT-3’).
Gene silencing with siRNAs. The siRNA for SMRT or NCoR was transfected using LipofectAMINE 2000 according to the manufacturer's instructions. The siRNA for TAFII30 was nucleofected using the Amaza Nucleofection System (Amaza Biosystems) according to the vendor's instruction. Knocking-down of SMRT, NCoR or TAFII30 was confirmed at both the mRNA level (by real time RT-PCR) and the protein level (by western blot). The siRNA sequence for SMRT is: 2584AAGGGUA UCAUCACCGCUGUG2604 (Yoon et al., 2003). The siRNA sequence for NCoR is: 2218AAUGCUAC UUCUCGAGAAACA2238 (Yoon et al., 2003). The siRNA sequence for TAFII30 is: CCGGAGATGCAGTGACTGGTTACTCGAGTAGTAACCAGTCAGTCACT GCATCTTTTTT (from Sigma MISSION™ shRNA). HeLa Cells were plated at 40% confluency and transfected with siRNA (75 μM) for SMRT or NCoR using LipofectAMINE 2000 (Invitrogen). Forty eight hours after transfection of siRNA, 400ng of FR-α P4-luciferase construct and 50 ng of ER expression plasmid were transfected using Fugene 6 (Roche) and incubated in media in the presence of the appropriate ligand as indicated for 48 h, and then harvested for luciferase assay. T47D-B cells were incubated for five days in the presence of the appropriate ligand after transfection of siRNA, and then FR-α expression was visualized by western blot. Hela cells were also nucleofected with human TAFII30 siRNA and 25ng or 50ng of ER expression plasmid and plated at 4x10^6 cells per in 10cm plates in phenol red-free MEM supplemented with FBS (10%), penicillin (100 units/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM). Twenty four hours after nucleofection, cells were grown in phenol red-free media supplemented with charcoal-stripped FBS (5% v/v), L-glutamine (2 mM), insulin (2
µg/ml), and transferrin (40 µg/ml) and treated with vehicle or 10nM E2 for 48h. Cells were harvested for luciferase activity assay 72 hours post nucleofection.

**RNA isolation, RT-PCR and real-time PCR.** Total RNA was prepared using the RNeasy Mini kit (Qiagen). Total RNA (200 ng) was reverse transcribed with random primers by using a reverse transcriptase kit (Applied Biosystems) according to the manufacturers’ protocol. The reverse transcription product was measured by quantitative real-time PCR using the Real-time PCR master mix (Applied Biosystems) in the 7500 Real Time PCR System (Applied Biosystems). The primers and TaqMan probe for SMRT, NCoR or luciferase were made by Integrated DNA Technologies, Inc. (Coralville, IA). The primers and the TaqMan probe for the SMRT gene are: TaqMan probe, 5’-6 FAM-CCGCCGCTCAGC GCCAA-TAMRA-3’; sense primer, 5’-GGGTAATAATGACCAGTGGAAGA-3’; antisense primer, 5’-TGGCATTCAGAGGT TTAAAGGC-3’ (Kershah et al., 2004). The primers and the TaqMan probe for NCoR are: TaqMan probe, 5’-6 FAM-CATAGACGTGATCATCAC CCGGC-TAMRA-3’; sense primer, 5’-GGAAGACTACCATTACTGCAAT ATAA-3’; antisense primer, 5’-CATCCTTGTCC GAGGCAATT -3’ (Kershah et al., 2004). The primers and the TaqMan probe for luciferase are: TaqMan probe, 5’-6 FAM-CATTTCGCAGCCTACCGTGTTGTTCC-TAMRA-3’; sense primer, 5’-AACGTAATTT GCTCAACAGTATGG -3’; antisense primer, 5’-TTGCAAACCCCTTTTTGGAAA -3’. The primers and the TaqMan probe for the human TAFII30 and control GAPDH gene were purchased from Applied Biosystems. All samples were measured in triplicate and normalized to GAPDH values.
**Western blots.** The blots were probed with primary rabbit antibodies followed by goat anti–rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase. The bands were visualized by enhanced chemiluminescence.

**Chromatin immunoprecipitation (ChIP) assay.** HeLa cells were transfected with ER expression plasmid or plasmid vector. Forty-eight hours after transfection, cells were treated with vehicle, $E_2$ (10 nM) or $E_2$ (10 nM) plus tamoxifen (1 μM) for 60 min, and then subjected to the cross-linking reaction using formaldehyde (1%). ChIP assays were performed as described previously (Hao et al., 2003) with the following modifications. ChIP signals were measured by real-time PCR analysis of chromatin-immunoprecipitated products. The optimal target sequence chosen to amplify the P4 promoter region was -171 to -80. The following primers and TaqMan probe were used for the real time PCR assay: TaqMan probe, 5′-FAM-TGGTGTCCTAATCCCTACCTTTCATT-TAMRA-3′; forward primer, 5′-CCCCCATCTCCCTCAGTTTT-3′; reverse primer, 5′-CCACCTGGAGAAGGCAATGA-3′. Each sample was tested in triplicate. For the non-target exon sequence 4000bp downstream of the P4 promoter in the FR-α gene (negative control), the following probes were used: TaqMan probe, FAM-CCTTGCCCAACCTTTCCATTTCTACTTCCCC-TAMRA-3′; forward primer, 5′-AGGTGCAGTGGGAGCT-3′; reverse primer, 5′-CATTGCACAGAACAGTGAGTG-3′.
RESULTS

The action of ER/E\textsubscript{2}/Tam on the P4 promoter does not require intermediate protein synthesis. We have previously reported that the P4 promoter of the FR-\(\alpha\) gene is repressed by ER and that E\textsubscript{2} promotes this repression whereas Tam reverses it (Kelley et al., 2003). The protein synthesis inhibitor, cycloheximide (CHX) was used to test whether the regulation of the P4 promoter and FR-\(\alpha\) gene expression by ER ligands could be indirect, mediated by a protein product(s) of one or more upstream target genes of ER. HeLa cells were transfected with an expression plasmid for ER and a P4 promoter-luciferase reporter plasmid; 48h later the effect of a 12h treatment with E\textsubscript{2}, alone or in combination with Tam, on the mRNA level of the luciferase reporter was tested either in the presence or in the absence of CHX (Fig. 1). The ability of CHX to inhibit protein synthesis was confirmed by the lack of luciferase activity in cells transfected for 12h with the P4 promoter-luciferase plasmid in the presence of CHX (data not shown). CHX was unable to affect repression of the luciferase mRNA expression by E\textsubscript{2} or its de-repression by Tam (Fig. 1), indicating that the regulation of the P4 promoter through ER did not require de novo protein synthesis.

Identification of the minimal determinant of negative ER regulation within the P4 promoter. The TATA-less P4 promoter of the FR-\(\alpha\) gene includes three non-canonical Sp1 binding elements (Fig. 2A) that cumulatively contribute to the promoter activity; the most proximal Sp1 element, however, is absolutely required for promoter activity (Saikawa et al., 1995). When, in the minimal P4 promoter, the entire Sp1 binding region
was replaced by a TATA box (Fig. 2A), the promoter was active (Fig. 2B). Like the P4 promoter, this promoter construct was repressed by E$_2$/ER and de-repressed by Tam (Fig. 2B), suggesting that the ER regulation of the P4 promoter did not require a Sp1 binding element.

Next, the portion of the core promoter comprising the initiator and flanking (InrF) sequence, in the P4 promoter was substituted with those of the SV40, thymidine kinase (TK) or rous sarcoma virus (RSV) promoters (Fig. 2A). The InrF sequence is defined here as the region between the TATA box or Sp1 element most proximal to the initiator and extending downstream of the initiator to the beginning of the luciferase reporter sequence (Fig. 2A). Thus the InrF sequence is devoid of TATA or Sp1 elements. All of the chimeric promoters showed basal promoter activity. However, whereas the P4/SV40$_{\text{InrF}}$ and the P4/TK$_{\text{InrF}}$ chimeras responded to ER regulation similar to the P4 promoter (Fig. 2C), the P4/RSV$_{\text{InrF}}$ chimera did not (Fig. 2C), suggesting that the nature of the InrF sequence and not upstream Sp1 or TATA elements accounts for core promoter selectivity in the repressive action of E$_2$/ER and de-repression by Tam.

**Repression of the core promoter by ER in the chromatin context and by endogenous ER.** The ability of the core promoter to be repressed by E$_2$ and derepressed by Tam in the chromatin context was tested by generating recombinant HeLa cells in which the following promoter-reporter constructs were stably integrated: P4 promoter-luciferase, P4/RSV$_{\text{InrF}}$-luciferase or ERE-TK-luciferase, which is an ERE-dependent promoter. As seen in Fig. 2D, in the chromatin context, the core P4 promoter was repressed by E$_2$ and
this repression was completely reversed by Tam. In contrast E₂ increased the activity of the ERE-TK promoter in a manner that was inhibited by Tam. The P4/RSV_{InrF} promoter chimera was not repressed by E₂. This pattern of regulation by E₂ and Tam is consistent with the promoter analysis described above using transient transfections.

To test whether endogenous ER could regulate the core P4 promoter similar to ectopically introduced ER, the ER-positive T47D-B cells were infected with lentivirus for chromosomal integration of P4 promoter-luc or ERE-TK-luc (Fig. 2E). In these cells also, E₂ repressed the P4 promoter and Tam reversed this repression; in contrast the ERE-TK promoter was activated by E₂ and this activation was blocked by Tam (Fig. 2E). Thus the action of transfected ER in HeLa cells reflects that of endogenous ER in T47D-B cells.

**Upstream elements strongly recruiting ER override core promoter repression by E₂/ER.** It has been established that ER may be recruited to promoters either by direct binding to a classical ERE or by association with DNA-bound Sp1. When a classical ERE was placed directly upstream of the minimal P4 promoter or the promoter in which the Sp1 element was replaced by a TATA box (Fig. 2A), E₂/ER enhanced the promoter activity and Tam inhibited this activation (Fig. 2F). Similarly, when the relatively weak Sp1 element in the basal P4 promoter was replaced by a cluster of six canonical Sp1 elements derived from the SV40 promoter (Fig. 2A), the promoter activity was increased by E₂/ER in a manner that was inhibited by Tam (Fig. 2F). The results indicate that recruitment of ER to upstream elements prevents core promoter repression by ER.
ER domains required for its action on the P4 promoter. Since the P4 promoter lacks a classical estrogen response element (ERE), it was of interest to identify the ER protein domains that were required for regulating the P4 promoter. Deletion mutants of ER lacking either the E/F domain or the A/B domain as well as an ER chimera (ER/GalDBD) in which the DNA binding domain (DBD) of ER was replaced by the Gal4-DNA binding domain (GalDBD) were tested (Fig. 3). The results showed that the E/F domain but not the A/B domain was required for the negative regulation of the P4 promoter and for de-repression by Tam (Fig. 3). Further, ER/GalDBD did not repress the P4 promoter (Fig. 3). Based on the known functions of ER domains, the results indicate that the ligand-independent trasactivation function (within the A/B domain) of ER is not required for regulating the P4 promoter. The DBD of ER is apparently required for repression of the P4 promoter.

Differential ligand effects on the association of ER with ERE vs. core promoter DNA fragments. Synthetic biotinylated DNA fragments corresponding to a classical ERE and core promoter fragments comprising the P4 promoter InrF either with or without an upstream TATA box (Figure 4A) were compared in terms of their ability to associate with ER by a in vitro DNA “pull down” assay (Fig. 4B). (Synthesis of a biotinylated P4 promoter fragment was not feasible due to length constraints). The biotinylated DNA fragments were incubated with nuclear lysates from HeLa cells transiently expressing ER in the presence of vehicle, E₂ or Tam followed by “pull down” of the proteins associated with the biotinylated DNA using immobilized streptavidin and western blot analysis of
the proteins. As expected, the ERE fragment specifically associated with ER and this association was increased by both E2 and Tam (Fig. 4B). The core promoter fragment also specifically associated with ER in a manner that was enhanced by E2 but in contrast to the ERE fragment, the association of ER was decreased by Tam (Fig. 4B). The InrF fragment alone (without the TATA box) did not associate with ER significantly above background levels (i.e., in the absence of a DNA probe) (Fig. 4B). These results indicate that ER is physically recruited to the core promoter in a manner that is promoted by E2 but inhibited by Tam. The results also indicate that in order to recruit ER, the entire core promoter that is required to form a pre-initiation complex, is needed.

**ER associates with the P4 promoter in situ in a ligand-dependent manner.** The preceding results suggested a direct action of ER on the FR-α P4 promoter by its recruitment to the core promoter. Therefore the ability of ER to directly associate with the P4 promoter in the endogenous FR-α gene was tested by the chromatin immunoprecipitation (ChIP) assay in which the relative degrees of association of ER with the promoter were quantified using real-time PCR (Fig. 5A). As seen in Figure 5A, ER associated with the P4 promoter in the chromatin context; this association was increased by E2 but decreased by Tam. Under these treatment conditions, the level of ER, detected by western blot, was not significantly altered (Fig. 5B).

**SMRT and NCoR associate with the P4 promoter in situ in an ER and ligand-dependent manner.** As noted previously (Kelley et al., 2003), over-expression of the ER co-repressor SMRT substantially enhanced the repressive effect of E2/ER on the P4
promoter but over-expression of a number of co-activators, including SRC-1, TIF-2, RAC-3, CBP and pCAF did not have an appreciable effect. On the other hand, an effect of the various co-regulators on de-repression by Tam was either absent or marginal (Kelley et al., 2003). Since SMRT appeared to play a role in the negative regulation of the P4 promoter by E2/ER, the possibility that similar to ER, the co-repressors SMRT and NCoR may associate with the P4 promoter in the endogenous FR-α gene was tested by the quantitative ChIP assay described above. Both SMRT (Fig. 5C) and NCoR (Fig. 5D) associated with the P4 promoter but this association required the presence of ER. Further, similar to ER, the association of SMRT or NCoR was increased by E2 and decreased by Tam (Fig. 5C and 5D). (In Fig. 5C, SMRT was introduced ectopically because the available antibody to SMRT could not efficiently immunoprecipitate the relatively low amount of endogenous SMRT and because we had previously shown that ectopic SMRT increases P4 promoter repression by ER).

**Functional role of SMRT and NCoR in mediating the effect of ER on the P4 promoter and on FR-α expression.** To further confirm a functional role for SMRT and NCoR in mediating ER regulation of the P4 promoter, the effect of decreasing the level of endogenous SMRT or NCoR on ER regulation of both the P4 promoter activity and expression of the endogenous FR-α gene was examined. In HeLa cells, siRNA designed to knockdown SMRT or NCoR substantially decreased SMRT or NCoR expression at both the mRNA (Fig. 6A) and protein (Fig. 6B) levels, in contrast to a scrambled siRNA control. Knockdown of either SMRT or NCoR substantially decreased the extent of ligand-dependent repression/de-repression of the P4 promoter and this effect was more
pronounced when both SMRT and NCoR were simultaneously knocked down (Fig. 6C). These observations are consistent with a role for SMRT and NCoR in mediating the repression by ER.

T47D-B cells express both FR-α and ER and were chosen to examine the effect of knocking down SMRT and NCoR on the regulation of the endogenous FR-α through the endogenous ER. In T47D-B cells the mRNA for SMRT or NCoR was decreased by the siRNAs for SMRT and NCoR, respectively (Fig. 7A); this was reflected by a decrease in the protein levels for SMRT and NCoR (Fig. 7B). The endogenous FR-α in T47D-B cells was substantially decreased by E₂ in a manner that was reversed by Tam (Fig. 7C). The combined knockdown of SMRT and NCoR abrogated this decrease in FR-α caused by E₂ and the addition of Tam did not significantly alter FR-α expression (Fig. 7C). This result demonstrates that SMRT and NCoR are required for repression/de-repression of the endogenous FR-α gene by ER ligands.

**Role of TAFII30 in mediating ER recruitment at the P4 promoter and its repression.** TAFII30 was tested as a candidate protein of the pre-initiation complex involved in the repressive association of ER with the P4 core promoter, since this protein is known to have the ability to directly associate with ER (Jacq et al., 1994). siRNA targeting TAFII30 substantially decreased the mRNA for TAFII30 in HeLa cells (Fig. 8A). The decrease in TAFII30 mRNA was reflected in a substantial decrease in the protein level (Fig. 8B). Knocking down TAFII30 caused a decrease in the basal P4 promoter activity suggesting a functional association of TAFII30 with the promoter (Fig. 8C).
8C). In contrast the knockdown did not appreciably affect the activity of the ERE-TK promoter (Fig. 8C), suggesting selectivity in the interaction of TAFII30 with the P4 promoter. Knockdown of TAFII30 substantially decreased P4 core promoter repression by E\textsubscript{2}/ER (Fig. 8D); in contrast, knocking down TAFII30 did not appreciably affect the activation of the ERE-TK promoter by E\textsubscript{2}/ER (Fig. 8E).

Knocking down TAFII30 also abolished the E\textsubscript{2}-induced association of ER with the P4 promoter in the chromosome context in situ as observed by the ChIP assay (Fig. 8F). It should be noted that in the ChIP assay in Fig. 8F, the brief treatment with E\textsubscript{2} followed complete knockdown of TAFII30 whereas in the promoter activity assay in Fig. 8D the treatment with E\textsubscript{2} was for 48h and concomitant with the progressive decrease in TAFII30 due to the tranfected siRNA; therefore the effect of the TAFII30 knockdown on the promoter repression by ER may be expected to be apparently incomplete. The results clearly indicate that the repressive association of ER with the core promoter characterized in the previous experiments is dependent upon the presence of TAFII30.

DISCUSSION

The minimal FR-α P4 promoter is negatively regulated by E\textsubscript{2}/ER in a manner that is reversed by Tam. This regulation is mediated by both ectopically introduced and endogenous ER. The degree of repression/de-repression of the minimal P4 promoter is similar to that of longer promoter constructs. The E\textsubscript{2}-dependent repression by ER also occurs in the chromosomal context as evident from an examination of stably integrated
promoter constructs, the association of ER and co-repressors with the chromosomal P4 promoter and the expression of endogenous FR-α. A classical response element for ER can not be identified within the P4 promoter. In addition to regulating genes by binding to classical hormone response elements, nuclear receptors have been shown to associate with DNA and repress genes by binding to elements termed “negative hormone response elements (nHREs)” (Aranda and Pascual, 2001; Moehren et al., 2004). Such elements have been characterized for glucocorticoid and thyroid hormone receptors, and typically occur near the transcription initiation sites of the target genes but may also occur in the 3' untranslated region. Further, in most cases, the hormone receptors bound to nHREs activate transcription in the absence of ligand and repress it in the presence of hormone.

Despite the requirement for the DNA binding domain of ER in repressing the P4 promoter, electrophoretic mobility shift assays using 40-mer synthetic oligonucleotide duplexes with 20 bp overlaps failed to reveal direct binding of ER to any segment within the entire InrF region of the P4 promoter (data not shown). Further, “DNA pull down” assays failed to show direct binding of ER to the InrF fragment of the P4 promoter in the absence of Sp1 or TATA elements, indicating the absence of a nHRE for ER in the P4 promoter. Nevertheless, several lines of evidence indicate a direct action of ER on the promoter; they include (i) the inability to prevent or alter this regulation by blocking \textit{de novo} protein synthesis using cycloheximide, (ii) specific association of ER with a core promoter fragment in nuclear extracts \textit{in vitro} in the presence of E$_2$ and inhibition of this association by Tam observed in a “DNA pull down” assay, (iii) E$_2$-induced association of ER with the P4 promoter region within the endogenous FR-α gene in the chromatin context \textit{in situ} and its inhibition by Tam by a quantitative ChIP assay and (iv) ER- and
ligand-dependent association of SMRT and NCoR with the P4 promoter in situ measured by the ChIP assay. The parallel functional effects of ER ligands on the P4 promoter activity and FR-α expression vs. the association of ER, SMRT or NCoR with the P4 promoter provide evidence of the functional nature of the physical associations of ER, SMRT and NCoR with the promoter. This is further supported by the relationship between SMRT or NCoR expression levels and (i) repression/de-repression of the P4 promoter activity by E₂/Tam and (ii) the decrease in endogenous FR-α upon treatment of ER-positive cells with E₂ and its reversal by Tam. The FR-α gene is thus a non-classical but direct target for negative regulation by E₂/ER and de-repression by Tam.

A minimal P4 promoter construct consisting of only a single Sp1 element that is essential for basal promoter activity and the downstream sequence (-36 to +33) (InrF) encompassing the transcription start site retained the ER responsiveness of the FR-α promoter. This sequence constitutes only the essential “core promoter” (Smale and Kadonaga, 2003) for a TATA-less gene. Replacing the Sp1 element in this construct with a TATA box retained basal promoter activity and converted it into a TATA-dependent core promoter. The TATA-dependent core promoter construct also retained the ER regulation of the P4 promoter, excluding a role for the Sp1 element in mediating the ER effect. Evidence that the promoter selectivity of repression by E₂/ER is determined by the nature of the InrF was obtained from the observation that the InrF of the P4 promoter could be replaced by that of the SV40 promoter or the TK promoter but not the RSV promoter without a loss of this repression. However, in an in vitro assay in the presence of nuclear proteins, only an intact core promoter but not the InrF alone could bind ER in a
ligand-dependent manner. This observation suggests that at least a part of the transcription pre-initiation complex needs to be formed to physically and functionally recruit ER to the core promoter.

Our studies show that the mode of recruitment of ER to a promoter clearly determines its functional consequence. Thus the introduction of either a classical ERE or a cluster of strong canonical G/C-rich (Sp1 binding) elements upstream of the core promoter overrides the repressive association of E₂/ER with a core promoter and results in its activation. In the absence of an upstream element that can recruit ER, the receptor had the ability to repress the basal activity of promoter constructs containing the InrF regions of different promoters (FR-α, TK, SV40) suggesting that ER may be generally repressive to a broad range of genes, by acting at the level of the core promoter. The repressive interaction of ER with the core promoter requires the DNA binding and E/F domains; whereas the E/F domain is known to contain the ligand binding pocket and co-regulator binding motifs, the role of the DNA binding domain in this context is unclear.

The lack of an appreciable effect of over-expressing known co-activators of ER (SRC-1, TIF-2, RAC-3, CBP and pCAF) on the repression of the FR-α P4 promoter by E₂/ER or its induction (de-repression) by Tam in our previous report (Kelley et al., 2003) indicates that the co-activators are not involved in the regulation of the FR-α gene through ER. In contrast, the effect of over-expression of SMRT, showed that the ER co-repressor potentiates repression of the P4 promoter by E₂/ER but does not determine the promoter activity in the presence of Tam (Kelley et al., 2003). A role for ER co-
repressors in the repression of the P4 promoter by E₂/ER is confirmed by the effects of knocking down SMRT or NCoR on ER regulation of both the P4 promoter and the endogenous FR-α gene expression. Our studies also show that SMRT and NCoR physically associate with the P4 promoter region of the endogenous FR-α gene in a manner that is (i) ER-dependent (ii) promoted by E₂ and (iii) inhibited by Tam. These observations, taken together with the functional data, imply that repression of the P4 promoter is mediated by the association of a complex containing ER and SMRT and/or NCoR with the core promoter and that de-repression by Tam is a passive process involving dissociation of ER, SMRT and NCoR.

Nuclear receptors may regulate transcription by interacting with general transcription factors in the pre-initiation complex (Schulman et al., 1995; Baniahmad et al., 1993; Sadovsky et al., 1995; Masuyama et al., 1997; Jacq et al., 1994; Fondell et al., 1993; Fondell et al., 1996; Tong et al., 1995; Mengus et al., 1997). Such interactions may be selective for the type of nuclear receptors or for the promoter. The co-repressors, SMRT and NCoR have also been shown to functionally associate with TAFs and TFIIB (Muscat et al., 1998; Wong and Privalsky, 1998). Although some degree of promoter selectivity is implied in these interactions of nuclear receptors and co-regulators based on the variable complement of TAFs, the actual recruitment of the receptors (and in turn the co-regulators) to the target promoter is believed to be mediated by cis-elements or DNA-bound proteins distinct from the core promoter. Thus, the classical targets for each nuclear receptor comprise a limited class of genes. The results in this study demonstrate that a core promoter is, in and of itself, adequate to recruit ER, SMRT and NCoR that
together repress it in a hormone-dependent manner and that the selectivity of this interaction is loosely related to features of the initiator and flanking sequence that are yet to be identified. Further, whereas the basal transcriptional activities of genes may be kept in check by E<sub>2</sub>/ER in this manner, gene repression by ER at the core promoter level is simply overridden in target genes that strongly recruit ER to other sites. Our studies demonstrate that TAFII30, which is present in a subset of TFIIID complexes and is a component of the pre-initiation complex known to associate directly with ER (Jacq et al., 1994), mediates repression in the conformational context of direct ER recruitment by the P4 core promoter. The observed requirement of the E/F domain of ER for its repressive action is also consistent with the previously noted ER domain specificity for binding to TAFII30. Such a mechanism may in part provide a basis to explain the recent findings of a relatively large number of genes that are repressed by estrogen in a manner that is reversible by Tam (Frasor et al., 2003; Frasor et al., 2004).

Consensus elements commonly found in core promoters include (i) the initiator encompassing the +1 position, (ii) an upstream TATA box, which in TATA-less promoters is commonly replaced by G/C-rich elements that bind Sp family proteins and (iii) the downstream promoter element (DPE) located at +28 to +32. Recent studies have revealed that these elements may occur in variable combinations giving rise to diversity among core promoters (Smale and Kadonaga, 2003). Additional sequence elements that are less characterized are also suspected to contribute to the variability in the organization of the core promoter (Smale and Kadonaga, 2003). It has been proposed that such a structural diversity among core promoters may permit variability in the patterns of gene
regulation by transcription factors acting at the level of assembly of the pre-initiation complex (Butler and Kadonaga, 2002; Smale, 2001; Smale and Kadonaga, 2003). This view of the core promoter offers a conceptual framework for the direct and repressive association of ER and SMRT or NCoR with the core promoters of a set of genes whose expression is increased by Tam by passive de-repression.
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Figure 1-II. Effect of inhibiting *de novo* protein synthesis on the action of ER on the FR-α -P4 promoter. Hela cells (10⁶) were transfected with 400 ng of FR-α -P4 promoter -luciferase (P4-luc) and 50 ng ER expression plasmid for 48 h. Then the cells were pretreated with either cycloheximide (CHX) (10 μM) or vehicle for 2 h followed by the introduction of E₂ (1nM) or tamoxifen (1μM) as indicated. Fourteen hours later, the cells were harvested for measurement of luciferase reporter mRNA by real-time RT-PCR. The ability of CHX to completely inhibit protein synthesis was confirmed by the lack of luciferase activity resulting from a 12h treatment with CHX of cells transfected with P4-luc during this period (data not shown). *P* values for the differences noted in the text were < 0.0001.
Figure 1-II.

Diagram showing reporter mRNA level (Ratio to Control) for different treatments over 0-14h and 2-14h time periods. The treatments include vehicle, CHX, vehicle, E2, E2+Tam, and CHX. The bars represent the mean with error bars indicating variability.
Figure 2-II. Regulation of various mutant and chimeric FR-α P4 promoter constructs by ER. Panel A: Schematic organization of FR-α promoter constructs. Panel B: HeLa cells (10⁶) were transfected with 400 ng of P4-luc, P4 min-luc or TATA-P4InrF-luc and co-transfected with 50 ng of either ER expression plasmid or the vector control. Cells were treated with vehicle, E₂ (1nM) or E (1nM) plus Tam(1μM) as indicated for the duration of the transfection (48h) after which they were harvested to measure luciferase activity. Panel C: HeLa (10⁶) cells were transfected with 400 ng of P4-luc, or the chimeric promoter luciferase constructs: P4/RSV \text{InrF}, P4/TK \text{InrF} or P4/SV40 \text{InrF}. The cells were co-transfected with 50 ng of either ER expression plasmid or the vector control. Cells were treated with vehicle, E₂ (1nM) or E (1nM) plus Tam(1μM) as indicated for the duration of the transfection (48h) after which they were harvested to measure luciferase activity. Panel D: HeLa cells containing stably integrated P4-luc, P4/RSV \text{InrF}-luc or ERE-TK-luc were transfected with ER by nucleofection and treated with vehicle, E₂ (1nM) or E₂(1nM) plus Tam(1μM) as indicated for 48h. The cells were then harvested to measure luciferase activity. Panel E: The ER-positive T47D-B cells were infected with lentiviruses expressing luciferase driven by either the FR-α P4 promoter or the ERE-TK promoter and treated with vehicle, E₂ (1nM) or E₂(1nM) plus Tam(1μM) as indicated for 48h. RNA was isolated from the cells and the mRNA level of luciferase was measured by real time RT-PCR. (The lentivirus expresses both luciferase and GFP. Because GFP interferes with the luciferase activity assay, the mRNA levels of luciferase were measured instead). Panel F: HeLa cells (10⁶) were transfected with 400 ng of ERE-P4-luc, ERE-TATA-P4InrF-luc or SV40(GC)₆-P4InrF-luc. ER expression plasmid (50ng) or the plasmid vector (50ng) was co-transfected with each promoter luciferase construct, as indicated.
Cells were treated with vehicle, E\(_2\) (1nM) or E (1nM) plus Tam(1\(\mu\)M) as indicated for the duration of the transfection (48h) after which they were harvested to measure luciferase activity. All values represent the mean ± standard deviation from three or more independent experiments. \(P\) values for the differences noted in the text were < 0.0001.
Figure 2-II

(a) Schematic representation of the regulatory regions of the estrogen receptor (ER) gene. The different promoters and enhancers are labeled, with the non-canonical Sp1 element indicated.

(b) Bar graph showing the promoter activity (Ratio to Control) for different conditions:
- P4
- P4 min
- TATA-P4InvF
- ERE-P4
- ERE-TATA-P4InvF
- P4/RSV InvF
- P4/TK InvF
- P4/SV40 InvF
- SV40(GO)6-P4InvF

(c) Bar graph showing the promoter activity (Ratio to Control) for different constructs:
- P4
- P4/RSV InvF
- P4/TK InvF
- P4/SV40 InvF

(d) Bar graph showing the chromosome context:
- P4
- P4/RSV InvF
- ERE-TK

(e) Bar graph showing the luciferase mRNA level for different cell lines:
- ER+ T47D-B cells
  - Vector+vehicle
  - ER+ vehicle
  - E2
  - E2+Tam

(f) Bar graph showing the promoter activity (Ratio to Control) for different constructs:
- ERE-P4
- ERE-TATA
- SV40(GO)6-P4InvF

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Figure 3-II. The action of ER protein domain mutants on the FR-α P4 promoter. HeLa cells (10⁶) were transfected with 400 ng of FR-α -P4 promoter-luciferase (P4-luc) and 50 ng of expression plasmid for a mutant ER (ER-ABCD) in which the EF domain was deleted, a mutant ER (ER-CDEF) in which the AB domain was deleted, or a chimeric ER (ER/GalDBD) in which the DNA binding domain (DBD) of ER was replaced by the Gal4 DBD. The transfected cells were treated with vehicle, E₂ (1nM) alone or E₂ (1nM) plus tamoxifen (1µM) for 48 h and then harvested for luciferase assays. Values represent the mean ± standard deviation from three or more independent experiments. P values for the differences noted in the text were < 0.0001.
Figure 3-II

![Graph showing promoter activity ratios for different conditions.](image-url)
**Figure 4-II.** Pull down assays for synthetic biotinylated promoter elements. Panel A: Schematic organization of the synthetic biotin-DNA probes. Panel B: HeLa cells were transfected with ER expression plasmid for 48h. The transfected cells were treated with vehicle, E$_2$ (1nM) or E$_2$ (1nM) plus tamoxifen (1μM) for 1h and then harvested to prepare nuclear lysates. The nuclear lysates were incubated in the presence of either vehicle or the appropriate ligands with biotinylated synthetic versions of the P4 promoter InrF, TATA-InrF or a canonical ERE. The DNA probes and associated proteins were pulled down using streptavidin sepharose beads, resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibody to ER. Lane 10 represents a negative control in which the nuclear lysate was treated with E$_2$ (1nM) but no DNA probe was present.
Figure 4-II

(a) TATA-InrF  
- Biotin  
- TATA box  
- P4 InrF (-35 to +33)  

InrF  
- Biotin  
- P4 InrF (-35 to +33)  

ERE  
- Biotin  
- ERE (TCAGGTCACTGACCTGA)  

(b) TATA-InrF  
- InrF  
- ERE  
- Input  

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Figure 5-II. Chromatin immunoprecipitation assay of the \textit{in situ} association of ER, SMRT or NcoR with the FR-\(\alpha\) gene. HeLa cells were transfected with either the ER expression plasmid or the vector (negative control) as indicated. Forty eight hours after transfection, cells were treated with vehicle, E\(_2\) (10nM) alone or E\(_2\) (10nM) plus tamoxifen (1\(\mu\)M) for 60 min and then subjected to ChIP as described under Materials and Methods. The relative amount of target genomic DNA fragment pulled down by antibody to ER (Panel A), SMRT (Panel C) or NcoR (Panel D) was quantified by real-time PCR. The values represent the mean of triplicates. Each assay was repeated at least 4 times, and concordant results were obtained. In Panel B, the ER expression level in transfected HeLa cells was analyzed by western blot. \(P\) values for the differences noted in the text were < 0.0001.
Figure 5-II
Figure 6-II. The effect of knocking down SMRT or NcoR on the action of ER on the FR-α P4 promoter. HeLa cells were transfected with scrambled siRNA (control), or siRNA for SMRT and/or NcoR. Forty eight hours after transfection, the cells were co-transfected with P4 promoter-luc and ER expression plasmid. The cells were treated with vehicle, $E_2$ (1nM) or E (1nM) plus Tam(1μM) for the duration of the second transfection (48h). At the end of the second transfection, the cells were harvested for measurement of mRNA levels of SMRT and NCoR by RT-PCR and real-time PCR (Panel A) or the protein expression levels of SMRT or NCoR by western blot (Panel B). At this time, the luciferase activity in the cells was measured to determine P4 promoter activity (Panel C). $P$ values for the differences noted in the text were < 0.001.
**Figure 7-II.** Effect of knocking down SMRT or NcoR in (ER-positive) T47D-B cells on regulation of endogenous FR-α by E₂ and Tam. T47D-B cells were transfected with scrambled siRNA (control) or siRNA for SMRT and NcoR. The cells were treated with vehicle, E₂(1nM) or E₂(1nM) plus Tamoxifen (1μM) at the time of transfection. Five days post-transfection, cells were harvested for measurement of the mRNA level for SMRT or NCoR by real time RT-PCR (Panel A), for western blot to visualize expression of SMRT, NCoR or tubulin (Panel B) and for western blot to visualize expression of FR-α or tubulin (Panel C).
Figure 7-II

(A) mRNA Level (Ratio to Control)

(B) Western Blot

(C) Western Blot
**Figure 8-II.** Effect of knocking down TAFII30 on P4 promoter repression by ER and its E$_2$-induced association with the promoter *in situ*. Panel A: HeLa cells were nucleofected with scrambled siRNA (control), or siRNA for TAFII30. 48h later total RNA was extracted from the cells and the mRNAs for TAFII30 and for GAPDH (control) were measured by real time PCR. The values for TAFII30 mRNA are normalized to the GAPDH control. Panel B: HeLa cells were nucleofected with scrambled siRNA (control), or siRNA for TAFII30. 48h later cell lysates were prepared and subjected to western blot analysis for the expression of TAFII30 or tubulin (control). Panel C: HeLa cells (10$^6$) were co-nucleofected with scrambled siRNA (control) or siRNA to TAFII30 together with either P4 promoter-luciferase plasmid (200ng) or ERE-TK promoter luciferase plasmid (200ng). 72h later the cells were harvested to measure luciferase activity in the cell lysates. Panel D: HeLa cells (10$^6$) were co-nucleofected with scrambled siRNA (control) or siRNA to TAFII30 and P4 promoter-luciferase plasmid (200ng) together with either ER expression plasmid (25ng) or a plasmid vector control (25ng). 24h after nucleofection the cells were treated with E$_2$ (1nM) for a further 48h and then harvested to measure luciferase activity in the lysates. Panel E: HeLa cells (10$^6$) were co-nucleofected with scrambled siRNA (control) or siRNA to TAFII30 and ERE-TK promoter-luciferase plasmid (200ng) together with either ER expression plasmid (25ng) or a plasmid vector control (25ng). 24h after nucleofection the cells were treated with E$_2$ (1nM) for a further 48h and then harvested to measure luciferase activity in the lysates. Panel F: HeLa cells (10$^6$) were co-nucleofected with either the ER expression plasmid (50ng) or the plasmid vector (negative control) together with either siRNA to TAFII30 or a scrambled siRNA (control). Forty eight hours later cells were treated with vehicle or E$_2$ (10nM) for 2h and
then subjected to ChIP as described under Materials and Methods. The relative amount of target genomic DNA fragment pulled down by antibody to ER and was quantified by real-time PCR. The values represent the mean of triplicates ± standard deviation. $P$ values for the differences noted in the text were < 0.0001.
Figure 8-II

(a) Expression of TAFII30 mRNA following siRNA treatment. Scrambled versus TAFII30 siRNA.

(b) Western blots showing TAFII30 and Tubulin levels.

(c) Relative luciferase units for P4 and ERE-TK promoters with Scrambled and TAFII30 siRNA, *P<0.0001.

(d) Promoter activity (Percent of Control) for P4 promoter, *P<0.0001.

(e) Promoter activity by E2/ER, fold activation by E2/ER.

(f) ER signal, Fold Difference.

Legend:

- E2 + Scrambled siRNA
- E2 + ER + Scrambled siRNA
- E2 + TAFII30 siRNA
- E2 + ER + TAFII30 siRNA
CHAPTER 4

**Hormonal Adjuvants may Encourage an Invasive Phenotype in Growth Adapted Breast Cancer Cells by Preventing Gene Repression by Estrogen: Identification of SERMs with Superior Therapeutic Potential**

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**Running Title:** Hormone-independent actions of the estrogen receptor

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ABSTRACT

Adjuvant treatments, including hormone depletion and tamoxifen antagonize estrogen-dependent growth of breast cancer cells primarily by preventing gene activation by estrogen (E$_2$). Less is known about the physiological and therapeutic significance of gene repression by E$_2$ and its antagonism by the adjuvant treatments. Using DNA Affymetrix microarray analysis it was determined that in MCF-7 cells, tamoxifen partially or fully blocked estrogen repression in most of the target genes. Among over 3,500 genes repressed by E$_2$ at 48h of treatment, about eighteen percent were known breast cancer associated genes. A comprehensive ontology analysis of this subset of genes revealed predominant associations with physiological functions that support an aggressive tumor phenotype correlated with poor prognosis, including invasiveness and metastasis, survival, angiogenesis, drug resistance and immune evasion. The ability of E$_2$ to repress this subset of genes in a tamoxifen-sensitive manner was confirmed by real time RT-PCR. Most of these genes were indirect targets of E$_2$ based on the need for new protein synthesis for the repressive effect. Using a library of estrogen receptor α (ER) ligands, we have identified inhibitors of gene activation by E$_2$ that also repress most of this subset of breast cancer associated genes. The results demonstrate a potential role for E$_2$ in restricting an aggressive phenotype in E$_2$-dependent tumors through gene repression. Hormonal adjuvant treatments may prevent this repression and encourage aggressive phenotypes in tumors cells that have acquired hormone-insensitivity for growth.
INTRODUCTION

In the clinical setting, drug response to breast cancer can be predicted largely on the basis of the estrogen receptor type α (ER) status of the tumor and the menopausal status of the patient (Dunnwald, Rossing et al. 2007). ER- positive breast cancer accounts for up to 75% of all breast cancer cases and ER expression in the primary tumor is usually an indicator of good prognosis (Dunnwald, Rossing et al. 2007). Patients with ER+ breast cancer benefit from hormonal adjuvant therapy for up to five years; however, over the course of treatment, disease progression occurs in approximately 50% of the patients due to the emergence of intrinsic or de novo resistance mechanisms (Ring and Dowsett 2004).

In breast cancer, intrinsic or acquired resistance results from the presence of subpopulations of cells within the tumor microenvironment with an aggressive growth phenotype and enhanced migratory capacity. The increased survival advantage of cells with a malignant phenotype over the remaining breast cancer cells characterized by a less aggressive phenotype ultimately leads to tumor recurrence, drug resistance and metastasis (Tredan, Galmarini et al. 2007).

In hormone-sensitive breast cancer cells, the tumorigenic actions of estrogen (E$_2$) are responsible for driving tumor cell growth through activation of estrogen responsive genes that support mitosis and cell cycle progression (Feigelson and Henderson 1996). E$_2$ is also known to repress the expression of genes encoding negative regulators of the cell cycle and tumor suppressor genes (Stossi, Likhite et al. 2006; Karmakar, Foster et al. 2009). Hormonal adjuvant therapies using tamoxifen and aromatase inhibitors suppress
E2 action and induce tumor regression in patients with hormone-sensitive breast cancer. In breast cancer cells, tamoxifen inhibits cell growth by antagonizing E2-induced gene activation, whereas aromatase inhibitors act by blocking peripheral and intratumoral E2 synthesis (Wong and Ellis 2004). In spite of the initial beneficial effects of hormonal adjuvant therapy, breast tumors often adapt to the lack of E2 or E2 signaling through the activation of alternative growth signaling pathways. In addition to the activation of hormone-refractory growth signaling pathways, breast cancer cells often adapt to tamoxifen treatment, by becoming dependent on tamoxifen for growth (Moy and Goss 2006).

In normal breast development E2 is believed to act on a subset of ER+ ductal cells to induce paracrine factors that in turn induce both proliferative and differentiation leading to ductal morphogenesis (LaMarca and Rosen 2007). Studies of the actions of E2 in breast cancer progression have overwhelmingly focused on the mechanisms by which E2 promotes and supports breast cancer cell proliferation (Clemons and Goss 2001). However, the paradoxical actions of E2 in inducing apoptosis and tumor regression in patients with hormone-refractory breast cancer who have failed hormonal adjuvant therapy reveals a different aspect of E2 action: as a tumor suppressor in hormone-refractory advanced breast cancer (Jordan, Lewis et al. 2005). The influence of E2 on physiological aspects of breast cancer cells that are distinct from proliferation have not been clearly elucidated. Furthermore, the proliferative actions of E2 on breast cancer cells are primarily related to gene activation by E2. However, gene expression analysis of MCF-7 breast cancer cells treated with E2 demonstrated that E2 down regulated the
majority of its target genes and in most cases, tamoxifen partially or fully opposed gene repression by E2 (Frasor, Danes et al. 2003). It was therefore of interest to examine the physiological correlates of gene repression by E2 in breast cancer cells. Altogether, the studies were intended to provide a better understanding of the clinical consequence of the loss of gene repression by E2 during hormone depletion and tamoxifen adjuvant therapies.

MATERIALS AND METHODS

Chemicals and reagents. Dulbecco’s minimum essential medium (DMEM), glutamine and penicillin/streptomycin/glutamine stock mix were purchased from Life Technologies, Inc. (Carlsbad, CA, USA). Fetal bovine serum (FBS) and charcoal-stripped FBS were from Invitrogen (Carlsbad, CA, USA). Reagents for real time PCR, primers and TaqMan probes for human ABCG2, ANXA1, BAG1, CCL5, CD24, CD55, CEACAM6, CITED2, CTGF, CTSF, CTSK, CTSS, CXCL2, DDR1, ERBB2, ERBB4, F2R, FSCN1, FZD7, ICAM1, ID1, ID3, IGFBP3, IL-6, IL-8, KLF5, KRT7, LIMK2, LYN, MAPK10, MSX2, NDRG1, PKCD, PKCZ, PASAP, RND3, SDC4, S100P, SPINT1, TACSTD2, TGFB3, TIMP3, TM4SF1, TSPAN31 and GAPDH were purchased from Applied Biosystems (Branchburg, NJ, USA). 17β-estradiol (E2) and 4-hydroxytamoxifen (OH-Tam) were purchased from Sigma Aldrich (Saint Louis, MO).

Cell culture. MCF-7 (American Type Culture Collection) cells were cultured in DMEM supplemented with FBS (10%), penicillin (100unit/ml), streptomycin (100μg/ml) and L-
glutamine (2mM). Hormone depleted MCF-7 cells were seeded in 6 well plates at 20% confluence in phenol-red free media supplemented with 5% charcoal-stripped FBS (v/v) and incubated at 37°C with 5% CO₂ for 48 hours. After 48 hours, the media was replaced with fresh phenol-red free media supplemented with 5% charcoal-stripped FBS (v/v) and L-glutamin (2mM) and the cells were treated with vehicle (ethanol), E₂ (1nM), 4-hydroxy-tamoxifen (100nM) or LY2158658 (100nM) for additional 48 hours. HeLa (American Type Culture Collection) cells were cultured in phenol red- free DMEM supplemented with FBS (10%), penicillin (100 units/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM). Three days before transfection and after transfection, HeLa cells were grown in phenol red-free media supplemented with charcoal-stripped FBS (5% v/v) and L-glutamine (2 mM).

**Luciferase assay.** HeLa cells were transfected with ERE-TK promoter or control vector DNA constructs in 6-well plates (Corning, New York, NY) using FuGENE 6 (Roche Diagnostics), according to the manufacturer’s protocol. E₂, OH-Tam or LY2158658 were used where indicated at the concentrations specified. Cells lysates were prepared and assayed for luciferase activity using luciferase substrate (Promega) in a luminometer (Lumat LB 9501; Berthold) according to the vendor’s protocol.

**RNA isolation, reverse transcription PCR and Real time PCR.** Total RNA from MCF-7 cells was isolated using the RNeasy mini kit (Qiagen, Maryland, MD,USA). Reverse transcription PCR reactions were performed using 500ng of total RNA and the high capacity complementary DNA Archive kit (Applied Biosystems, Branchburg, NJ,
USA) according to the vendor’s protocol. cDNA was measured by quantitative real time PCR in the 7500 StepOne Plus Real time PCR System (Applied Biosystems). Primers and TaqMan probes obtained from the Applied Biosystems inventory. All samples were measured in triplicate and normalized to GAPDH values.

**mRNA profiling.** The Affymetrix DNA microarray analysis was performed as a full service global gene expression study at the transcriptional profiling core facility of the Cancer Institute of New Jersey. Total RNA samples were used to generate labeled cRNAs, which were hybridized to human U133 Plus2.0 Affymetrix microarrays. The expression data were analysed initially using Affymetrix GeneChip Operating Software to create CEL files. The CEL files were imported into the Bioconductor program affylmGUI (Wettenhall, Simpson et al. 2006). The probe set level intensities were quantified and normalized using robust multiarray averaging and quantile normalization. Differential expression between treatments was determined using the limma linear modeling method, and the significance of differences were ranked by the moderated $t$-statistic.

**Statistical analyses.** Experimental values are presented as mean ± s.d. The statistical significance of differences ($P$-value) between values being compared was determined using analysis of variance. In all cases, the differences noted in the text are reflected by a $P$-value of <0.05.
RESULTS

Estrogen dependent proliferation of breast cancer cells and its antagonism by tamoxifen are primarily associated with target genes activated by estrogen. Affymetrix DNA microarray analysis was used to determine changes in the mRNA profiles of MCF-7 cells resulting from hormone depletion, E₂ treatment, tamoxifen treatment or combined treatment with E₂ and tamoxifen for 48h. Ontology analysis of the hormonally regulated gene sets was performed using the DAVID bioinformatics Resources 2008 (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). Of a total of 590 annotated genes (Supplemental Table 1) activated by E₂ by at least 2-fold, tamoxifen antagonized gene activation by E₂ by at least 50 percent in 305 of the genes (Supplemental Table 2). The E₂ activated genes predominantly supported cell cycle and mitosis, with an enrichment score of 46.60 and a Benjamini score of 4.5E-49. The tamoxifen-sensitive E₂ activated genes also predominantly supported cell cycle and mitosis with an enrichment score of 55.28 and a Benjamini score of 3.0E-63. In contrast, the gene sets that was repressed by E₂ by at least 30 percent (Supplemental Table 3), failed to show significant enrichment in favor of genes known to either positively or negatively impact cell growth and survival. Whereas the data on E₂ activated genes is consistent with the findings reported by others (Frasor, Danes et al. 2003), further studies were undertaken to evaluate the physiological significance of the genes repressed by E₂ as discussed below.
The estrogen repressed genes set in MCF-7 cells is enriched for genes overexpressed in association with clinical progression of ductal carcinoma in situ to an invasive phenotype. Of a total of 2,470 annotated genes that were repressed by E2, tamoxifen blocked the repression by at least 50 percent in 82 percent of the genes (Supplemental Table 4). Whereas, hormone depletion by definition completely opposed gene repression by E2, tamoxifen prevented gene repression by E2 in the vast majority of its target genes.

In an elegant study, Schuetz et.al. (Schuetz, Bonin et al. 2006) have established the gene overexpression profile associated with progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) using laser dissected epithelial cells obtained from matched DCIS/IDC samples from 9 patients. Comparison of the E2 repressed genes identified in MCF-7 cells in our study with the top 10 percent of the invasiveness associated genes from Schuetz et al. (Schuetz, Bonin et al. 2006) showed a highly significant overlap with a P value of $8.7 \times 10^{-3}$ for genes repressed by E2 by at least 30 percent (Figure 1A) and the much lower P value of $4.7 \times 10^{-8}$ for genes repressed by E2 by at least 50 percent (Figure 1B). Thus, in MCF-7 cells, E2 represses a significant number of genes associated with clinical progression of DCIS to an invasive phenotype.

Functional analysis of estrogen repressed genes in MCF-7 cells reveals enrichment for genes that support an aggressive phenotype in breast cancer. In order to obtain a clearer understanding of the potential functional impact of E2 repressed genes in breast cancer, all of the genes currently known to be associated with breast cancer were first comprehensively identified from the larger pool of E2 repressed genes. Complementary approaches were used to identify the breast cancer associated genes. In one approach, the
genes occurring in a previously published list of 1,000 breast cancer genes (the BC1000 list) (Witt, Hines et al. 2006) were identified. In a second approach, MILANO (Microarray Literature-based Annotation) (Rubinstein and Simon 2005) was used to thoroughly scan the literature database (GeneRIF and Medline through PubMed) to identify relevant research articles in which individual genes repressed by E$_2$ on the microarray list were associated with breast cancer. After running the list of genes through the MILANO program, the program generates an annotation table containing the number of publications in which the term ‘breast cancer’ as well as the name of each gene on the microarray list occurs in the text with a cut off at 4 publications per gene. A list of 467 genes was obtained in this manner, representing approx. 19 percent of the E$_2$ repressed genes. The literature was then manually scanned to obtain updated information on the specific functions of each gene in breast cancer; in this manner, 196 genes (Table 1) were found to have well established functions related to breast cancer as indicated in Figure 2. Since some of the 196 genes are known to belong to more than one functional category indicated in Figure 2, the numbers of genes placed in the individual groups in Figure 2 add up to 232.

As seen in Figure 2, among genes functionally associated with breast tumor invasion and metastasis, the E$_2$ repressed genes overwhelmingly supported this phenotype. Among genes influencing breast tumor cell survival and growth, a greater number of the E$_2$ repressed genes supported the phenotype. Additionally, E$_2$ repressed genes included those known to support drug resistance, angiogenesis and immune evasion in breast tumors and none that opposed these phenotypes.
The repression by E$_2$ of most of the genes in Figure 2 initially identified by Affymetrix DNA microarray analysis was further confirmed by real time RT-PCR (Figure 3). In virtually all of these genes, tamoxifen at least partially blocked the repression by E$_2$ (Figure 3).

The results demonstrate a strong functional bias of E$_2$ repressed genes towards an aggressive phenotype associated with breast cancer progression. The repression of these genes is fully prevented by hormone depletion and either partially or fully prevented by tamoxifen.

**Sustained gene repression by estrogen occurs by both direct and indirect mechanisms.** E$_2$ target genes that required de novo protein synthesis for repression by E$_2$ were identified using cycloheximide to block protein synthesis during 24 hours of treatment with E$_2$. Of the E$_2$ repressed genes in MCF-7 cells, the repression was prevented by cycloheximide treatment in 27 percent of the genes (Figure 4A). Among the E$_2$ repressed genes that showed a significant decrease in mRNA levels within the 24 hours duration of the experiment, repression of a similar proportion (32 percent) of genes was blocked by cycloheximide treatment among the gene set in Figure 3 as determined by real time RT-PCR (Figure 4B). Therefore, the direct target genes of E$_2$ repression are represented by only a fraction of the genes identified above as those functionally associated with an aggressive phenotype in breast cancer.
DISCUSSION

Our results are consistent with the published literature that in hormone-sensitive breast cancer cells, a large proportion of E2 target genes are repressed by the hormone (Frasor, Stossi et al. 2004). Although the results of this study as well as previous reports observed a strong link between gene activation by E2 and breast cancer cell proliferation, the functional significance of gene repression by E2 on the physiology of breast cancer cells is not at once apparent from standard gene ontology analyses. Gene repression by E2 has thus far largely been investigated in the context of the regulation of growth inhibiting tumor suppressor genes. In the present study, the E2 repressed gene pool was examined by an initial identification of breast cancer associated genes using complementary approaches followed by an updated literature survey of the known functions of the individual genes in this subset. This method of analysis revealed that gene repression by E2 could potentially mitigate an aggressive phenotype in breast cancer cells by inhibiting the expression of many genes known to support invasiveness and metastasis, compared to a relatively small number known to oppose this phenotype. Additional functions of E2 repressed genes included drug resistance, angiogenesis and immune evasion, all characteristics associated with progression of breast cancer. Although more E2 repressed genes encoded positive than negative regulators of cell growth and survival, there was not as strong a bias towards this phenotype compared to invasiveness and metastasis; it appears rather that the influence of E2 on cell proliferation is determined overwhelmingly through target genes that are activated by the hormone. The majority of these genes were
indirect targets of E₂ action, since they required *de novo* protein synthesis for repression by E₂.

The differential effects (supporting vs. opposing) of E₂ on physiologically distinct aspects of breast tumor development, as suggested by the gene ontology analysis above, is not inconsistent with the physiological role of E₂ in the normal breast development where the hormone induces both cell proliferation and differentiation (LaMarca and Rosen 2007; LaMarca and Rosen 2008). Indeed, dissected of its effects on growth, E₂ is known to have anti-tumor effects as evident from the pro-apoptotic effect of E₂ on MCF-7 cells growth adapted to the absence of E₂ (Song, Mor et al. 2001), an observation that possibly underlies the success of clinical regimens of hormonal therapy in breast cancer, where E₂ is administered to patients with aromatase inhibitor-resistant tumors (Ellis, Gao et al. 2009; Munster and Carpenter 2009). The ontology analysis of gene repression by E₂ in this study however, extends such observations to a negative effect of E₂ on the general invasive and metastatic potential of breast cancer cells, including cell motility, adhesion, extracellular matrix degradation, epithelial to mesenchymal transition and angiogenesis.

The molecular mechanisms involved in the transition from a hormone-responsive phenotype towards a more aggressive hormone-refractory phenotype are poorly understood. (Significantly, the subset of E₂ repressed genes overlapped a set of genes consistently upregulated in invasive ductal carcinoma specimens relative to matched *in situ* ductal carcinoma specimens from the same patients). Therefore, the repression by E₂ of genes associated with breast tumor progression has significant implications in
understanding the long term effects of hormonal adjuvant therapies, including hormone depletion and tamoxifen, which oppose the actions of E₂. Whereas our study is consistent with previous reports that the antiproliferative effects of the adjuvant treatments are overwhelmingly mediated by blocking gene activation by E₂, the consequence of antagonizing gene repression by E₂ by the same treatments would predictably be detrimental by encouraging a more invasive phenotype in tumor cells that have acquired hormone independence for growth. By this reasoning, a superior hormonal adjuvant should antagonize gene activation by E₂ while it also represses critical E₂ repressed genes. At the very least, identification of such agents should provide chemical biological tools to establish the relationship between specific genotropic effects of E₂ signaling or tamoxifen to tumor physiology.
REFERENCES


Figure 1-III. Comparison of E\(_2\) repressed genes in MCF-7 cells with genes upregulated in association with clinical progression of ductal carcinoma \textit{in situ} (DCIS) to invasive ductal carcinoma (IDC). Affymetrix DNA microarray analysis was used to determine genes repressed by E\(_2\) (1nM) in MCF-7 cells at the end of 48 h of treatment. The gene sets that were repressed by \(\geq 30\) percent (Panel A) or \(\geq 50\) percent (Panel B) were compared for overlap with the top 10 percent genes upregulated in association with progression of DCIS to IDC in 9 paired clinical tumor specimens as reported by Schuetz \textit{et al.} 2006. \(P\) values for the overlap were calculated using Fisher’s exact test.
Figure 1-III.

A.

2020
E$_2$ repressed
($\geq$30\% of genes)

1646
Top 10 percent up
regulated in IDC
vs DCIS

261

$P = 8.7 \times 10^{-3}$

B.

434
E$_2$ repressed
($\geq$50\% of genes)

1815
Top 10 percent up
regulated in IDC
vs DCIS

92

$P = 4.7 \times 10^{-4}$
Figure 2-III. Gene ontology of E₂ repressed genes in relation to progression of breast cancer. Affymetrix DNA microarray analysis was used to determine genes repressed by E₂ (1nM) in MCF-7 cells at the end of 48 h of treatment. Breast cancer associated genes were identified within this group of genes by comparison with the BC1000 list (Witt et al. 2006) and also by scanning the literature database (GeneRIF and Medline through PubMed) using the MILANO (Microarray Literature-Based Annotation) (Rubinstein and Simon 2005) to identify publications in which the text contained the term “breast cancer” and the name of each gene; the genes were selected on the basis of a cut off at 4 publications for each gene. The literature for each selected gene was then manually scanned to assign one or more functions. The figure indicates the numbers of genes assigned to functional categories covering all aspects of breast cancer progression.
Figure 2-III.

Functional categories

- Invasion/metastasis (migration, EMT, adhesion, ECM degradation)
  - Associated with: Poor prognosis
  - Associated with: Good prognosis
- Support cell/tumor growth/survival
- Negative regulation of cell growth or survival
- Angiogenesis
- Drug resistance
- Immune evasion

Number of genes
Figure 3-III. Confirmation of Affymetrix DNA microarray analysis of selected E$_2$ repressed genes in MCF-7 cells. MCF-7 cells were treated with E$_2$ (1nM) for 48 h and the total RNA purified. The mRNA levels corresponding to the E$_2$ repressed genes that were assigned to the functional category of tumor invasion and metastasis in figure 2 were measured by real time RT-PCR and normalized to the corresponding values for GAPDH. Forty five of the 65 genes that were in this category in Figure 2 were analyzed based on the commercial availability of inventoried TaqMan® probes.
Figure 3-III.

![Graph showing relative mRNA levels with significance markers.](image-url)
Figure 4-III. Effect of cycloheximide on gene repression by E$_2$ in MCF-7 cells.

Hormone depleted MCF-7 cells were treated with either E$_2$ (1nM) or vehicle for 24 h. In each case, the cells were pre-treated with either cycloheximide (Chx) (10µM) or vehicle for 2 h followed by the continued presence of Chx or vehicle for the next 24 h. Total RNA was then purified. Affymetrix DNA microarray analysis was used to identify E$_2$ repressed genes (Panel A). The numbers of genes repressed by E$_2$ in the absence or in the presence of Chx are indicated in Panel A. The results of the Affymetrix analysis were validated by real time RT-PCR for a limited number of genes (Panel B).
Figure 4-III.

A. Total number of genes repressed by $E_2 = 3,069$
- $E_2$ repressed
- NOT blocked by Chx = 648 genes
- BLOCKED by Chx = 2,421 genes

B. Relative mRNA Level
- No ligand
- $E_2$

B. Relative mRNA Level
- No ligand
- $E_2$

E₂ repression
BLOCKED by Chx

E₂ repression
NOT blocked by Chx
Table I-III. Functional classification of E2 repressed genes in breast cancer.

Invasion/metastasis (migration, EMT, adhesion, ECM degradation):

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<thead>
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<td>PSAP</td>
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<td>PTGER4</td>
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<tr>
<td>CTSK</td>
<td>INSR</td>
<td>PRKCD</td>
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Inhibition of invasion and metastasis:

NDRG1  
P13    
PTCH   
SIM2   
SPINT1 
TGFβ3  
TIMP3  
TNS    
TP73L  

Support cell/tumor growth/survival:

<table>
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</tr>
<tr>
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<td>TXN</td>
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<tr>
<td>ETNK1</td>
<td>XPA</td>
</tr>
<tr>
<td>HSPBAP1</td>
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</tbody>
</table>
Negative regulation of cell growth or survival:

AHRR  CST6  PAWR  
APC   FHIT  PDK3  
ARID4A FOXA1  PRKCH  
BAK1  FOXO1  PTPN13  
BTG2  HES1  RHOBTB2  
BIK   IGF2R  RXRA  
CASP9 INHBB  SMAD4  
CCNG2 IRF1  STAT6  
CDK6  ITM2B  TEP1  
CDKN1A MCL1  TGFBR2  
CEBPD MST1  TGFBR2  
CFLAR NQO1  TNFSF10

Angiogenesis:

ANG   MIF  
CCL2  NRP1  
CCL5  TIM4SF1  
CD74  VEGFA  
EFNA1  VEGFC  
FGFR2  ZFP56L1  
IL8   ZFP56L2

Drug resistance:

ABCC1  DUSP1  
ABCC3  DUSP6  
ABCC4  HSFBAP1  
ABCC5  IL6  
ABCG1  MAPK12  
ABCG2  MVP  
AHR   PGFEP1  
ARSD  PRKCD  
BCAR3  PSAP  
CYP2C8 RPS6KA2  
DPYD

Immune evasion:

CCL5  
CLU  
NT5E  
TNFRSF21
CONCLUSION

The development of resistance to hormonal adjuvant therapies is currently a major clinical problem in the treatment of breast cancer. Decades of work have uncovered major mechanisms by which breast cancer cells could acquire resistance to the adjuvant therapies and some of them have been extended to clinical contexts with clear benefits. A well known example of resistance to hormonal adjuvant therapy is the amplification or overexpression of HER-2, through which the tumor cells bypass hormonal signaling pathways. The result of this finding has led to the development of HER-2 targeted therapies. It must be emphasized that despite such progress in understanding and addressing resistance to hormonal adjuvants, there has been a relatively modest improvement in the treatment of patients with advanced breast cancer and the frequency of resistance to hormonal adjuvants has remained the same. Therefore, there is a critical need for new approaches in understanding and targeting drug resistance mechanisms in breast cancer.

The central premise of this Ph.D. dissertation is that a better understanding of early events leading to the various mechanisms of resistance to hormonal adjuvants will lead to improvements in the outcome of hormonal adjuvant therapy in breast cancer. In other words, addressing the general process leading up to drug resistance in order to reduce the frequency of resistance may have a broader impact than attempts to target individual resistance mechanisms. Accordingly, Chapters 2, 3 and 4 describe novel mechanistic studies of transcriptional signaling of ER through which current hormonal adjuvants may actually enable breast cancer cells to progressively acquire genetic or epigenetic changes
associated with drug resistance (Chapter 2). Alternatively, our findings suggest that the hormonal adjuvants may enable the expression of an aggressive (invasive and metastatic) phenotype in cells that have either inherent or acquired resistant to the adjuvants (Chapter 4). Additionally, the studies reveal novel non-classical mechanisms of E_2/ER signaling (Chapters 2, 3 and 4) and also suggest new and potentially superior approaches in hormonal adjuvant therapy of breast cancer.

Chapter 2 highlights a potentially significant mechanism of hormone-independent transcriptional action of ER in hormone-sensitive breast cancer cells. This action of ER is clearly a major contributor to the ability of hormone-sensitive breast cancer cells to maintain a basal level of proliferation under conditions of hormone-depletion. This effect of apo-ER occurred primarily through supporting the cell division cycle. Remarkably, this action of apo-ER was also rather insensitive to OH-Tam. In a tumor environment this slow proliferation must be offset by cell death, resulting in an overall tumorstatic effect. Since a basal level of cell division is an essential pre-condition for progressive events leading to the eventual development of resistance of breast tumors to hormonal adjuvant therapy, understanding the mechanism of the hormone-independent effects of ER in hormone-sensitive cells is important.

In hormone-sensitive breast cancer cells, the well established ER-RAR axis has been best characterized in the context of ligand effects (estrogen, retinoids, OH-Tam + retinoids). The studies in Chapter 2 however establish that in hormone-sensitive cells that are depleted of hormone or treated with OH-Tam, a major mechanism by which ER
supports the cell cycle is by supporting the basal expression of RARα1. This mechanism was remarkable for the following reasons. First, apo-ER regulated the α1 subtype of RAR but not RARs -β or - γ. Second, most of the common target genes of apo-ER and apo-RARα1 including all of the genes involved in the cell division cycle were insensitive to ATRA. These findings suggest that a major molecular mechanism by which apo-ER supports basal cell division in hormone-sensitive breast cancer cells may not be sensitive to conventional RAR ligands (agonists), but would be predictably opposed by specific inactivators or down-regulators of RARα1.

The studies in Chapter 2 further indicate that multiple molecular mechanisms must underlie the downstream action of apo-RARα1 on target genes in the context of mediating the effects of apo-ER. Theapo-ER/apo-RARα1 axis regulates genes in both a positive and a negative manner to support cell division; both sets of target genes were enriched for associated chromatin sites of RAR binding, suggesting that RARα1 must act on these target genes by direct as well as indirect mechanisms. RAR belongs to the ClassII subfamily of nuclear receptors which typically, in their ligand-free (apoprotein) form, maintain a transcriptionally repressed state of target genes activated by the corresponding agonists. However, only a small fraction of genes regulated by the apo-ER-RARα1 axis appeared to have the hallmarks of the classical mechanism of action of RARα1. Therefore, apo-RARα1 must act by non-classical mechanisms on most of the target genes, including those with associated RAR binding sites.
Finally the studies in Chapter 2 have a clear clinical significance. RARα is consistently present in the nucleus in breast tumors and its expression levels correlate with that of the proliferation marker, ki-67. The functional RARα isoform in MCF-7 cells and that identified in most breast tumors was exclusively of type 1, an isoform that is believed to be genetically redundant. The structural divergence of the two RARα isoforms arising from alternative promoter usage and alternative splicing includes differences in functional sub-domains which may enable their differential targeting with pharmacological agents. The mechanistic model elucidated in this study would predict that agents that specifically target the α1 subtype of RAR for functional inhibition or degradation will enhance current hormonal adjuvant therapies of ER+ breast cancer. This approach may have fewer side effects than SERDs due to a redundancy of RAR subtypes in other tissues. Studies are underway to test this concept in pre-clinical models of hormone-sensitive breast cancer.

The discovery in Chapter 3 of a novel mechanism of gene repression by E₂ using the FRα gene as a model was an important prelude to all of our studies of gene repression by E₂ in breast cancer. The minimal FR-α P4 promoter was negatively regulated by E₂/ER in a manner that was reversed by OH-Tam. Several lines of evidence indicated a direct action of ER on the P4 promoter. The parallel functional effects of ER ligands on the P4 promoter activity and FR-α expression vs. the association of ER, SMRT or NCoR with the P4 promoter provide evidence of the functional nature of the physical associations of ER, SMRT and NCoR with the promoter. The FR-α gene is thus a non-classical but direct target for negative regulation by E₂/ER and de-repression by OH-Tam.
The results of the studies described in Chapter 4 are consistent with the published literature that in hormone-sensitive breast cancer cells, a large proportion of E\(_2\) target genes are repressed by the hormone. Although the results of this study as well as previous reports observed a strong link between gene activation by E\(_2\) and breast cancer cell proliferation, the functional significance of gene repression by E\(_2\) on the physiology of breast cancer cells is not at once apparent from standard gene ontology analyses. Gene repression by E\(_2\) has thus far largely been investigated in the context of the regulation of growth inhibiting tumor suppressor genes. In the present study, the E\(_2\) repressed gene pool was examined by an initial identification of breast cancer associated genes using complementary approaches followed by an updated literature survey of the known functions of the individual genes in this subset. This method of analysis revealed that gene repression by E\(_2\) could potentially mitigate an aggressive phenotype in breast cancer cells by inhibiting the expression of many genes known to support invasiveness and metastasis, compared to a relatively small number known to oppose this phenotype. Additional functions of E\(_2\) repressed genes included drug resistance, angiogenesis and immune evasion, all characteristics associated with progression of breast cancer. Although more E\(_2\) repressed genes encoded positive than negative regulators of cell growth and survival, there was not as strong a bias towards this phenotype compared to invasiveness and metastasis; it appears rather that the influence of E\(_2\) on cell proliferation is determined overwhelmingly through target genes that are activated by the hormone. The majority of these genes were indirect targets of E\(_2\) action, since they required \emph{de novo} protein synthesis for repression by E\(_2\).
The differential effects (supporting vs. opposing) of E₂ on physiologically distinct aspects of breast tumor development, as suggested by the gene ontology analysis above, is not inconsistent with the physiological role of E₂ in the normal breast development where the hormone induces both cell proliferation and differentiation. Indeed, dissected of its effects on growth, E₂ is known to have anti-tumor effects as evident from the pro-apoptotic effect of E₂ on MCF-7 cells growth adapted to the absence of E₂, an observation that possibly underlies the success of clinical regimens of hormonal therapy in breast cancer, where E₂ is administered to patients with aromatase inhibitor-resistant tumors. The ontology analysis of gene repression by E₂ in this study however, extends such observations to a negative effect of E₂ on the general invasive and metastatic potential of breast cancer cells, including cell motility, adhesion, extracellular matrix degradation, epithelial to mesenchymal transition and angiogenesis.

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antagonizing gene repression by $E_2$ by the same treatments would predictably be detrimental by encouraging a more invasive phenotype in tumor cells that have acquired hormone independence for growth. By this reasoning, a superior hormonal adjuvant should antagonize gene activation by $E_2$ while it also represses critical $E_2$ repressed genes. At the very least, identification of such agents should provide chemical biological tools to establish the relationship between specific genotropic effects of $E_2$ signaling or OH-Tam to tumor physiology.
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