MOLECULAR INTERACTIONS BETWEEN ENDOGENOUS AND EXOGENOUS FACTORS: REGULATION OF BRCA-1 TUMOR SUPPRESSOR GENE EXPRESSION IN BREAST CANCER CELLS

by

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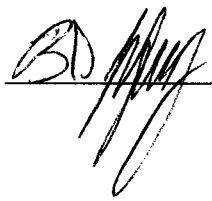
SIGNED: 

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ABBREVIATIONS

ANF	α-naphthoflavone
B[a]P	Benzo[a]pyrene
BPDE	7*r*,8*t*-Dihydroxy9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
BRCA-1	BReast CAncer-1
CBP/VBP	CREB/Vitellogenin Binding Protein
E2	17-β-estradiol
EMSA	electrophoretic mobility shift assay
ER-α	estrogen receptor-α
ER+/ER-	estrogen receptor positive/negative
ERE	estrogen response element
ERU	estrogen response unit
RLU	Relative Luciferase Units
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SERM	selective estrogen receptor modulator
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

ABSTRACT

This dissertation focuses on the *central hypothesis* that in breast cancer cells containing the estrogen receptor-α (ER-α+) and wild-type p53, the BRCA-1 tumor suppressor gene is positively regulated by the steroid hormone estrogen and negatively regulated by Aromatic Hydrocarbon Receptor (AhR) ligands which damage DNA. In this dissertation, we demonstrate that BRCA-1 promoter activity is reduced by the DNA damaging agent Benzo[a]pyrene in breast cancer cells containing both a functional estrogen receptor and p53 pathway. In addition, our data suggests that exposure of MCF-7 breast cancer cells to estrogen stimulates transcription from the BRCA-1 5′ flanking region, and this increase in transcription is paralleled by an increase in estrogen receptor-α interaction at the BRCA-1 promoter between –46 → -14 upstream of exon 1b. We report that in both untreated and estrogen-treated MCF-7 cells, a transcriptional complex, which we have termed an "Estrogen Responsive Unit" (ERU), containing AP-1, Sp1, and CREB family members, forms at the same –46 → -14 region which binds ER-α. Moreover, we show that wild-type p53 is required for estrogen induction of BRCA-1 and overexpression of a dominant-negative mutant variant of p53 can prevent this induction. Finally, we show that overexpression of wild-type p53 is able to disrupt the estrogen receptor interaction with the BRCA-1 ERU under both basal and estrogen-induced conditions while mutant p53 is only able to disrupt this interaction when estrogen is present. Taken together, these data suggest that loss of function of either the estrogen receptor-α or p53 signaling pathways may result in an inability for BRCA-1 regulation to occur and may in turn be a risk factor in the etiology of sporadic breast cancer.

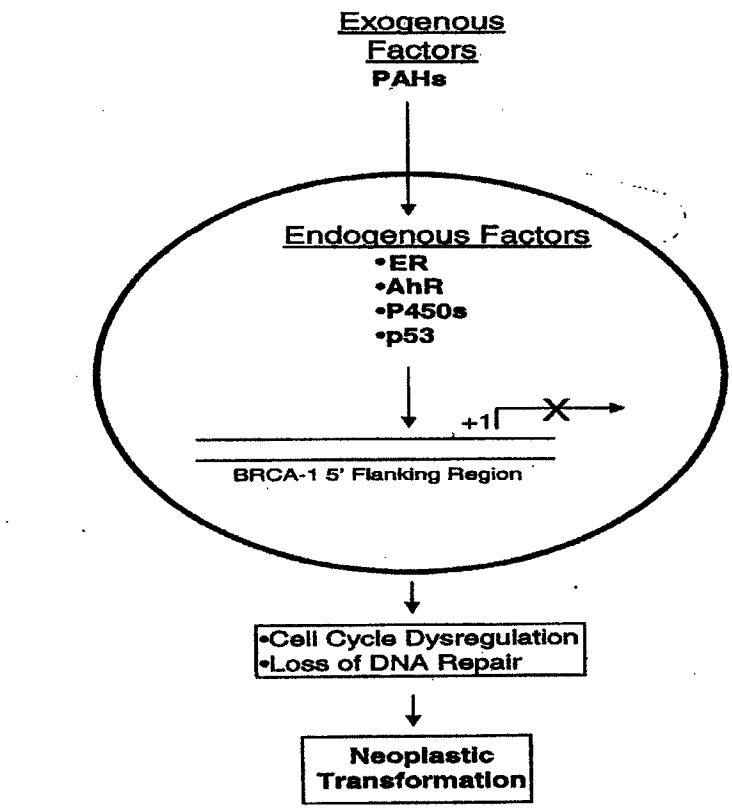


Fig. 1. PAHs as potential risk factors in breast carcinogenesis. Exposure to PAHs may target the expression of regulatory proteins involved in detoxification and DNA repair, thus altering the ability of cells to maintain genomic integrity. The susceptibility to PAHs may be influenced by the cellular complement of endogenous factors including the ER and AhR receptor, enzymes of the P450 family, and p53. These factors can modulate the expression of responsive genes (*BRCA-1*). Possible outcomes may be loss of expression of *BRCA-1* and DNA repair capacity, dysregulation of cell cycle control, and neoplastic transformation.

This dissertation is *novel and significant* because to date, many of the studies published in the BRCA-1 field tend to focus on mutations in the BRCA-1 coding sequence, which only account for approximately 5-10% of breast cancer cases; this report, however, focuses on how mutations in the p53 coding sequence or loss of ER-α function may lead to aberrant regulation of BRCA-1 which in turn could have consequences similar to those caused by BRCA-1 mutations.

Objective/Hypothesis: The *objectives* of this project were: 1). To examine whether or not exposure to PAHs, in particular B[a]P and its primary biotransformation product BPDE, disrupts BRCA-1 expression via a mechanism dependent on the estrogen receptor-α and wild-type p53; 2). Investigate if the presence of wild-type p53 and ERα are both required for estrogen-induced transcription from the BRCA-1 5′ regulatory region to occur; and 3.) To investigate and characterize the putative responsive element(s) in the BRCA-1 promoter which interact with p53 and the estrogen receptor to modulate expression of BRCA-1 in the presence of PAHs or estrogen.

CENTRAL HYPOTHESIS

The central hypothesis of this proposal is that regulation of BRCA-1 gene expression by environmental PAHs (B[a]P/BPDE) and/or the steroid hormone estrogen is mediated through a pathway which requires functional estrogen receptor-α and p53 pathways and that this regulation is occurring at specific elements contained within the BRCA-1 promoter region. We predict that 1). The PAH Benzo[a]pyrene and its biotransformation product BPDE will elicit an increase in p53, which in turn will result in decreased BRCA-1 expression in cells containing both wild-type p53 and ER-α. 2). The estrogen receptor-α interacts with wild-type p53 protein in a transcription complex at the 5′ flanking region of the BRCA-1 gene to upregulate gene expression through a non-classical ER-mediated mechanism not involving estrogen response elements (EREs). 3). Increased wild-type, but not mutant p53 protein levels can disrupt ER-α binding at non-ERE sites in the BRCA-1 promoter with a consequent loss regulation of BRCA-1 expression.

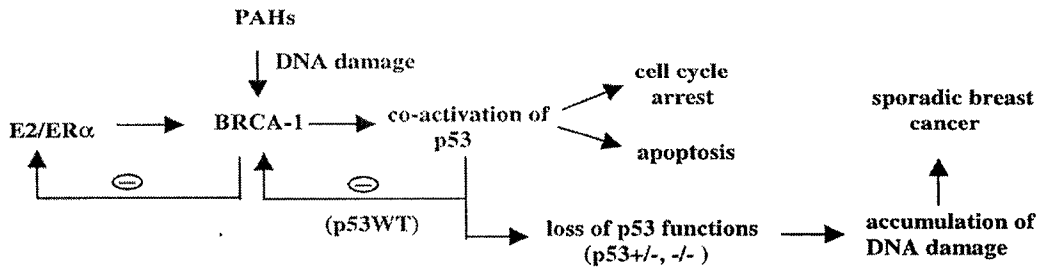


Figure 2. Proposed model: Under normal conditions, a DNA damaging agent (PAH) initially induces BRCA-1 which leads to the stabilization of p53 and decreased estrogen receptor-α function, which via a negative feedback loop, represses BRCA-1. Activation of this pathway may lead to either cell cycle arrest for repair of DNA damage to occur, or apoptosis if the damage is irreparable. In cells expressing mutant p53, this negative feedback loop is not functional and consequently BRCA-1 expression can no longer be modulated by either estrogen or DNA damaging agents. The consequences of loss of BRCA-1 regulation could include normal progression thru the cell cycle in the presence of DNA damage, loss of certain DNA repair capabilities, accumulation of mutations in other genes, and eventual neoplastic transformation.

SPECIFIC AIMS

1. Investigate the requirements for estrogen receptor-α and wild-type p53 protein on transcriptional regulation of BRCA-1 in breast and colon cancer cells exposed to the polycyclic aromatic hydrocarbon DNA damaging agent (Benzo[a]pyrene (B[a]P) or its metabolite BPDE):

2. Investigate the regulation of BRCA-1 promoter activity by estrogen in cells with varying estrogen receptor-α and p53 status.

3. Identify and characterize potential non-estrogen response element (non-ERE) sites within the BRCA-1 promoter which may contribute to the estrogen- and B[a]P/BPDE-mediated regulation of BRCA-1 promoter activity via interaction between ER-α and p53.

Project Relevance and significance to Cancer:

This proposal is significant because to date, no mutations in the BRCA-1 gene have been associated with the etiology of sporadic breast cancer, although lower levels of BRCA-1 message and protein have been found in sporadic breast tumor tissue as compared to non-tumor breast tissue. Based on this fact, we hypothesize that epigenetic factors may

contribute to the lowering of BRCA-1 levels which in turn could be a risk factor in the etiology of sporadic breast cancer. Since sporadic breast cancer accounts for approximately 90-95% of all breast cancer cases, we feel that elucidation of the molecular mechanisms underlying the regulation of BRCA-1 is of extreme significance. This project will examine the contribution of environmental PAHs and the steroid hormone estrogen as epigenetic effectors of BRCA-1 expression and the combinatorial role of the ER-α and p53 pathways to this regulation. Understanding of these molecular mechanisms may contribute to the overall understanding of the etiology of certain types of sporadic breast cancer and could have potential therapeutic implications for women with varying p53 mutational status exposed to PAHs and/or exogenous hormones.

Innovation

This project is *novel* in that it proposes to address the combinatorial requirement for functional p53 and estrogen receptor-α on regulation of BRCA-1 promoter activity in breast cancer cells. To date, no studies have been published which conclusively describe:

1. Whether or not specific mutations in the p53 coding sequence are able to alter the response of the BRCA-1 promoter to DNA damaging agents B[a]P/BPDE.

2. Whether or not estrogen can stimulate transcription from a BRCA-1 promoter construct containing the transcriptional starts sites for exon 1a and 1b, but no consensus estrogen response elements.

3. The identification and characterization of non-ERE elements located within the BRCA-1 promoter which may confer responsiveness to B[a]P/BPDE or estrogen via a transcriptional complex requiring both p53 and ER-α.

Chapter 1: Introduction

The BRCA-1 Gene and Breast Cancer

The BRCA-1 gene. The BRCA-1 tumor suppressor gene, identified in 1994, encodes for a phosphoprotein of 1,863 amino acids (Miki et al., 1994). Mutations in the coding sequence of BRCA-1 predispose individuals to the development of primary breast and ovarian carcinomas (Futreal et al., 1994). Although the function of BRCA-1 is still under investigation, several lines of evidence indicate that the BRCA-1 protein acts as a transcription factor localized to the nucleus, which participates in homologous recombination and transcription-coupled repair of oxidative damage (Gowen et al., 1998; Scully et al., 2000; Karran et al., 2000). Association of the BRCA-1 protein with the RNA polymerase II holoenzyme, which is necessary for transcription from DNA, suggests that BRCA-1 participates in the regulation of transcription (Gowen et al, 1998). In mitotic and meiotic cells, BRCA-1 interacts with the DNA repair protein, rad51 (Sharan et al., 1997; Scully et al., 1997). The rad51 protein is involved in repairing double-stranded breaks and recombination-linked repair, offering one paradigm that BRCA-1 may be involved in maintaining the integrity of the genome (Kinzler and Vogelstein, 1997; Andres et al., 1998). Normal expression of BRCA-1 is associated with cell growth retardation and tumor inhibition (Holt et al., 1996; Somasundaram et al., 1997), whereas depressed expression of BRCA-1 contributes to accelerated growth (Thompson et al., 1995; Rao et al., 1996; Larson et al., 1997). The BRCA-1 protein is expressed in a cell-cycle dependent manner (Chen et al., 1996) and peaks at the G1/S

boundary (Rajan et al., 1996). One mechanism by which BRCA-1 may induce cell cycle arrest is through interactions with a variety of molecules including the tumor suppressors p53, p21, and Rb (Fig. 3). Regulation of phosphorylation of BRCA-1 during the cell cycle or in response to DNA damage may also contribute to the function of BRCA-1 (Thomas et al., 1997). The structural organization of the BRCA-1 promoter region underlies its complex regulation and may provide insight to its mechanism of tissue- and cell-specific regulation of expression, as well as why BRCA-1 mutations have been found to be specifically associated with breast and ovarian cancers. Investigations of the 5′ regulatory region of BRCA-1 have shown that this region contains two distinct transcriptional start sites (exon 1a and exon 1b) although translation always begins from exon 2 (Xu, 1995). Although exons 1a and 1b are usually co-transcribed, exon 1a is predominantly expressed in mammary tissue, suggesting tissue-specific regulation of this promoter (Fig. 4). Interestingly, although BRCA-1 message and protein levels are increased in breast and ovarian cancer cells exposed to estrogen, no functional estrogen response element (ERE) consensus sequences have been identified in exon 1a (Marks, 1997, Romagnolo, 1998). As with many other genes which interact with BRCA-1 such as estrogen receptor-α and p53, BRCA-1 recruits the transcriptional coactivators CBP/p300 for enhancement of its transcription (Pao, 2000). While mutations in BRCA-1 display high penetrance and confer a high probability that women carrying the mutated BRCA-1 will develop breast cancer, familial cases represent only 5-10% of the breast cancers. This contrasts with a large percentage of breast tumors that contain mutated p53 (Hill and Sommer, 2002).

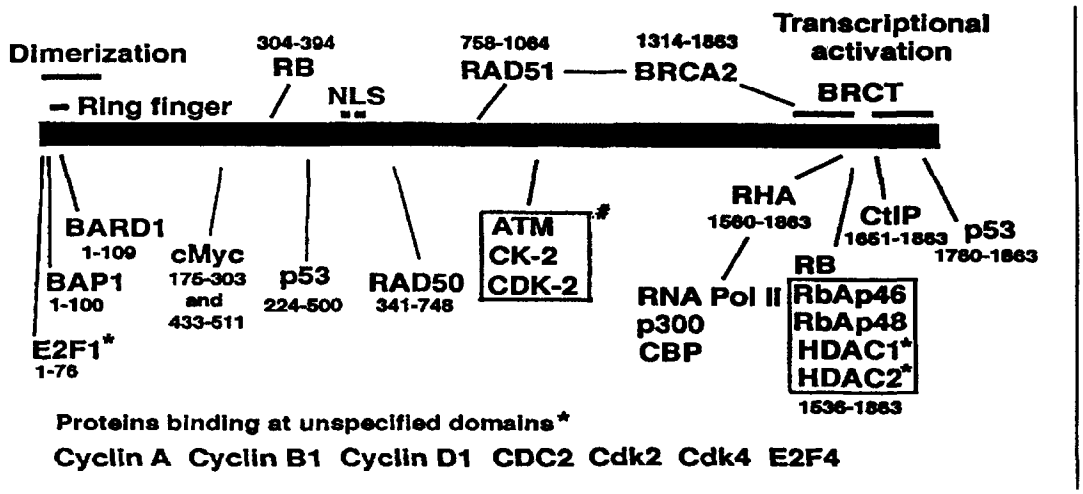


Figure 3. The BRCA-1 protein can interact with a variety of partners including cell cycle proteins, DNA repair proteins, transcription factors, and transcriptional co-factors (From Deng and Brodie, 2000).

On the other hand, sporadic breast cancers express lower levels of BRCA-1 than those observed in normal tissue (Thompson et al., 1995) suggesting that regulatory factors other than mutations in the BRCA-1 gene may contribute to loss of BRCA-1-mediated functions (Rebbeck, 2002). In addition to its contribution to DNA repair (Paull et al., 2001), BRCA-1 may exert mammary-specific functions in response to stimulation by estrogen. This concept is supported by evidence that BRCA-1 represses ER-α-mediated transcriptional activity, whereas BRCA-1 increases p53 protein levels and transcriptional activity (Somasundaram et al., 1999). In turn, p53 may modulate BRCA-1 through a feedback loop (Ouchi et al., 1998; MacLachlan et al., 2000; Arizti et al., 2000; Jeffy et al., 2002a, 2002b). Loss of BRCA-1 in cells containing wild-type p53 has been demonstrated to inhibit the activation of genes involved in DNA repair and cell cycle progression, but not apoptosis, suggesting that that BRCA-1 and p53 may co-modulate certain DNA repair functions (MacLachlan, 2002).

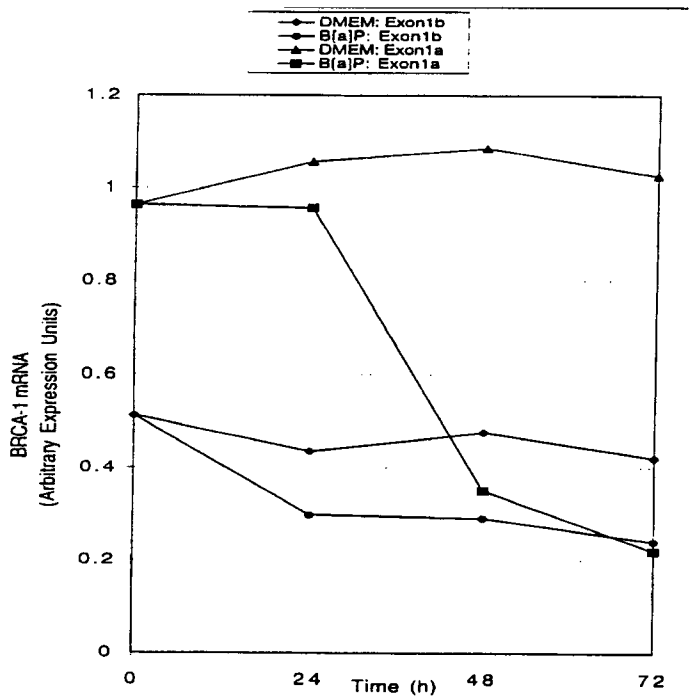


Figure 4. BRCA-1 mRNA levels are decreased in MCF-7 (ER-α+/p53wt) breast cancer cells treated with B[a]P. Transcription in this cell line is higher from exon 1a than exon 1b, and B[a]P treatment causes a more significant reduction in mRNA levels from exon 1a than 1b.

p53, DNA Damage, and Breast Cancer

Polycyclic Aromatic Hydrocarbons as risk factors in breast carcinogenesis. Cigarette smoke, diet, and environmental pollution vehicle a complex mixture of compounds including polycyclic aromatic hydrocarbons (PAH), aromatic amines, and nitrosamines, all of which after metabolic activation induce DNA damage and consequently, p53 levels (Dipple et al., 1999; Szeliga et al., 1997; Gorleska-Roberts, et al., 2002). Of the many substances present in tobacco smoke and grilled meats, B[a]P is considered a prototype PAH, and classic DNA damaging agent and complete carcinogen (Russo et al., 2002; Hecht, 2002). B[a]P is a ubiquitous pollutant found in amounts of 10 ng per cigarette contributing about 200 ng/d for a pack-a-day smoker (Scherer et al., 2000). Food ingestion is also a significant source of exposure to B[a]P. The daily dietary intake of B[a]P has been estimated to range from 120 to 2800 ng/d (Hattemer-Frey et al., 1991) with average values approximating 600 ng/d (Scherer et al., 2000). Catabolism of B[a]P can generate reactive diol-epoxides, which have been shown to form stable DNA adducts at mutational hotspots in the p53, HPRT, and Harvey-ras genes, disrupt transcription, and the binding affinity of Sp1 and E2F transcription heterodimers to DNA (Denissenko et al., 1996; Butler et al., 1997; MacLeod et al., 1995, Wei et al, 1993). In a wide variety of cell types, B[a]P enters the cell and binds with high affinity to the aromatic hydrocarbon receptor (AhR). The ligand-activated AhR translocates to the cell nucleus where it can then interact with xenobiotic responsive elements in the promoter regions of responsive genes to alter transcription. Via this mechanism, B[a]P/AhR can upregulate expression of cytochrome p450 1A1 (CYP1A1) which in turn will lead to the biotransformation of

B[a]P to the highly mutagenic diol-epoxide 7r,8t-dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (Ruddon, 1995) (Figure 5). BPDE has been shown to preferentially form DNA covalent (+) trans adducts at the N^2 position in guanine which may cause the DNA polymerase to insert an adenine across from it during replication (Denissenko, 1996). In the subsequent round of replication, a thymine is inserted across from the A with the end result being a G→T transversion mutation becoming fixed. Defective repair of B[a]P:DNA adducts has been related to occurrence of transversion mutations in the p53 tumor suppressor gene and the incidence of various types of cancer including lung cancer, head and neck cancers, and esophageal carcinomas (Denissenko et al., 1996).

While conflicting epidemiological evidence exists as to whether or not cigarette smoking or exposure to environmental PAHs may increase the risk of developing sporadic breast cancer, ample molecular data have been collected to demonstrate that: 1. Suspected mammary mutagens such as PhIP and IQ have been isolated from human breast milk obtained from lactating women, which suggests that these compounds can be systemically delivered to the mammary gland (Thompson, 2002), 2. Statistically significant levels of PAH-DNA adducts are found in breast tumors as compared to benign breast tissue samples (Rundle, 2002, Li, 1996), and 3. A higher prevalence of p53 mutations have been found in breast tumors from smokers vs non-smokers (36.5% vs 23.6%, adjusted OR=2.11) and the tumor tissue from smokers contains higher levels of PAH signature mutations (G:C→T:A) than the non-tumor tissue (Conway, 2002).

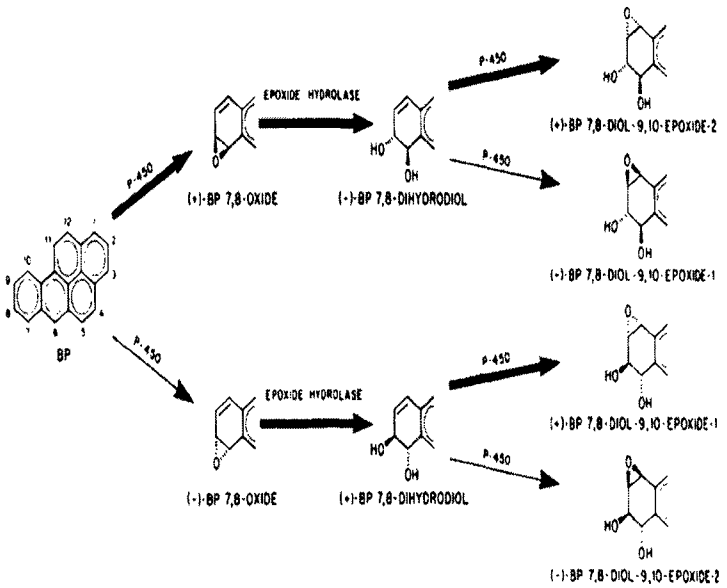


Figure 5. Biotransformation of B[a]P to BPDE (From Ruddon, 1995)

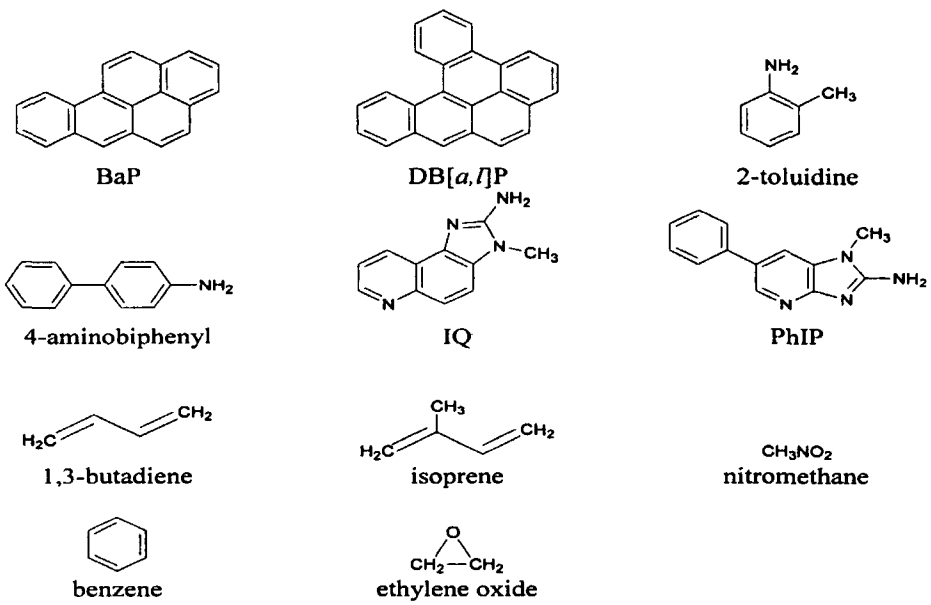


Figure 6. Structures of some environmental carcinogens which have been demonstrated to induce mammary tumors in animal studies (Hecht, 2002).

The p53 gene, environmental carcinogenesis, and breast cancer. The p53 gene, known as 'The guardian of the genome' is one of the most widely studied human tumor suppressor genes. Mutations in the coding sequence of p53 have been identified in approximately 60% of all human cancers (Denissenko, 1996) and 30% of breast cancers (Olivier, 2001) with the majority of mutations being missense point mutations located in the DNA binding domain of the protein (Sigal, 2000) (Figure 8).

The human p53 gene, localized to chromosome 17p13 (Olivier, 2001), contains 10 exons which encode for a protein of 53 kDa (391 amino acid residues) with residues 102-292 comprising the DNA binding domain in which the majority of mutations have been found to occur (Cho, 1994) (Figure 7). Many p53 mutations act in a dominant-negative fashion, in which expression of mutant p53 can inhibit function of wild-type p53 present in the same cell, and this dominant-negative effect can be target-gene specific (Monti, 2002). p53 protein has been shown to have a diverse range of functions including acting as a transcription factor, activating genes involved in cell cycle arrest in response to DNA damage, apoptosis, proliferation, and DNA synthesis (Greenblatt, 1994, Olivier, 2001). The vast majority of p53 mutations in the DNA binding domain are associated with both loss of wild-type and gain of oncogenic functions (Sigal, 2000, Ruddon, 1995). A large number of published studies have concluded that cells containing wild-type 53 which are subjected to treatment with DNA damaging agents, show an increase in p53 protein levels which in turn can lead to cell cycle arrest so that damage can be repaired or apoptosis, if the damage is too extensive to be repaired.

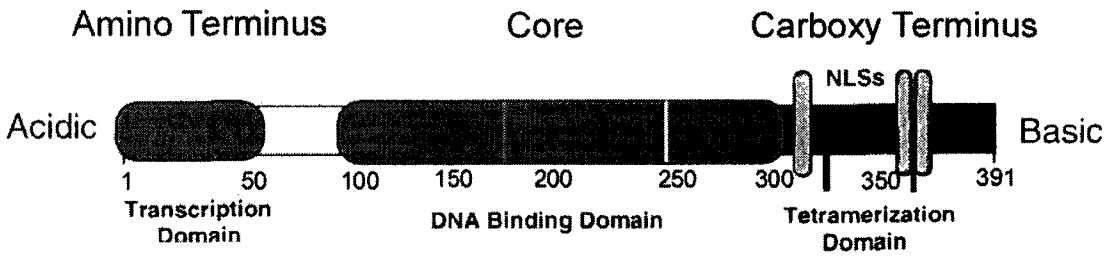


Figure 7: Amino acid structure of the p53 protein

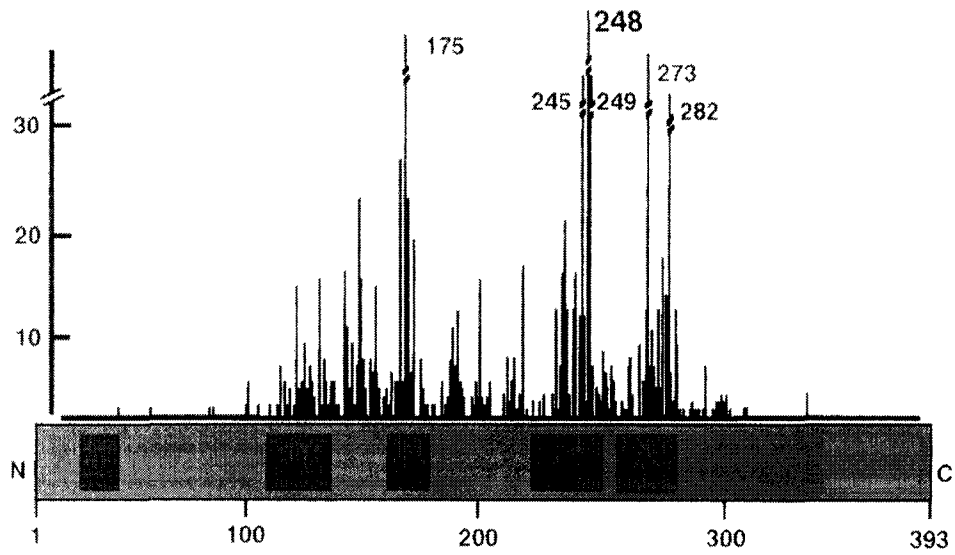


Figure 8: Mutational hotspots in the p53 gene. Amino acid number is shown on the x axis and frequency is shown on y axis.

Loss of p53 function through mutation has been associated with decreased DNA repair, inhibition of apoptosis, and accumulation of mutations in other genes. A wide variety of environmental DNA damaging agents have been shown to induce mutations in the p53 coding sequence, including but not limited to polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene/BPDE (Denissenko, 1996), DMBA (Medina, 2002), chrysene and chrysene derivatives (Smith, 2000) and other environmental carcinogens such as aflatoxin B1 (Olivier, 2001). The types, spectrum, and prevalence of mutations induced by PAHs in the p53 gene have been extensively characterized in numerous publications.

Endogenous factors of susceptibility to PAHs: AhR and ER pathways.

At the cellular level, PAHs activate multiple and overlapping signal transduction pathways, which must be regarded as an integral part of a cellular network (Shackelford et al., 1999). PAHs can induce tumor initiation and promotion through several, likely combinatorial, mechanisms including negative effects on transcription of tumor suppressor genes (Denissenko et al., 1996), transcriptional activation of protooncogenes (Bral et al., 1997), and activation of enzymes that oxidize procarcinogenic PAHs to reactive metabolites (Aust et al., 1981). In mammalian models, responsiveness to PAHs is mediated, among other factors, by the aromatic/aryl hydrocarbon receptor (AhR) pathway. The AhR is a steroid/nuclear type receptor which acts as a ligand-activated transcription factor by binding to xenobiotic responsive elements (XRE=5′-GCGTG-3′) in the promoter regions of responsive genes including cytochrome p4501A1 (CYP1A1) (Krishnan, 1995).

PAHs exert a number of pleiotropic responses including mammary tumors in rodents (Ronai et al.,1994), dermal toxicity, immune reactivity, and disruption of endocrine functions (Krishnan et al., 1995; Hoivik et al., 1997). Many investigations have dealt with cross-talk between the AhR and ER pathways and disruption of estrogen responses by AhR ligands in breast cancer cells (Krishnan et al., 1995; Safe and Krishnan, 1995). The susceptibility to PAHs has been attributed to the concomitant expression of the ER and AhR (Safe and Krishnan, 1995), and transcription factors or receptors that bind to the ER (Dohr et al., 1995; Spink et al., 1998).

Regulation of Endogenous BRCA-1 Expression by B[a]P and BPDE

In detailing the function of BRCA-1 as a tumor suppressor gene, it is imperative to assess the relationships between regulation of cell proliferation and BRCA-1 expression. Growth stimulation of ERα+ MCF-7 breast cancer cells (containing wild-type BRCA-1 and p53) with estrogen correlated with increased expression of BRCA-1 (Gudas et al., 1995; Marks et al., 1997). The antiestrogen ICI-182780 inhibited estrogen-induced cell proliferation and BRCA-1 expression confirming the participation of the ER pathway (Romagnolo et al., 1998).

Similarly, BRCA-1 levels accumulated rapidly over a period of 12 h with higher amounts of B[a]P (5 μM), and declined to near basal levels (24 h) in conjunction with accumulation of high levels of p53 and p21. Experiments with the AhR antagonist α-NF confirmed the involvement of the AhR in the B[a]P-dependent repression of BRCA-1 (Jeffy et al., 2000), and corroborated the proposed inverse relationship between BRCA-1 and p53 (MacLachlan et al., 2000).

Regulation of Gene Transcription by Estrogen and the Estrogen Receptor

The exposure to PAHs has been shown to contribute to DNA damage, cell growth arrest (Khan et al., 1997), accumulation of p53 (Khan et al., 1998), and disrupt the expression of estrogen-inducible genes (Safe et al., 1999). These effects are influenced by the intensity and duration of exposure (Wei et al., 1991). Our published data indicated that BRCA-1 protein levels were increased 2.5-fold over a period of 72 h by non-cytotoxic doses (100 nM) of B[a]P, whereas higher concentrations (0.5 to 1.0 μM) reduced BRCA-1 below basal levels (Jeffy et al., 2000).

Classical vs Non-classical mechanisms of estrogen-mediated gene transcription

The steroid hormone 17-β-estradiol (hereafter referred to as estrogen or E_2) has been shown to enhance transcriptional activity of responsive genes (Nilsson, 2001. In 1962, a steroid/nuclear receptor, the estrogen receptor, was identified as a protein which could selectively bind estrogen and act as a homodimeric ligand activated transcription factor (Jensen, 1962). This estrogen receptor, now referred to as ER-α, originally was shown to act through a classical steroid hormone signaling pathway in which the ligand activated receptor can bind to *cis* elements, termed estrogen response elements (EREs-consensus sequence = 5′-GGTCAnnnTGACC-3′) in promoter regions of certain genes (Driscoll, 1998) including vitellogenin, *c-fos*, *c-jun*, pS2, cathepsin D, and choline acetyltransferase (Hyder, 1999). In 1995, a second variant of the estrogen receptor, ER-β, was identified from rat prostate cDNA library (Nilsson, 2001). Although ER-β has

been shown to have a high degree of homology to ER-α and can bind estrogen and consensus EREs with similar affinity to ER-α, tissue-specific distribution of these receptors has been reported with the ERβ being most highly expressed in the male genitourinary tract (Hyder, 1999, Nilsson, 2001). Both of these ER subtypes have been found to recruit transcriptional cofactors (coactivators/corepressors) (Figure 9a). The precise complement of cofactors recruited to the estrogen receptor depends on a variety of factors including the type of ligand bound to the receptor (estrogen or antiestrogen) (Jakacka, 2001). Both ER-α and ER-β have been found to bind a class of anti-estrogen-like compounds known as selective estrogen receptor modulators (SERMs) such as the ER agonist/antagonist Tamoxifen, the antiestrogen Raloxifene, or the pure antiestrogen ICI 182780 (Jordan, 2001). All of these aforementioned compounds have been determined to competitively bind to the estrogen receptor to inhibit ER-mediated gene transcription (Jordan, 2001). In light of the fact that not all estrogen responsive gene promoters have been found to contain EREs, alternative mechanisms of estrogen-mediated transcription have been investigated. These investigations have suggested that these non-classical mechanisms, which do not require ER binding directly to DNA, may act by binding to other transcription factor complexes including Sp1 (Porter, 1997), NF-κB, and AP-1 family members, including Fra-1 (Jacacka, 2001, Weatherman, 2001, Paech, 1997, Phillips, 1998). Additionally, ER has been found to act through interactions with other transcription factors such as the GATA-1 and GATA-2 family members and CREB (Heyworth, 1999), CREB, the aromatic hydrocarbon receptor

(Wormke, 2000) as well as a variety of co-activator and co-repressor proteins such as CBP/p300 p160/SRC, TRAP/DRIP complexes, RIP140, N-Co-R, SMRT, and L7-SPA (Nilsson, 2001, Kushner, 2000) (Figure 9b). Of particular importance to this dissertation is evidence of interaction between the estrogen receptor and the p53 protein. The estrogen receptor has been shown to physically interact with p53 both *in vivo* and *in vitro* with a consequence of repressing transcriptional activity of the ER (Yu, 1997). This interaction between p53 and ER occurs at multiple sites on the ER-α protein and can interfere with the ER ability to bind to EREs or other proteins in an ER-mediated transcription complex (Liu, 1999).

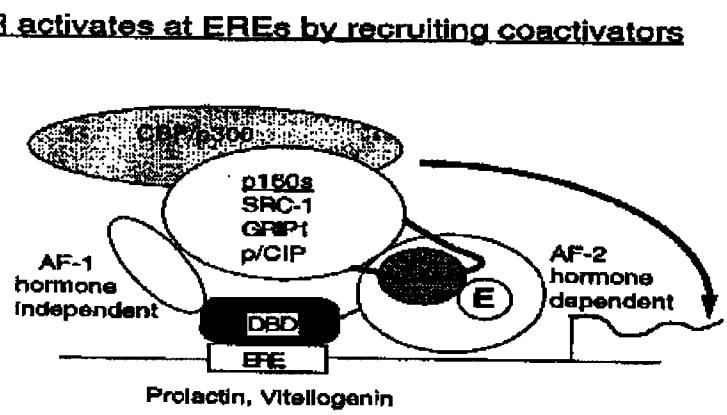


Figure 9(a). Classical model of estrogen-mediated transcription. Estrogen (E) binding to transactivation function AF-2 of the estrogen receptor allows ER binding to an ERE by recruiting co-activators such a CBP/p300 and p160 family members (From Kushner, 2000)

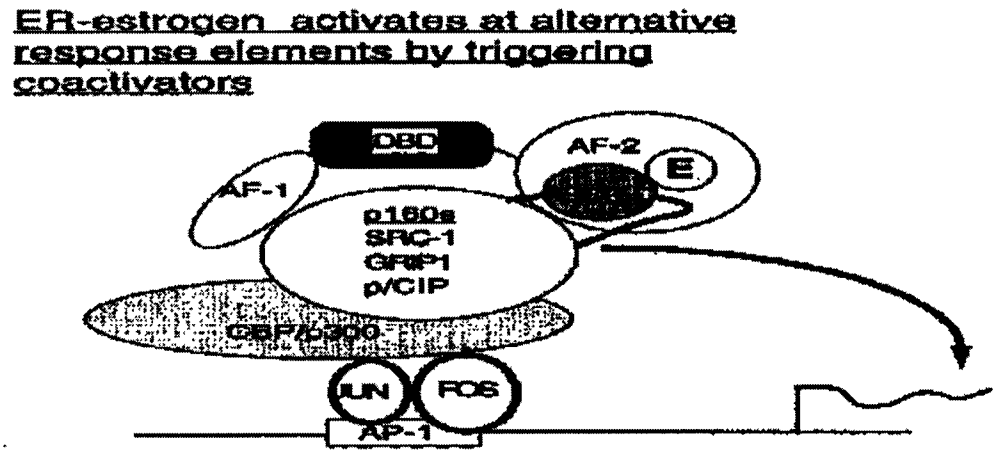
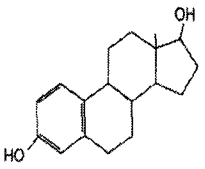


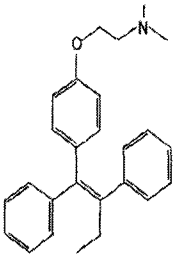
Figure 9 (b). Estrogen receptor α can act indirectly at an AP-1 site by interacting with the same coactivators recruited by Jun and Fos proteins (From Kushner, 2000).

Estrogens, SERMs, and Breast Cancer

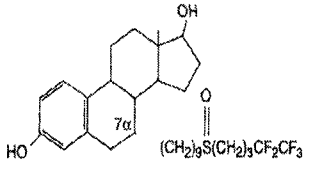
Epidemiological and animal studies have suggested that cumulative lifetime exposure to endogenous estrogens, as determined by early onset of menarche or late menopause , or exogenous estrogens such as oral contraceptives, may be significant risk factors in the etiology of sporadic breast cancer (Spears, 2002). Conversely, selective estrogen receptor modulators such as Tamoxifen and Raloxifene, in addition to certain phytoestrogens (i.e. genistein) have been shown to act as antagonists of the estrogen receptor activity and have been investigated at therapeutic agents in breast cancers which are positive for the estrogen receptor. Both ER-α and ER-β have been found to bind a class of anti-estrogen-like compounds known as selective estrogen receptor modulators (SERMs) such as the ER agonist/antagonist Tamoxifen, the antiestrogen Raloxifene, or the pure antiestrogen ICI 182780 (Jordan, 2001) (Figure 10). Many investigations have been undertaken to elucidate the molecular mechanisms of activation of the estrogen receptor under both ligand-dependent and independent conditions. Interestingly, since BRCA-1 transcription has been shown to be increased by estrogen, and increased BRCA-1 has been associated with an increase in repair of oxidative DNA damage, some which may be induced by estrogen metabolites, it would seem intuitive that estrogen would have a protective effect against breast cancer. A paradigm has arisen recently to explain this apparent disparity: The potential for estrogen to act as a risk factor may be dependent upon the mutational status of the cells exposed to estrogen. In a normal, uninitiated cell, estrogen treatment may have a protective effect by increasing BRCA-1



17-β-estradiol



tamoxifen



ICI 182780 (steroidal antiestrogen)

Figure 12.11. Diagram of estrogen/tamoxifen action in cells. Estrogen (E_2) or tamoxifen (TAM) binds to the hormone-binding domain of the estrogen receptor (ER), which dimerizes through protein:protein interactions. The ER dimer then binds to specific DNA sequences, termed *estrogen-response elements* of estrogen-responsive genes, thereby influencing gene expression. Depending on the ligand bound, either stimulation or inhibition of growth is seen. (From Fuqua, 1994, with permission.)

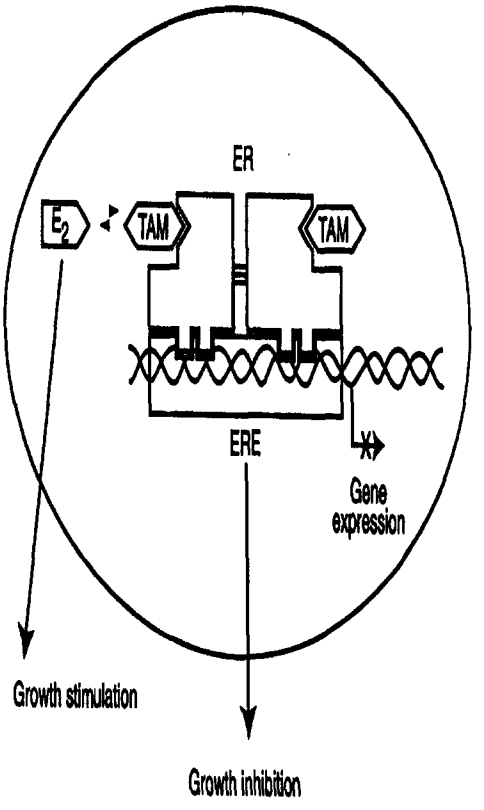


Figure 10 (From Tannock & Hill Basic Science of Oncology, p283-4)

DNA repair activity. In contrast, in mammary epithelial cells which have accumulated mutations or have become malignantly transformed, exposure to estrogen can lead to increased proliferation of these cells and the increase in BRCA-1 levels induced by estrogen may increase cell survival and decrease apoptosis, leading to tumor growth. In fact, as up to 30% of sporadic breast tumors contain mutations in the p53 gene, the mitogenic effects of estrogen without the ability of BRCA-1 levels to be increased, could lead to rapid clonal expansion of these cells.

Regulation of the BRCA-1 gene by Estrogen. While mutations in BRCA-1 display high penetrance and confer a high probability that women carrying the mutated BRCA-1 will develop breast cancer, familial cases represent only 5-10% of the breast cancers. On the other hand, sporadic breast cancers express lower levels of BRCA-1 than those observed in normal tissue (Thompson et al., 1995) suggesting that regulatory factors other than mutations in the BRCA-1 gene may contribute to loss of BRCA-1-mediated functions (Rebbeck, 2002). Although the function of BRCA-1 is still under investigation, several lines of evidence indicate that the BRCA-1 protein acts as a transcription factor localized to the nucleus, which participates in homologous recombination and transcription-coupled repair of oxidative damage (Gowen et al., 1998; Scully et al., 2000; Karran et al., 2000). Normal expression of BRCA-1 is associated with cell growth retardation and tumor inhibition (Holt et al., 1996; Somasundaram et al., 1997), whereas depressed expression of BRCA-1 contributes to accelerated growth (Thompson et al., 1995; Rao et

al., 1996; Larson et al., 1997). The BRCA-1 protein is expressed in a cell-cycle dependent manner (Chen et al., 1996) and peaks at the G1/S boundary (Rajan et al., 1996). Although one study has suggested that BRCA-1 induction by estrogen is indirect and is linked to the mitogenic effects of estrogen, the conclusions from this study rest on the facts that *de novo* protein synthesis is required for BRCA-1 upregulation by estrogen and that there is no estrogen receptor binding to putative BRCA-1 EREs. Since publication of this study in 1997, new mechanisms of estrogen-mediated transcription have been elucidated, including ER acting through non-ERE sites and that *de novo* synthesis of transcription factors and co-factors may be required, both of which suggest that further investigation of BRCA-1 induction by estrogen are necessary. Other studies have offered contradictory data which indicate that reduced levels of BRCA-1 correspond with enhanced estrogen-mediated cell proliferation and decreased apoptosis (Annab, 2000). Although BRCA-1 message and protein levels are increased in breast and ovarian cancer cells exposed to estrogen, no estrogen response element consensus sequences have been identified in the BRCA-1 promoter region or exon 1a (Romagnolo, 1998). Instead, however, studies have shown that constitutive regulation of BRCA-1 promoter activity may be, in part, modulated by transcription factors, including CREB and ATF-1 (Thakur, 1999, Atlas, 2001). BRCA-1 has also has been shown to recruit the transcriptional coactivators CBP/p300, which also can interact with ER-α, for enhancement of its transcription which may in turn lead to inhibition of estrogen receptor activity (Pao, 2000). This fact is extremely significant to understanding breast cancer etiology and progression because this interaction between BRCA-1 and the

estrogen receptor leading to loss of cellular responses to estrogen can have many therapeutic implications, especially in the use of SERMs, whose activity is dependent on that of the ER. Additionally, alteration of ER activity due to epigenetic disregulation of BRCA-1 expression can lead to increased survival and decreased apoptosis of cells harboring DNA damage. This concept is supported by evidence that BRCA-1 represses ER-α-mediated transcriptional activity through direct physical interactions as well as through the transcriptional coactivator p300 (Somasundaram et al., 1999, Fan 1999, Fan 2002, Fan 2001, Zheng 2001).

ER activation by transcriptional cofactors and phosphorylation

The estrogen receptor has been found to recruit transcriptional coactivators and corepressors for the enhancement or repression of transcription when activated by binding of various ligands or phosphorylation through 2nd messenger pathways. The precise complement of cofactors recruited to the estrogen receptor can vary greatly and depends on a variety of factors including the type of ligand bound to the receptor (estrogen or SERM) and which particular transcription factors it is acting through (Jakacka, 2001). Investigations have suggested that these non-classical mechanisms, which do not require ER binding directly to DNA, may act by binding of the estrogen receptor to other transcription factor complexes including Sp1 and AP-1 family members, including Fra-1, Jun, and Fos family members and CREB family members (Porter, 1997, Jacacka, 2001, Weatherman, 2001, Paech, 1997, Phillips, 1998, Heyworth, 1999). Activation of a transcriptional complex interacting with the estrogen receptor at a non-ERE by E2 can occur through various mechanisms. In the promoter region of the cyclin

D1 gene, estrogen receptor has been found to interact with c-*jun* and ATF-2 at a CRE site, as well as with Sp1 at an Sp1 binding site in the same region to induce expression by estrogen (Castro-Rivera, 2001). A similar mechanism has been found for estrogen activation of the bcl-2 promoter by ER interaction with both Sp1 and an ATF-1/CREB-1 complex (Dong, 1999). In addition to activation of these complexes by ligand binding to the ER, phosphorylation of individual transcription factors through 2nd messenger pathways has also been shown to play a significant role in estrogen induction. Phosphorylation of CREB through an ER-mediated pathway involving the MAP kinase ERK, has been shown to be necessary for its activation by estrogen at CREB binding sites (Wade, 2003). Additional studies have demonstrated that activation of the jun-N-terminal kinase (JNK) signal transduction pathway may have an involvement in tamoxifen-induced apoptosis (Mandlekar, 2000). While agonists of the ER bind to the DNA binding domain of the protein, the result of this receptor-ligand interaction can be that the AF-2 domain at the C-terminal region of the ER protein may become exposed due to a conformational change of the receptor and transcriptional co-activators are recruited, leading to the acetylation of the surrounding histones. The AF-2 domain of the ER contains three LXXLL motifs (NR-boxes) which can bind co-activators such as members of the p160/SRC family (SRC-1, SRC-2/GRIP1, SRC-3) upon agonist binding (Nilsson, 2001). In addition to interacting with the estrogen receptor, the p160/SRC family of co-activator proteins also has been shown to bind to CBP/p300 and CARM1,

which respectively possess acetyltransferase and methyltransferase activity which may assist in chromatin/histone remodeling with the result being increased transcription (Nilsson, 2001). Additional reports have found a family of transcriptional co-activators (TRAPs/DRIPs) which may be acting as a sort of scaffolding protein for tethering the ER to the basal transcription factors which are interacting directly with the DNA (Nilsson, 2001). In contrast, histone deacetylation mediated by the recruitment of co-repressors (N-CoR, SMRT, mSin3a, HDAC2, RIP140) to the ER by receptor antagonists may lead to transcriptional repression of ER-mediated gene expression. Recent investigations have suggested that reduction of co-repressor levels is associated with tamoxifen resistance in ER+ MCF-7 cells (Nilsson, 2001). Although recruitment of co-factors which modify local chromatin structure has been shown to be a major factor in regulating estrogen-mediated transcription of target genes, additional post-translational modifications, primarily phosphorylation, of the ER also are involved in regulation of its activity. Investigations have demonstrated that ER activity can be modulated through various ligand-dependent and –independent signal transduction pathways which lead to phosphorylation of the ER at specific serine and threonine residues. Second messenger pathways can be involved in the activation of ER by phosphorylation, and these include phosphorylation of Ser118 by MAP kinase pathways, Ser167 through the PI3K or Akt pathways, Ser 236 by activation of the PKA pathway, and Thr 311 by the p38 MAPK pathway (Lannigan, 2003, Lee, 2002). Phosphorylation of Thr 311 on the ER by the p38 MAPK pathway has been shown to be involved in transcriptional co-factor recruitment to the ER and ER nuclear translocation, while mutation of this residue has been

associated with inhibition of the mitogenic effects of estrogen (Lee, 2002). Additionally, phosphorylation of the ER upon ligand binding also can occur at Ser104/106 by the CyclinA/CDK2 pathway, which may suggest one mechanism by which estrogen may potentiate some of its mitogenic effects (Lannigan, 2003).

Chapter 2: Materials and Methods

Cell Culture

MCF-7, HBL-100, MDA-MB-231 and ZR75.1 breast cancer, and BG-1 ovarian cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HCT-116 and HCT-116p53ko colon cancer cells were a generous gift from the laboratory of Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Sigma Chemical, St. Louis, MO) containing phenol-red and supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) as previously described (Jeffy, 1999). For experiments involving estrogen treatment, cells were conditioned in phenol red-free DMEM (Sigma Chemical, St. Louis, MO) containing 10% charcoal stripped/dextran treated FBS (Hyclone Laboratories, Logan, UT) for three days prior to induction with estrogen. For experiments, the following chemicals were used:

Actinomycin D: Sigma Chemical, St. Louis, MO

α-naphthoflavone (ANF): Sigma Chemical, St. Louis, MO

Anabasine: Sigma Chemical, St. Louis, MO

Aphidicolin: Calbiochem, La Jolla, CA

Benzo[a]pyrene (B[a]P): Sigma Chemical, St. Louis, MO

Benzo[e]pyrene (B[e]P): Sigma Chemical, St. Louis, MO

7r, 8t-Dihydroxy-9t, 10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE): Midwest Research Institute, Kansas City, MO

Colchicine: Sigma Chemical, St. Louis, MO

17-β-estradiol (Estrogen): Sigma Chemical, St. Louis, MO

3-Methylcholanthrene (3-MC): Sigma Chemical, St. Louis, MO

Nicotine: Sigma Chemical, St. Louis, MO

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): Midwest Research Institute, Kansas City, MO

Measurements of Endogenous BRCA-1 Expression

A. *Semiquantitative RT-PCR*

For measurement of endogenous BRCA-1 transcription, MCF-7 cells were plated at a density of 1 x 10^6 cells/60mm dish (x2). Approximately 24 hours after plating, cells were treated with the various compounds for various lengths of time. At the desired time-point, media was removed and cells were washed 2x with cold DPBS. For RNA extraction, 500µl TRI-reagent (Molecular Research Corporation) was added to each dish, cells were scraped, and replicate lysates (2x) were combined. RNA was then extracted with phenol/chloroform followed by isopropanol precipitation followed by a wash with ice cold 80% ethanol. RNA integrity was assayed by loading 500ng of total RNA onto a 1.5% agarose gel containing EtBr followed by gel electrophoresis and visualization of the 18s and 28s ribosomal RNA subunits. For reverse transcription, 400 ng of total RNA was incubated with random hexamer primers, reverse transcription buffer (Ambion inc.), Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and ribonuclease inhibitor (Life Technologies) for 1 hour at 42 degrees C. Following the reverse transcription reaction, enzymes were heat inactivated for 5 minutes at 95 degrees C. PCR

was performed as described previously and control experiments were performed to ensure that amplification was within the linear range (Jeffy, 1999). For amplification of the internal standard 18s ribosomal RNA (488 bp), we used the competimer module from the QuantumRNA kit (Ambion Inc., Austin, TX). For PCR amplification from cDNA, BRCA-1 oligonucleotides DR1-F (5′-AGCTCGCTGAGACTTCCTGGA-3′) and DR6-R (5′-CAATTCAATGTAGACAGACGT-3′) were used to generate a fragment of 712 bp spanning BRCA-1 exons 1-8. Authenticity to the published BRCA-1 sequence (GenBank accession number U1460) was verified by direct sequencing. Expression levels were visualized by agarose gel electrophoresis using an Alpha Imager (Alpha Innotech, Inc., San Diego, CA) and BRCA-1 levels were corrected for 18s internal standard (BRCA-1/18s). To verify efficacy of PAH treatments, cytochrome p4501A1 (CYP1A1) mRNA levels were measured by RT-PCR using oligonucleotides A1A-F: 5′-TAACATCGTCTTGGACCTCTTTG-3′ and A1A-R: 5′-GTCGATAGCACCATCAGGGGT-3′.

B. Ribonuclease Protection Assay

Total cellular RNA was extracted using a guanidinium thiocyanate procedure (Puissant, 1990). The integrity of the total RNA was confirmed by electrophoretic analysis of ribosomal 28S and 18S subunits. Changes in BRCA-1 mRNA were measured by ribonuclease protection assay as previously described (Romagnolo, 1998) using the Hybspeed kit (Ambion Inc., Austin, TX). Breifly, a DNA fragment of 162 bp encoding

a portion of BRCA-1 exon 15 was amplified using the DF15 (5′-ATGATAGGTGGTACATGCACA-3′) forward and DF14 (5′-CTAGATCTTGCCTTGGCAAGT-3′) reverse oligonucleotides.

The polymerase chain reaction (PCR) fragment, which was previously cloned in the antisense orientation into the pTriplescript vector (Ambion Inc., Austin, TX), was found to be identical to the GenBank BRCA-1 sequence by direct sequencing (DNA Sequencing Facility, Arizona Research Labs, The Univeristy of Arizona). In vitro transcription from this construct (Maxiscript kit, Ambion, Inc., Austin, TX) generated a riboprobe that hybridized with BRCA-1 mRNA to protect a fragment of the expected length following treatment with ribonucleases. As an internal control, we transcribed a riboprobe for human cyclophilin by using the pTRI-cyclophilin template (Ambion, Inc., Austin, TX), which upon digestion with RNase protects a fragment of 103 bp. BRCA-1 mRNA was quantitated by phosporimage analysis (STORM, Molecular Dynamics) in arbitrary units corrected for expression of cyclophilin mRNA (BRCA-1/cyclophilin).

C. Western Blotting

Western blotting was performed as previously described (Gudas, 1995). Protein extracts were prepared by lysing cells in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. Cell extracts wee normalized to protein content and separated by 4-12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, the blots were blocked with Tris-buffered saline containing 10% dried milk and 0.1% Tween-20. BRCA-1 immunoblotting was performed using the BRCA-1 (Ab2), p21 (Ab1), or p53 (Ab2) antibody (Oncogene

Research Products, Cambridge, MA), which was raised against an amino-terminal fusion protein, diluted 1:50 in 2% milk in Tris-buffered saline with Tween-20. The immunoblots were incubated with anti-BRCA-1 or p53 antibodies for 2h at room temperature and then with a goat-anti-mouse horseradish peroxidase secondary antibody (GAM-HRP, Biorad). Normalization of western blots was performed by incubating with an anti-actin-1 antibody (Sigma, St. Louis). The immunocomplexes were visualized using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Generation of pGL3 BRCA-1

Genomic DNA was extracted from ovarian cancer BG-1 cells (American Type Culture Collection) and used for polymerase chain reaction (PCR) amplification of a 1.69 kb fragment of the BRCA-1 5′ flanking region. For amplification of the fragment from genomic DNA, the forward oligonucleotide DRPR-f-KpnI (5′-ATCGGTACCGCATTCTGAACCACAGACTCT-3′) and reverse oligonucleotide LH9-R-BglII (5′-ACTAGATCTACCTCATGACCAGCCGACGTT-3′) were used. The primers were designed with KpnI and BglII linkers, which after restriction digestion with the indicated enzymes, generated KpnI and BglII-compatible cloning sites. The authenticity to the BRCA-1 sequence deposited in GenBank (Accession number HSU37574) was confirmed by direct sequencing of the PCR product, which contained both exon 1A and 1B transcriptional start sites. The digested BRCA-1 promoter fragment was cloned into the pGL3 Basic vector (Promega Corporation, Madison, WI),

which had been previously digested with KpnI and BglII to create ends compatible with the BRCA-1 promoter fragment. Overnight ligation at 14 °C of the digested BRCA-1 promoter fragment with the pGL3 Basic was performed using T4 DNA ligase to generate the pGL3 BRCA-1 promoter-reporter construct (Figure 11).

Transient Transfections

ER+ breast cancer MCF-7 cells (5 x 10^5 cells/well in a 6-well plate) were transiently transfected using Lipofectamine Plus reagent (Invitrogen) with 10μg pGL3 BRCA-1 alone or in combination with 3μg of a dominant-negative plasmid containing specific p53 mutations. 48 hours after transfection, cells were treated with either 5μM B[a]P or 500nM BPDE for 24 hours. To measure promoter activity, luciferase reporter assays were performed using a Turner Designs 20/20 luminometer in conjunction with the Luciferase Assay Kit (Promega). For normalization of transient transfection efficiency, 50ng of a Renilla luciferase reporter vector constitutively driven by the CMV promoter, (pRL-TK, (Promega)) was co-transfected with each plasmid. As a positive control for B[a]P treatment, a plasmid containing four xenobiotic responsive elements in tandem upstream of the CYP1A1 promoter and driving luciferase expression (P1A1-4x-LUC) was transfected into MCF-7 cells receiving the treatment with B[a]P. All transfections, treatments, and luciferase readings were performed in triplicate with the results being expressed as the average of the three readings with standard error shown. Results are expressed as Relative Luciferase Units (RLU) corrected for the Renilla internal standard (luciferase/renilla).

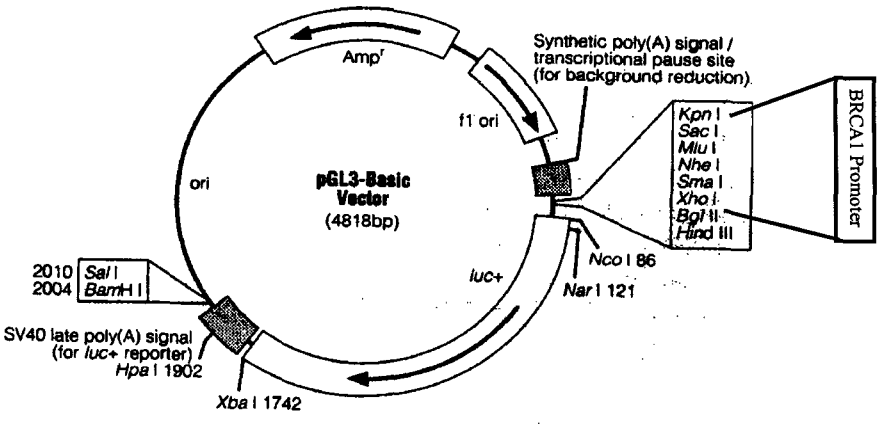


Figure 11. pGL3-BRCA-1 was generated by cloning a 1.7 kb fragment 5' from the BRCA-1 +1start site into the pGL3-Basic vector (Promega)

Transient transfections of 1μg/well (n=3) of a positive control luciferase reporter vector containing three tandemly arranged EREs (p3xERE-generous gift from Steve Safe, Texas A&M University) was used to verify efficacy of estrogen treatment.

MCF-7 cells were transiently transfected as described above with pGL3 BRCA-1 and pRL-TK alone or in combination with 3μg of the following p53 dominant-negative expression plasmids:

a. pCMV-WTp53 (wild-type p53-gift from B. Vogelstein)
b. pCMV-empty (no p53 gene inserted-gift from B. Vogelstein)
c. pCMV-p53mut175 (gift from B. Vogelstein)

These specific p53 mutations were selected for investigation because they have been found to either be hotspots for BPDE adduct formation, are highly associated with sporadic breast cancer, or both. These mutations are dominant-negative acting when expressed with concomitantly in cells containing wild-type p53, and have been shown to differentially regulate certain p53-responsive genes.

To generate these mutations, site-directed mutagenesis was performed according to the QuikChange Site-Directed Mutagenesis Kit (Stratagene) protocol. Briefly, specific forward and reverse oligonucleotides containing the desired mutations surrounded by the native flanking base pairs were ordered (Sigma-Genosys). PCR was performed using the pCMV-WTp53 vector as template with the mutation-containing oligonucleotides used as primers and amplification will be carried out with the *Pfu*Turbo DNA polymerase.

Amplified PCR product was then digested with the DpnI restriction enzyme which only digests the methylated parental strands leaving only the plasmid containing the introduced mutations. The plasmids carrying the desired mutations were used to transform *E. coli*, strain DH5α (Invitrogen). Bacterial cultures were grown under standard conditions and plasmids were harvested using Qiagen plasmid purification kits. Mutations were verified by direct sequencing (Arizona Research Laboratories, DNA Sequencing Facility, University of Arizona), and transient transfections and luciferase assays were performed as described previously. pGL3-BRCA-1 (10μg/well) was co-transfected with 50ng/well of the Renilla expression vector pRL-TK into HCT-116 and HCT-116-p53KO (generous gifts from B. Vogelstein) cancer cells and luciferase activity was measured as described above. p1A1-4x-LUC was transfected independently (n=3 wells) as a positive control for B[a]P or TCDD treatment efficacy as described above. ER-α+/p53 wild-type MCF-7 and ER+-α/p53mutant ZR75.1(aa 152 Pro→Leu) breast cancer cells were cultured in charcoal-stripped/dextran treated DMEM + 10% charcoal-stripped fetal bovine serum for three days for the purpose of depleting exogenous hormones and upregulating the estrogen receptor. On the third day in charcoal-stripped media/serum, cells were plated at a density of 5 x 10^5 cells per well in a 6-well plate and transient transfections of pGL3 BRCA-1 were performed as described previously. 48 hours after transfections, cells were induced with either fresh charcoal-stripped media/serum or charcoal-stripped media/serum containing 10nM estrogen. Cells were

harvested for luciferase assay at 12, 24, 48, and 72 hours after induction. Luciferase activity was measured as previously described. As a control for estrogen efficacy, 1 μg per well (n=3) of a vector containing three EREs arranged in tandem upstream of a luciferase reporter gene (p3xERE) was co-transfected and received the same treatments as above

DNA Binding/Electrophoretic Mobility Shift Assays

Double stranded oligonucleotides containing the core sequences and flanking regions for the potential sites of ER-α action were generated for DNA binding/EMSA analysis:

NC #1-F: 5′-TCGTATTCTGAGAGGCTGCTG-3′
NC #1-R: 5′-CAGCAGCCTCTCAGAATACGA-3′

NC #2-F: 5′-AGCTCGCTGAGACTTCCTGG-3′
NC #2-R: 5′-CCAGGAAGTCTCAGCGAGCT-3′

NC #3-F: 5′-AACCTGAGAGGCGTAAGGCGTT-3′
NC #3-R: 5′-AACGCCTTACGCCTCTCAGGTT-3′

BRCA-1 ERU-F: 5′-GGGTAGGGGCGGAACCTGAGAGGCGTAAGGCGTT-3,
BRCA-1 ERU-R: 5′-AACGCCTTACGCCTCTCAGGTTCCGCCCCTACCC-3′

5 picomoles of each of these complementary oligonucleotides were annealed to each other to create double-stranded DNA for binding and supershift analysis. Briefly, each set of double stranded oligonucleotides was 5′ end labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (Stratagene). Nuclear extracts were prepared according to the protocol of Wilson, et al. 1997. Briefly, 5 x 10^5 cells per well in a 6-well plate were

plated (x 12 wells = 6 x 10^6 cells total per treatment group). Cells were cultured in charcoal- stripped/dextran treated DMEM (Sigma) + 10% charcoal stripped fetal bovine serum (Hyclone) for 3 days before plating and remained in charcoal-stripped media/serum for 24 hours after plating. After 24 hours, the media was removed and replaced with charcoal-stripped media/serum containing either no treatment, 5μM B[a]P, 10nM E_2, or 5μM B[a]P + 10nM E_2. After 24 hours, cells were washed with cold PBS, followed by harvesting with trypsin. Cells were then pelleted and resuspended in 3 mL ice-cold HED buffer (25mM HEPES, 1.5mM EDTA, 1mM DTT) and placed on ice for 10 minutes. Cells were pelleted at 3000 x g for 10 minutes and resuspended in 1 mL of cold HEGD buffer (25mM HEPES, 1.5mM EDTA, 10% glycerol, 1mM DTT). Resuspended cells were homogenized using a teflon pestle drill apparatus and homogenate was pelleted at 3000 x g for 10 minutes. The pellet containing the nuclear proteins was then resuspended in HEGDK+ (HEGD buffer + 0.5 M KCl) with a volume approximately equal to the size of the pellet. The homogenate then was centrifuged for 1 hour at 4 °C at 26,000 x g, and the resultant supernatant containing the nuclear proteins was transferred to a new tube for quantitation with a BCA assay. Nuclear extracts were stored in aliquots at –80 °C. DNA binding and gel shift analysis was performed using the Gel Shift Assay Systems Kit (Promega). In brief, 10μg of nuclear extracts were incubated with binding buffer (containing poly dI-dT), water, and 3μg of desired antibody for 2 hours at 4 °C. Next, previously labeled double-stranded oligonucleotides were added to the reaction and incubated for 20 minutes at room temperature. Samples were then loaded onto a 6% polyacrylamide gel (37.5:1 bis:acrylamide, 40%) and were

run at 200 V for 1.5-2 hours. Gels were dried on a gel dryer and analyzed by phosphor screen imaging on a STORM phosphorimager (Molecular Dynamics). Appropriate controls, such as a lane containing labeled oligonucleotide with no nuclear extracts (free oligo) and a sample incubated with 100X cold oligonucleotide were included. A typical gel loading scheme is listed below:

Lane 1. Free oligo
Lane 2. DMEM nuclear extract + labeled oligo
Lane 3. Treatment nuclear extract + labeled oligo
Lane 4. DMEM nuclear extract + labeled oligo + 3μg non-specific antibody
Lane 5. Treatment nuclear extract + labeled oligo + 3μg non-specific antibody
Lane 6. DMEM nuclear extract + labeled oligo + 3μg specific antibody
Lane 7. Treatment nuclear extract + labeled oligo + 3μg specific antibody
Lane 8. DMEM nuclear extract + 100X unlabeled oligo + labeled oligo
Lane 9. Treatment nuclear extract + 100X unlabeled oligo + labeled oligo

Antibodies tested in supershift analysis:

We investigated whether or not any of the antibodies listed below can cause a clearing or supershift of the previously identified DNA-protein complexes. Based on the degree of homology of the sites contained in our oligonucleotides to consensus transcripton factor binding sequences identified by TRANSFAC and the fact that these proteins are all known to interact with the estrogen receptor-α at non-EREs, we tested the following antibodies:

1. Estrogen Receptor Alpha: MA1-310 (Affinity Bioreagents)
2. Estrogen Receptor Beta: sc-6820 (Santa Cruz)
3. p53: Ab-6 OP43 (Oncogene Research Products)

4. c-Jun: sc-45 (Santa Cruz)
5. JunB: sc-46 (Santa Cruz)
6. JunD: sc-74 (Santa Cruz)
7. c-Fos: sc-52 (Santa Cruz)
8. FosB: sc-48 (Santa Cruz)
9. Fra-1: sc-183 (Santa Cruz)
10. Fra-2: sc-171 (Santa Cruz)
11. ATF-1: sc-270 (Santa Cruz)
12. ATF-2: sc-187 (Santa Cruz)
13. GATA-1: sc-265 (Santa Cruz)
14. GATA-3: sc-268 (Santa Cruz)
15. CBP: sc-7300 (Santa Cruz)
16. CREB-1: sc-240 (Santa Cruz)
17. Nrf-2: sc-722 (Santa Cruz)
18. AhR: MA1-513 (Affinity Bioreagents)
19. Normal mouse IgG Ab was used as a control for non-specific shifting/clearing

We selected these particular antibodies (company and catalog numbers) based on the fact that they have been shown to effectively shift or clear the protein of interest in a variety of published studies.

Flow Cytometry

Flow cytometry was performed as previously described (Andres, 1998). Briefly, cells were harvested with trypsin and washed 3x with ice cold PBS. The cells were then treated with RNase and stained in Krishnan's Buffer containing 50μg/mL propidium iodide in PBS. Cell cycle distribution profiles were recorded with a FACscan (Becton-Dickinson, Franklin Lakes, NJ) using a CELLQuest program. Data were analyzed with the MODFIT.2 software at the Flow Cytometry Core Facility at the Arizona Cancer Center.

Chapter 3: Environmental Factors: Regulation of BRCA-1 Expression by the Aryl Hydrocarbon Receptor (AhR) Ligands B[a]P/BPDE and TCDD

Dose-Dependent Effects of B[a]P on Cell Viability and BRCA-1

Previous reports from our laboratory have shown that treatment of ER-α+ MCF-7 breast and BG-1 ovarian cancer cells with B[a]P reduces BRCA-1 mRNA levels (Jeffy, 1999). For this report, we wanted to further characterize this phenomenon by first examining the effects of B[a]P on MCF-7 cell viability and BRCA-1 protein levels. Our data suggest a strong correlation between cell number and levels of BRCA-1 protein (Fig. 12A, B) in cells treated with varying concentrations of B[a]P. MCF-7 cells treated with 0.05 to 0.5 μM B[a]P proliferated at the same rate as control cells, even though BRCA-1 protein levels were reduced by three-fold at concentrations of 0.5 μM B[a]P.

This observation indicates that non-cytotoxic concentrations of B[a]P might abrogate the expression of BRCA-1 in circumstances of chronic exposure. However, acute doses of 1 and 5 μM B[a]P respectively reduced cell viability 3.5- and 10-fold after 72 hours (Fig 12A). BRCA-1 protein was reduced six-fold in the presence of 1 μM B[a]P, whereas at 5 μM B[a]P, BRCA-1 immunocomplexes were virtually undetectable. These data lend support to our previous findings that non-lethal doses of B[a]P might reduce BRCA-1 protein, whereas at concentrations higher than 0.5 μM, the loss of BRCA-1 is paralleled by decreased cell viability. The finding that 20% to 30% of cells did not succumb to treatment with cytotoxic levels of B[a]P, but contained little or no BRCA-1, triggered our interest in examining the dynamic changes in BRCA-1 and cell cycle kinetics in this sub-cell population.

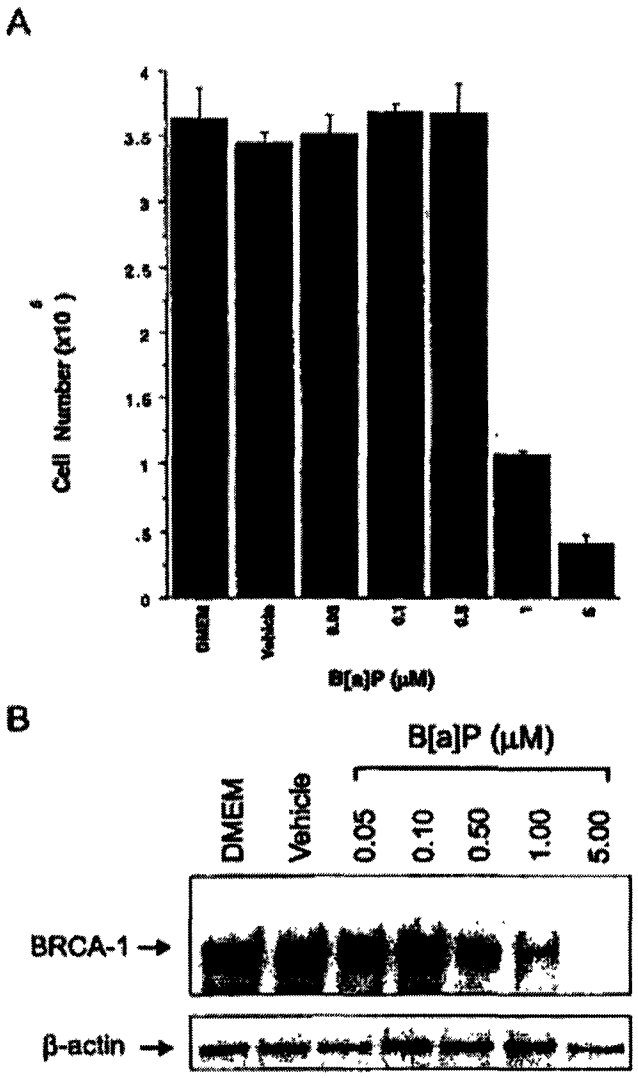


Figure 12: Effects of B[a]P on MCF-7 cell viability (A) and BRCA-1 protein levels (B).

We performed Western blot analysis of cell extracts obtained from asynchronous MCF-7 cells cultured in control medium or medium supplemented with 5 μM B[a]P for various periods of time (Figure 13). In both control and B[a]P-treated cells, BRCA-1 protein levels peaked at twelve hours. This induction was attributed to stimulated expression of BRCA-1, which is characteristic of rapidly proliferating cells. Whereas BRCA-1 protein levels returned to basal levels in control cells by 24 hours, the presence of B[a]P drastically reduced BRCA-1 levels at both 48 and 72 hours. Treatment with 5 μM B[a]P for 12 hours induced a significant increase in p53, which was further augmented at 24 hours, and remained significantly higher than the amount detected in control cells. The accumulation of p53 in B[a]P treated cells was accompanied by an increase in the levels of the cyclin-dependent kinase inhibitor p21 between 12 and 24 hours. In contrast, there were no detectable changes in the cellular content of p27 in either the presence or absence of B[a]P. Cellular levels of the p53 regulatory protein, mdm2, remained unchanged in throughout the experiment in MCF-7 cells cultured in DMEM/F12, whereas mdm2 accumulated between 12 and 24 hours after treatment with B[a]P. Consistent with the existence of a p53-BRCA-1 feedback loop, when comparing the temporal profiles of expression of BRCA-1 and p53, the accumulation of p53 at 12 hours in B[a]P-treated cells preceded the reduction in expression of BRCA-1 by approximately 12 hours. At 48 and 72 hours after treatment of MCF-7 cells with 5 μM B[a]P, BRCA-1 protein was nearly undetectable whereas cellular levels of p53 and p21 remained high (Figure 13).

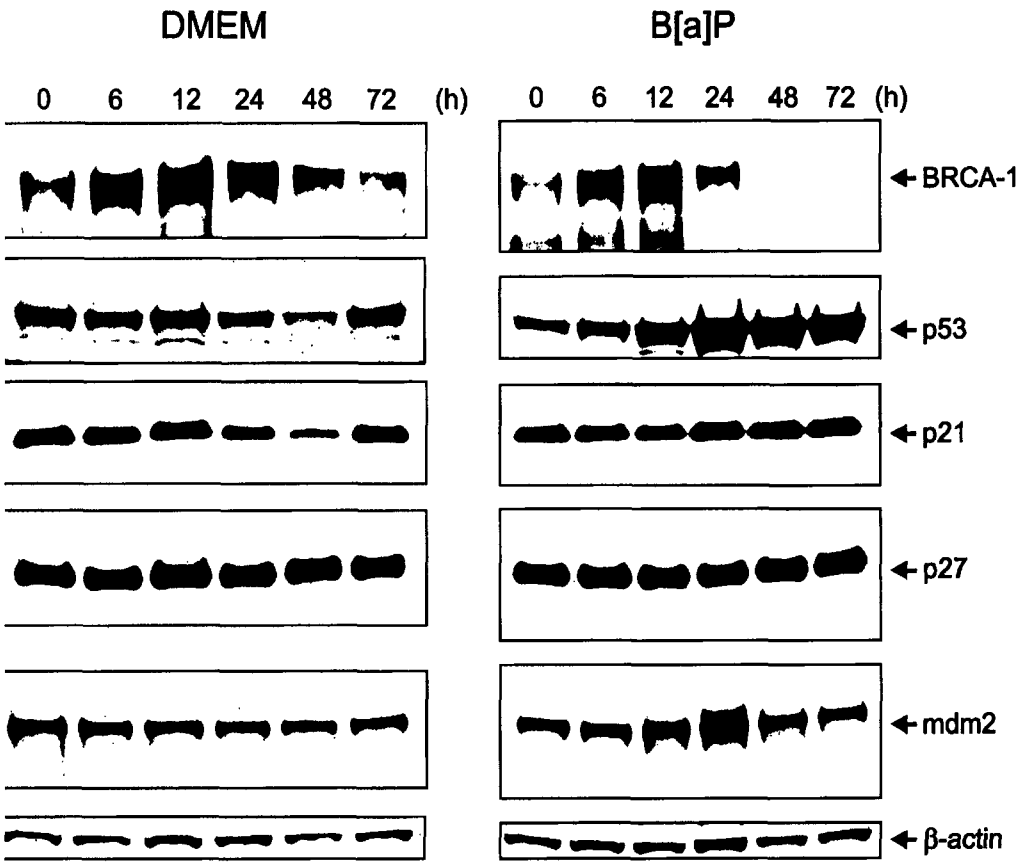


Figure 13: Western blotting was performed using lysates from MCF-7 cells treated with DMEM (A) or B[a]P (B) for various amounts of time.

B[a]P Alters Cell Cycle Kinetics

The concomitant loss of BRCA-1, along with the changes in p53 and p21 proteins, prompted us to examine the effects of B[a]P on cell cycle progression. Cell cycle distribution was measured by flow cytometric analysis of propidium iodide stained asynchronous MCF-7 cells either untreated or treated with 5 μM B[a]P. Treatment of asynchronous MCF-7 cells with B[a]P, when 55% of cells were in G0/G1, induced within 24 hours a significant increase in the number cells in S-phase of the cell cycle as compared to control cells (53.1% vs. 32.0%) (Figure 14). The accumulation in S-phase was paralleled by a reduction in the fraction of cells in G0/G1 (36.3% vs. 54.0%) and a 2.5- and 4.0-fold increase in the percentage of cells positioned in G2/M at 48 and 72 hours respectively (Data not shown). Thus, loss of BRCA-1 and stabilization of p53 and p21 correlated with pausing of cells in S-phase and subsequent arrest in G2/M. Because levels of BRCA-1 normally peak in S-phase, we next wished to characterize the effects of B[a]P on levels of BRCA-1 protein and cell cycle progression in MCF-7 cells synchronized in S-phase. After synchronization with 1μg/ml aphidicolin for 24 hours, cells were released from S-phase arrest by replacing the culture medium with fresh DMEM/F12 plus 10% fetal bovine serum or medium supplemented with 5μM B[a]P. The data depicted (Figure 14) showed that at 12 hours post release, the addition of B[a]P induced arrest in S-phase (36.1% vs. 21.2%) and reduced the fraction (27.4%) that progressed to G2/M compared with cells (42%) cultured in basal DMEM/F12 medium. However, while a significant percentage of cells released into control medium transitioned to G0/G1 (72.4%) by 24 hours and assumed the characteristic asynchronous

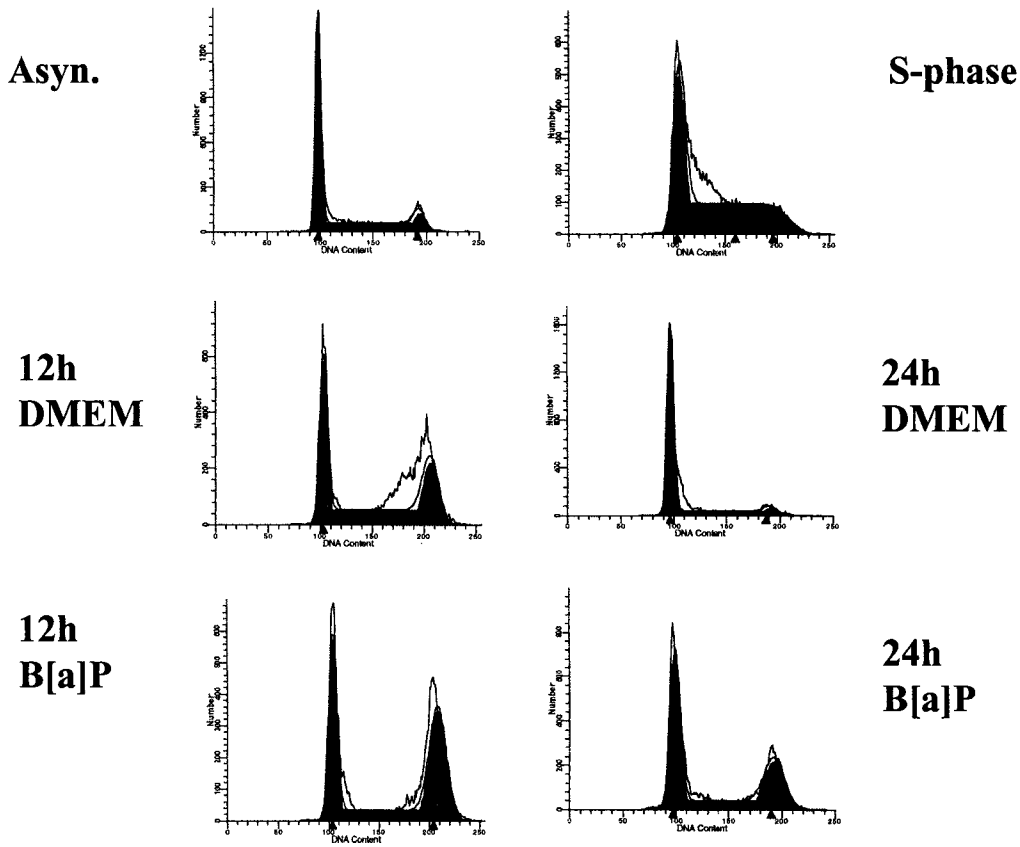


Figure 14: MCF-7 cells were arrested in S-phase using aphidicolin and were subsequently released into media ± 5μM B[a]P for 12 or 24 hours. Cell cycle progression was analyzed by flow cytometry.

distribution (figure 14), the treatment with B[a]P sustained significant accumulation in G2/M (29.4% vs. 5.6%). Overall, B[a]P appeared to alter normal cell cycling by lengthening the transition through S and inducing subsequent arrest in G2/M. This inference was confirmed by a G2/M trapping experiment in which cells previously synchronized in S-phase with aphidicolin were released into media containing colchicine (0.25μM) to prevent cycling beyond G2/M. These experiments illustrated that, upon treatment with B[a]P, cells progressed to G2/M with an approximate 12 hour delay as compared with cells released in control DMEM/F12 (Figure 15). In fact, in the presence of B[a]P plus colchicine, 44.0% of cells were positioned in S-phase at 12 hours compared with only 17.6% when cells were treated with colchicine alone. Nevertheless, flow cytometry profiles confirmed that by 24 hours after release, a significant percentage of cells treated with B[a]P plus colchicine had escaped S-phase arrest and occupied the G2/M window at levels (63.5%) similar to those elicited by colchicine (57.0%). Therefore, although B[a]P induced S-phase arrest, this checkpoint was relaxed since cells resumed cycling to G2/M within a 24-hour interval. The synchronization in S-phase with aphidicolin elicited the accumulation of BRCA-1 protein, as indicated by Western blotting of protein extract (Figure 16). The release from S-phase into basal DMEM was accompanied at 12 hours by a slight reduction in BRCA-1, whose levels increased at 24 hours when 51.5% of the cells were in S-phase. These dynamic changes in BRCA-1 are consistent with the fact that BRCA-1 levels fluctuate during cell cycle progression and that BRCA-1 protein is most abundant during S-phase.

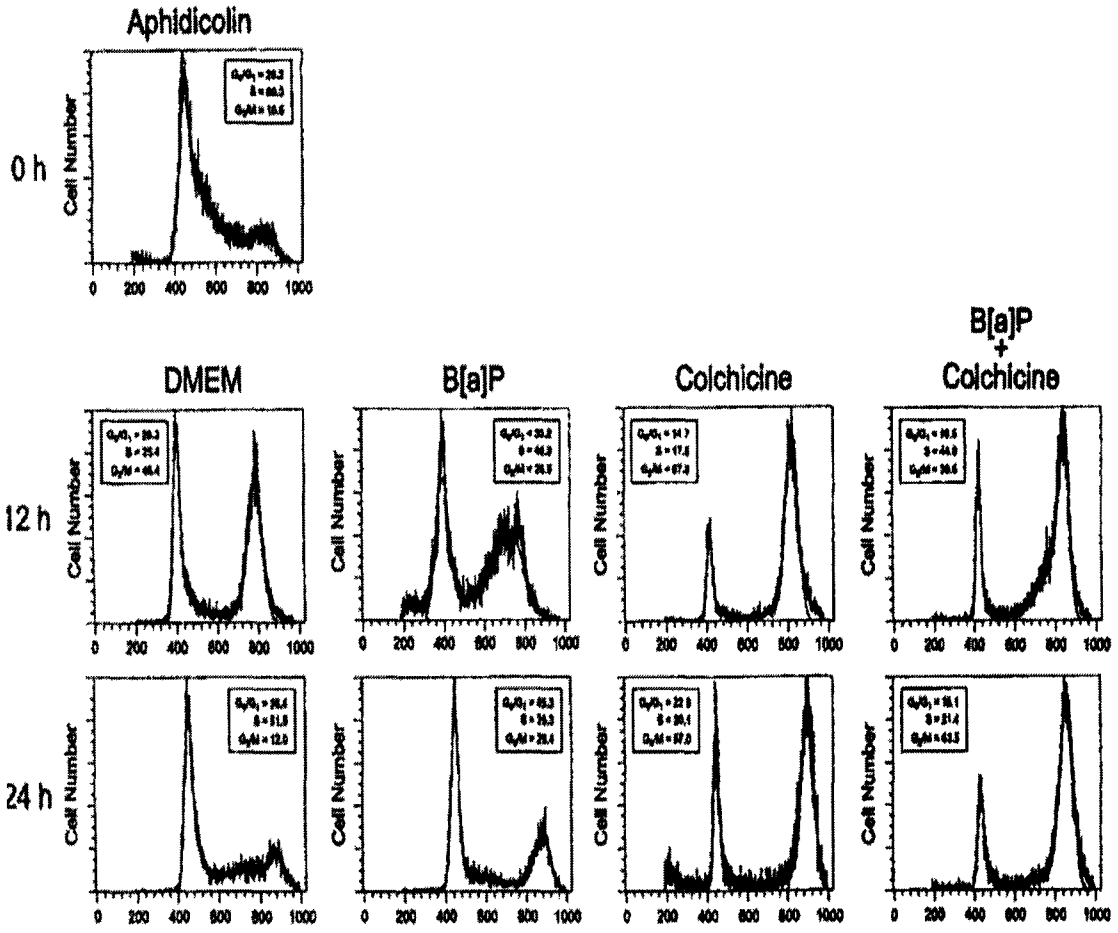


Figure 15: MCF-7 cells were arrested in G2/M with colchicine and were released into media ± B[a]P for 12 or 24 hours and were analyzed by flow cytometry.

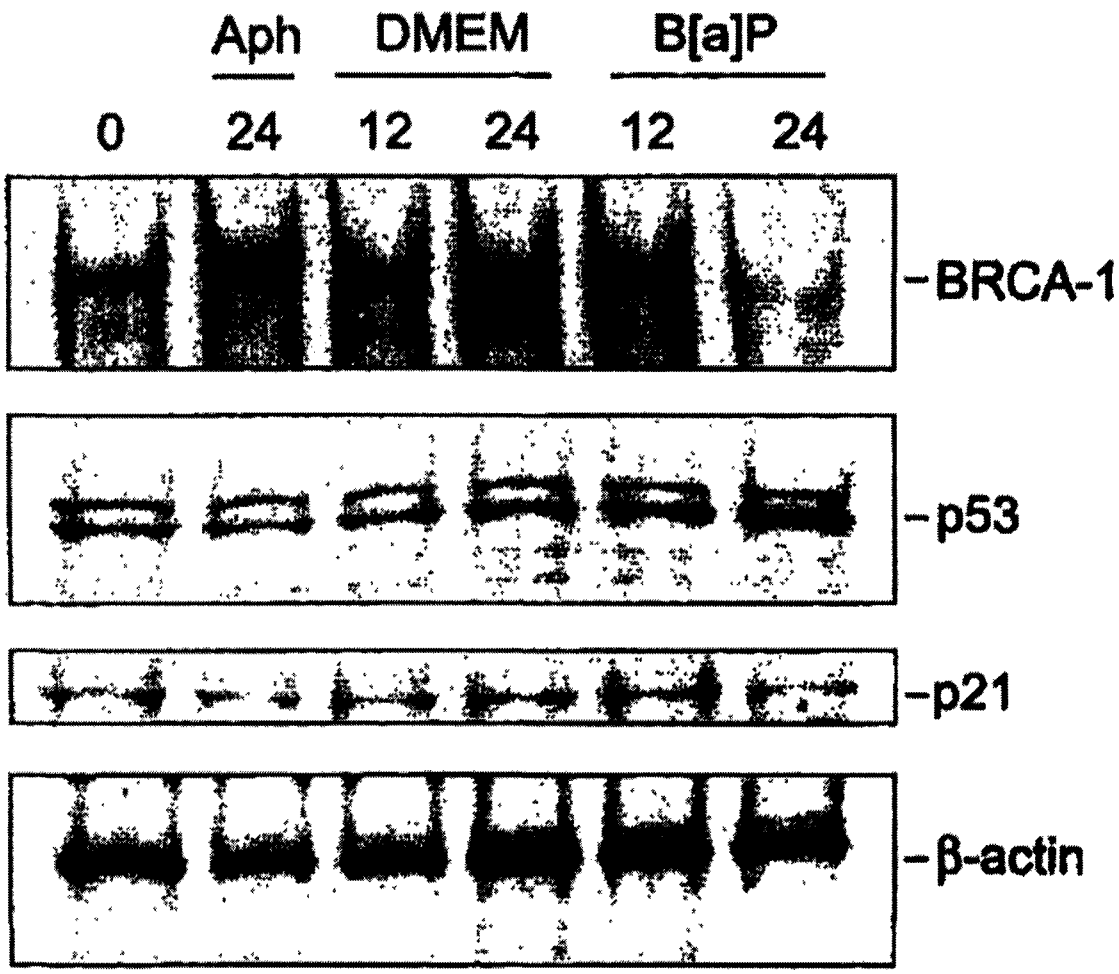


Figure 16: MCF-7 cells were arrested in S-phase of the cell cycle with aphidicolin and subsequently released into media ± B[a]P for 12 or 24 hours. Western blotting for BRCA-1, p53, and p21 was performed using these lysates. β-actin blotting was performed as an internal control.

In contrast, BRCA-1 levels were significantly reduced after treatment with B[a]P for 24 hours, whereas p53 levels increased. Interestingly, the accumulation of p53 did not yield a corresponding increase in p21, whose levels remained unchanged at 12 and 24 hours after release from S-phase. These data indicated that cells containing increased levels of p53 paused transiently in S-phase, but subsequent transition to G2/M occurred with reduced cellular levels of BRCA-1.

α-Naphthoflavone Counteracts the Loss of BRCA-1 Expression and Disruption of Cell Cycle Kinetics

We next wished to obtain direct evidence of the involvement of the Aromatic Hydrocarbon Receptor (AhR) pathway in the disruption of cell cycle kinetics by B[a]P. Therefore, we tested whether or not co-treatment with the AhR antagonist, α-naphthoflavone (ANF), prevented the perturbations in cell cycle kinetics induced by B[a]P, and restored BRCA-1 expression to basal levels. The co-treatment with ANF did indeed abrogate the transient arrest of MCF-7 cells in S-phase and the subsequent accumulation in G2/M, suggesting that the AhR pathway mediated the disruptive effects of B[a]P on cell cycle progression. In addition to restoring normal cell cycle distribution (Figure 17), the co-treatment with ANF counteracted the loss of BRCA-1 protein and prevented the accumulation of p53 induced by B[a]P (Figure 18). Taken together, these data confirmed that the changes in cell cycle kinetics as well as the fluctuations in BRCA-1 and p53 involved the participation of the AhR pathway, can be inhibited by an AhR antagonist. Furthermore, these findings confirm the existence of an inverse relationship between BRCA-1 and p53 status with regard to exposure to B[a]P.

ANF prevents accumulation in S- and G2/M

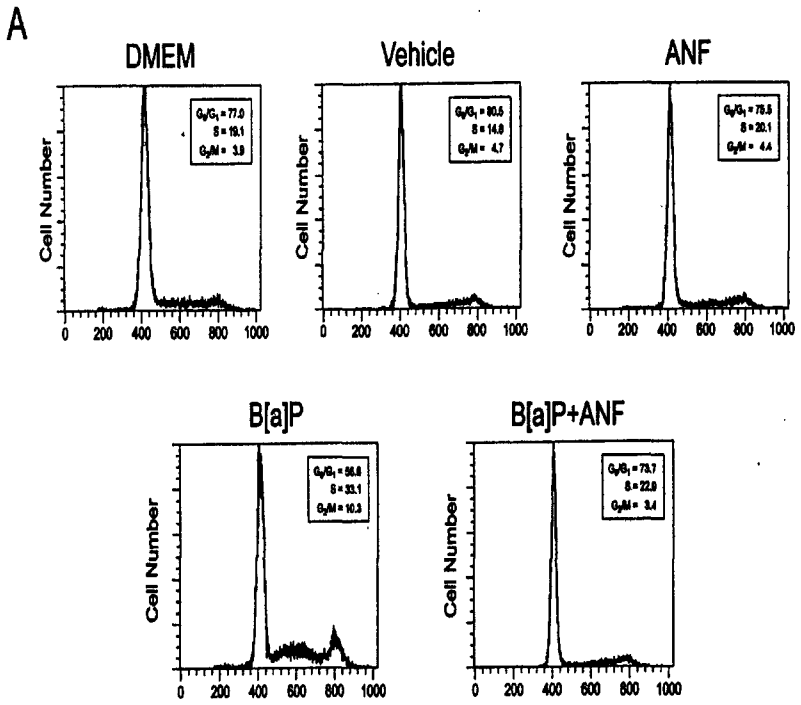


Figure 17: MCF-7 cells treated with B[a]P for 24 hours showed an increased percentage of cells in S-phase as compared to untreated control. Pretreatment with ANF followed by treatment with ANF + B[a]P was able to prevent the S-phase accumulation.

BPDE Induces S-Phase Arrest and Reduces the Potential for BRCA-1 Expression

In previous studies (Jeffy, 1999), we reported that B[a]P, but not 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), reduced the expression of BRCA-1 mRNA in ER-α+ breast and ovarian cancer cells. The fact that TCDD binds with high affinity to the AhR, but is not metabolized to a more reactive compound, led us to consider the possibility that downregulation of BRCA-1 may be mediated, at least in part, by metabolites of B[a]P which are capable of inducing DNA damage. Therefore, we investigated further the effects of the B[a]P biotransformation product, 7*r*,8*t*-Dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), on cell cycle progression and expression of BRCA-1. Flow cytometry analysis of MCF-7 cells documented that, similar to B[a]P, BPDE was able to induce a dose-dependent arrest of cells in S-phase (Figure 19). The fraction of cells positioned in S-phase at 24 hours increased from 28% for the DMEM control to 50% for MCF-7 cells treated with 500 nM BPDE (Figure 19A). The BPDE-dependent accumulation in S-phase was paralleled by a reduction in the percentage of cells positioned in G0/G1 (from 60% to 44%) and G2/M (from 10% to 5%). RT-PCR analysis from total cellular RNA revealed that treatment with increasing amounts of BPDE elicited a dose-dependent loss of BRCA-1 transcripts (Figure 19B), which were nearly undetectable at concentrations equal or greater than 500nM BPDE.

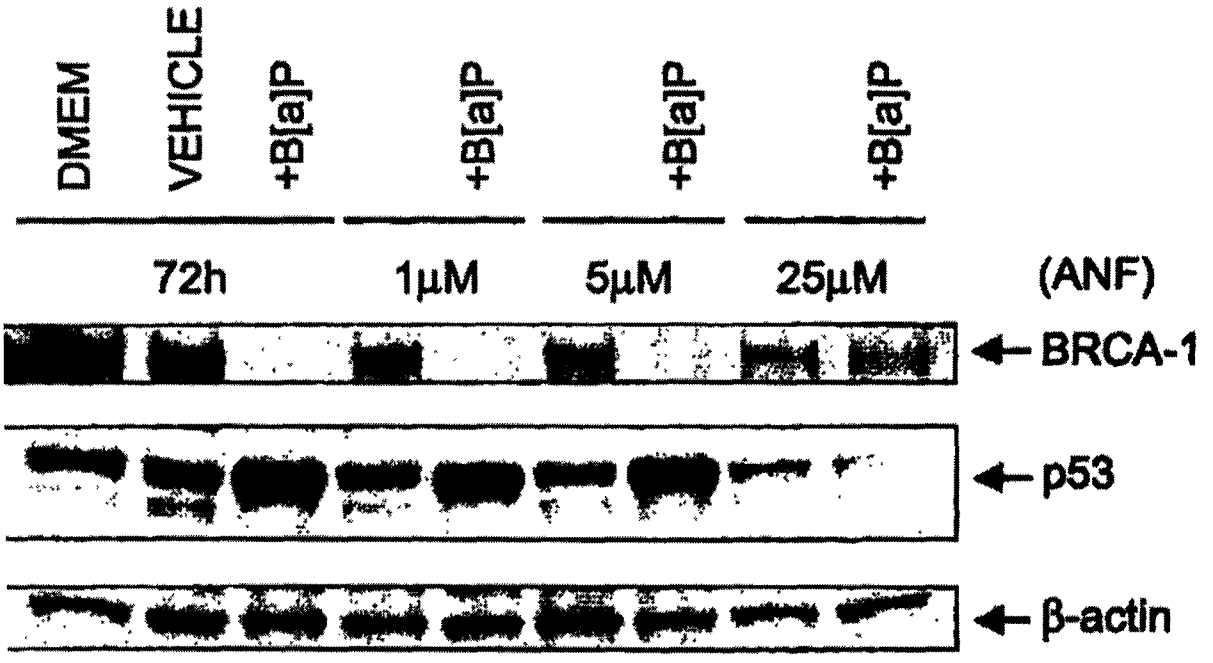


Figure 18: Titration effect of ANF on B[a]P-induced changes in BRCA-1 and p53 protein levels. 25μM ANF is able to prevent the loss of BRCA-1 and accumulation of p53 induced by 5μM B[a]P in MCF-7 cells.

BPDE induces S-phase arrest and reduces the potential for BRCA-1 expression in MCF-7 cells

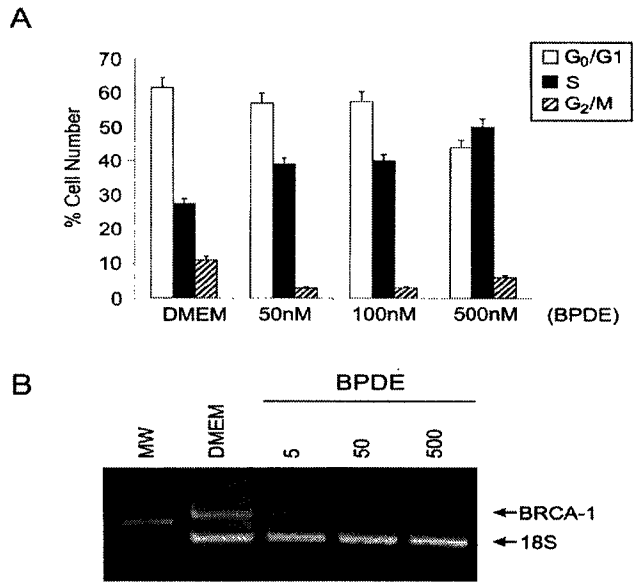


Figure 19: Effects of B[a]P titration on MCF-7 cell cycle distribution (A) and BRCA-1 mRNA levels (B)

The effects of BPDE on BRCA-1 expression were further examined in a washout experiment in which MCF-7 cells were pre-treated with 500nM BPDE for 0.5, 1, 6, and 12 hours. Then, after washing out of the media containing the BPDE, cells were cultured in fresh DMEM/F12 plus 10% fetal bovine serum up to 72 hours. At the end of the incubation period, we analyzed the levels of BRCA-1 mRNA in total RNA and protein in cell lysates. While at 72 hours the levels of BRCA-1 mRNA in control cells were similar to those observed in cells harvested at the time of induction (data not shown), BRCA-1 transcripts were reduced five-fold in cells pre-treated with BPDE for 6 or 12 hours (data not shown). Similarly, BRCA-1 protein levels were reduced significantly by the pre-treatment for 6 to 12 hours with BPDE (data not shown). The loss of BRCA-1 at these time points was paralleled by an accumulation of p53 and p21 proteins. Because the half-life of BPDE in solution is approximately 5 to 20 minutes (MacLeod, 1982), we discounted the likelihood that residual BPDE may have been responsible for the reduction in BRCA-1 mRNA and protein. In fact, cells were washed twice and then cultured in fresh medium for at least 60 hours after removal of BPDE. Based on these considerations, we concluded that the short-term exposure to BPDE exerted a signature effect by reducing the potential for BRCA-1 expression and increasing the cellular levels of p53 and p21 proteins.

Effects of B[a]P, BPDE, and TCDD on BRCA-1 mRNA Expression Profiles

RT-PCR analysis of total RNA from MCF-7 cells revealed that treatment with B[*a*]P and BPDE lowered BRCA-1 mRNA levels (Fig. 20A). These changes were accompanied by up-regulation of the *CYP1A1* gene, which encodes for a member of the P450 family of metabolizing enzymes. The accumulation of CYP1A1 mRNA confirmed the functionality of the AhR pathway in MCF-7 cells, although B[*a*]P was more effective than BPDE in elevating the content of CYP1A1 transcripts. Treatment with B[*a*]P increased the levels of BAX-α mRNA, whereas transcripts for Bcl-2 were reduced, suggesting that B[*a*]P inversely regulated the expression of Bax-α and Bcl-2. However, neither the Bax-α nor the Bcl-2 mRNA level was affected by BPDE (Figure 20A). These distinct expression patterns, which showed both gene induction as well as repression, emphasized the fact that loss of BRCA-1 mRNA in cells treated with B[*a*]P or BPDE did not stem from a general effect on the transcriptional machinery. Expectedly, treatment with BPDE significantly reduced BRCA-1 protein, whereas the cellular p53 and p21 levels were elevated (Fig. 20B, C). In contrast, treatment with 10 nM TCDD did not alter BRCA-1, p53, or p21 protein levels, but elicited the accumulation of CYP1A1 mRNA levels (data not shown).

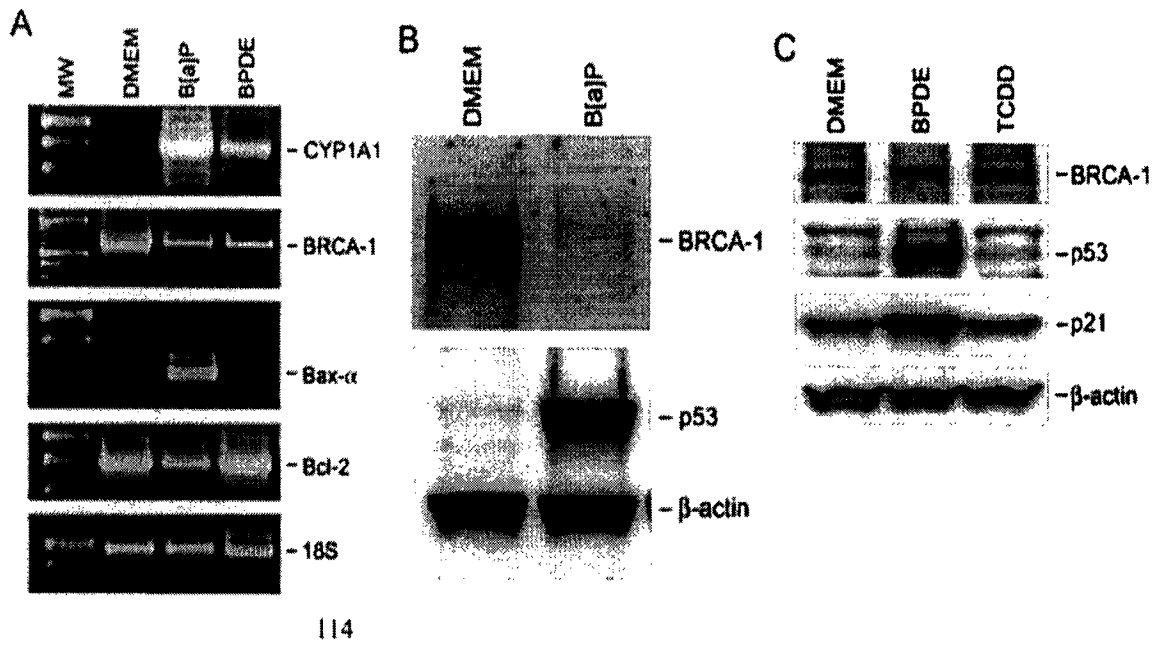


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Figure 20: Effects of B[a]P and BPDE on CYP1A1, BRCA-1, Bax, and Bcl-2 mRNA from MCF-7 cells. 18s is shown as an internal control for RT-PCR (A). B[a]P treatment of MCF-7 cells for 24h results in the loss of BRCA-1 and accumulation of p53 proteins. β-actin is shown as an internal control for Western analysis (B). BPDE but not TCDD induces loss of BRCA-1, and accumulation of p53 and p21 proteins in MCF-7 cells (C).

B[a]P Does Not Compromise BRCA-1 mRNA Stability but Reduces Promoter Activity

The expression data indicated that B[*a*]P and BPDE activated multiple, perhaps overlapping, signal transduction pathways. In this context, we were interested in determining whether inhibition of BRCA-1 expression by B[*a*]P resulted from reduced stability of BRCA-1 mRNA. Data from RNase protection assay (Fig. 21A) experiments revealed that, compared with DMEM, the levels of BRCA-1 mRNA corrected for the cyclophilin mRNA were reduced 3.0-fold by treatment of MCF-7 cells with B[*a*]P.

To examine the effects of B[*a*]P on BRCA-1 mRNA stability, we compared the rate of decay of BRCA-1 transcripts in control and B[*a*]P-treated cells. After MCF-7 were precultured for 24 h in DMEM/F12 containing 10% FCS with or without B[a]P, culture medium was replaced with fresh DMEM containing 5 μg/ml actinomycin D to inhibit the production of new transcripts in the presence or absence of B[*a*]P. Kinetics of disappearance were assessed by calculating at 3, 6, 9, 12, and 18 h the relative levels of BRCA-1 mRNA corrected for the cyclophilin mRNA. The temporal changes depicted in Fig. 21B document that the kinetics of disappearance of the protected BRCA-1 fragment followed a first-order process that was not influenced by B[*a*]P. In fact, the half-life of the BRCA-1 transcript was 12 h in both control and B[*a*]P-treated cells. These results suggested that loss of BRCA-1 expression in cells treated with B[*a*]P was likely not a result of increased degradation of BRCA-1 mRNA and prompted further investigations to assess whether B[*a*]P interfered with regulation of transcription at the BRCA-1 promoter.

B[a]P Does Not Alter BRCA-1 mRNA Stability

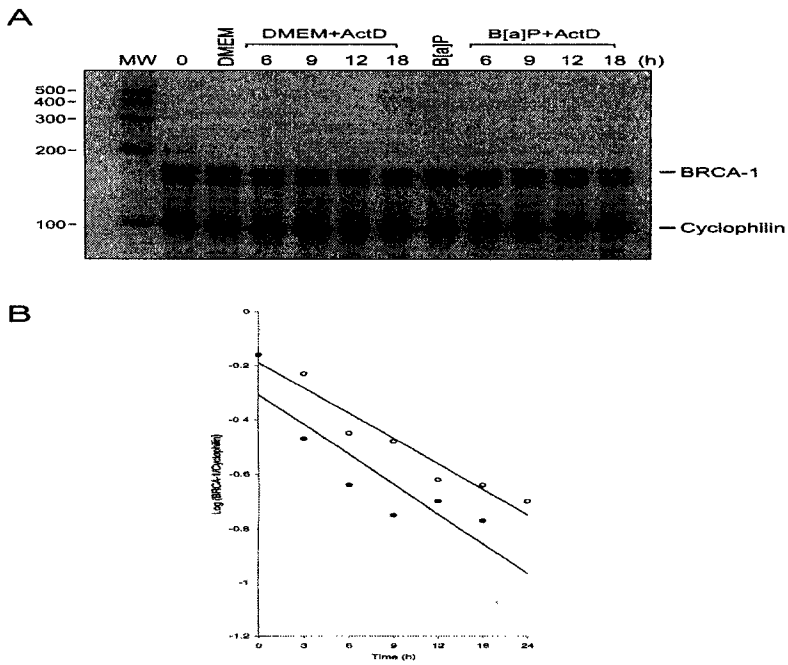


Figure 21: Total RNA was harvested from MCF-7 cells treated with actinomycin D ± 5μM B[a]P for various times. Ribonuclease protection assay results show BRCA-1 and cyclophilin (internal standard) protected fragments (A) and semi-logarithmic rates of decay (expressed as BRCA-1/cyclophilin mRNA) (B).

To investigate this, we obtained genomic DNA from BG-1 ovarian cancer cells and amplified a 1.69kb fragment of the BRCA-1 promoter containing the transcriptional start sites for both exons 1a and 1b. Following amplification, we cloned the BRCA-1 promoter fragment into the pGL3 Basic luciferase-reporter vector (Promega) to generate the BRCA-1 promoter-reporter vector, pGL3 BRCA-1. Fig. 22A diagrams the luciferase activity detected in MCF-7 cells transfected with pGL3-BRCA-1 in the presence or absence of B[*a*]P. Compared with the RLU measured in cells transfected with the empty pGL3Basic vector, luciferase activity increased, although not proportionally, 16.0- and 22.0-fold in cells transfected with 5 or 10 μg of pGL3-BRCA-1. However, after treatment with B[*a*]P for 24 hours, the reporter activity was reduced by 2.2- and 2.0-fold in MCF-7 cells transfected with 5 or 10 μg of the pGL3-BRCA-1 vector, respectively. The RLU detected after transfection with 1 μg of the internal pGL3 Control vector were not influenced by treatment with B[*a*]P and were 10.0-fold higher than those measured in cells transfected with the pGL3Basic lacking a promoter element. In parallel experiments (Fig. 22B), we assessed the dose-dependent effects of B[*a*]P in MCF-7 cells transfected with 10 μg of the pGL3-BRCA-1 vector. A B[*a*]P concentration of 0.5 μM did not influence RLU, whereas doses of 1 and 5 μM B[*a*]P significantly reduced luciferase activity by 1.5- and 1.6-fold, respectively. The reporter activity in control cells transfected with the positive control p1A1-4X-LUC was 4.0-fold higher than that produced by the p1A1-LUC vector lacking the four XREs and was increased an additional 10-fold in the presence of B[*a*]P (Fig. 22C).

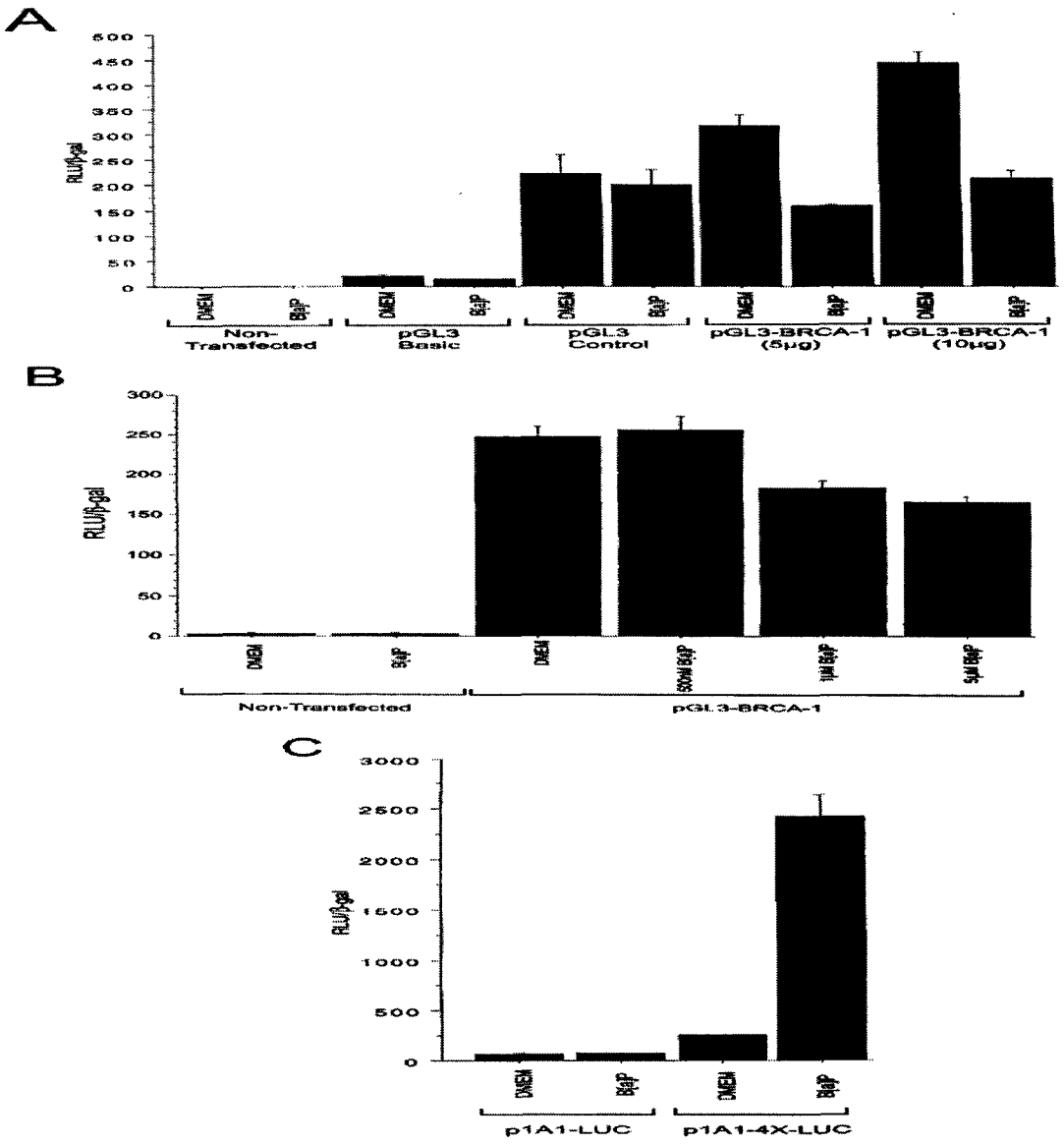


Figure 22. MCF-7 cells were transiently transfected (pGL3 Basic (empty), pGL3 Control (SV40 promoter), and pGL3 BRCA-1) and treated with 5μM B[a]P for 24h (A). MCF-7 cells were transiently transfected with pGL3 BRCA-1 and treated with increasing amounts of B[a]P for 24h (B). Control vectors p1A1-LUC (CYP1A1 promoter lacking XREs) and p1A1-4X-LUC (CYP1A1 promoter with 4 tandemly arranged XREs) were transfected into MCF-7 cells treated with 5mM B[a]P for 24h (C).

BPDE but not TCDD Represses BRCA-1 Promoter Activity

The data shown in Fig. 20C illustrated that, at least at the concentration (10 nM) used in this study, TCDD did not lower BRCA-1 protein levels. It should be pointed out that in previous studies, increasing the concentration of TCDD from 10 nM to 1000 nM affected neither BRCA-1 mRNA nor protein content in MCF-7 cells, although cell viability was reduced by 50 and 80% with 10 and 1000 nM TCDD, respectively. Because the affinity of TCDD for the AhR is 100-fold higher than that of B[*a*]P but TCDD is not metabolized to a more reactive form, we envisioned that activation of the AhR pathway may be necessary but not sufficient for B[*a*]P-mediated repression of BRCA-1 transcription. Rather, we formulated the hypothesis that genotoxic products of B[*a*]P bioactivation, possibly BPDE, contributed to down-regulation of BRCA-1. To test this contention, we compared the effects of BPDE (100 and 500 nM) and TCDD (10, 100, and 500 nM) on BRCA-1 promoter activity in cells transiently transfected with the pGL3-BRCA-1 construct. In previous studies, concentrations up to 1.2 μM BPDE were used to investigate repair of DNA damage. However, we used lower concentrations, ranging from 100 to 500 nM BPDE, which in our hands have been effective in promoting S-phase arrest and loss of BRCA-1 expression in MCF-7 cells. The results shown in Fig 23A indicate that treatment for 24 h with 100 or 500 nM BPDE inhibited by 1.5- and 2.2-fold, respectively, transcription from the BRCA-1 promoter. In contrast, the activity of the BRCA-1 reporter construct was not affected by treatment with TCDD at any of the concentrations tested in this study (Fig. 23B).

BPDE but not TCDD decreases pGL3-BRCA-1 activity in MCF-7 cells (A,B), TCDD & B[a]P induce p1A1-4XRE-Luc positive control (C)

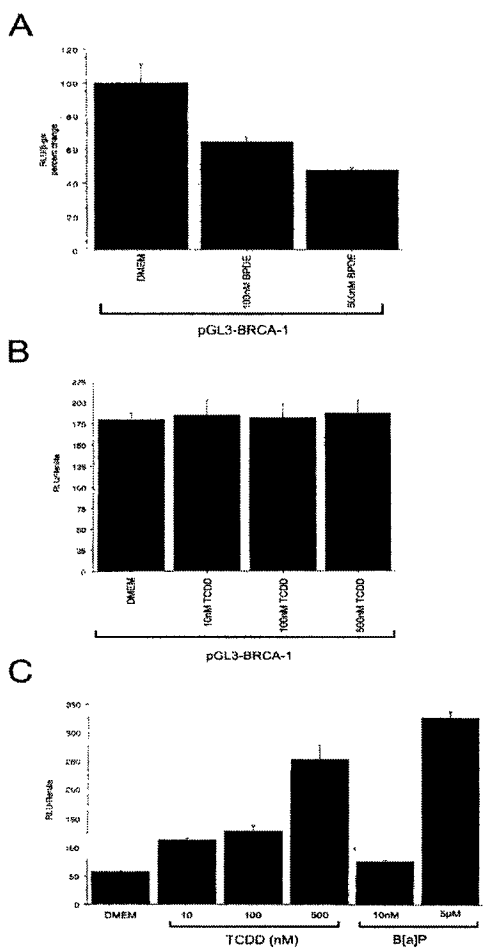


Figure 23. 10μg of pGL3 BRCA1 was transiently transfected into MCF-7 cells which were subsequently treated with increasing amounts of BPDE (A) or TCDD (B) for 24h. 1μg of Positive control vector p1A1-4X-LUC was transiently transfected into MCF-7 cells which were subsequently treated with increasing amounts of TCDD or B[a]P to determine equitoxic doses (C).

TCDD had no effects on BRCA-1 levels: AhR-binding may not be sufficient for repression of BRCA-1

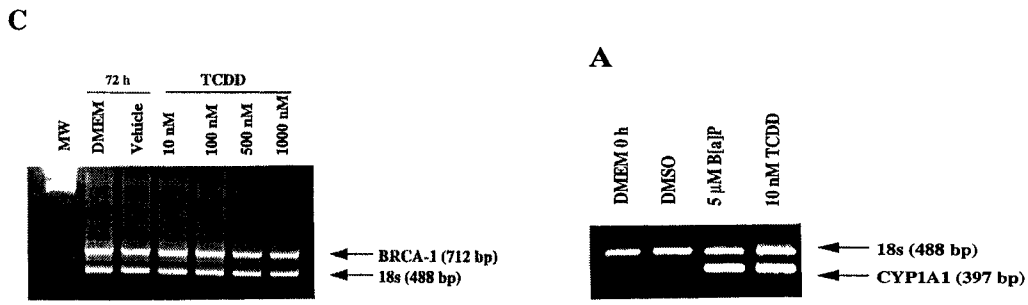


Figure 24: Effects of TCDD titration on BRCA-1 mRNA (RT-PCR) from MCF-7 breast cancer cells (C). RT-PCR Positive control CYP1A1 mRNA from MCF-7 cells treated with either 5µM B[a]P or 10nM TCDD (A).

The efficacy of the TCDD treatment was confirmed by evidence that it stimulated a dose-dependent increase in the reporter activity of the positive control, p1A1-4X-LUC (Fig. 23C). TCDD concentrations of 10 nM stimulated by 2.0-fold the activity of the p1A1-4X-LUC promoter compared with that measured in cells treated with equimolar concentrations of B[*a*]P. This effect was attributed to the higher binding affinity of TCDD for the AhR. A TCDD concentration of 500 nM gave a response comparable to that obtained with 5 μM B[*a*]P (Figure 23C). In addition, 5μM B[a]P and 10nM TCDD were able to induce CYP1A1 mRNA to comparable levels (Figure 24). These cumulative data suggested that the AhR is not involved directly in BRCA-1 repression but that the metabolite BPDE, or factors regulated by BPDE, contribute, at least in part, to B[*a*]P-dependent inhibition of BRCA-1 transcription.

Repression of BRCA-1 Promoter Activity by B[a]P and BPDE Requires Functional p53

On the basis of our published observation that the AhR antagonist αα-naphthoflavone counteracted the S-phase arrest and loss of BRCA-1 expression induced by B[*a*]P while preventing the accumulation of p53, we questioned whether the acquisition of p53 functions in MCF-7 cells treated with B[*a*]P contributed to repression of BRCA-1 promoter activity. To test this hypothesis, we cotransfected MCF-7 cells with a plasmid containing a cassette encoding for p53 mutated at position 175 (Arg to His) under the control of the cytomegalovirus promoter (pCVM53mut) subcloned into pCMV (plasmids were gifts from Dr. Bert Vogelstein, The Johns Hopkins University School of Medicine,

Baltimore, MD and made available by Dr. J. Martinez, The University of Arizona, Tucson, AZ).

The cotransfection of the empty pCMV (data not shown) or pCMV53mut vectors with pGL3-BRCA-1 did not influence BRCA-1 luciferase reporter activity in cells cultured in control medium. In contrast, the concomitant transfection of pGL3-BRCA-1 with pCMV53mut175, which encodes mutant p53, prevented the loss of BRCA-1 promoter activity (2.0-fold) induced by B[*a*]P (Figure 25A). Positive evidence that the pCMV53mut construct expressed p53 was obtained by Western blot analysis (Fig. 25B). In control medium (DMEM), p53 levels were low in nontransfected cells or cells transfected with the empty pCMV vector, whereas p53 increased significantly in the presence of B[*a*]P. Conversely, accumulation of p53 was observed in cells cultured in DMEM after transfection with the pCMV53mut vector. The intensity of the p53 immunocomplex increased further after treatment with B[*a*]P, presumably because of coincident immunodetection of endogenous and recombinantly expressed p53. We next examined the effects of BPDE on activity of the BRCA-1 reporter construct (Fig. 26A). The RLU detected in cells transfected with pGL3-BRCA-1 were reduced 1.8-fold by BPDE. In contrast, cotransfection with pCMV53mut restored luciferase activity to control levels. Similar results were obtained after cotransfection of a vector encoding for the human papilloma virus E6 protein, which prevented the loss of reporter activity elicited by BPDE (data not shown). Treatment with BPDE reduced BRCA-1 protein, whereas p53

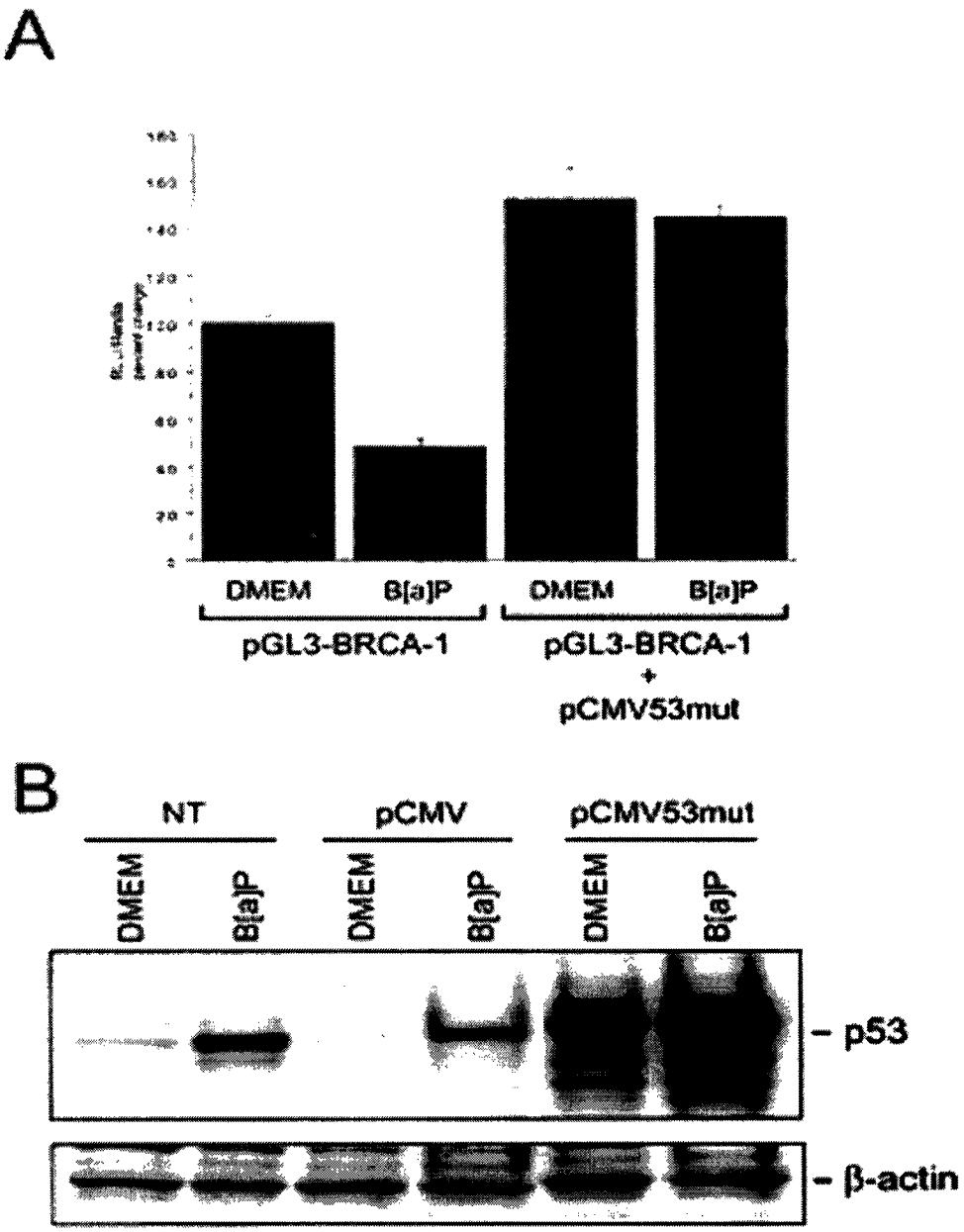


Figure 25: pGL3 BRCA-1 was transiently transfected into MCF-7 cells either alone or along with a dominant-negative p53 vector (pCMV-p53mut175) harboring a mutation in the cDNA corresponding to amino acid 175 (R→H). Transfected cells were either untreated or treated with 5μM B[a]P for 24h (A). MCF-7 cells were either non-transfected (NT), mock-transfected (pCMV) or transfected with pCMV-p53mut175, and treated with B[a]P for 24h. Western blotting for p53 was performed. (B).

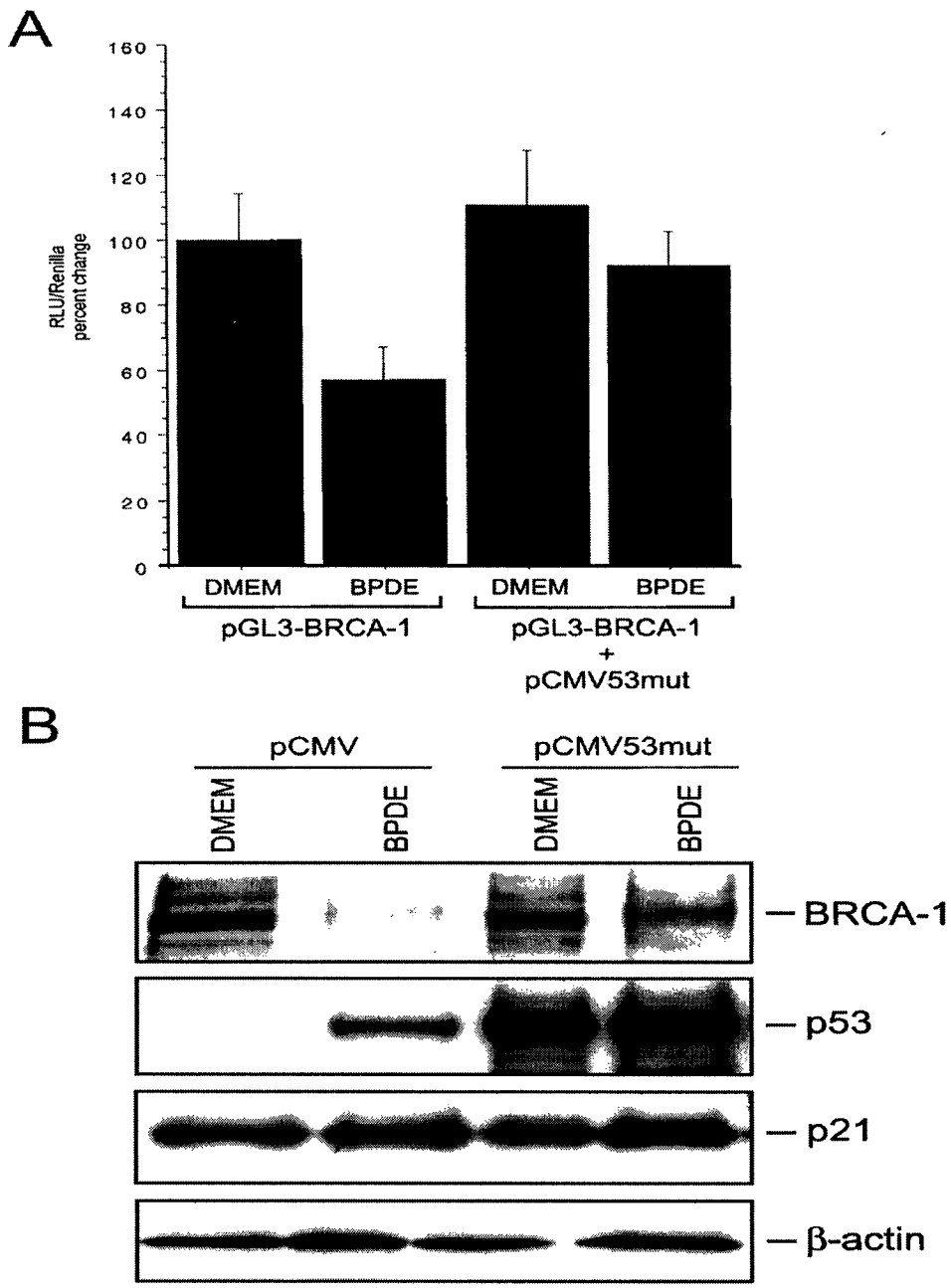


Figure 26: pGL3 BRCA-1 was transiently transfected into MCF-7 cells either alone or along with a dominant-negative p53 vector (pCMV-p53mut175) harboring a mutation in the cDNA corresponding to amino acid 175 (R→H). Transfected cells were either untreated or treated with BPDE for 24h (A). MCF-7 cells were either mock-transfected (pCMV) or transfected with pCMV-p53mut175, and treated with BPDE for 24h. Western blotting for p53 and p21 was performed (B).

and p21 levels were increased in cells transfected with the empty pCMV vector (Data not shown). However, in cells transfected with pCMV53mut, we detected constitutive expression of p53, whose levels were increased further by BPDE. More importantly, BRCA-1 protein was restored almost to control levels in cells expressing p53mut and treated with BPDE. The cellular content of p21 was elevated by BPDE in cells transfected with pCMV or pCMV53mut, but it was not altered by expression of exogenous mutant p53 in cells cultured in DMEM. To further test the hypothesis that the metabolite BPDE inhibits BRCA-1 transcription via an effect that is mediated by p53, we examined the regulation on BRCA-1 transcriptional activity in ZR75.1 breast cancer cells, which contain mutated p53 (^{152}Pro→Leu). The functionality of the AhR pathway in ZR75.1 cells was confirmed by evidence of transcriptional activation of the positive control, p1A1-4X-LUC construct, in the presence of 5 μM B[*a*]P (7.0-fold) and 500 nM TCDD (10.0-fold; Fig. 27A). Therefore, it appeared that ZR75.1 cells were more responsive than MCF-7 cells to stimulation with equimolar concentrations of TCDD or B[*a*]P. A slight increase (1.5-fold) in RLU was also observed in ZR75.1 cells transfected with p1A1-4X-LUC and treated with 500 nM BPDE. In contrast, transfection experiments with pGL3-BRCA-1 indicated that TCDD had no effects on BRCA-1 promoter activity (Fig. 27C), whereas RLU were increased 1.5-fold in ZR75.1 cells treated with 500 nM B[*a*]P (Fig. 27B) or 50 and 100 nM BPDE (Fig. 27D). This increase in transcriptional activity of the BRCA-1 promoter was similar to the increase observed in MCF-7 cells cotransfected with dominant-negative p53 (Figs. 25A and 26A). Overall, these findings indicated that

transcription of BRCA-1 was not repressed by B[*a*]P, BPDE, or TCDD in ZR75.1 breast cancer cells containing mutated p53.

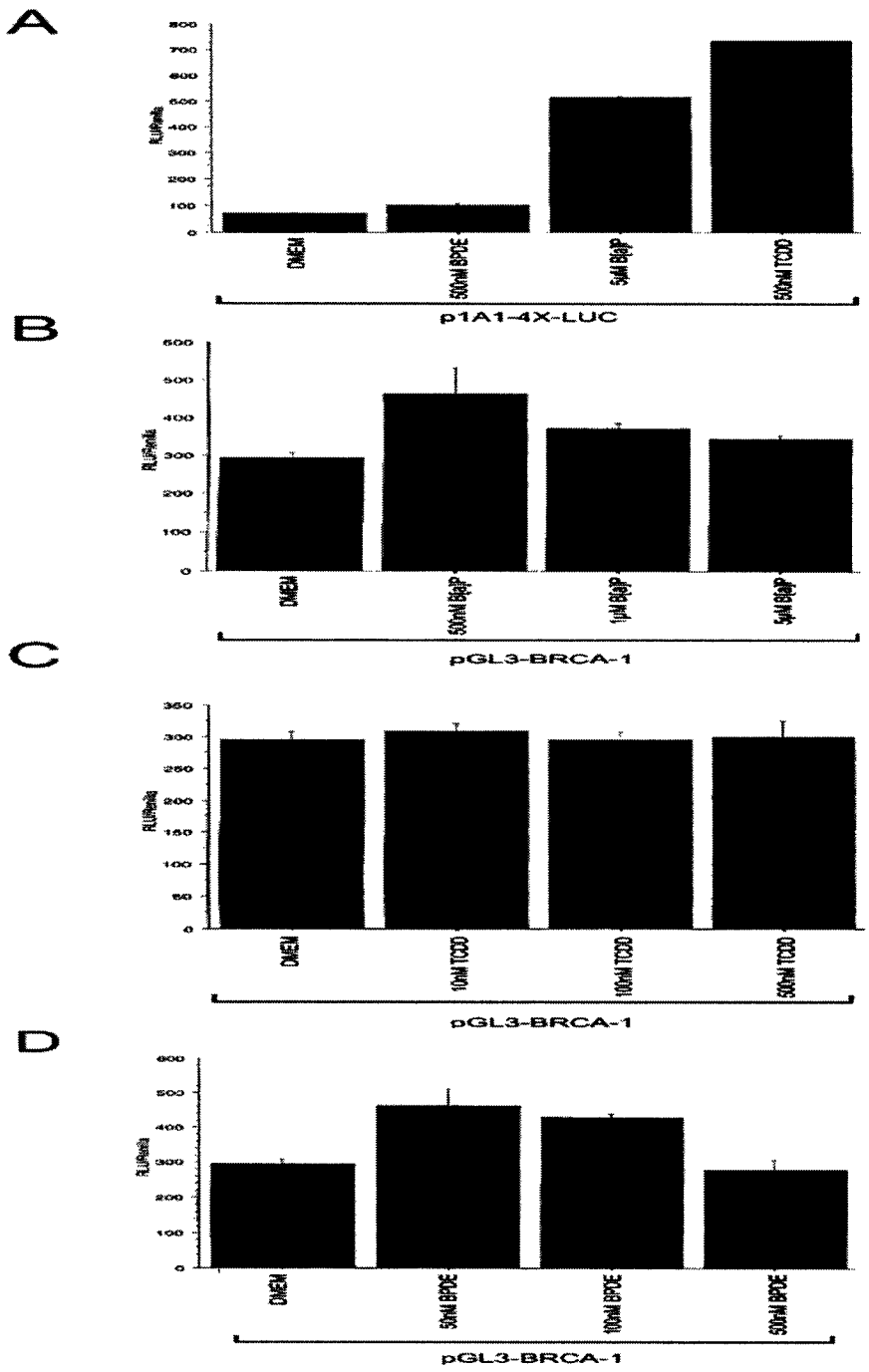


Figure 27. p1A1-4X-LUC positive control was transiently transfected into ZR75.1 breast cancer cells subsequently treated with BPDE, B[a]P, or TCDD (A). pGL3 BRCA-1 was transiently transfected into ZR75.1 breast cancer cells treated with increasing amounts of B[a]P (B), TCDD (C), or BPDE (D) for 24h.

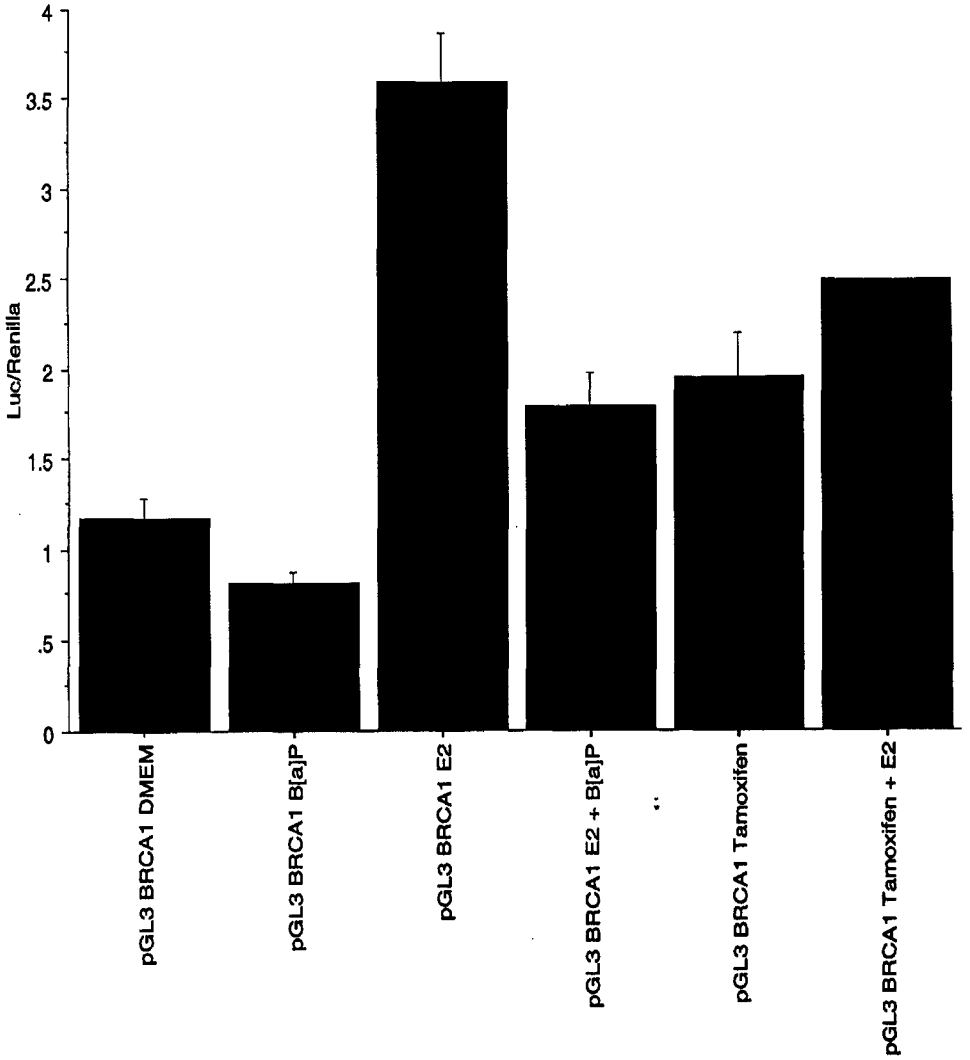
Chapter 4: Endogenous Factors: Regulation of BRCA-1 Expression by 17-β-estradiol

Transcription From a 1.69 kb BRCA-1 Promoter-Reporter Construct Is Induced by Estrogen and Inhibited by Tamoxifen in MCF-7 Breast Cancer Cells

In the previous chapter, we have demonstrated that a reporter vector driven by a 1.69 kb fragment of the BRCA-1 5′ flanking region can be downregulated by polycyclic aromatic hydrocarbon DNA damaging agents (B[a]P/BPDE) in ER-α+ cell lines. Previously, we have also shown that BRCA-1 mRNA levels increase when ER-α+ breast and ovarian cells are exposed to estrogen (Jeffy, 1999, Romagnolo, 1998). To confirm that estrogen treatment of ER-α+ breast cancer cells also leads to increased protein levels, we performed western blotting for BRCA-1 on lysates from MCF-7 cells untreated or treated with 10nM estrogen for 24 hours. The increase in BRCA-1 promoter activity was matched by a similar accumulation of endogenous BRCA-1 protein following estrogen treatment for 24 hours (data not shown). Because of the fact that BRCA-1 mRNA and protein levels are increased when ER-α+ MCF-7 cells are exposed to estrogen, we investigated whether or not the BRCA-1 promoter-reporter construct (pGL3 BRCA-1) would be able to be induced by estrogen when transfected into this cell line. Treatment of transfected cells with 10nM estrogen for 24 hours induced a 3-fold increase in BRCA-1 promoter activity (Figure 28). Interestingly, although the estrogen-mediated increase in BRCA-1 transcription has previously been linked to increased mitogenesis or an

enriched population of cells in S-phase of the cell cycle, pGL3 BRCA-1 activity was not increased by estrogen at any time point prior to 24 hours even though cell cycle progression is affected by estrogen at earlier times (data not shown). This suggests that more rapid cell cycle progression may not be the only factor in E2-mediated regulation of BRCA-1 and that a direct mechanism may exist. To further confirm the role of the estrogen receptor-α in regulation of BRCA-1 promoter activity, we pre-treated MCF-7 cells for 2 hours with 5μM tamoxifen and subsequently replaced the media with either tamoxifen alone or tamoxifen + 10 nM E2. While treatment for 24 hours with 5μM tamoxifen alone gave a slight induction of BRCA-1 promoter-reporter activity, a two hour pre-treatment followed by co-treatment with tamoxifen + estrogen for an additional 24 hours showed an almost complete inhibition of BRCA-1 E2-mediated induction (Figure 28). A positive control luciferase reporter vector driven by three tandemly arranged EREs (p3xERE) showed a 10-fold increase in activity following treatment with 10nM estrogen in these cells (data not shown). Taken together, these data suggest that positive regulation of the BRCA-1 promoter by estrogen occurs in ER-α+ MCF-7 cells within 24 hours of treatment and is paralleled by an accumulation of endogenous BRCA-1 protein levels and that this is not necessarily coupled to the mitogenic effects of estrogen.

Figure 28: pGL3 BRCA-1 was transiently transfected into MCF-7 cells treated with B[a]P, estrogen (E2), estrogen + B[a]P, Tamoxifen, or Tamoxifen + estrogen for 24 hours. Luciferase results are shown as the ratio of luciferase/renilla.



Estrogen Receptor-α is Required for BRCA-1 Induction by Estrogen

Based on the fact that BRCA-1 message and protein can be detected in a wide variety of ER+ and ER- cell lines, we and others have previously reported that maintenance of basal levels of BRCA-1 expression is not ER-α-dependent (Jeffy, 1999). However, we have found that when ER-α negative cell lines including HeLa cervical and HCT-116 colon cancer cells are transiently transfected with pGL3 BRCA-1, estrogen treatment fails to induce promoter-reporter activity (Fig. 29, 30). In order to determine whether or not there is a requirement for ER-α in estrogen-mediated BRCA-1 transcription, we transiently co-transfected a vector encoding the cDNA for ER-α (pHEO) along with pGL3 BRCA-1 into ER-α-negative HeLa cervical cancer cells followed by treatment with 10nM E2 for 24 hours. Luciferase data clearly demonstrated that while transfection of ER-α into this cell line did not affect basal pGL3 BRCA-1 levels, the presence of ER-α was sufficient to restore estrogen responsiveness to pGL3 BRCA-1 as well as to a positive control reporter vector driven by 3 tandemly arranged estrogen response elements (p3xERE) (Figure 29B). To further confirm the requirement for ER-α as a mediator of estrogen induction of BRCA-1 promoter activity, we next transiently co-transfected pHEO and pGL3 BRCA-1 into the HCT-116 colon cancer cell line, which expresses ER-β but not ER-α.

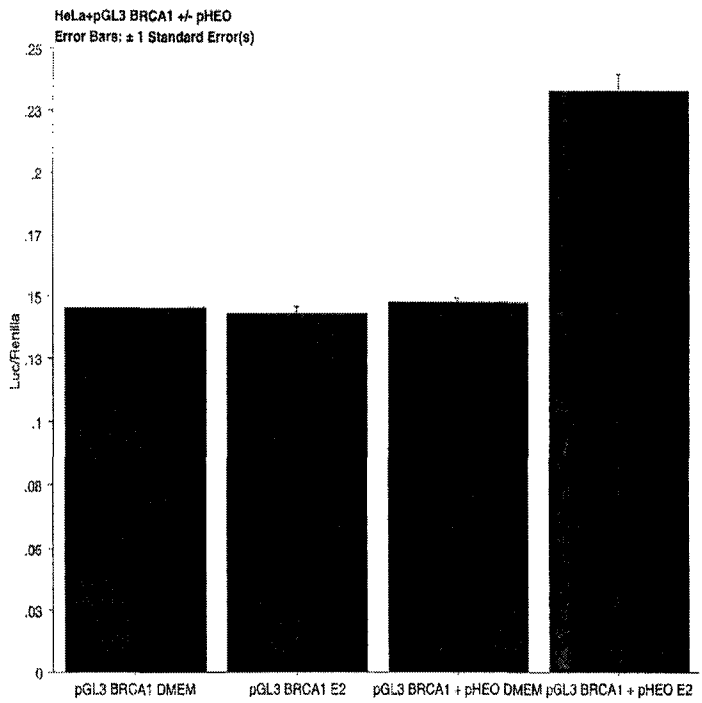


Figure 29. pGL3 BRCA-1 was transfected either alone or co-transfected with pHEO into ER-negative HeLa cells. Transfected cells were either untreated or treated with estrogen for 24h.

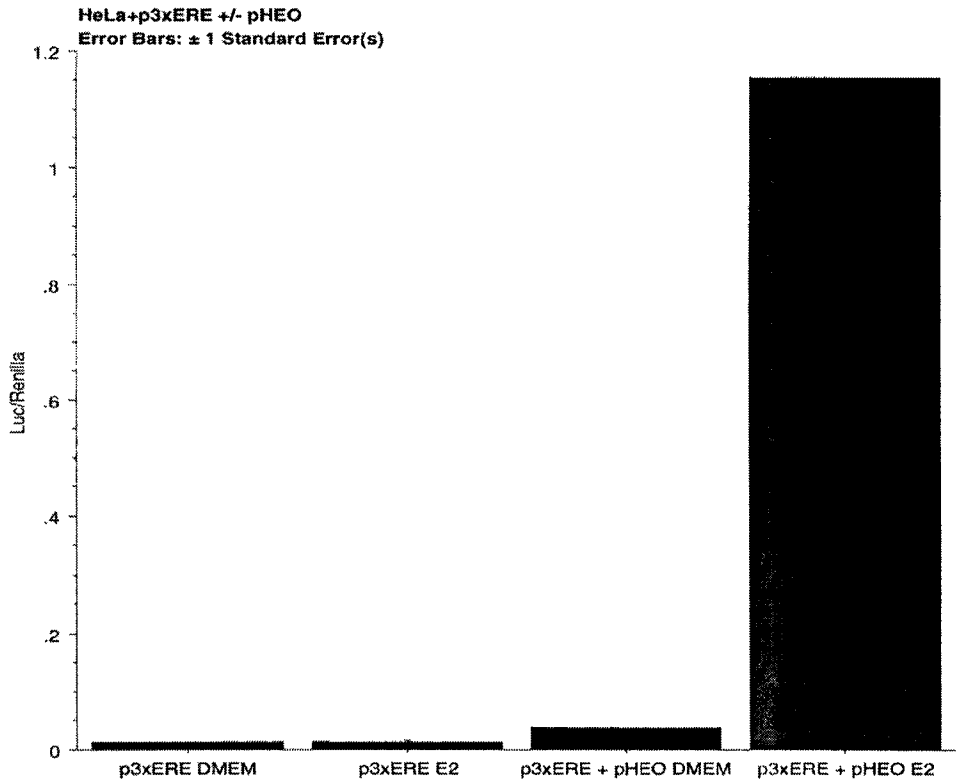


Figure 29B. Positive control vector for estrogen efficacy (p3xERE) was transfected into HeLa cells ± pHEO. Transfected cells were untreated or treated with 10nM estrogen for 24h.

While pGL3 BRCA-1 activity was not altered by estrogen treatment in cells which did not receive the ER-α vector, transient expression of 3μg of pHEO per 5 x 10^5 cells caused a 2.1-fold increase in basal and a 3.3-fold increase in estrogen-induced pGL3 BRCA-1 activity (Figure 30). Luciferase measurements suggest that not only is ER-α expression necessary for BRCA-1 promoter induction by estrogen, but that the amount of ER expression vector transfected into this cell line may be a determining factor in determining the fold induction. Transient transfection of 0.5μg/well of pHEO was not sufficient to restore BRCA-1 promoter sensitivity to E2 while 1, 3, and 6 μg of the plasmid showed an increased response to 10 nM E2 based on the amount of plasmid transfected (1.2, 1.5, 1.6-fold respective induction-Figure 31). Interestingly, transient transfection of pHEO was sufficient to lead to an increase in pGL3 BRCA-1 basal activity in the absence of treatment suggesting a relationship between ER-α levels and BRCA-1 expression.

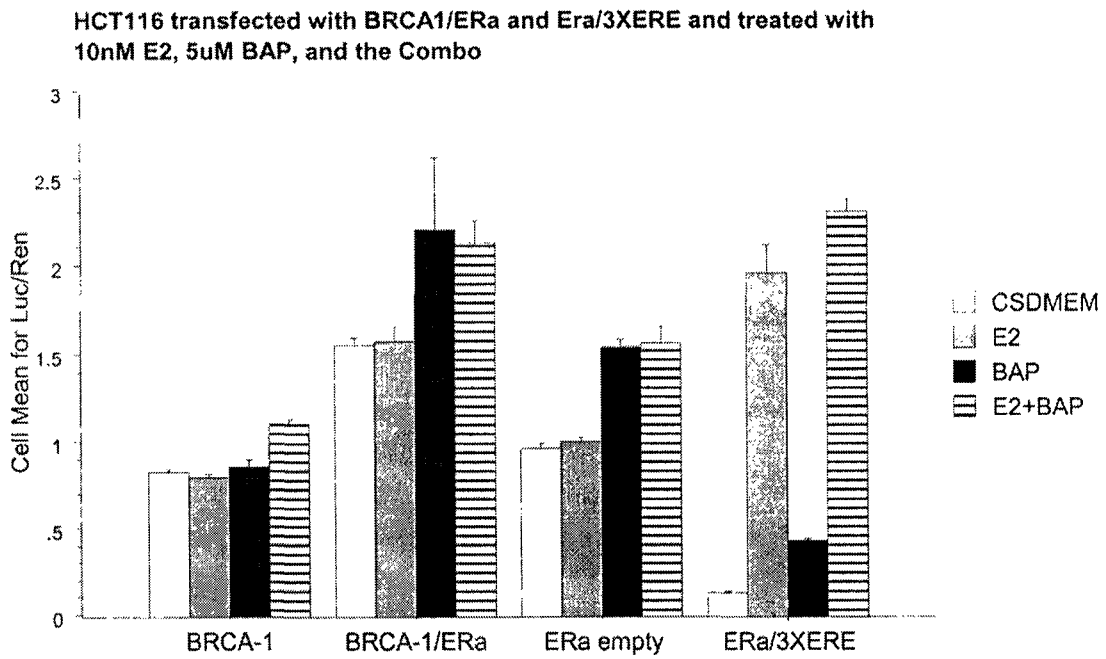

HCT116 transfected with BRCA1/ERa and Era/3XERE and treated with 10nM E2, 5uM BAP, and the Combo
Cell Mean for Luc/Ren
3
2.5
2
1.5
1
.5
0
BRCA-1
BRCA-1/ERa
ERa empty
ERa/3XERE
CSDMEM
E2
BAP
E2+BAP

Figure 30.

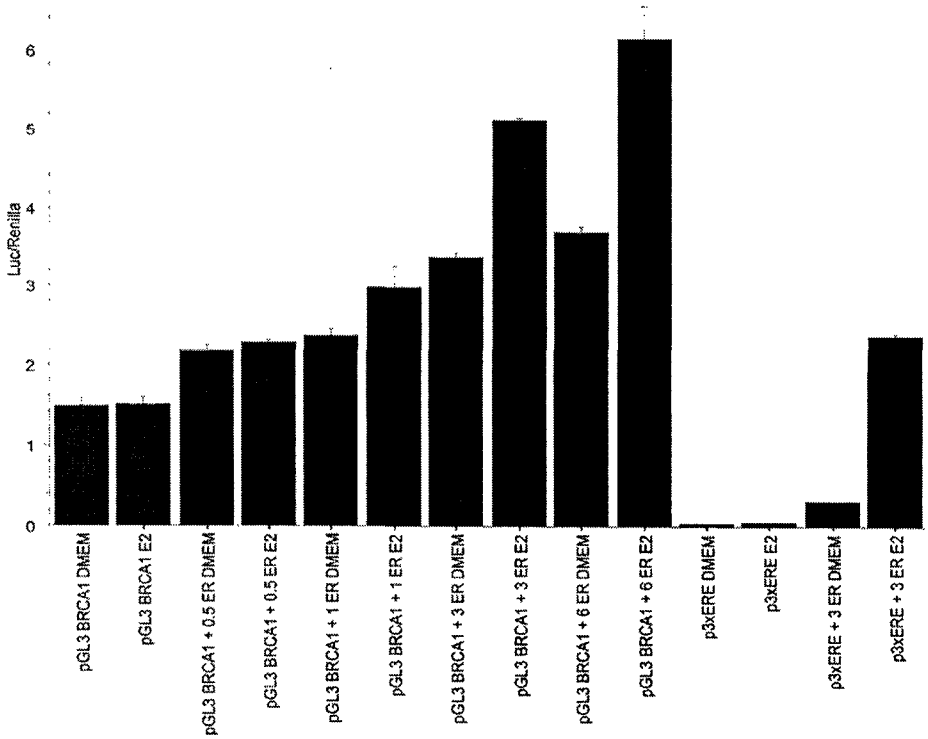


Figure 31. HCT-116 cells were transiently co-transfected with 10μg of pGL3 BRCA-1 and increasing amounts of pHEO. p3xERE was co-transfected along with 3μg of pHEO. Transfected cells were treated with 10nM E2 for 24h.

Estrogen Receptor-α Interacts With the BRCA-1 Promoter at A Non-ERE Site

Since the classical model of increased transcription of responsive genes to estrogen requires the liganded ER to translocate to the cell nucleus, which can lead to transactivation of the promoter through ER interactions with an estrogen response element (ERE) we attempted to identify possible ERE consensus sequences within the pGL3 BRCA-1 construct. Due to the fact that no regions with significant homology to ERE consensus sequences could be found in our BRCA-1 promoter construct, we began investigating alternative, non-classical mechanisms of estrogen-mediated gene transcription. Through visual inspection of the BRCA-1 promoter sequence followed by consensus sequence searching via the TRANSFAC/Match transcription factor database (www.gene-regulation.com), we identified a potential region (-46→-14 upstream of BRCA-1 exon 1b) containing binding sites for proteins known to have the ability to mediate estrogen-mediated transcription at non-ERE sites including Sp1, AP-1, and CREB family members. This site was found to contain a high degree of sequence homology (core consensus sequence matrix score ≥ 0.7) to each of these proteins, which have been shown to interact with the estrogen receptor-α (Figure 32). In addition to the results of computer-assisted homology searching, we chose to investigate the significance of this site due to the fact that others have reported interactions between combinations of Sp1, AP-1, and CREB family members with the ER-α to enhance E2-mediated transcription in various

"BRCA-1 AP-1" sequence was analyzed by Tranfac Database to determine potential binding proteins

Inspecting sequence BRCA1AP13_1 [?] (1 - 22):

V$GATA1_03	1 (+)	0.750	0.751	aacctGAGAggcgt
V$GATA1_04	2 (+)	0.750	0.758	acctGAGAggcgt
V$GKLF_01	5 (+)	0.937	0.782	tgagaggcgtAAGG
V$ATF_01	6 (-)	0.750	0.757	gccTTACgcctctc
V$DELTAEF1_01	6 (-)	0.754	0.756	ttacGCCTctc
V$VBP_01	8 (-)	1.000	0.800	cTTACgcctc
V$CREB_02	9 (-)	0.774	0.751	cgccTTACgccc

A total of 22 basepairs was scanned.

Figure 32

promoters, including the loricrin, cyclin D1, bcl-2, and progesterone receptor genes, suggesting that these protein-protein interactions may be a common mechanism underlying estrogen-mediated transcription (Jang, 2002, Petz, 2002, Dong, 1999, Sabbah, 1999, Castro-Rivera, 2001) (Figure 33). As we believe that the interaction between Sp1, AP-1, and CREB along with the ER-α may be required to act together as a functional unit to enhance E2-mediated transcription of BRCA-1, we have termed our putative binding region an "Estrogen Response Unit" or "ERU". The location of the potential BRCA-1 ERU is located in the region between -46 → -14 upstream of exon 1b and contains the sequence (Figure 34):

5′-GGGTAGGGGGCGGAACCTGAGAGGCGTAAGGCGTT-3. After identifying this potential non-classical ER binding domain in the BRCA-1 promoter, we designed double-stranded oligonucleotides containing this region and performed DNA binding/gel shift assays to determine whether or not this was a *bona fide* protein binding site. Using nuclear extracts from MCF-7 cells either untreated or treated with 10nM estrogen for 24h, we saw significant binding to the BRCA-1 AP-1 oligonucleotide (Figure 35 Band B, Lanes 2-5). In order to determine if the protein complex bound to the BRCA-1 AP-1 contained the ER-α, we incubated the nuclear extracts with an antibody for the ER-α and observed a supershift, suggesting its presence in the complex (Figure 35, Band A, Lanes 6-11). Antibodies for ER-β were unable to cause a shift at this site (data not shown). To further confirm that the ER-α was indeed binding to this site within the

Sp1, AP-1, CREB, and Estrogen Receptor show functional synergism in estrogen induction of a variety of genes

	Sp1	AP-1	CREB
Consensus:	**GGGCGG**	**CTGAG**	**TGACGTCA**
	(Kardassis, 1999)	(Petz, 2002)	(Sabbah, 1999)

	-46	-14 upstream of exon 1b
BRCA-1	5′-GGGTAGGGGCGGAACCTGAGAGGCGTAAGGCGT-3′	
ERU	3′-CCCATCCCCGCCTTGGACTCTCCGCATTCCGCA-5′	

E2-Responsive Promoter	Sp-1	AP-1	CREB	
p21:	GGGCGG			(Kardassis, 1999)
bcl-2	GGGCTGG			(Dong, 1999)
α6 Integrin	GGGCGG	TGACGT	TCGTCA	(Nishida, 1997)
Vit. D Receptor	GGGCGGG	TGACACA		(Qi, 2002)
Cyclin D1			CGTCA	(Sabbah, 1999)
Pr Receptor		TGAGTGA		(Petz, 2002)
c-Fos		CTGAG/GTGAC		(Weisz, 1990)
GnRH		TGACATA		(Cheng, 2003)

Figure 33

BRCA-1 AP-1 and Estrogen Response Unit (ERU) oligonucleotides were used to determine the identity and to characterize the transcription factor binding profiles to the BRCA-1 promoter in extracts from untreated or estrogen-treated MCF-7 cells

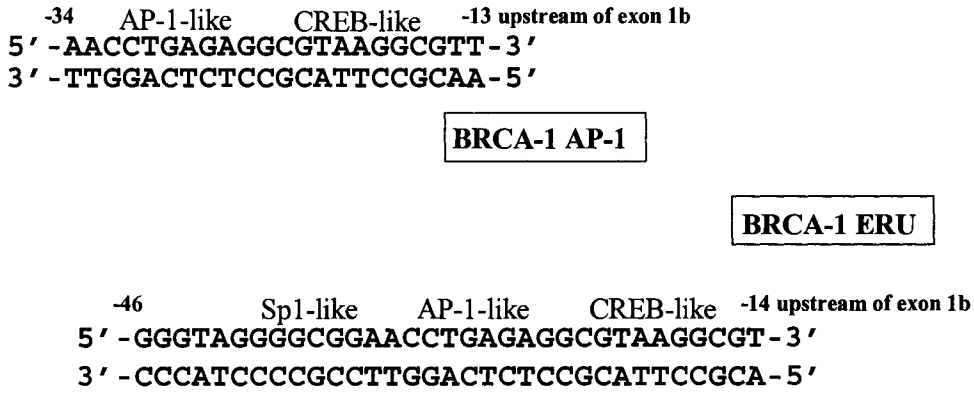


Figure 34

BRCA-1 promoter, we employed a DNA binding-protein purification protocol using concatamerized double stranded BRCA-1 ERU oligonucleotide-linked magnetic beads (Roche) to isolate and identify proteins that were binding to the BRCA-1 ERU region. DNA binding followed by protein elution was performed using nuclear extracts from MCF-7 cells ± E2 for 24 hours. Western blotting for the ER-α was performed on the complement of proteins that were eluted from this concatamerized oligonucleotide and confirmed that the ER-α was indeed binding to the BRCA-1 ERU site and E2 treatment increased ER-α binding to this oligonucleotide (Figure 36).

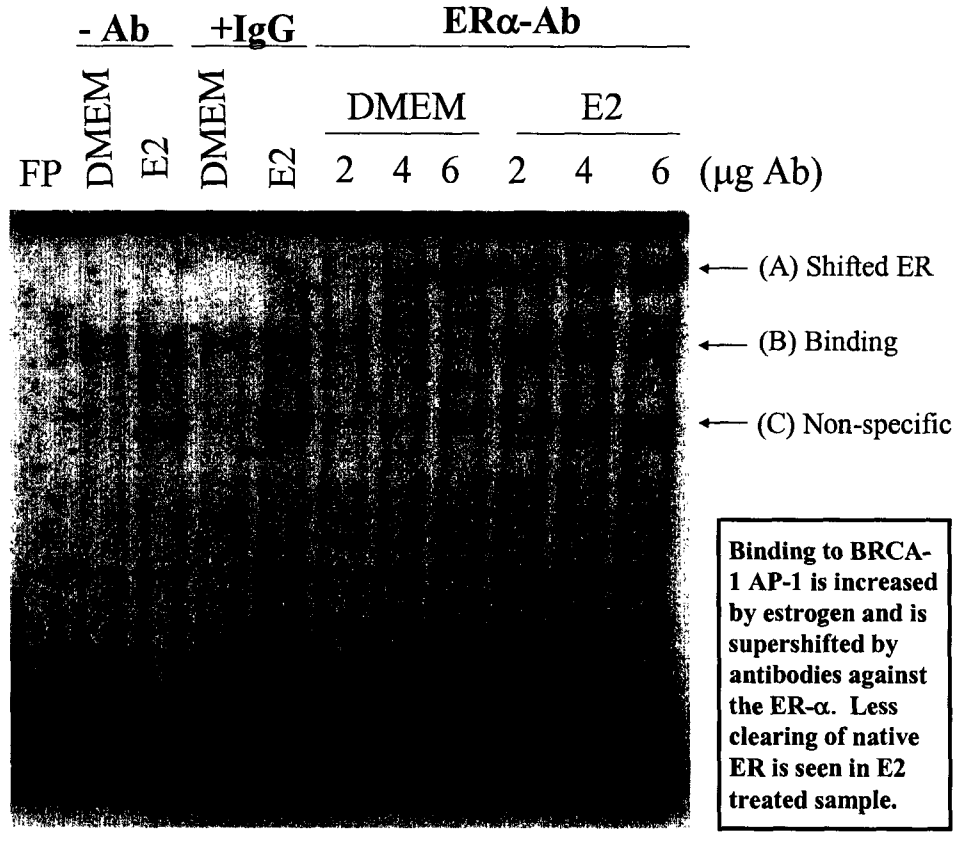

- Ab
+IgG
ERα-Ab
DMEM
E2
DMEM
E2
DMEM
E2
FP
2 4 6 2 4 6 (μg Ab)
(A) Shifted ER
(B) Binding
(C) Non-specific
Binding to BRCA-1 AP-1 is increased by estrogen and is supershifted by antibodies against the ER-α. Less clearing of native ER is seen in E2 treated sample.

Figure 35

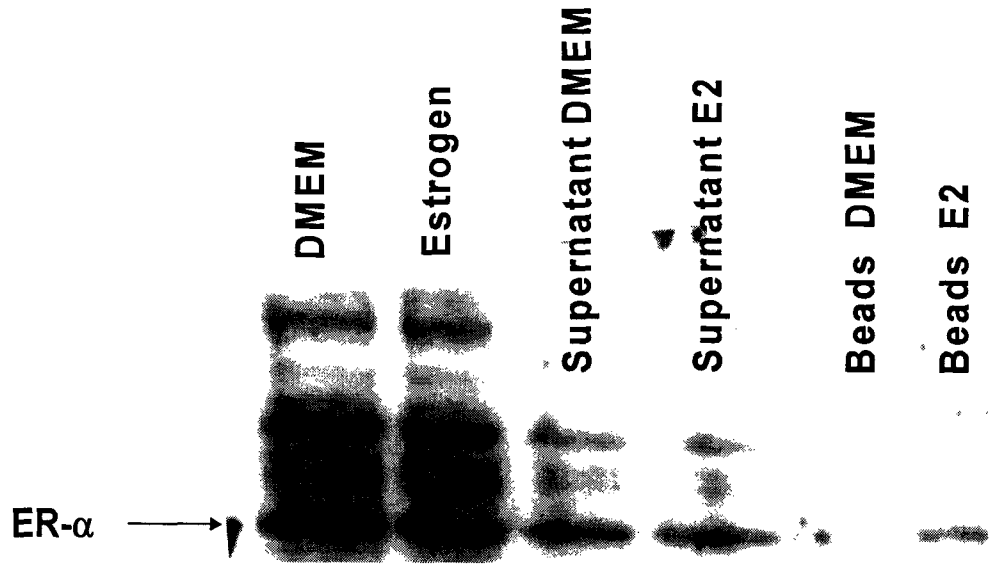


Figure 36. BRCA-1 AP-1 oligonucleotide/DNA binding-protein purification procedure was employed to purify proteins interacting with the oligonucleotide. Western blotting for ER-α was performed on the eluate.

Estrogen Receptor-α Interacts With AP-1, Sp1, and CREB Family Members at the -46 → -14 Region of the BRCA-1 Promoter to Form an "Estrogen Response Unit"

Due to the fact that ER has been shown to modulate transcription of responsive genes through interactions with Sp1, AP-1, and CREB and because we had identified a region of the BRCA-1 promoter which shows homology to consensus binding sequences for all of these transcription factors, we investigated whether or not we could identify which particular proteins were present at this region under various conditions. Since ER-α has been shown to interact with Sp1 to enhance estrogen-mediated transcription from responsive genes, we first performed EMSA for the Sp1 family of transcription factors using the annealed BRCA-1 ERU oligonucleotides which contain a putative Sp1 binding site (⁻41-GGGGCGG-⁻35). While we were unable to obtain a supershift using nuclear extracts from MCF-7 cells either untreated or estrogen-treated using antibodies for Sp2 and Sp4, a shift was observed with Sp1 and Sp3 antibodies indicating their presence in the transcriptional complex which can bind to the BRCA-1 ERU (Fig. 37).

The intensity of the Sp1 or Sp3 shift was not altered by estrogen treatment suggesting that these factors may be constitutively present. Next, in order to discern the identity of other transcription factors that were binding to BRCA-1 ERU along with Sp1/3 and the ER-α, we identified a potential cyclic AMP response element (⁻24-GCGTA-⁻20) 11 base pairs 3′ of the potential Sp1/3 binding site, also known to interact with the ER-α via CREB, we performed gel shift analysis and confirmed that CREB-1 was also present

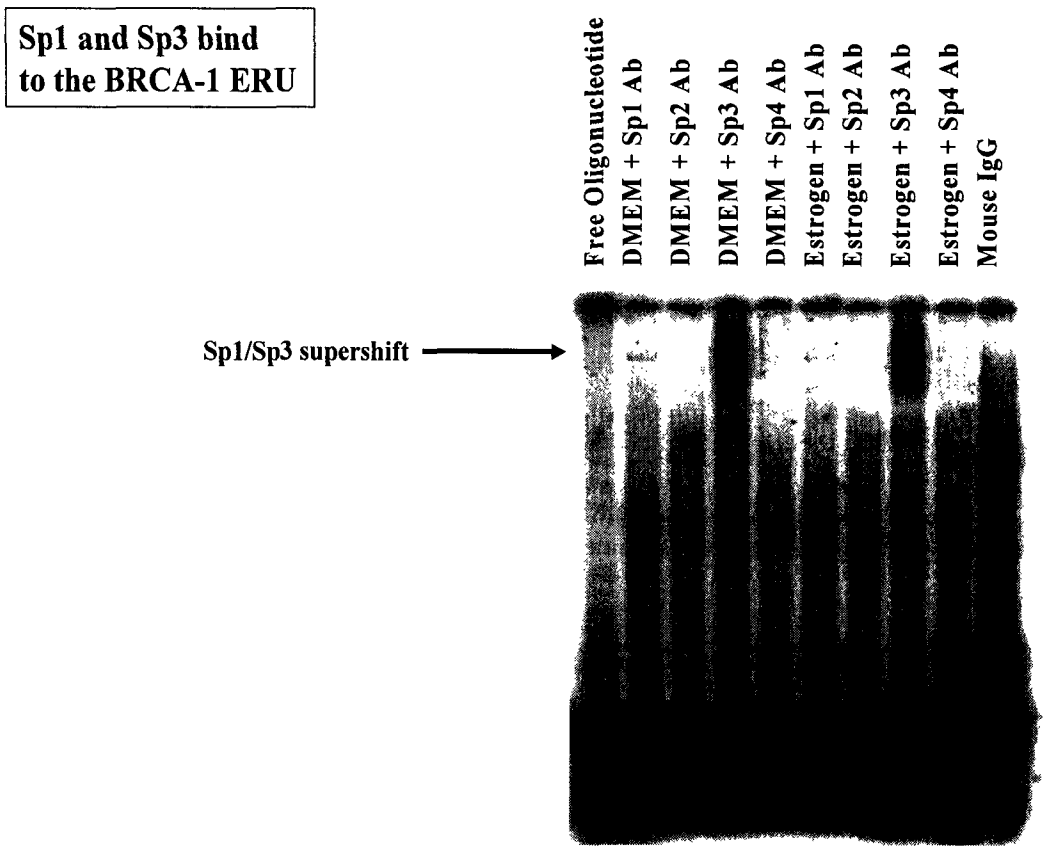
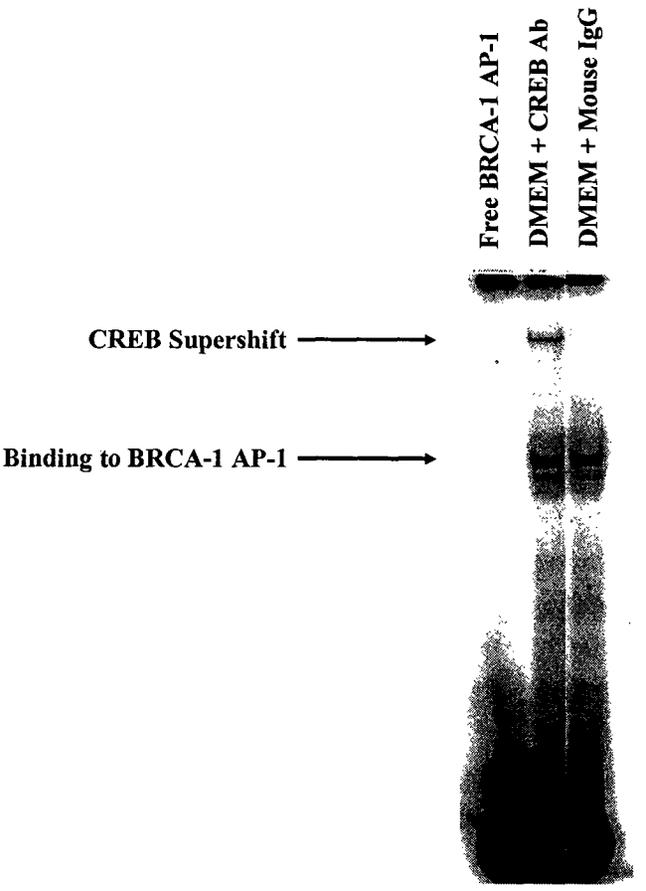


Figure 37: EMSA was performed on MCF-7 nuclear extracts untreated or treated for 24h with estrogen using the BRCA-1 ERU oligonucleotide. Antibodies for the Sp family members were used for supershift analysis.

(Figure 38) and its levels were not altered by treatment with estrogen in both the BRCA-1 AP-1 and ERU oligonucleotides (Data not shown). In order to ascertain whether or not the BRCA-1 promoter contains a *bona fide* AP-1 binding domain through which the ER could be interacting, electrophoretic mobility shift assays were performed using antibodies for various AP-1 family members known to interact with the ER-α at AP-1 binding sites. Within the BRCA-1 5′ flanking region, located directly between the Sp1 and CREB binding sites, we identified a potential AP-1 domain (¯31-CTGAG-¯27) with homology to a consensus AP-1 motif. Gel shift results using MCF-7 nuclear extracts from cells ± E2 for 24h showed that c-Jun, JunB, FosB, and Fra-2 were able to bind to the BRCA-1 AP-1 and ERU oligonucleotides under these conditions (Figure 39). Additionally, we were able to obtain weaker supershifts using antibodies to JunD and c-Fos (Figure 39). Unlike Sp1, 3, and CREB, minor differences in the intensity of the AP-1 family member bands were observed following estrogen treatment. The most significant differences observed were slight decreases in JunB and increased c-Fos (Figure 39). In order to determine activity of the AP-1-like site in the BRCA-1 ERU, oligonucleotides containing the desired base pair changes were generated (Sigma-Genosys) and were used to introduce specific mutations via PCR into the pGL3 BRCA-1 promoter sequence according to the QuikChange Site-Directed Mutagenesis Kit protocol (Promega). Base pair changes in pGL3 BRCA-1 were introduced into the AP-1-like region (TGAG → acta) of the BRCA-1 ERU and were verified by direct sequencing (Figure 40).



CREB binds to the BRCA-1 ERU under using nuclear extracts from MCF-7 cells

Figure 38: EMSA for CREB was performed on BRCA-1 AP-1 oligonucleotide using MCF-7 nuclear extracts.

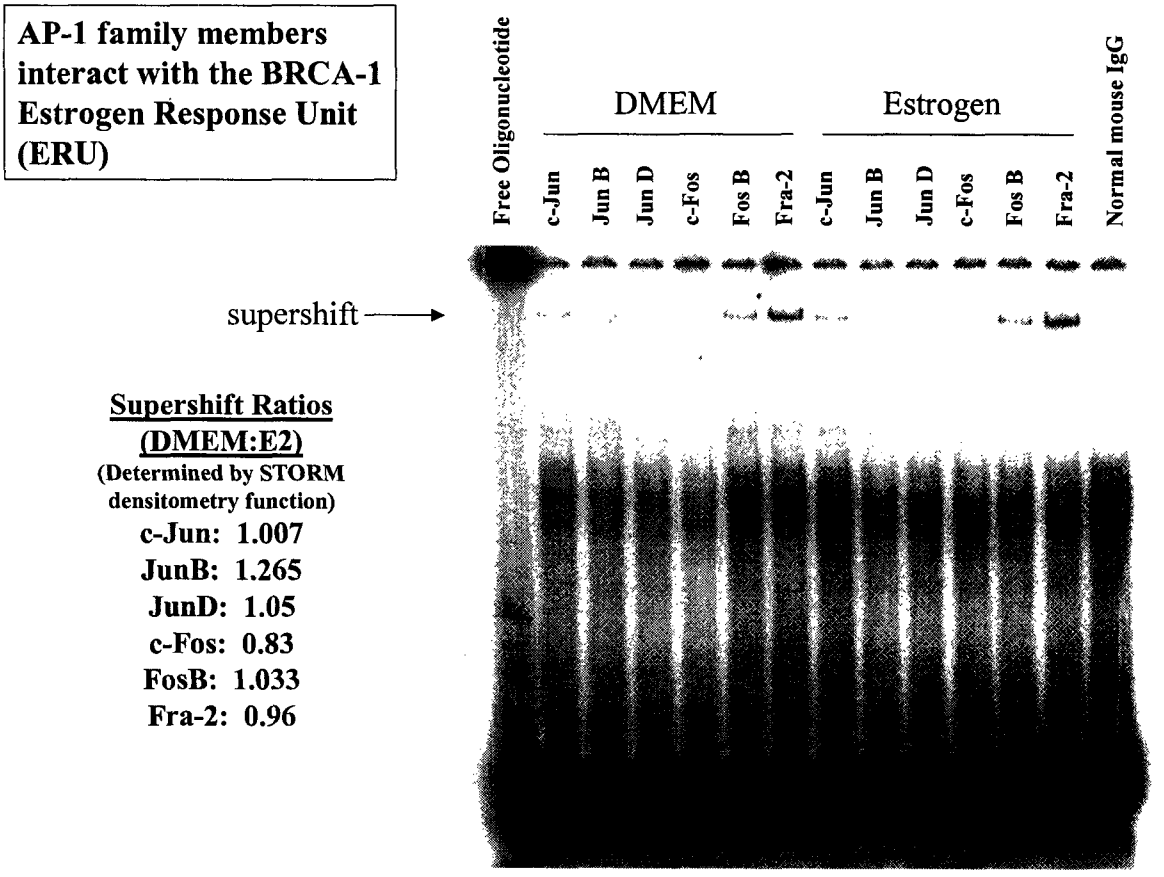
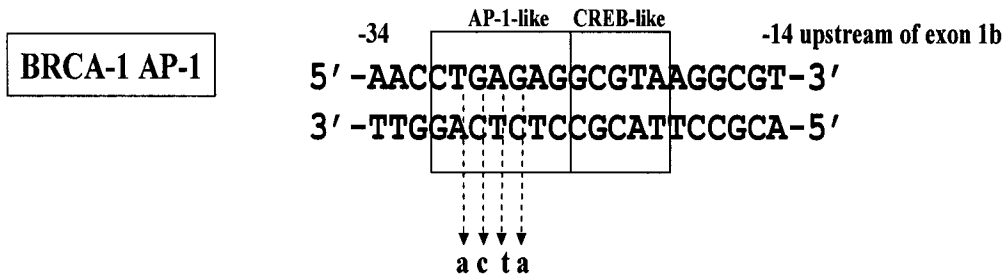


Figure 39. EMSA for AP-1 family members was performed using the BRCA-1 ERU oligonucleotides and nuclear extracts from MCF-7 cells untreated or treated with 10nM estrogen for 24h.

Transient transfection of MCF-7 cells with this mutant vector and analysis by luciferase analysis demonstrated that mutation of four base pairs comprising the AP-1-like binding motif within the BRCA-1 ERU site was sufficient to nearly completely abrogate both basal and estrogen-induced BRCA-1 promoter activity (Figure 41B). Finally, in order to confirm the role of AP-1 in regulation of BRCA-1 transcription, we transfected a dominant-negative c-Jun variant (Tam67) into MCF-7 cells along with pGL3 BRCA-1 and treated with 10nM E2 for 24h. Luciferase analysis indicated that not only did Tam67 prevent estrogen induction of pGL3 BRCA-1, but basal transcription was reduced by nearly half (Figure 43). Finally, in order to determine whether or not these three binding domains were actually binding transcription factors, we employed an approach of using an excess of various unlabeled BRCA-1 ERU oligonucleotides containing mutations in each of the various domains for cold competition (Figure 44). By incubating excess (100x) unlabeled cold oligonucleotides containing mutations in the desired regions with nuclear extracts from untreated and estrogen treated MCF-7 cells prior to incubation with the ^{32}P labeled oligonucleotides, we expected that the specific transcription factors present in the nuclear extracts would preferentially bind to the excess cold oligonucleotides and would therefore not be present when the labeled oligonucleotides was later added. While we anticipated that the pre-incubation of nuclear extracts with unlabeled wild-type ERU oligonucleotides would compete for factors and severely reduce binding to our labeled



Site-directed mutagenesis of pGL3 BRCA-1: The BRCA-1 oligonucleotide contains AP-1 and CREB-like binding motifs. Mutations (lower case) were introduced into the AP-1-like sequence in pGL3 BRCA-1, resulting in nearly complete loss of basal and estrogen-induced reporter activity.

Figure 40

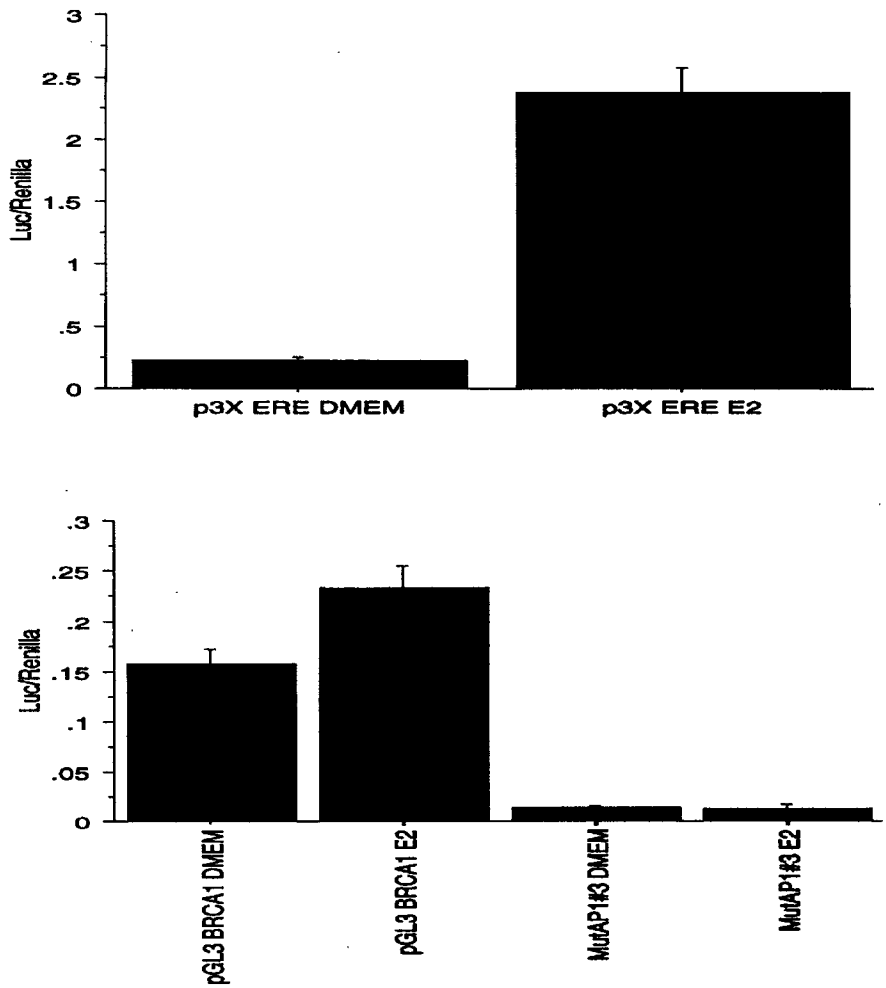


Figure 41. pGL3 BRCA-1 or pGL3 BRCA-1 mutAP-1 was transfected into MCF-7 cells. Transfected cells were untreated or treated with 10nM estrogen for 24h.

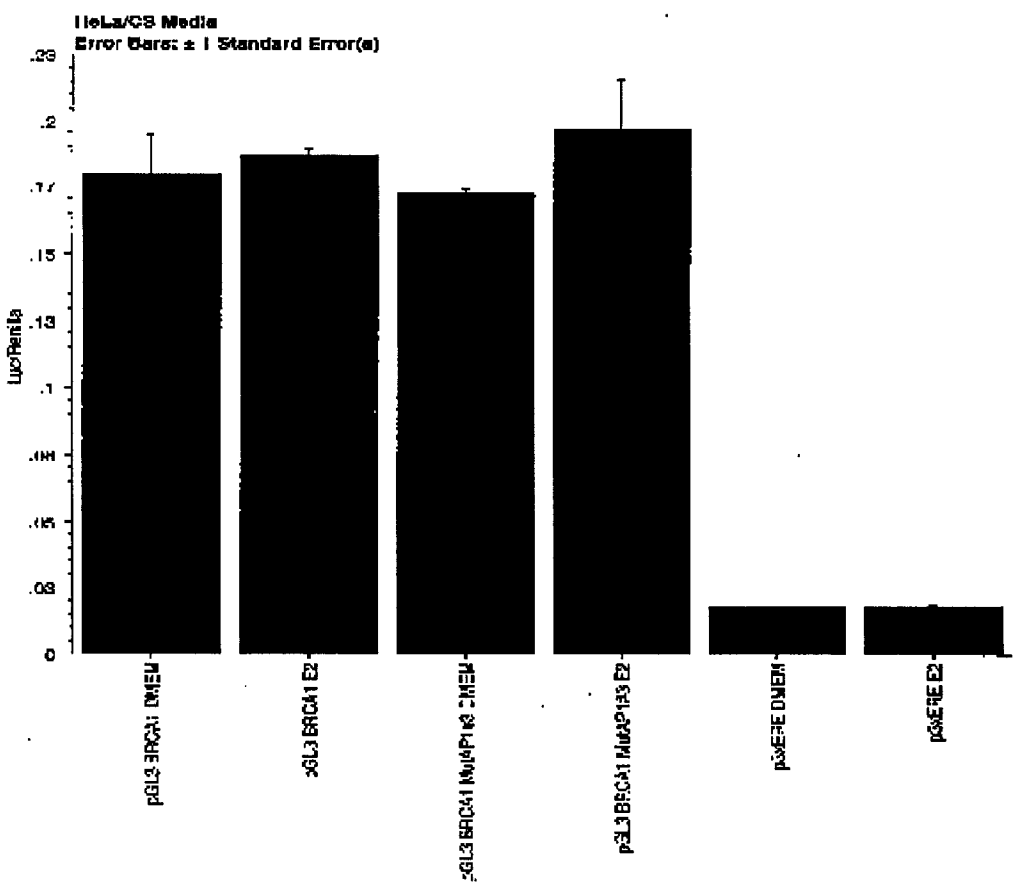


Figure 42. pGL3 BRCA-1 or pGL3 BRCA-1 mutAP-1 was transfected into HeLa cells. Transfected cells were untreated or treated with 10nM estrogen for 24h. p3xERE was used as a control for estrogen efficacy.

Dominant-negative c-Jun (Tam67) inhibits estrogen induction and reduces basal activity of pGL3 BRCA-1 in ER+ MCF-7 cells suggesting role of AP-1 in regulation of BRCA-1 transcription

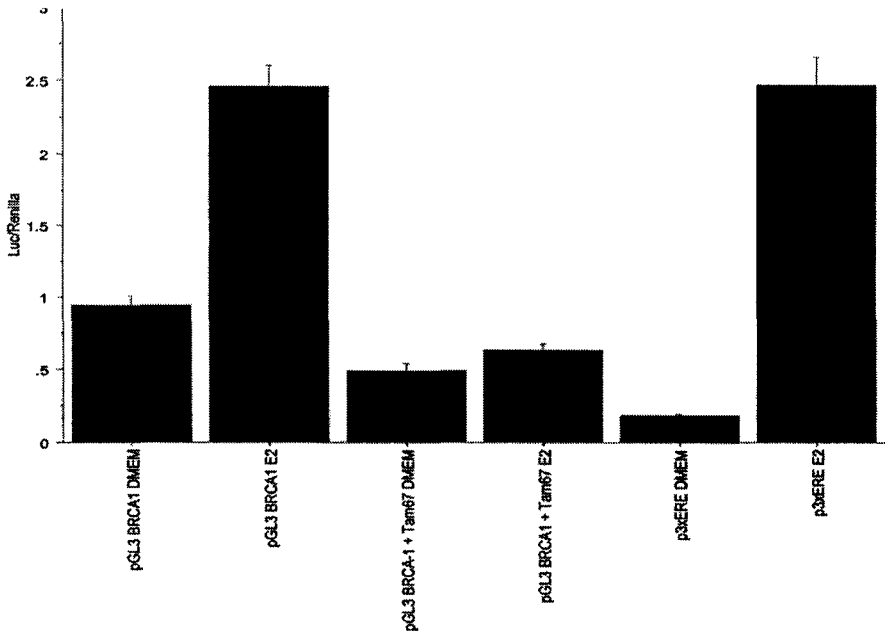


Figure 43

oligonucleotides, we predicted that the unlabeled mutant oligonucleotides would not entirely outcompete the labeled BRCA-1 ERU oligonucleotides and binding would still be observed. As predicted, we observed that the competition with unlabeled wild-type ERU oligonucleotides was indeed able to significantly reduce binding of transcription factors. In addition, we found that competition with unlabeled oligonucleotides which contained mutations in either the Sp1, AP-1, or CREB motifs were unable to effectively outcompete binding to the labeled wild-type oligonucleotides (Figure 45). These observations suggest to us that each of these three binding domains located within the BRCA-1 ERU are indeed *bona fide* binding domains for transcription factors. Based on these cumulative data, we suggest that BRCA-1 transcription induced by estrogen may require the assembly of a transcriptional complex which includes Sp1, AP-1 family members, CREB, and the ER-α at the region of the BRCA-1 promoter located between nucleotides –46 and –14 upstream of the exon 1b transcriptional start site.

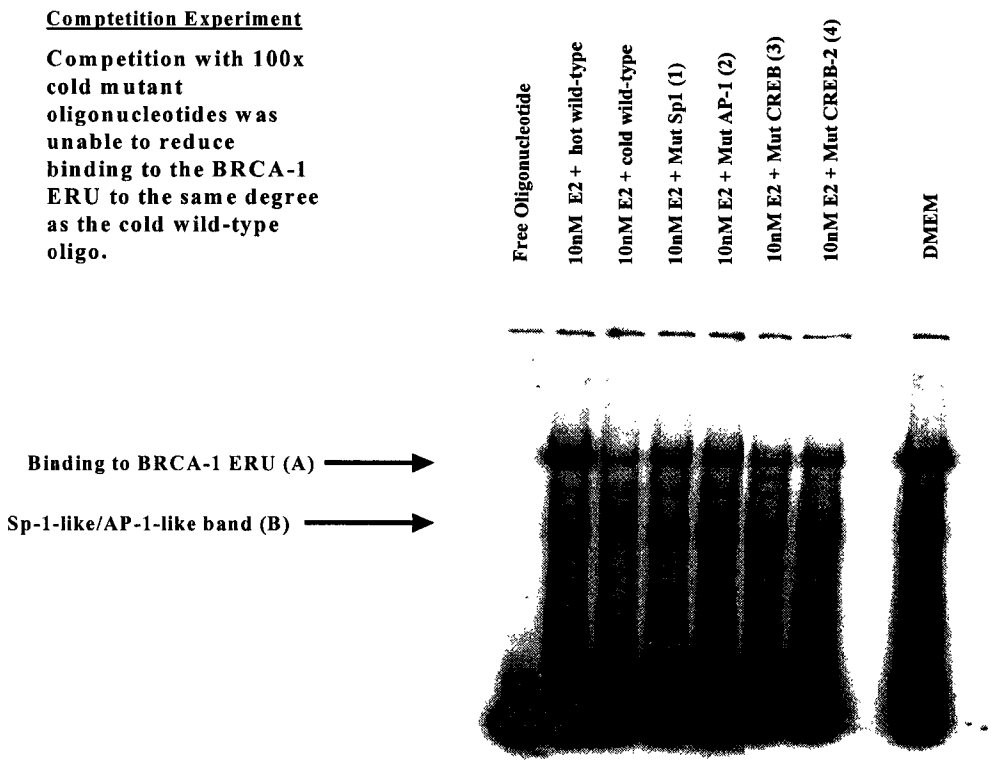
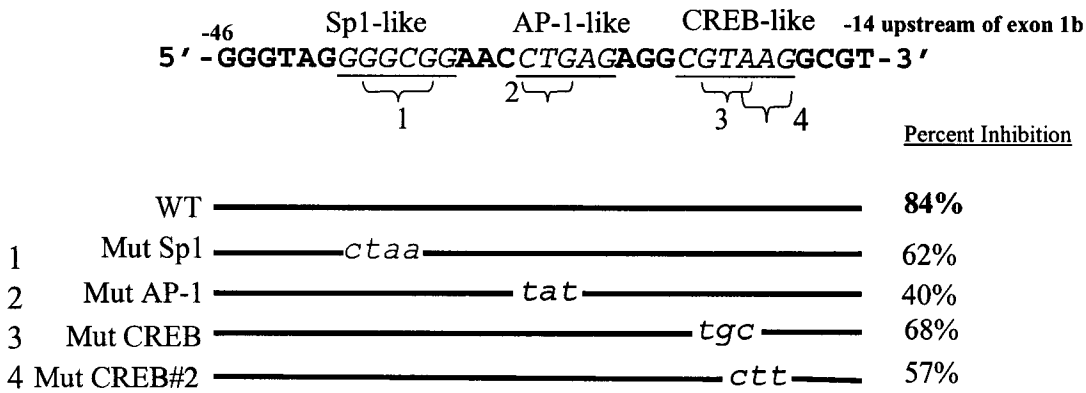

Comptetition Experiment
Competition with 100x cold mutant oligonucleotides was unable to reduce binding to the BRCA-1 ERU to the same degree as the cold wild-type oligo.
Free Oligonucleotide
10nM E2 + hot wild-type
10nM E2 + cold wild-type
10nM E2 + Mut Sp1 (1)
10nM E2 + Mut AP-1 (2)
10nM E2 + Mut CREB (3)
10nM E2 + Mut CREB-2 (4)
DMEM
Binding to BRCA-1 ERU (A)
Sp-1-like/AP-1-like band (B)

Figure 44



A 100x molar excess of unlabeled wild-type or mutant oligonucleotides were individually incubated with nuclear extracts from untreated or estrogen-treated MCF-7 cells prior to the addition of ^{32}P-labeled wild-type BRCA-1 ERU oligonucleotide. The competition with cold oligonucleotides mutated in the Sp-1-, AP-1-, and CREB-like motifs was unable to reduce binding to the BRCA-1 ERU as completely as wild-type cold oligonucleotide suggesting that these are *bona fide* transcription factor binding domains.

Figure 45

Proposed Model: BRCA-1 transactivation by E2 may require assembly of a transcriptional complex in the BRCA-1 5′ Estrogen Response Unit (-46 → -14 upstream of exon 1b) composed of Sp1, CREB, and AP-1 family members which can then interact with the ER-α. Alteration of the complement of co-activators/co-repressors present in the the cell under various treatements (agonists vs. antagonists) may partially explain the action of these compounds on transcriptional enhancement.

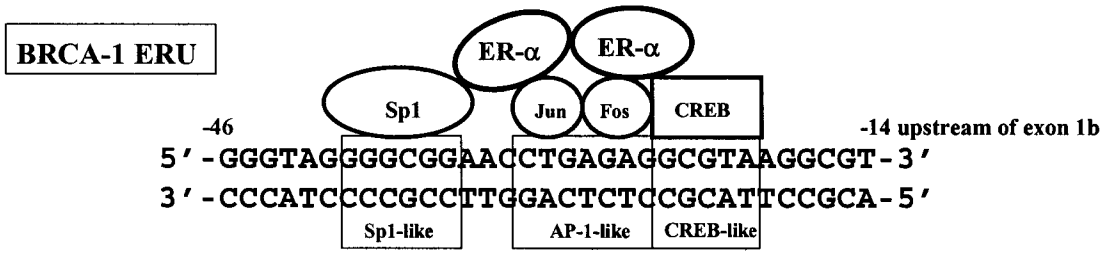


Figure 46

Chapter 5: Molecular Interactions Between Endogenous Factors: Regulation of BRCA-1 Promoter Activity by p53 and Estrogen Receptor-α

p53 and Associated Transcriptional Co-factors Interact with the BRCA-1 ERU

Since it is well established that p53 can alter levels of endogenous BRCA-1 message and protein as well as physically disrupt the ER-α binding from an estrogen responsive promoter (Liu, 1999), we investigated whether or not p53 protein interacts with the BRCA-1 ERU. Because previous investigations have demonstrated that overexpression of p53 can downregulate BRCA-1 and that treatment of MCF-7 cells with the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (B[a]P) elicits an accumulation of endogenous p53 as well as disrupts estrogen-mediated transcription of BRCA-1, we treated MCF-7 cells with either B[a]P, E2, or B[a]P + E2 for 24 h, harvested nuclear extracts, and performed EMSA using a p53 antibody to determine if p53 was interacting with the complex at the BRCA-1 ERU under these conditions. Although we were unable to obtain a clear supershifted band, the p53 antibody was able to cause a clearing of the band representing the binding to the BRCA-1 ERU from untreated or estrogen-treated cells although an equal amount of p53 antibody was unable to clear the band from the B[a]P-treated cells to the same degree (Fig. 47). To confirm the presence of p53 at this site, we utilized a DNA binding-protein purification procedure using concatamerized BRCA-1 AP-1 oligonucleotides linked to magnetic beads, followed by western blotting for p53 from the eluate. Western analysis showed that p53 interacts with the BRCA-1 ERU and that more p53 is present at this site following treatment of the cells with 10nM E2 for 24h.

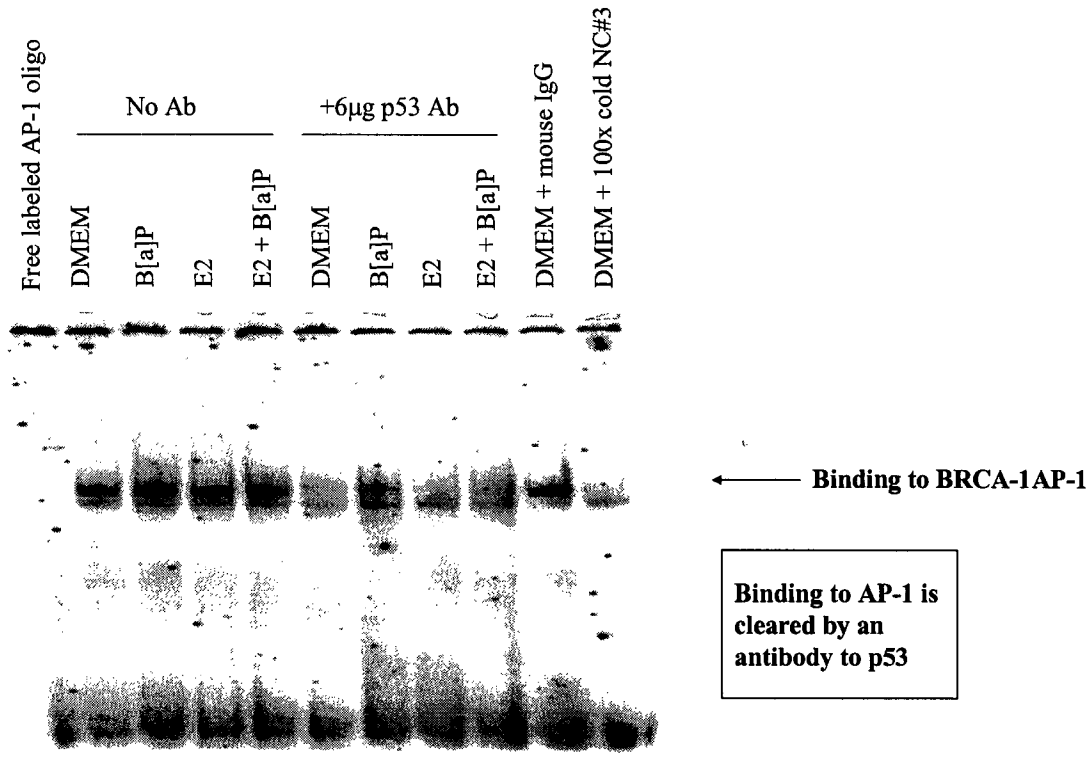
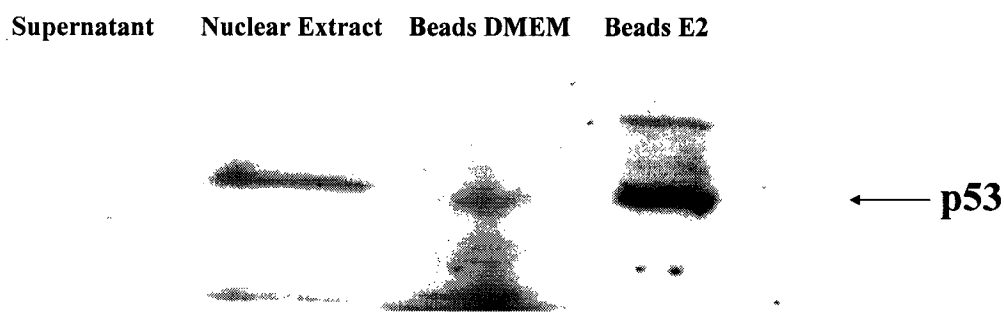


Figure 47. Antibody clearing assay for p53 was performed using the BRCA-1 AP-1 oligonucleotide and nuclear extracts from MCF-7 cells either untreated or treated with 5μM B[a]P, 10nM estrogen, or 5μM B[a]P + 10nM estrogen for 24h.

Figure 48. Eluate from AP1#3 oligos was run on 10% TG gel and western blotting for p53 was performed



In addition, we were able to determine that estrogen treatment for 24h was able increase the amount of p53 binding to the BRCA-1 AP-1 oligonucleotides (Figure 48). Our recent findings, along with the results of others, suggest that high levels of p53 are able to disrupt ER binding to EREs or other proteins in a transcriptional complex. In line with our expectations, increased binding to the BRCA-1 ERU was seen in extracts from B[a]P-treated cells as compared to control and this increased binding was less able to be cleared by the p53 antibody, suggesting that greater amounts of p53 protein bind to the ERU region of the BRCA-1 promoter when a DNA damaging agent is present.

Since it has been demonstrated that p53 may mediate some of its effects as a transcription factor by interacting with transcriptional co-factors, we performed EMSA analysis to determine if we could identify any of these proteins. Although we were able to supershift both p300 and mSin3a, there was no difference in the intensity of the shifted bands in the untreated versus the E2-treated samples (Figure 49). Together, these data suggest to us that p53 interacts with the BRCA-1 ERU and that treatment with estrogen or the DNA damaging B[a]P increases the amount of p53 at this site.

Transcriptional Cofactors mSin3a and p300 are present at AP1#3 in MCF-7 cells

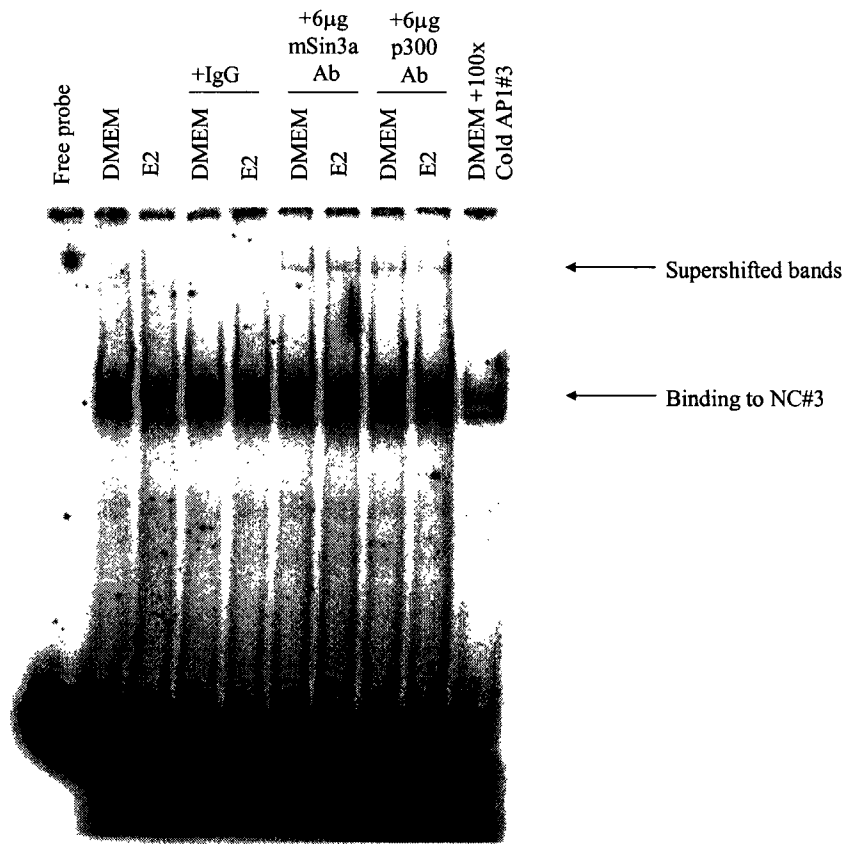


Figure 49. EMSA for mSin3a and p300 was performed using the BRCA-1 AP-1 oligonucleotide and nuclear extracts from MCF-7 cells ± 10nM estrogen for 24h.

Overexpression of Wild-Type p53 Decreases BRCA-1 Promoter Activity and Reduces Estrogen Receptor-α Binding to the BRCA-1 ERU

Since basal levels of both pGL3 BRCA-1 promoter activity and endogenous BRCA-1 protein levels were unaltered by transient transfection of the mutant p53 constructs, we investigated whether or not wild-type p53 may be required for both estrogen-induced as well as maintenance of basal BRCA-1 transcription. Interestingly, when we transfected 3μg of an expression vector containing the cDNA for wild-type p53 (pCMV-p53wt) into MCF-7 cells, both BRCA-1 promoter activity (pGL3 BRCA-1) and endogenous protein levels were significantly decreased in either E2-treated or untreated cells suggesting that increased levels of wild-type p53 may repress basal as well as induced BRCA-1 expression (Figure 51A, B). In investigating a potential mechanism for reduced transcription from the BRCA-1 promoter, we determined through EMSA analysis that overexpression of wild-type p53 in MCF-7 cells decreased ER-α binding to the BRCA-1 ERU under both basal and estrogen-induced conditions (Figure 50). This contrasts with overexpression of mutant p53 aa175, which we have shown disrupts ER-α binding at this site in both untreated and estrogen-treated cells.

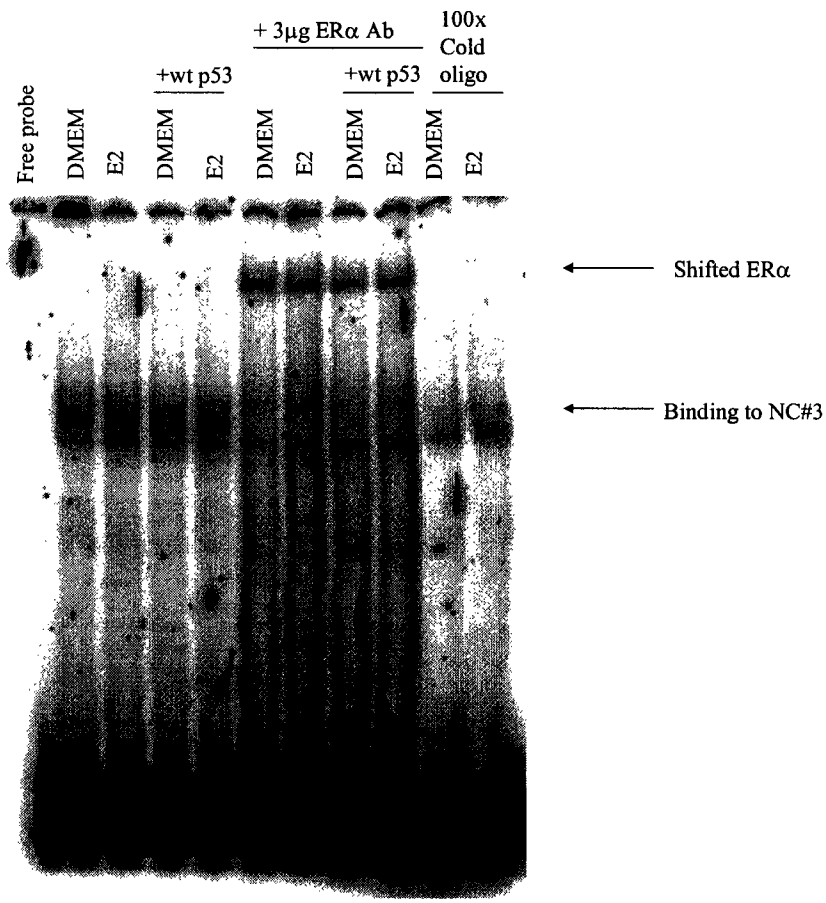


Figure 50. EMSA for ER-α was performed using nuclear extracts from MCF-7 cells transfected with pCMV-wtp53. Cells were either untreated or treated with 10nM estrogen for 24h.

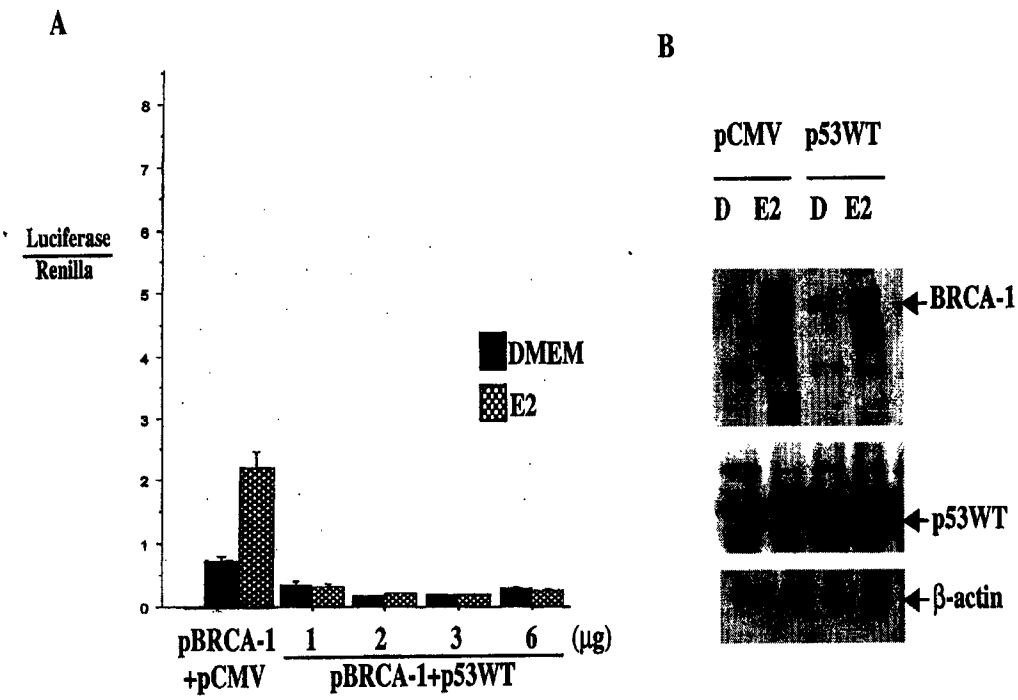


Figure 51. MCF-7 cells were transiently co-transfected with pGL3 BRCA-1 and either pCMV or pCMV-wtp53 and were harvested for luciferase analysis (A) or western analysis (B).

Overexpression of Mutant p53 Eliminates Estrogen-mediated Induction of BRCA-1 Promoter Activity Without Altering Basal Transcription

Because of published evidence that p53 can repress BRCA-1 via a negative feedback loop and that wild-type p53 can negatively regulate estrogen receptor signal transduction pathways, we characterized the response of pGL3 BRCA-1 to estrogen in an ER+ breast cancer cell line containing a mutant variant of p53 (ZR75.1, aa152 Pro→Leu). Interestingly, while E2 treatment was able to significantly induce an estrogen-responsive positive control vector containing three tandemly arranged EREs (p3xERE) in this cell line, pGL3 BRCA-1 showed no change in activity when subjected to identical treatment (Data not shown). These cumulative data suggested to us that a functional ER signaling pathway may be necessary, but not sufficient to lead to estrogen induction of BRCA-1. In order to determine if mutations in p53 could lead to loss of estrogen responsiveness of pGL3 BRCA-1, we transfected a dominant-negative acting mutant p53 vector driven by the CMV promoter (pCMV-p53mut175-gift from Jesse Martinez) in combination with pGL3 BRCA-1 into MCF-7 cells. As seen previously in MCF-7 cells, pGL3 BRCA-1 alone was induced by E2 treatment, but the cells which were co-transfected with the various pCMV-p53mut vectors did not show any increase in BRCA-1 promoter-reporter activity following treatment with estrogen (Figure 52). Moreover, when MCF-7 cells were transfected with pCMV-p53mut175 and treated with estrogen, analysis by western blotting did not detect an increase in endogenous BRCA-1 protein in contrast to the non-transfected or mock-transfected controls (Data not shown).

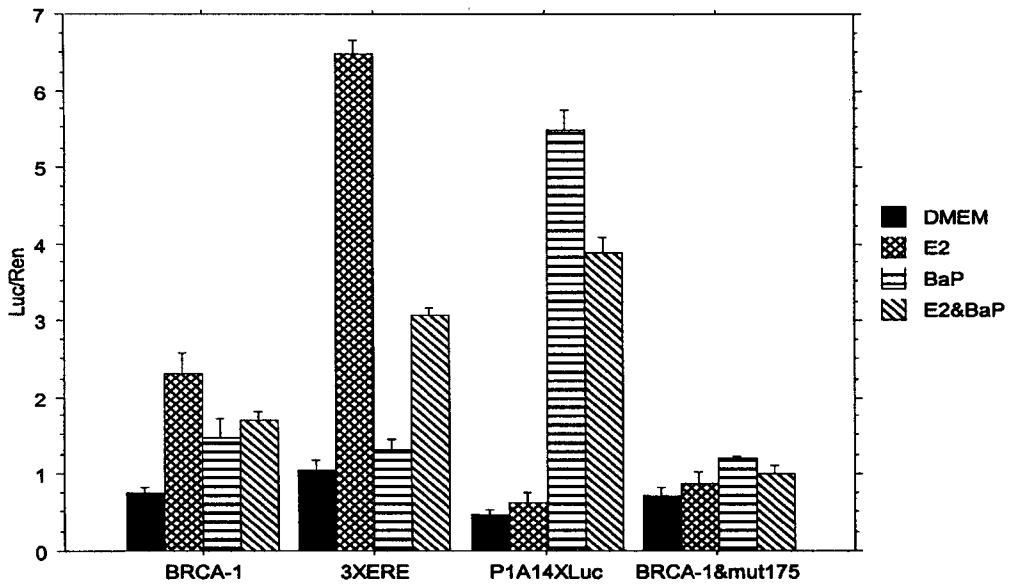


Figure 52. pGL3 BRCA-1 was co-transfected with pCMV-p53mut175 into MCF-7 cells. Transfected cells were treated for 24h with 10nM estrogen, 5μM B[a]P, or 10nM estrogen + 5μM B[a]P for 24h prior to harvesting for luciferase analysis.

In both western and luciferase assays, basal levels of BRCA-1 were unaffected by expression of the mutant variant of p53. DNA binding/gel shift analysis for ER-α using nuclear extracts from MCF-7 cells transfected with pCMV-p53mut175 yielded results consistent with luciferase and western data. Our data suggest that overexpression of pCMV-p53mut175 in MCF-7 cells treated with estrogen disrupts estrogen receptor-α interaction at the BRCA-1 ERU site in the BRCA-1 promoter while no reduction is seen in untreated cells (Fig. 53).

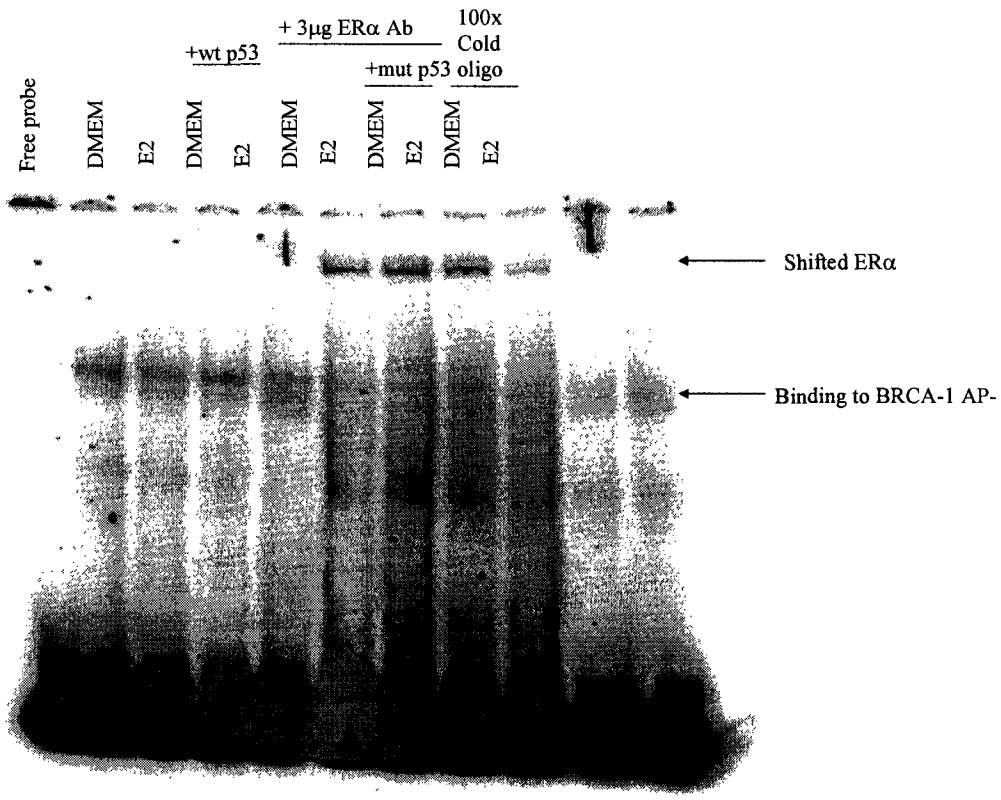


Figure 53. EMSA for ER-α was performed using nuclear extracts from MCF-7 cells transfected with pCMV-mutp53aa175. Cells were either untreated or treated with 10nM estrogen for 24h.

Both Wild-Type p53 and Estrogen Receptor-α are Required for BRCA-1 Induction by Estrogen in the HCT-116 Colon Cancer Cell Line

After determining that both ER-α and p53 were binding to the ERU site in the BRCA-1 promoter and that this site had functional significance as determined by site-specific mutagenesis/luciferase assay, we investigated the requirement for both ER-α and p53 on E2-mediated transcription of BRCA-1 by reconstituting a functional ER-α/p53/BRCA-1 signaling pathway in the HCT-116 colon cancer cell line. We used a variant of the HCT-116 colon cancer cell line (HCT-116-p53KO-gift from Bert Vogelstein) to study BRCA-1 promoter activity because this cell line lacks functional ER-α and is p53-null. As expected, E2 treatment failed to cause an increase in pGL3 BRCA-1 reporter activity in this cell line (Figure 54A). In order to determine if reconstituting a functional ER signaling pathway in these cells would restore BRCA-1 promoter sensitivity to estrogen, we transfected the HCT-116-p53KO cells with various amounts of ER-α (pHEO) and a wild-type p53 expression vector (pCMV-p53wt) alone or in combination along with pGL3 BRCA-1. Estrogen treatment of the HCT-116-p53KO cells co-transfected with only the ER-α (3μg) and pGL3 BRCA-1 vectors showed only a 1.2-fold induction (Figure 54). However, when we transfected 500ng of a vector containing the cDNA for wild-type p53 along with 3μg ER-α expression vector and pGL3 BRCA-1 into the HCT-116-p53KO cells and treated with E2, a 2.8-fold induction was seen. Additionally, the combination of 500ng wild-type p53 vector plus 3μg of the ER-α plasmid resulted in a lowering of basal (7-fold reduction) and estrogen-treated (3.2-fold reduction) pGL3

BRCA-1 reporter activity. By performing titration experiments in which various amounts of wild-type p53 and ER-α expression vectors were co-transfected into HCT-116-p53ko cells, we were able to determine that a ratio of 500ng of wild-type p53 plasmid to 3μg of ER-α expression plasmid allowed for maximal estrogen inducibility of pGL3 BRCA-1 in these cells (Figure 54A). Transfection of these amounts of wild-type p53 and ER-α vectors were not only able to establish a functional signaling pathway in this cell line, but also reduced pGL3 BRCA-1 relative luciferase activity to a level similar to that seen in MCF-7 cells. Transient transfection of a dominant-negative vector expressing mutant p53 (mut175 R→H) along with the ER-α vector into this cell line prevented estrogen induction of pGL3 BRCA-1 (Figure 54B).

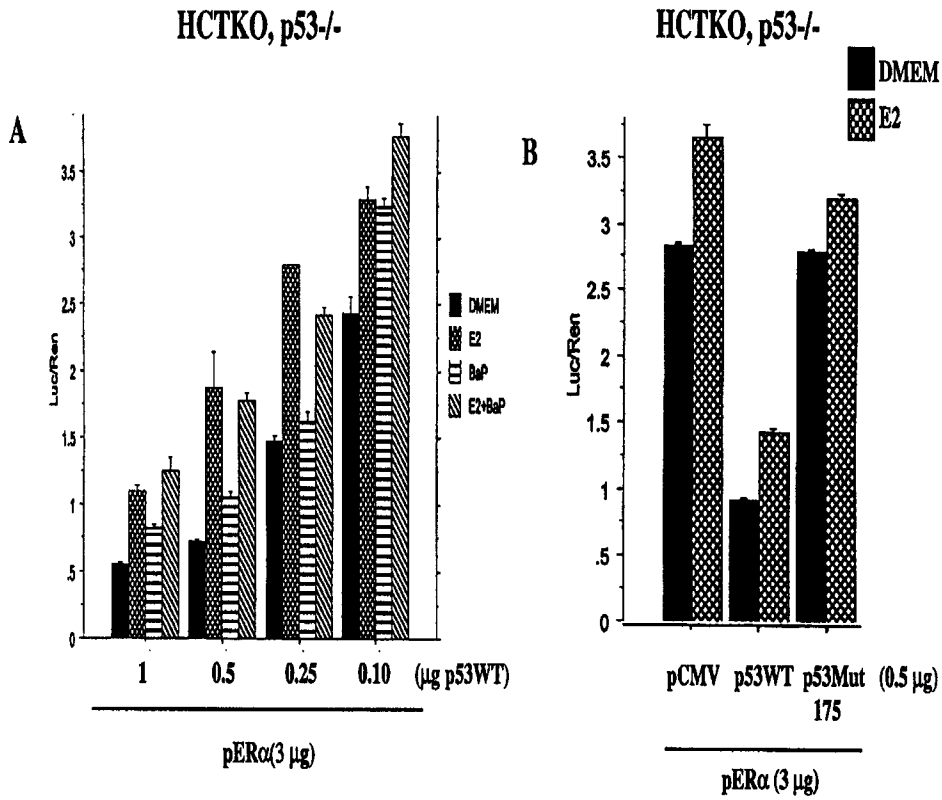


Figure 54. 3µg of pHEO + increasing amounts of pCMV-p53wt were co-transfected into HCT-116p53ko cells. Transfected cells were treated for 24h with 10nM estrogen, 5µM B[a]P, or 10nM estrogen + 5µM B[a]P for 24h prior to harvesting for luciferase analysis (A). Control vector (pCMV), 500ng of pCMV-p53wt or pCMV-p53mut175 were co-transfected along with pGL3 BRCA-1 into HCT-116p53ko cells ± 10nM estrogen for 24h (B).

Chapter 6: Summary and Conclusions

In this dissertation, we have investigated molecular interactions between endogenous and exogenous factors which play a role in regulation of BRCA-1 tumor suppressor gene expression in breast and colon cancer cells. The central hypothesis of this dissertation is listed below:

Central Hypothesis

Regulation of BRCA-1 expression by the polycyclic aromatic hydrocarbon B[a]P or its biotransformation product BPDE, and/or the steroid hormone 17-β-estradiol, is occuring via a mechanism requiring both the estrogen receptor-α and a functional p53 pathway. To achieve this goal, three specific aims were addressed:

1. Investigate the requirements for estrogen receptor-α and wild-type p53 protein on transcriptional regulation of BRCA-1 in breast and colon cancer cells exposed to the polycyclic aromatic hydrocarbon DNA damaging agent (Benzo[a]pyrene (B[a]P) or its metabolite BPDE).

2. Investigate the regulation of BRCA-1 promoter activity by estrogen in cells with varying estrogen receptor and p53 status.

3. Identify and characterize potential non-estrogen response element (non-ERE) sites contained within the BRCA-1 promoter which may contribute to the estrogen- and

B[a]P/BPDE-mediated responsiveness of BRCA-1 promoter activity via interaction between ER-α and p53.

A summary of the major results and conclusions from each specific aim are listed below:

Specific Aim #1: Summary of Results

1. The activity of a BRCA-1 promoter-reporter construct, pGL3 BRCA-1, is downregulated by B[a]P and BPDE treatment in ER-α+/p53 wt MCF-7 but not ER-α+ ZR75.1 breast cancer cells containing mutant p53.

2. Co-transfection of pGL3 BRCA-1 and either a dominant negative mutant p53 (mut aa 175) or HPV E6 expression vector into MCF-7 cells prevents reduction of promoter activity induced by B[a]P or BPDE. Overexpression of this variant of mutant p53 also inhibits B[a]P/BPDE-mediated loss of endogenous BRCA-1 mRNA and protein

3. pGL3 BRCA-1 activity is decreased by B[a]P in ER-α negative HeLa cells co-transfected with an ER-α expression vector. pGL3 BRCA-1 activity is not altered by B[a]P treatment in non-transfected HeLa cells.

4. The AhR ligand TCDD, which does not induce p53, has no effect on pGL3 BRCA-1 in MCF-7 cells.

Specific Aim #2: Summary of Results

1. A reporter plasmid driven by a 1.69 kb fragment of the BRCA-1 5' flanking region (pGL3-BRCA-1) is induced by estrogen in cells containing both wild-type p53 and the estrogen receptor-a (MCF-7) but not in cells lacking either (HeLa, HCT-116, HCT-116 p53ko).

2. Overexpression of wild-type p53 in ER+ MCF-7 cells represses both basal and estrogen-induced pGL3 BRCA-1 activity.

3. Overexpression of a dominant-negative p53 mutant (175 R→H) is able to prevent estrogen stimulation of pGL3 BRCA-1 without altering basal levels.

4. Transient expression of the ER-α in HeLa and HCT 116 cells or of ER-α and wild-type p53 in HCT 116 p53ko cells enables pGL3 BRCA-1 induction by estrogen. Transfection of various dominant-negative mutant p53 vectors with wild-type ER-α into HCT 116 p53ko cells prevents estrogen induction of pGL3 BRCA-1.

5. The level of estrogen-mediated induction of pGL3 BRCA-1 can be modulated by varying the ratio of wild-type p53 to estrogen receptor-α.

Specific Aim #3: Summary of Results

1. Both BRCA-1 AP-1 and ERU oligonucleotides contain *bona fide* transcription factor binding sites

2. Mutation of the AP-1-like domain in pGL3 BRCA-1 abrogates both basal and estrogen-induced BRCA-1 promoter activity in MCF-7 cells

3. Overexpression of dominant-negative c-Jun (Tam67) decreases pGL3 BRCA-1 basal and estrogen-induced activity in MCF-7 cells

4. Sp1, AP-1, and CREB family members bind to the BRCA-1 ERU

5. FosB shows greater binding and JunB less binding to the BRCA-1 ERU using extracts from estrogen treated vs untreated MCF-7 cells

6. Estrogen receptor-α is able to interact with the transcriptional complex assembling at the BRCA-1 ERU and higher levels of binding are seen in extracts from estrogen-treated vs untreated MCF-7 cells

7. Overexpression of wild-type p53 in MCF-7 cells reduces BRCA-1 promoter activity under basal and estrogen-induced conditions while overexpression of p53mut175 only reduces estrogen induction without altering basal levels.

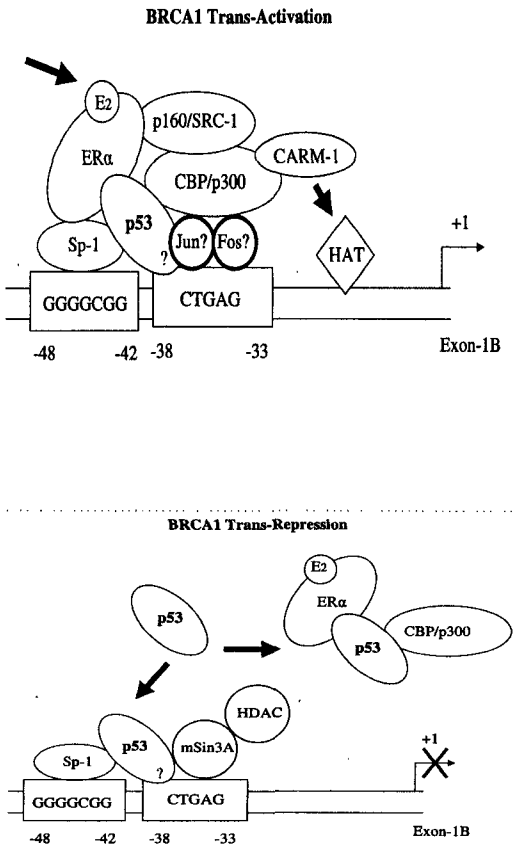
8. Overexpression of wild-type p53 in MCF-7 cells reduces ER-α binding to the BRCA-1 ERU under basal and estrogen-induced conditions while overexpression of p53mut175 disrupts ER-α binding only under estrogen-induced conditions.

Conclusions

Specific Aim #1: BRCA-1 promoter activity is reduced by B[a]P and BPDE, but not TCDD, in cells which express wild-type p53 and estrogen receptor-α.

Specific Aim#2: Estrogen receptor-α and wild-type p53 are required for induction of BRCA-1 promoter activity by estrogen.

Specific Aim#3: The estrogen receptor-α interacts with the BRCA-1 promoter at an Estrogen Response Unit (ERU) which binds Sp1, AP-1, and CREB family members.



Proposed model of transcriptional complex

Top: Basal levels of wild-type p53 lead to stabilization of the transcriptional complex and recruitment of co-activators when liganded estrogen receptor is present.

Bottom: High levels of wild-type p53 disrupt estrogen receptor from complex and lead to recruitment of co-repressors

Figure 55A (top) and B (bottom). Proposed models of BRCA-1 transactivation and transrepression.

In this dissertation, we have shown that regulation of BRCA-1 transcription by estrogen may require interaction between Sp1 and AP-1 family members with the estrogen receptor-α at a non-ERE site in the BRCA-1 promoter region. Our data suggests that exposure of MCF-7 breast cancer cells to estrogen stimulates transcription from a 1.69 kb fragment of the BRCA-1 5′ flanking region and this increase in transcription is paralleled by an increase in estrogen receptor-α interaction at the BRCA-1 promoter between –46 → -14 upstream of exon 1b. Additionally, while this region of the BRCA-1 promoter does not share homology with an estrogen response element (ERE), it is similar to consensus Sp1, AP-1, and CREB binding motifs (all > 0.7), all of which bind proteins known to interact with the estrogen receptor. We report that in both untreated and estrogen-treated MCF-7 cells, a transcriptional complex, which we have termed an "Estrogen Responsive Unit" (ERU), which can contain c-Jun, JunB, FosB, Fra-2, and CREB, forms at the same –46 → -14 region which binds ER-α. Additionally, either overexpression of a dominant-negative variant of c-Jun (Tam67) or mutation of the AP-1-like motif in pGL3 BRCA-1 abrogates BRCA-1 promoter-reporter activity in MCF-7 cells and reduces binding to the BRCA-1 ERU. Although it was surprising to us that we did not observe any difference in binding intensity of any of the AP-1 or CREB family members in untreated versus estrogen-treated cells, we believe that the assembly of the complex may be a time- and ligand-dependent event and we are currently investigating this hypothesis in our laboratory. Other studies have shown that constitutive assembly of a transcriptional complex in promoter regions may be necessary for maintenance of basal

transcription. Although E2 treatment of MCF-7 cells for 24 hours leads to an increase in ER-α at the BRCA-1 ERU, we did not see any difference in levels of any other transcription factors bound to the BRCA-1 ERU in basal or estrogen-treated cells. Interestingly, many different AP-1 proteins, including c-Jun, JunB, JunD, c-Fos, FosB, and Fra-2, were all found to have the ability to bind to the BRCA-1 ERU under conditions of treatment with estrogen. We have shown that in MCF-7 cells treated with 10nM E2 for 24h, c-Jun, JunB, FosB, and Fra-2 have the highest binding intensity to the BRCA-1 ERU, while JunD and c-Fos only bind weakly. We believe that since this region contains an element with high homology to an AP-1 site, it is possible for multiple Jun-Jun or Jun-Fos combinations to have the ability to assemble *in vitro* and the precise complement of factors assembling *in vivo* may depend on the ligand and length of treatment. The increase in BRCA-1 transcription induced by estrogen and its inhibition with Tamoxifen is likely a complex event that involves many mechanisms, including the recruitment of transcriptional co-activators and –repressors to the ER for modulation of the surrounding chromatin. In this dissertation, we present evidence that the transcriptional cofactor CBP/p300 can be found associating with the BRCA-1 ERU under basal and estrogen-induced conditions in extracts from MCF-7 cells. Agonists of the ER can result in the AF-2 domain of the ER protein becoming exposed due to a conformational change of the receptor leading to co-activator recruitment and acetylation of the surrounding histones. The AF-2 domain of the ER contains three LXXLL motifs (NR-boxes) which can bind co-activators such as members of the p160/SRC family (SRC-1, SRC-2/GRIP1, SRC-3) upon agonist binding (Nilsson, 2001). In addition to

interacting with the estrogen receptor, the p160/SRC family of co-activator proteins also has been shown to bind to CBP/p300 and CARM1, which respectively possess acetyltransferase and methyltransferase activity which may assist in chromatin/histone remodeling and increased transcription (Nilsson, 2001). Additional reports have found that other co-activators (TRAPs/DRIPs) may be acting as a scaffolding protein for tethering the ER to the transcription factors which are interacting directly with the DNA (Nilsson, 2001). In contrast, histone deacetylation mediated by the recruitment of co-repressors (N-CoR, SMRT, mSin3a, HDAC2, RIP140) to the ER by receptor antagonists may lead to transcriptional repression of ER-mediated gene expression (Huang, 2002) (Figure 55A, B). Recent investigations have suggested that reduction of co-repressor levels in breast cancer cells is associated with tamoxifen resistance (Lavinsky, 1998). Investigations have demonstrated that ER activity can be modulated through various ligand-dependent and –independent signal transduction pathways which lead to phosphorylation of the ER at specific serine and threonine residues (Nilsson, 2001). Second messenger pathways can be involved in the activation of ER by phosphorylation, and these include phosphorylation of Ser118 by MAP kinase pathways, Ser167 through the PI3K or Akt pathways, Ser 236 by activation of the PKA pathway, and Thr 311 by the p38 MAPK pathway (Lannigan, 2003, Lee, 2002). Phosphorylation of Thr 311 on the ER-α by the p38 MAPK pathway has been shown to be involved in transcriptional co-factor recruitment to the ER, while mutation of this residue has been associated with inhibition of the mitogenic effects of estrogen (Lee, 2002). Phosphorylation of the ER upon ligand binding also can occur at Ser104/106 directly by the CyclinA/CDK2

pathway, which may suggest one mechanism by which estrogen may potentiate some of its mitogenic effects (Lannigan, 2003). We are currently investigating the role of recruitment of transcriptional co-factors to the BRCA-1 ERU under conditions of various p53 levels and mutational status, as well as in the presence of various ER ligands and therapeutic SERMs on regulation of BRCA-1 transcription.

One of the controversies surrounding regulation of BRCA-1 expression is whether or not the increase in BRCA-1 expression induced by estrogens is direct or is possibly an artifact of increased mitogenesis. To address this, we transiently transfected pGL3 BRCA-1 into ER-α negative HCT116 colon cancer cells and treated with B[a]P. Although B[a]P treatment inhibited HCT-116 proliferation, BRCA-1 message and protein levels were increased which suggests that upregulation of BRCA-1, as least by B[a]P, is not due to increased cell proliferation (Data not shown). Additionally, we have previously reported that co-treatment of MCF-7 cells with 10nM E2 and 5μM B[a]P prevented an accumulation of BRCA-1 message although cell number continued to increase (Jeffy, 1999). Taken together, these results suggest that BRCA-1 transcription induced by estrogen is not necessarily always linked to increased mitogenesis and likely occurs through ER-α/transcription factor interactions at the BRCA-1 promoter. Since BRCA-1 transcription is increased by estrogen, and increased BRCA-1 has been associated with an increase in repair of oxidative DNA damage, some which may be induced by carcinogenic estrogen metabolites, it would appear that E2 would have a protective effect against breast cancer (Gowen, 1998, Romagnolo, 1998). The potential for estrogen to act as a risk factor may be dependent upon the mutational status of key

tumor suppressor genes or oncogenes whose products may contribute to regulation of BRCA-1 expression, contained within the specific cells exposed to estrogen. In a normal, uninitiated cell, estrogen treatment may have a protective effect by increasing BRCA-1 protein levels and consequently, its DNA repair activity. In contrast, in mammary epithelial cells which have accumulated mutations in key genes or have become malignantly transformed and have low levels of BRCA-1, exposure to estrogen can lead to increased proliferation of these cells with reduced DNA repair capacity, leading to tumor progression.

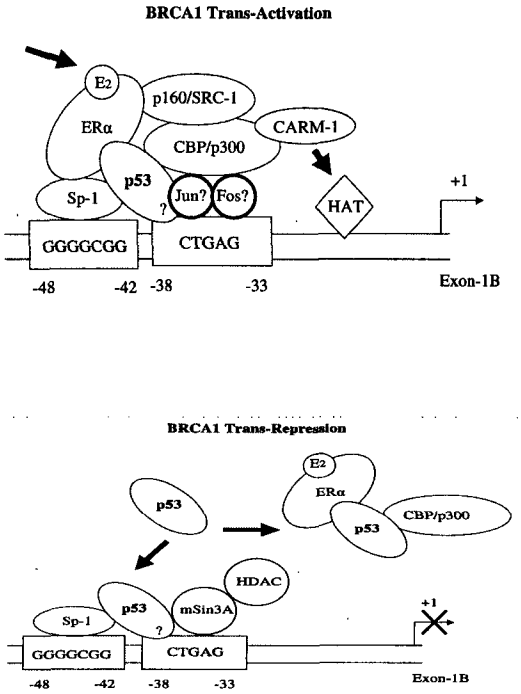
In this dissertation, we have demonstrated that normal regulation of the BRCA-1 promoter by estrogen may require an interaction between Sp1, AP-1, and estrogen receptor-α proteins. Our data suggests that ER-α is interacting with a complex of transcription factors at a non-ERE binding site in the BRCA-1 5′ regulatory region (BRCA-1 ERU) and that the exact complement of Sp and AP-1 family members in this complex may be dependent on the type of ER ligand. Taken together, our data suggests that deregulation of the wild type BRCA-1 response to estrogen can occur when p53 mutations are present or when cells lose estrogen receptor function. We believe that these findings may have significant implications in regards to breast cancer therapeutics as well as understanding mechanisms underlying the etiology of sporadic breast cancer in women who do not harbor BRCA-1 mutations.

In this dissertation, we also have shown that regulation of BRCA-1 transcription by estrogen may require both functional estrogen receptor and p53 pathways. We are currently investigating the role of recruitment of transcriptional co-factors to the BRCA-1

ERU under conditions of various p53 levels and mutational status, as well as in the presence of various ER ligands and therapeutic SERMs on regulation of BRCA-1 transcription. This paper has many significant implications for understanding the etiology and therapy of sporadic breast cancers. One of the questions arising from this report is the role of mutant p53 in the regulation of BRCA-1 transcription by estrogen. Since p53 has been shown to be mutated in over 30% of sporadic breast tumors, we believe that the fact that various mutant p53 proteins can prevent BRCA-1 induction by estrogen is highly significant. In our laboratory, we are currently investigating some of the mechanisms that may be underlying this phenomenon including the difference in co-factor recruitment to the BRCA-1 ERU by either wild-type or mutant p53. In addition, we are examining the physical interaction between the ER-α and wild-type p53 under various conditions to ascertain the precise mechanism of the disruption of the ER-α from the BRCA-1 ERU. We believe that one possible explanation for why overexpression of wild-type but not mutant p53 can disrupt basal BRCA-1 transcription is that high levels of wild-type, low levels of wild-type, and any mutant p53 can all recruit different sets of transcriptional co-factors for modulation of ERU activity.

One of the controversies surrounding regulation of BRCA-1 expression is whether or not the increase in BRCA-1 expression under various treatments was direct or simply an artifact of increased mitogenesis. To address this, we transiently transfected pGL3 BRCA-1 into ER-α negative/p53wt HCT116 colon cancer cells and treated with B[a]P. Although B[a]P treatment inhibited HCT-116 proliferation, BRCA-1 message and protein levels were increased which suggests that upregulation of BRCA-1, as least by B[a]P, is

not due to increased cell proliferation (Data not shown). Additionally, we have previously reported that co-treatment of MCF-7 cells with 10nM E2 and We suggest that ER-α is interacting with a complex of transcription factors at a non-ERE binding site in the BRCA-1 5′ regulatory region (BRCA-1 ERU) and that overexpression of wild-type p53 can disrupt the ER-α physical interaction with these factors at a specific site in the BRCA-1 promoter. Additionally, we have shown that basal levels of wild-type p53 may be required for estrogen induction of BRCA-1 and overexpression of wild-type p53 can repress both basal and estrogen-induced BRCA-1. Taken together, our data suggests that deregulation of the wild type BRCA-1 response to estrogen can occur when p53 mutations are present or when cells lose estrogen receptor function. We believe that these findings may have significant implications in regards to breast cancer therapeutics as well as understanding mechanisms underlying the etiology of sporadic breast cancer in women who do not harbor BRCA-1 mutations.



Proposed model of transcriptional complex

Top: Basal levels of wild-type p53 lead to stabilization of the transcriptional complex and recruitment of co-activators when liganded estrogen receptor is present.

Bottom: High levels of wild-type p53 disrupt estrogen receptor from complex and lead to recruitment of co-repressors

Figure 55A, B

Future Directions

1. Investigate the role of phosphorylation of transcription factors (c-jun, CREB, ER-α) binding to the BRCA-1 ERU under treatment with various ER ligands.

2. Examine the activation of the transcriptional complex assembled at the BRCA-1 ERU through various estrogen-activated signal transduction pathways.

3. Determine the functional role of transcriptional co-regulatory proteins (co-activators/repressors) at the BRCA-1 ERU and investigate effects of estrogen treatment on modulation of local chromatin acetylation and de-acetylation as a mechanism for transcriptional enhancement/repression.

4. Further investigate BRCA-1 and p53 functional interactions in a mouse mammary xenograft model system in which combinations of ER-α-, p53-, and BRCA-1-null mammary epithelial cells will be transplanted into the cleared mammary fat pad of wild-type mice.

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