COMPARATIVE ACTIVATION OF ESTROGEN RECEPTOR α (ERα) BY ENDOCRINE DISRUPTORS

A Dissertation

by

FEI WU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Biochemistry

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Approved by:

Chair of Committee,	Stephen Safe
Committee Members,	James Sacchettini
	Gary Kunkel
	Robert Burghardt
Head of Department,	Gregory Reinhart

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ABSTRACT

Comparative Activation of Estrogen Receptor α (ERα) by Endocrine Disruptors.

(December 2007)

Fei Wu, B.S., Fudan University

Chair of Advisory Committee: Dr. Stephen Safe

Estrogen receptor α (ERα) is a ligand activated transcription factor. Many widely used synthetic compounds and natural chemicals can activate ERα. The compounds investigated in this study include 17β-estradiol (E2), diethylstilbestrol (DES), antiestrogens ICI 182,780, 4-hydroxytamoxifen, the phytoestrogen resveratrol, and the xenoestrogens bisphenol A (BPA), nonylphenol (NP), octylphenol (OP), endosulfan, kepone, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) and 2,3,4,5-tetrachlorobiphenylol-4-ol (HO-PCB-Cl_4). With the exception of the antiestrogens, all the compounds induced transactivation in MCF-7 or MDA-MB-231 cells transfected with wild-type ERα and a construct (pERE_3) containing three tandem estrogen responsive elements (EREs) linked to a luciferase gene. However, these compounds differentially activated C-terminal deletion mutants of ERα. For example, neither E2 nor DES induced transactivation in MCF-7 transfected with ERα(1-537) due to partial deletion of helix 12 of ERα; however, OP, NP, resveratrol, kepone and HPTE induced this ERα mutant, demonstrating that the estrogenic activity of these synthetic compounds do not require activation function 2 (AF-2) of ERα.

This study also investigated the effects of xenoestrogens on activation of reporter gene activity in MCF-7 and MDA-MB-231 cells transfected with a construct (p$Sp1_3$) containing three tandem GC-rich Sp binding sites linked to the luciferase gene. In MCF-7 cells, antiestrogen-induced activation of ERα/Sp1

required the zinc finger motifs of ERα, whereas activation by estrogen and some xenoestrogens activation, such as endosulfan, NP and OP required the H12 of ERα. In contrast, xenoestrogens, such as HPTE, BPA, kepone and HO-PCB-Cl_4, significantly induced transactivation of all four ERα deletion mutants tested in this study. Moreover, RNA interference assays demonstrated structure-dependent differences in activation of ERα/Sp1, ERα/Sp3 and ERα/Sp4.

The *in vivo* activities of E2, ICI 182,780, BPA and NP were further investigated in a transgenic mouse model containing $pSp1_3$ transgene. All the compounds induced luciferase activity in the mouse uterus; however activities observed in the penis and testis of male and stomach of both male and female mice were structure-dependent,.

These results demonstrate that various ER ligands differentially activate ERα in breast cancer cells and transgenic mice, and their activities are dependent on ERα variants, promoter-, cell-context and selective use of different Sp proteins, suggesting these structurally diverse compounds are selective ER modulators (SERMs).

DEDICATION

To

My husband Jiajun Lu
My wonderful parents Zhengang Wu and Dongmei Liu
My sister and brother-in-law Jing Wu and Qiang Liu
My mother-in-law Aifeng Chen
For their unconditional love, support and guidance

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CHAPTER I

INTRODUCTION

1.1. Estrogen Receptor

1.1.1. Overview of Nuclear Receptor

The estrogen receptor (ER) is a member of the nuclear receptor (NR) superfamily, which is a class of ligand binding intracellular transcription factors. NRs play an important role in embryonic development, reproduction, cellular homeostasis, metabolism and other biological processes [3]. In general, NRs consist of conserved structural modules that behave independently and based on their sequence alignment NRs can be divided into six classes [4]. Table 1 outlines different receptors within each class along with their ligands and response elements. Receptors in each class bind different ligands and exert their functions in a diverse manner. Unbound steroid hormone receptors are inactive and associated with heat shock proteins. Upon ligand binding, these receptors dissociate from heat shock proteins and form nuclear complexes that bind their cognate specific response elements in target gene promoters. The protein-DNA interactions are accompanied by recruitment of other transcription regulators and gene transcription is initiated. In contrast, thyroid hormone receptors bind DNA as heterodimers, usually with retinoid X receptor (RXR). Orphan receptors are a class of NRs that have no known endogenous ligands, and include the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [5, 6]. Orphan receptors exhibit multiple functions; for example PXR and CAR are xenobiotic sensors that induce the expression of cytochrome p450 enzymes, whereas dosage-sensitive sex reversal adrenal hypoplasia critical region on chromosome X gene 1 (DAX-1) [7] and short heterodimer partner (SHP) [8, 9] are NRs that inhibit expression of multiple genes.

This dissertation follows the style and format of FEBS Letters.

Table 1 Human nuclear receptor families of transcription modulators. Modified from [2-4]

Subfamily	Group	Receptor	Subtypes	Denomination	Ligand	RE	Dimer
I. Thyroid hormone receptor-like	TR	TR	α, β	Thyroid hormone receptor	Thyroid hormone (T3)	Pal, DR-4, IP	H
	RAR	RAR	α, β, γ	Retinoic acid receptor	Vitamin A and related compounds	DR-2, DR-5, Pal, IP	H
	PPAR	PPAR	α, β/δ, γ	Peroxisome proiferator-activated receptor	Benzotriene B4, thiazolidinediones (TZDS), polyunsaturated fatty acids	DR-1	H
	Rev-erb	Rev-erb	α, β	Reverse Erb	Orphan	DR-2, hemisite	M, D
	ROR	ROR	α, β, γ	Retinoid-related orphan receptor	Orphan	Hemisite	M
	LXR-like	LXR	α, β	Liver X receptor	Oxysterols	DR-4	H
		FXR		Farnesoid X receptor	Bile acids, fexaramine, lanosterol	DR-4, IR-1	H
	VDR-like	VDR		Vitamin D receptor	1-25-dihydroxy vitamin D3	DR-3, IP-9	H
		PXR		Pregnane X- receptor	Pregnanes; xenobiotics	DR-3	H
		CAR	α, β	Constitutive androstane receptor	Androstanes; 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene; xenobiotics	DR-5	H
II Retinoid X Receptor-like	HNF-4	HNF-4	α, γ	Hepatocyte nuclear factor-4	Fatty acyl-CoA thioesters	DR-1, DR-2	D
	RXR	RXR	α, β, γ	Retinoid X receptor	9-cis-retinoic acid	Pal, DR-1	D
	TR	TR	2, 4	Testicular receptor	Orphan	DR-1 to 5	D, H
	PNR/ TLP	TLX		Human homologue of Drosophila tailless gene	Orphan	DR-1, hemisite	M, D
		PNR		Photoreceptor-specific nuclear receptor	Orphan	DR-1, hemisite	M, D
	COUP/ EAR	COUP-TF	α, β, γ	Chicken ovalbumin upstream promoter-	Orphan	Pal, DR-5	D, H

Table 1 (Continued)

Subfamily	Group	Receptor	Subtypes	Denomination	Ligand	RE	Dimer
				transcription factor			
		EAR2		EARA-related 2	Orphan		
III Steroid hormone receptors	ER	ER	α, β	Estrogen receptor	Estrogen	Pal	D
	ERR	ERR	α, β, γ	Estrogen related receptor	Orphan	Pal, hemisite	M, D
	3-ketosteroid receptor	GR		Glucocorticoid receptor	Cortisol	Pal	D
		MR		Mineralocorticoid receptor	Aldosterone		
		PR		Progesterone receptor	Progesterone	Pal	D
		AR		Androgen receptor	Testosterone, androgen	Pal	D
IV Nerve growth factor IB-like	NGFIB/ NURR1/ NOR1	NGFIB	α, β, γ	Nerve growth factor IB	Orphan	Pal, DR-5	M, D, H
		NURR1		Nuclear receptor related 1	Orphan		
		NOR1		Neuron-derived orphan receptor 1	Orphan		
V Steroidogenic factor-like	SF1/ LRH1	SF1	α, β	Steroidogenic factor 1	Oxysterols	Hemisite	M
		LRH1		Liver receptor homolog 1	Orphan		
VI Germ cell nuclear factor-like	GCN1	GCN1		Germ cell nuclear factor	Orphan	DR-0	D
Miscellaneous	DAX/ SHP	DAX-1		Dosage-sensitive sex reversal 1	Orphan		
		SHP		Short heterodimer partner	Orphan		
	2DBD-NR	2DBD-NR		Nuclear receptor with two DBD	Orphan		

Abbreviations:

M, monermer; D, homodimer; H, heterodimer; DR, direct repeart; Pal, palindrome; IP, inverted palindrome

NRs are characterized by the similarities of their common structural domains. A typical NR contains a variable N-terminal region (A/B), a highly conserved and centrally located DNA-binding domain (DBD) (C), a variable and flexible hinge region (D), a moderately conserved C-terminal ligand binding domain (LBD) (E), and a variable C-terminal region (F) (Fig. 1). The DBD consists of two highly conserved cysteine-rich zinc finger motifs and is the most conserved region. These two zinc finger motifs spanning ~60-70 amino acids are common to the entire NR family with the exception of two divergent members DAX-1 [5] and SHP [6, 7]. The LBD has several critical functions. First, the LBD contains an interior binding pocket specific for its cognate ligand. Second, the LBD contains activation function 2 (AF-2), which is ligand-dependent and is necessary for recruiting coregulatory proteins. Finally, the LBD is also a critical factor in the formation of stable NR dimers that bind tightly to their specific DNA sequences

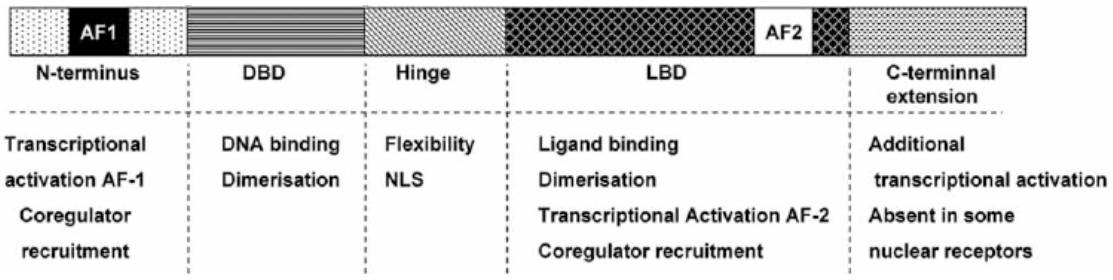

Figure 1. Schematic layout of nuclear receptor structure [1, 8, 9].

termed response elements (REs) [10]. REs consist of two copies of a PuGGTCA core sequences and REs can be palindromic, direct repeats, inverted repeats or hemisites [11]. There are also a large number of gene promoters that do not

have REs, but are regulated by NRs through DNA-independent interactions, suggesting there are multiple NR-mediated transcriptional regulation pathways [12, 13]. Most NRs contain another activation function domain (AF-1) in the N-terminal region. Unlike AF-2 which is embedded within the LBD and activated upon ligand binding, AF-1 is a ligand-independent activation domain and shares less structure conservation (<15%) across the NR superfamily and subfamilies. The lack of sequence homology in AF-1 may explain how closely related steroid receptors can bind to similar REs *in vitro*, yet differentially regulate gene promoters containing the same REs *in vivo*. Even though the AF-1 function is a ligand-independent transcriptional activator, it can also functionally synergize with AF-2 [14].

The high resolution structural analysis of isolated NR domains (e.g. core DBD and LBD) has provided important insights on their properties. Nevertheless, these receptors function as complete proteins. Studies of specific mutations suggest that in the holoreceptors, intramolecular domain-domain interactions are important for complete function. Evidence shows that different regions of NRs cooperate during ligand and DNA binding. Studies on the PR have shown that binding to REs results in allosteric communication between the DBD and N-terminal regions, and the DBD stabilizes the N-terminal structure and facilitates binding with coregulatory proteins [14, 15]. Agonist-induced N- and C-interdomain interactions have been reported for the ER [16], AR [17], and PR [18], but not for the GR [19]. However, it is still unclear how each domain of the receptor interacts to generate holoprotein function

Activities of NRs can be regulated by at least three other mechanisms. Ligand-independent transcriptional activity has also been reported for several nuclear receptors which is regulated by covalent modifications including phosphorylation, acetylation and ubiquitination. Secondly, cross talk between NRs and other transcription factors can also modulate NR functions and this

may contribute to their complex roles in cancer. For example, enhanced insulin-like growth factor (IGF)/ER cross talk promotes breast cancer progression [20]. Finally, some NR are also involved in nongenomic pathways that occurs within a few minutes after addition of ligand.

1.1.2. Estrogen Receptor (ER)

Estrogens induce their responses through binding the ER, which is a ligand-dependent transcription factor. ERα was first identified in the 1960s [21-24], and was shown to be a ligand-activated transcription factor [25]; ERα was cloned in 1986 [26-28]. For a long time it was believed that only a single form of ER existed. However, in 1996, multiple laboratories independently discovered a second type of ER, namely ERβ [29-31]. ERα and ERβ are encoded by two different genes and gene ablation studies in mice show that two subtypes have redundant and distinct functions.

1.1.2.1. Physiological Functions

Estrogens are steroid hormones that have profound effects on physiological processes in mammals, including but not limited to reproduction, cardiovascular health, bone integrity, adipogenesis, and behavior [32]. Initially no loss-of-function mutations of the ER gene were observed in normal mammalian tissue. Therefore it is surprising that disruption of ERα is not lethal [33], since it was generally assumed that ER was essential for survival, fertility, and female sexual differentiation and development and any disruption in the pathway of estrogen synthesis and/or action would be lethal [34]. ERα knock-out (αERKO) mice survive and do not exhibit any abnormal phenotype until they are adults. Adult female αERKO mice exhibit complete infertility and have no breast tissue development, indicating the important role of ERα for reproductive capability and breast development. ERβ knock-out (βERKO) mice are also viable [35] but exhibit unique phenotypes compared to αERKO mice, indicating the different

physiological functions of the two ER subtypes. βERKO females are fertile and exhibit normal sexual behavior, but have fewer and smaller litters than wild-type mice due to fewer corpora lutea. They have normal breast development and also lactate normally. Therefore, ERβ is essential for normal ovulation efficiency, but is not essential for female sexual differentiation, mammary gland development, fertility or lactation [35]. During ovarian cancer development, there is a selective ERβ loss in cysts and ovarian carcinomas [36], further indicating the predominant role of ERβ in ovarian function. Female double knock-out of both ERα and ERβ (αβERKO) mice [37] exhibit normal reproductive tract development but are infertile. The αβERKO female mouse uterus is similar to that observed in αERKO mice which show hypoplastic uteri and no response to estrogen treatment. The αβERKO mammary gland phenotype resembles that of αERKO females, indicating the predominant role of ERα in mammary tissue. However, αβERKO ovaries exhibit a unique sex reversal phenotype, which resembles the male testis seminiferous tubules and expresses the testis specific genes: Sox9 and SGP-2. Therefore, both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary.

Estrogen receptors not only play an important role in females, but also have significant functions in male reproductive tissues. Male αERKO mice have decreased fertility and low sperm counts, low testis weight and decreased sperm motility [33, 38], indicating that ERα is required for male fertility and that the absence of ERα is detrimental to spermatogenesis, sperm function, and mating performance. Male βERKO mice are fertile and have normal sexual behavior [35, 39]. Therefore, only ERα is important for male reproductive activity and sexual differentiation. αβERKO males exhibit a complete disruption of sexual behavior with an 80% reduction in sperm counts [37, 39, 40].

Estrogens also have important roles in maintenance of bone tissue and in the cardiovascular system, and these responses are ER-dependent. Female αERKO mice have shorter length of the appendicular skeleton and normal axial skeletal growth. However, males have lower bone density [41]. Female βERKO mice have higher bone density whereas males display no bone abnormalities compared to wild type animals [42]. Low levels of estrogen significantly suppress the carotid arterial response to injury in ovariectomized mice [43]. There is a high expression of ERβ in the cardiovascular system and it has been suggested that ERα mediates the major cardiovascular effects of estrogens [44, 45]. However, neither αERKO nor βERKO mice have gross or histologic cardiovascular abnormalities [33, 46] and knockout of ERα or ERβ alone does not affect estrogen-dependent protection against vascular injury, indicating that ERα and ERβ may play redundant roles in estrogen-mediated cardioprotection [47, 48]. Furthermore, estrogen and ERs may also play a significant role in carcinogenesis, especially in the breast and female reproductive tract.

1.1.2.2. Coded Genes and Protein Structures

ERα and ERβ are not isoforms, but distinct proteins encoded by two different genes, located on different chromosomes. The full length human ERα has 595 amino acids with an approximate molecular mass of 66 kDa. The gene is approximately 6.3 kb with 9 exons, and located at chromosome 6q [49]. There are multiple promoter and regulatory regions in the 5' UTR of the human ERα, but there is a single open reading frame [50, 51]. Numerous studies have reported the discovery and characterization of naturally occurring splicing variants and mutations of the ERα mRNA [52, 53], which is expressed in many tissues, including the breast, endometrium, and pituitary tissues, as well as smooth muscle cells and peripheral blood mononuclear cells. hERα36 variant lacks both transcriptional activation functions of full length ERα, but retains its

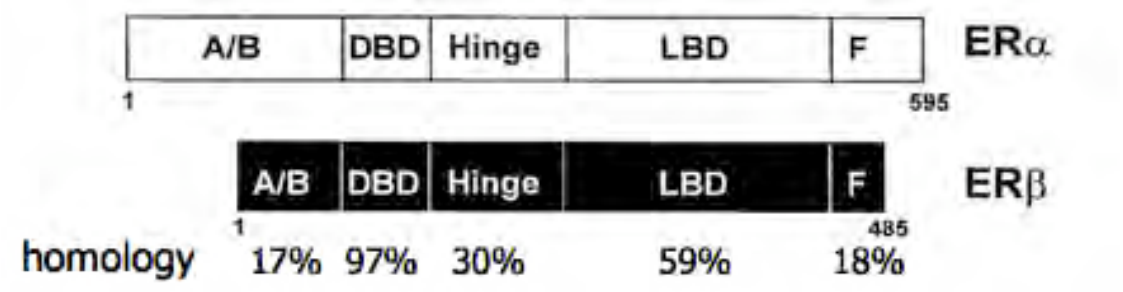

Figure 2. Schematic structure and homology of ERα and ERβ [1, 64].

DBD, partial dimerization and LBD domains [54]. This variant is overexpressed in MDA-MB-231 and MDA-MB-436 ER negative breast cancer cells and predominantly associated with the plasma membrane where it activates estrogen-dependent and antiestrogen-dependent ER nongenomic pathways and stimulates cell proliferation [55]. The human full length ERβ has 530 amino acids with molecular mass of 54 kDa. The major differences in size between the two forms of ER are due to the truncated N-terminal region of ERβ, which has a much shorter and weaker A/B domain than ERα (Fig. 2). This gene is mapped at chromosome 14q and the translated exons span approximately 40 kb [56]. A variety of ERβ mRNA isoforms have been identified, including alternative 3'-exons (ERβ2, ERβ3, ERβ4, ERβ5) and over 10 exon deletion ERβ variant mRNAs with deletion of single or multiple exons (e.g. ERβΔ2, ERβΔ5/6) [53, 56] [57]. Some of these mRNA isoforms code for ERβ proteins with impaired ligand or DNA binding or altered cofactor interaction surfaces [58-60]. A large number of naturally occurring splicing variants of both ERα and ERβ have been identified in cancer tissues, including breast and endometrial cancer. For example, deletion ERβ variants ERβΔ5 and ERβΔ6 were markedly (80%) decreased in tumor tissues compared to matched normal tissues [61]. However, several studies have been unable to demonstrate differences in ERβ variant

expression levels in diseased tissues and normal controls, suggesting that these variants may also play an important role in normal physiological processes [62, 63].

1.1.2.3. Tissue Distribution

The most significant difference between the two ER subtypes lies in their tissue distribution. ERα has a broad expression pattern, whereas ERβ exhibits more tissue-specific expression patterns. In humans, ERα mRNA is predominantly expressed in the uterus and liver [65-67], whereas the highest ERβ mRNA levels are found in colon, brain, prostate and small intestine [68]. High expression has also been observed in lung, spleen, pituitary gland, blood leukocytes, bone marrow, colon, testis, and ovary [56]. An increasing number of studies have reported discrepancies in expression patterns of the two ER subtypes among different species [66, 69]. For example, ERβ mRNA is detected in the pituitary of the rat [70] and human [71], but not in mouse [66]. Even though ERα and ERβ are expressed in some of the same tissues, including mammary gland, brain, testis, prostate, kidney, heart, and skeletal muscle, for the most part they do not exist in the same cell type. For example, ERα and ERβ are localized in the stroma and epithelium in rat prostate respectively [29].

1.1.2.4. Transcriptional Activity

ERα and ERβ have different transcriptional activities which are dependent on ligand structure, promoter context and cell types. ERα and ERβ have opposite actions on cyclin D1 regulation [36]. ERβ down-regulates cyclin D1 gene expression, whereas ERα increases cyclin D1 levels. ERβ may negatively regulate cell proliferation and have a protective role in prostate, breast, ovarian and colon [72-75]. In the prostate, loss of ERβ results in hyperplasia of the prostatic epithelium [76]. Induction of apoptosis by ERβ contributes to the

decreased proliferation of ovarian cancer cells [77]. Therefore, ERβ plays a proapoptotic role in ovarian cancer development. The βERKO mouse uterus exhibits increased cell proliferation and an exaggerated response to E2 [78] and therefore loss of ERβ enhances ERα-mediated proliferation of hormone-dependent cancer cells. ERβ exhibits reduced transactivation in most cells compared with ERα and AF-1 may play an important role in their different transcriptional activities. ERα/β chimeric proteins show similar transcriptional activity with its AF-1 origin. When both proteins are coexpressed in selective tissues, they can form functional heterodimers. The order of DNA binding affinity is ERα homodimers ≈ ERα/β heterodimers > ERβ homodimers [79]. The role that heterodimers play in estrogen signaling, specifically in mediating the antagonistic effects of ERβ on ERα transcriptional activity is unclear.

The following sections will primarily focus on the properties and functions of ERα.

1.1.3. Structure of DBD and LBD of ERα

The DBD (a.a.180-a.a. 262 of ERα) is the most highly conserved domain among members of the nuclear receptor superfamily. It consists of two zinc fingers each of which has four cysteines tetrahedrally coordinated to a bound zinc ion. Three key amino acids in the so-called P box located at the C-terminal side of the first zinc finger of ERα play a key role in distinguishing between ERE and GRE [80], and largely determine the specificity of ERα binding to an ERE. Results of nuclear magnetic resonance (NMR) imaging and X-ray crystallography [6, 81] show that the P box of each ERα binds to the ERE at the DNA major groove. The D-box in the second zinc finger acts as the dimer interface and has the nuclear localization signal [82, 83]. The DBD alone cannot form an ERα dimer, but requires association with a palindromic ERE [81]. The interaction of one ERα DBD monomer to one of two ERE half-sites facilitates binding of the second

monomer to the second half-site. Both the RE spacer and the sequence are responsible for stabilizing the ERα interactions. DNase I protection and protease sensitivity assays show that the structure of a RE can modulate ERα structure and influence its activity. Therefore the RE is also an allosteric modulator of ERα [84, 85].

The E domain is the largest region of the receptor (Fig. 1) and it contains overlapping domains for ligand binding, transcriptional activation (AF-2), receptor dimerization and heat shock protein binding [86-88]. The LBD is wedge-shaped and folded into a three-layered sandwich composed of 12 α-helices (H1-H12) and a single β sheet (S1/S2) [6, 81, 89] [90, 91]. Hormone recognition is achieved through a combination of specific hydrogen bonds and van der Waals interactions that involve highly conserved residues, and this facilitates interaction of the non-polar estrogen ligand in the binding cavity. The crystal structures of the LBD of ERα bound to 17β-estradiol (E2), the selective antagonists raloxifene and tamoxifen, the pure antagonist ICI and the synthetic agonist DES show that agonists and antagonists all bind to the same site within the core of the LBD but induce distinct conformations of the LBD [92]. The combination of the specific polar and non-polar interactions accounts for the ability for ERα to selectively recognize a wide range of structurally diverse ligands with high affinity. The ligand binding site is formed by residues from H3, H6-H8 and the preceding loop, H11, H12 and the S1/S2 hairpin. Even though the ERα agonist and antagonist induce different responses, the bound LBD surface residues are similar and none of the ligands directly contact H12. Therefore, the major effect of the LBD conformation on its transcriptional activity is from the orientation of H12, which is highly sensitive to the nature of the bound ligand. In the agonist bound LBD complex, H12 seals the ligand cavity and provides the hydrophobic environment for AF-2 function. This promotes recruitment of coactivators and receptor interactions with the special motifs of coactivators [93]. While in the antagonist bound LBD complex, H12 rotates 120˚ and occupies the binding site

for coactivators [91]. This suggests a mechanism for antagonism in which H12 and the coactivator compete for a common binding site. Different H12 position of antagonist bound ERα can be induced by three classes of antagonists (Fig. 3) [90]. The first class constitutes a series of synthetic compounds, such as raloxifene and 4-hydroxytamoxifen which are characterized by a hydrophobic core that mimics E2 with an extended side chain of varying length. The phenolic core fits into the hydrophobic cavity, and the bulky side chain is too large to be contained within the cavity and protrudes between H3 and H11. As a result, H12 is unable to bind across the LBD in the agonist orientation. And the coactivator binding sites on H12 are blocked and gene transcription is not initiated. Another class of ERα antagonists is typified by the phytoestrogen genistein. The difference between genistein and raloxifene is that genistein's smaller size without a bulky tail allows it to be buried within the ligand binding cavity. Genistein fails to make appropriate contacts within the binding cavity. H12 adopts a position shifted by 25˚ from the antagonist conformation observed in the raloxifene complex [91]. The third class of ERα antagonists includes ICI 182,780 which contains a bulky tail, and is unable to fit in the ERα binding pocket and inactivate AF1 or AF2. ERα bound to ICI 182,780 induces a distinct conformation in which H12 is not stably associated with the LBD. As a result, the AF2 domain is highly accessible in the fully antagonized state and may favor efficient corepressor recruitment.

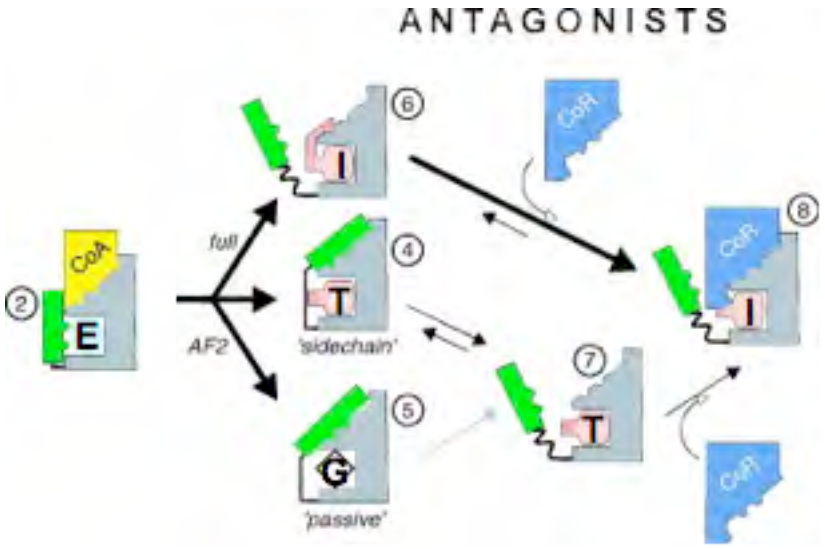

Figure 3. Structural basis of ER activation. The position of H12 (green) with respect to ER LBD (grey) is shown. Upon E2 binding, H12 of ER completely seals the ligand cavity and recruit the coactivators (CoA)②⑦⑧, however, ICI 182, 780 fails to fit in the ERα bidning pocket because of its bulky tail, and H12 is unable to seal the pocket ⑥. 4-OHT phenolic core fits into the ER binding cavity, but H12 protrudes between H3 and H11 because of its bulky tail ④. Genistein fails to make appropriate contacts with the binding cavity, H12 adopts a position shifted by 25° ⑤ from the conformation observed in ④. E: E2, I: ICI 182,780, T: 4-hydroxytamoxifen, G: genestein. Adapted from [90].

The current 3D structures are based only on the LBD of ERα bound to ligands, and other domains of ERα will also influence the conformation of the LBD and affect ERα-dependent transactivation. The construct containing the entire LBD used in X-ray crystal analysis only contains about 30% of the affinity of intact ERα for E2, indicating the importance of other domains for stabilizing ligand-binding. The evidence for direct physical interactions between AF1 and AF2 also supports this possibility. Therefore, the agonism of raloxifene and tamoxifen may be dependent on AF-1 and tissue-specific expression of AF-1 coactivators. However, little is known about the three dimensional structure of intact, full length ERα, due to interdomain flexibility and the unstructured A/B domain. In

future studies, the crystal structure of the full length ERα is necessary to investigate the crosstalk between different regions of ERα.

1.1.4. ERα-Mediated Transactivation

1.1.4.1. Genomic Action of ERα

1.1.4.1.1. Classical EREs

In the classical model, ERα is inactive due to association with heat shock protein 90 (hsp90), which protects unliganded ERα from degradation and keeps the receptor in an inactive state but ready for binding ligands. Upon ligand binding, ERα undergoes a conformational change, forms a homodimer or heterodimer with ERβ, binds to its target gene promoter, recruits other coregulators, and induces gene transcription (Fig. 4). The first ERE was identified in the proximal promoter region of the Xenopus vitellogenin A2 gene [94]. ERα binding to an ERE promotes DNA bending toward the major groove, which exposes DNA and facilitates interactions between coregulatory proteins and the transcription machinery bound to different sites; ERα-ERE binding also promotes DNA looping. Recent reports show that ERα is constitutively bound to its ERE, but remains inactive in the absence of ligand [95]. In the presence of an ERα agonist, corepressors dissociate from the DNA-bound ERα complex and are exchanged for coactivators to activate transcription.

1.1.4.1.2. ERα/AP-1

ERα can regulate gene expression without directly binding to DNA, but can act through protein-protein interactions with other DNA-bound transcription factors. Kushner and coworkers have demonstrated hormone-dependent induction of genes through activator protein 1 (AP-1), an ubiquitous transcription factor

composed of c-Jun and Fos proteins [13, 96, 97]. E2-responsive genes that are regulated by ERα/AP-1 interactions include ovalbumin, IGF-I, collagenase, and cyclin D1. ERα interacts with c-Jun and amino acids 259-302 in the hinge domain of ERα are required for interaction with c-Jun and Fos [98]. E2 activates AP-1 regulated genes in ER-positive MCF-7, ZR-75 breast cancer cells, but represses AP-1 target genes in other cells, such as ER-negative MDA-MB-231 cell [99]. In Hela cells, both estrogens (E2, DES) and antiestrogen (ICI) stimulate ERα transcriptional activity [13]. However, DES and E2 are unable to stimulate an AP-1 site through ERβ. In contrast, ICI and raloxifene activate ERβ transcriptional activity at AP-1 sites. Therefore, ERα and ERβ respond differentially to the estrogens and antiestrogens at AP-1 sites.

Activation of ERα/AP-1 can be either AF-dependent or AF-independent [97]. Estrogen-activated ERα/AP-1 is AF-dependent and requires both AF1 and AF2 of ERα. The same protein-protein contacts are required to activate AP-1 at EREs. However, the AF-1 of ERα strongly inhibits antiestrogen responses through AP-1 sites; therefore, antiestrogens activate ERβ which has weak AF-1 activity through an AF-1-independent mechanism [100]. Even though the DBD of ERα is not required for hormonal activation through AP-1 [96], both zinc finger structures of the ERα DBD are important for response through AP-1 sites [101]. The DBD may exert inhibitory effects on ERα/AP-1 activity possibly by controlling interactions with a modulating repressor, since DBD mutations (ERαK206A/G) cause super-activation of AP-1 through activation function dependent pathways [102].

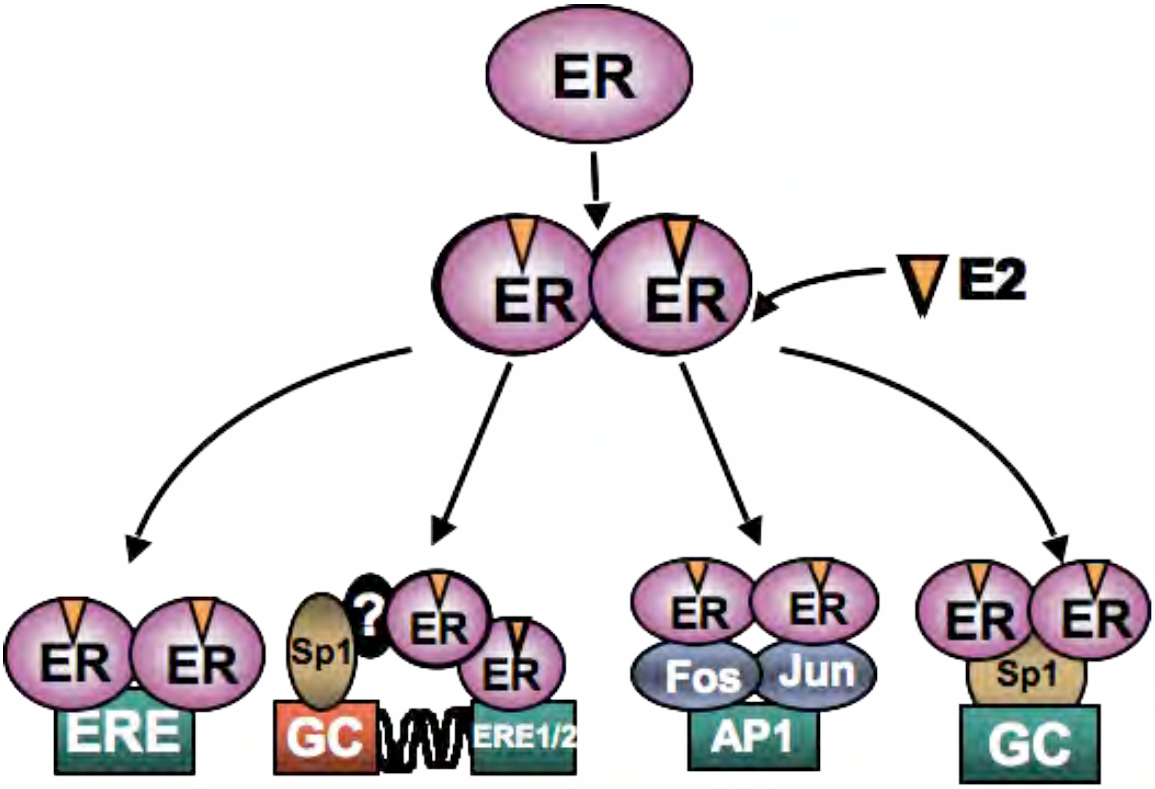

Figure 4. ERα genomic pathway.

1.1.4.1.3. ERα/Sp

There are two mechanisms of E2-mediated transactivation that involve ERα and Sp proteins. One pathway includes a DNA-dependent mechanism in which ERα binds to an ERE half site ($ERE_{1/2}$) flanked by a GC-rich sequence (GGGGCGGGG) for Sp binding and a DNA-independent mechanism in which ERα directly binds Sp proteins.

Analysis of the c-myc promoter identified a 116 bp E2-responsive region containing a non-consensus $ERE_{1/2}$ for ERα binding and a GC-rich sequence for specificity protein 1 (Sp1) binding ($ERE_{1/2}N_xSp1$) [103]. A similar motif was identified and involved in the E2-responsiveness in the proximal promoter region of the creatine kinase B, cathepsin D, Hsp27 and TGFα gene [104-107]. In gel

mobility assays, a broad retarded band was observed after incubation of nuclear extracts of breast cancer cells with oligonucleotides containing $ERE_{1/2}N_xSp1$ sequences from the cathepsin D, TGFα and Hsp27 gene promoter [108].

Deletion of the $ERE_{1/2}$ does not abrogate E2 responsiveness of Hsp27 and c-fos promoter constructs suggesting that the GC-rich box alone is sufficient for E2-responsiveness [12, 104, 109]. In transient transfection assays, in breast cancer cells E2 induces reporter gene activity with constructs containing three tandem GC-rich sites [12]. E2 also induces transactivation in cells cotransfected with a DBD deletion mutant of ERα, suggesting that ERα binding to DNA is not required for this induction and only Sp1 or other Sp proteins interact with its DNA binding site. This represents a novel DNA-independent pathway of ERα action which involves interactions with Sp proteins [12, 110, 111]. Further studies show that E2 can induce many estrogen responsive genes through interactions with Sp proteins. In breast cancer cells, promoter analysis has identified genes involved in DNA synthesis, nucleotide metabolism, and cell cycle progression that are dependent on ERα/Sp interactions with critical GC-rich gene promoter sites [112]. These genes include c-fos [113], RARα, insulin-like growth factor binding protein 4 (IGFBP4), bcl-2 [114], E2F1 [115, 116], adenosine deaminase (ADA) [117], cathepsin D [118], thymidylate synthase [119], DNA polymerase α (DNAPα) [120], Cad [121], cyclin D1 [122], epidermal growth factor receptor (EGFR) [123], low density lipoprotein receptor (LDLR), vascular endothelial growth factor (VEGF) [124, 125] and VEGF2 receptor (VEGFR2) [126, 127].

Although both ERα and ERβ form complex with Sp1 protein in vitro, only ERα increases gene expression in cells transfected with a consensus GC rich luciferase construct (GC_3-Luc), whereas ERβ exhibits minimal or decreased reporter gene activity and these responses are ligand and cell specific [111]. ERα and ERβ bind the C-terminal domain of the Sp1 protein, while multiple regions of ERα are important for interactions with Sp1. In MCF-7 cells, E2, 4-

OHT and ICI 182,780 induce ERα transactivation through interactions with Sp1, whereas these ligands did not activate ERβ/Sp-dependent response [111]. AF1 is important for the interaction of ERα with Sp1, and this region may mediate an association with other proteins that are important for activation of ERα/Sp1.

Numerous studies have shown interactions between ERα and Sp1 [111, 128], and ERα interacts with the zinc finger motifs of Sp1. Sp1 interacts with multiple regions of ERα [111]. The reason for multiple regions of ERα involved in Sp1 interactions may be due to the multiple docking sites provided by Sp1 tetramers. However, the C-terminal F domain of ERα [129] and the AF1 domain of ERα [111] are responsible for E2 induced activation of GC-rich promoters. The DBD of ERα is required for activation of GC-rich promoters by antiestrogens 4-hydroxytamoxifen and ICI 182,780, but not for activation by E2 [128]. H12 is important for ERE-mediated E2 responsiveness; however, antiestrogen and estrogen activate GC-Luc in cells transfected with an ERα variant containing three point mutations in H12. Immunostaining shows that Sp1 facilitates ERα recruitment to GC-rich promoters and ERα colocalizes with Sp1 [127]. ChIP assays show that that ERα/Sp1 are associated at the same promoter region of estrogen responsive genes [127] and FRET assays further confirm that estrogen-dependent ERα/Sp1 interactions [128].

Other transcription factors may also play an important role in ERα/Sp1-mediated transactivation and the mechanisms of regulation are dependent on promoter and cell context. For example, in MCF-7 cells, induction of E2F1 by E2 is regulated by ERα/Sp1/NFY cooperation with both GC-rich and NFY sites, whereas in ZR-75 cells, E2F1 is activated by genomic action of ERα/Sp1 and nongenomic cAMP-dependent phosphorylation of NFYA [115].

E2 differentially regulates gene expression through interaction with Sp proteins, depending on promoter and cell context. VEGF expression is induced by E2 in

ZR-75 breast cancer cells [124], but is inhibited in HEC1A endometrial cancer cells [130]. The role of Sp proteins in estrogen-mediated transactivation in breast cancer cells will be further discussed in detail in section 1.3.4.4.

1.1.4.1.4. ERα Mediated Gene Repression

ERα can also mediate gene repression by tethering with other transcription factors. For example, the repression of IL-6 by E2 is mediated through the interaction of ERα with NF-κB and CCAAT/enhancer binding protein β (C/EBPβ) [131, 132]. The repression of erythropoiesis by E2 involves the interaction of ERα with transcription factor GATA [133].

1.1.4.2. Nongenomic Pathways of E2-Induced Gene Expression

Several studies show that E2 rapidly induces some genes through activation of kinases [134-136], and this is mediated through membrane associated ERα or a G protein-coupled seven-transmembrane-spanning receptor [137]. There is currently controversy over the ability of classical ERs to recapitulate GPR30-mediated signaling mechanisms and *vice versa*. Membrane associated ERα is a transmembrane intracellular protein that mediates rapid cell signaling [138] and can lead both to altered functions of proteins in the cytoplasm and to regulation of gene expression. Rapid ligand-dependent activation of kinases by ERα is an important and critical pathway of E2-dependent responses. Membrane ERα signaling involves kinase cascades, calcium, and other second messengers that impact transcription (Fig. 5). Palmitoylation [139] or phosphorylation [140] may target classical ERs to the cytoplasmic face of the plasma membrane. ERα at the plasma membrane associates with Src through an adaptor protein, called modulator of nongenomic activity of estrogen receptor (MNAR), and promotes the ligand-dependent interaction of ERα with Src kinase, leading to an increase in Src kinase activity and enhanced MAPK signaling. Other adaptor proteins

may also be involved in connecting the ERα-Src complex and these include caveolin-1, Shc, ras, the p85α subunit of PI3K and cytoskeletal protein p130 Cas.

The molecular mechanisms of nongenomic activation by ERα are complex and are dependent on the cell type, availability of the signal transduction molecules and downstream targets.

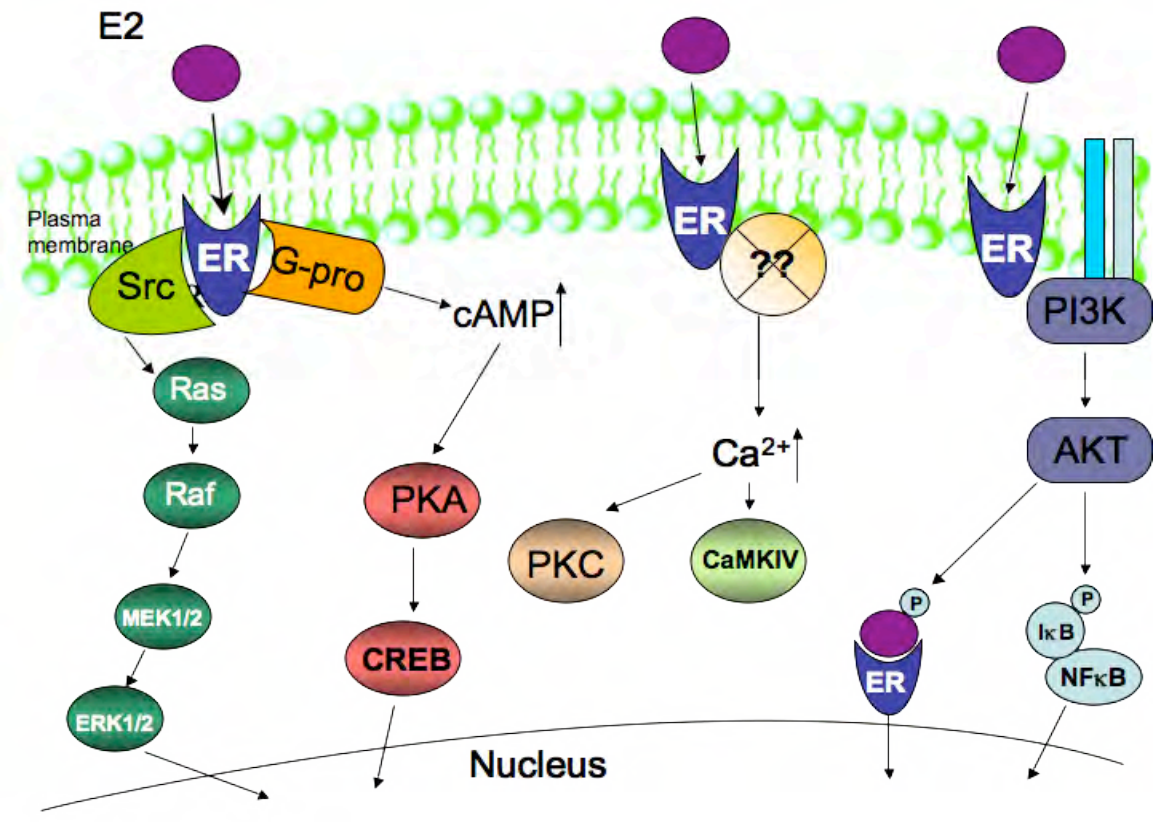

Figure 5. ERα nongenomic pathway.

1.1.4.2.1. Mitogen Activated Protein Kinase (MAPK)

E2 can stimulate the proline-directed, threonine/serine kinase, extracellular-regulated kinase (ERK). ERK is among the most extensively investigated MAPKs, and serves as the prototype for understanding MAPK signaling. ERK is rapidly activated by E2, resulting from activation of Ras, MEK1/2, Raf and Src stimulation in MCF-7 breast cancer cells (Fig. 5) [141]. The activation of MAPK contributes to E2-induced proliferation [142] and survival of MCF-7 cells [143], and the ERα antagonist ICI 182,780 can inhibit this activation. Other MAPKs, such as JNK and p38 can also be activated via membrane ERα [143-145].

1.1.4.2.2. Phosphatidylinositol 3-Kinase (PI3K)

In endothelial cells and MCF-7 breast cancer cells, E2 stimulates PI3K, leading to activation of Akt kinase and induction of nitric oxide. Activation of PI3K through ERα may be mediated by direct association with p85, the PI3K regulatory subunit. Src is another possible component of this ERα/p85 complex and it functions as an enhancer.

1.1.4.2.3. Protein Kinase A (PKA)

Activation of G protein coupled receptor (GPCR) induces adenylate cyclase activity, which catalyzes the conversion of ATP to cAMP. In breast cancer cells E2 rapidly induces cAMP levels [146], which in turn activate PKA and downstream targets, such as CREB and NFκB [147]. However, the mechanism of E2-dependent action of this pathway is still unknown.

1.1.4.2.4. Protein Kinase C (PKC)

PKC is a family of protein kinases consisting of approximately 10 isoforms, that require Ca^{2+}, diacylglycerol (DAG) and phospholipid for activation [146]. PKCs

consist of a regulatory domain and a catalytic domain connected by a hinge region. Although PKC has been identified as a target for nongenomic actions of ERα, little is known about its mechanism [148]. Activation of PKC by E2 is either dependent or independent of ERα, and cannot be inhibited by the antiestrogen ICI 182,780 but by tamoxifen [149].

1.1.4.2.5. Calcium/calmodulin-dependent Protein Kinase IV (CaMKIV)

Calcium/calmodulin-dependent kinase is primarily regulated by the Ca^{2+}/ calmodulin complex. CaMKIV functions as a monomer, and phosphorylation mediated by CaM kinase kinases (CaMKKs) is required for the full function of CaMKIV. E2 induces an influx of Ca^{2+} within seconds [150, 151], and in MCF-7 cells, E2 stimulates CaMKIV activity and only CaMKIV but not CaMKII is activated through an ERα-dependent nongenomic pathway [152].

1.1.4.3. Ligand-Independent ERα Transactivation

ERα can be stimulated in a ligand-independent manner by modulation of different agents/kinase pathways including peptide growth factors [153], interleukin-2, cAMP [154], insulin [155] and activators of MAPK and PKA such as neurotransmitters and cyclins [156]. The ligand-independent activity of ERα is a result of phosphorylation, which is important for its function, including protein-protein interactions, nuclear translocation, DNA binding and transcriptional activation. For example, phosphorylation of serine 167 is important for DNA binding by the receptor [157]. Growth factors activate ERα transactivation through the MAPK pathway[136, 153, 155, 158], and ERα is regulated by phosphorylation of several conserved serine residues within AF-1 (Fig. 6).

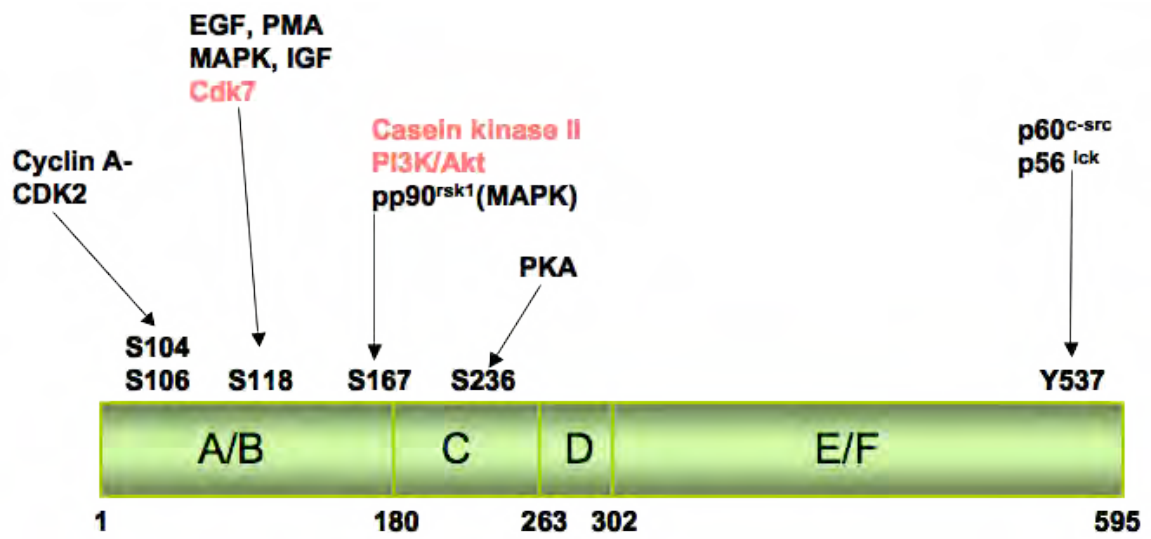

Figure 6. ER α can be phosphorylated at multiple sites

Activation of ERα via phosphorylation at multiple sites by different kinases is indicative of crosstalk between growth factor signaling and ERα. Increased growth factor signaling may account for the loss of E2 dependence, thereby causing resistance to tamoxifen treatment during breast cancer chemotherapy. In most cases, phosphorylation enhances the transcriptional activity of ERα. However, PKA-mediated phosphorylation of Ser 236 inhibits ERα dimerization. ERα phosphorylation also impacts the effects of SERMs; for example, Ser305 phosphorylation of ERα converts tamoxifen from an antagonist to an agonist in breast cancer [159], and this site can be phosphorylated by either PKA [159] or p21-activated kinase 1 [160].

Phosphorylation of ERα primarily occurs on serine residues in the N-terminal region. Ser 118 and Ser 106 are highly conserved residues in ERα from different species, whereas Ser 104 appears to be conserved only in mammals. EGF can induce Ras-MAPK phosphorylation of Ser 118 of human ERα [161, 162]. *In vivo* studies indicate that EGF can imitate the effects of E2 in the murine female reproductive tract [163]. αERKO mice lack uterotropic response to EGF,

indicating the involvement of ERα in mediating the effects of EGF [164]. Inhibition of EGF in the mammary gland blocks E2-induced expression of PR and development of the terminal end buds, indicating crosstalk between EGF signaling and ERα. ERβ can also be phosphorylated by MAPK and phosphorylation of Ser106 and Ser124 of mouse ERβ enhances recruitment of SRC-1 and increases ERβ transcriptional activity [165]. Cyclins and their kinases, cyclin-dependent kinases (cdks) regulate cell cycle progression. Ser104 and Ser106 in human ERα are phosphorylated by the cyclin A-cdk2 complex [166], enhancing transcriptional activity of ERα in both the absence and presence of ligand, and this may be important for cell proliferation. Another phosphorylation site in AF-1 of ERα is Ser167 which is mediated by activation of a different MAPK pathway involved in p90 ribosomal S6 kinase (RSK) [167]. Ser167 can also be phosphorylated by casein kinase II and AKT in vitro [168]. In general, phosphorylation of Ser residues in the AF1 domain of ERα appears to influence recruitment of coactivators, releasing inhibitory effects from the LBD resulting in enhanced ERα-mediated transcription.

Induction of cAMP by forskolin, okadaic acid, and cholera toxin activates PKA by inducing the release of the regulatory element from the catalytic subunit and stimulates ligand-independent transcriptional activities of ERα through phosphorylation of the DBD, which is distinct from growth factor induced AF-1 phosphorylation [168]. Phosphorylation of Ser236 in the DBD of ERα by PKA inhibits receptor dimerization.

1.1.4.4. Summary of Complex ERα-Dependent Activities

Regulation of gene expression by ERα is a multifactorial and complex process, involving both genomic and nongenomic actions (Fig. 7), which ultimately enhance ERα-dependent transactivation through both ligand binding and/or phosphorylation or by activation of other transcription factors. Specific gene

responses are determined by different factors, such as transcription factors bound to a specific gene promoter, the cellular localization of ERα, expression levels of various coregulator proteins and other signal transduction components and the nature of extracellular stimuli.

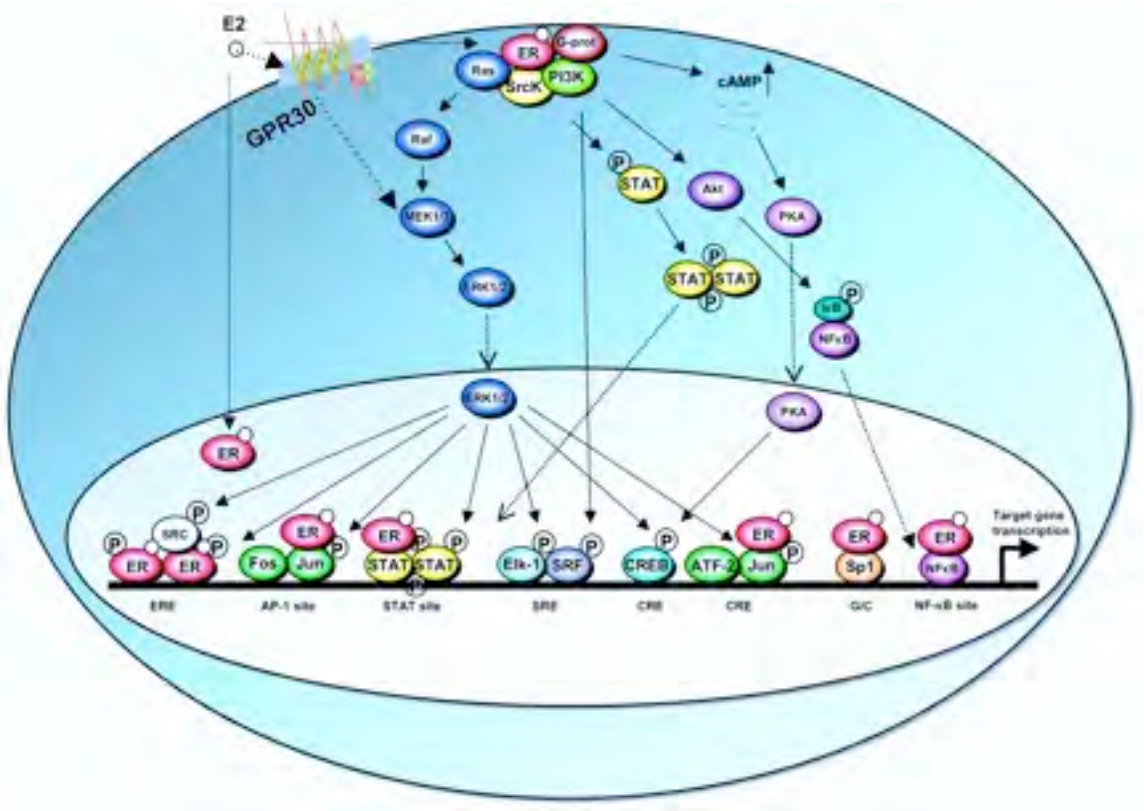

Figure 7. Schematic illustration of the convergence of ERα-mediated genomic and nongenomic actions on target genes [169].

1.1.5. Cofactors and Other Interacting Proteins

ERα functions in concert with other transcriptional cofactors including chromatin-modifying complexes and regulatory proteins. Depending on their functions, these regulatory proteins are grouped into three classes: coactivators, which activate transcription; corepressors, which repress transcription; and

cointegrators, which do not directly activate gene expression but act as bridge factors to integrate coactivators and corepressors with the transcription machinery. They are all referred to as cofactors.

1.1.5.1. Coactivators

Coactivators are molecules recruited to receptor-bound DNA complexes by ligands or other stimulators that enhance gene expression. Approximately 200 coactivators that modulate transactivation of the 48 NRs have been identified. In this thesis, only coactivators specific to NRs are discussed. Coactivators are involved in several steps in the activation of gene expression, including transcription initiation, elongation, termination, nuclear transportation, mRNA alternative splicing and post-transcriptional regulation.

The steroid receptor coactivator family (p160/SRC) are coactivators that primarily interact with NRs. All three members: SRC-1, SRC-2, and SRC-3 (Fig. 8) have three conserved LXXLL motifs (where L is leucine and X is any amino acid, they are so-called NR boxes) that directly interact with NRs.

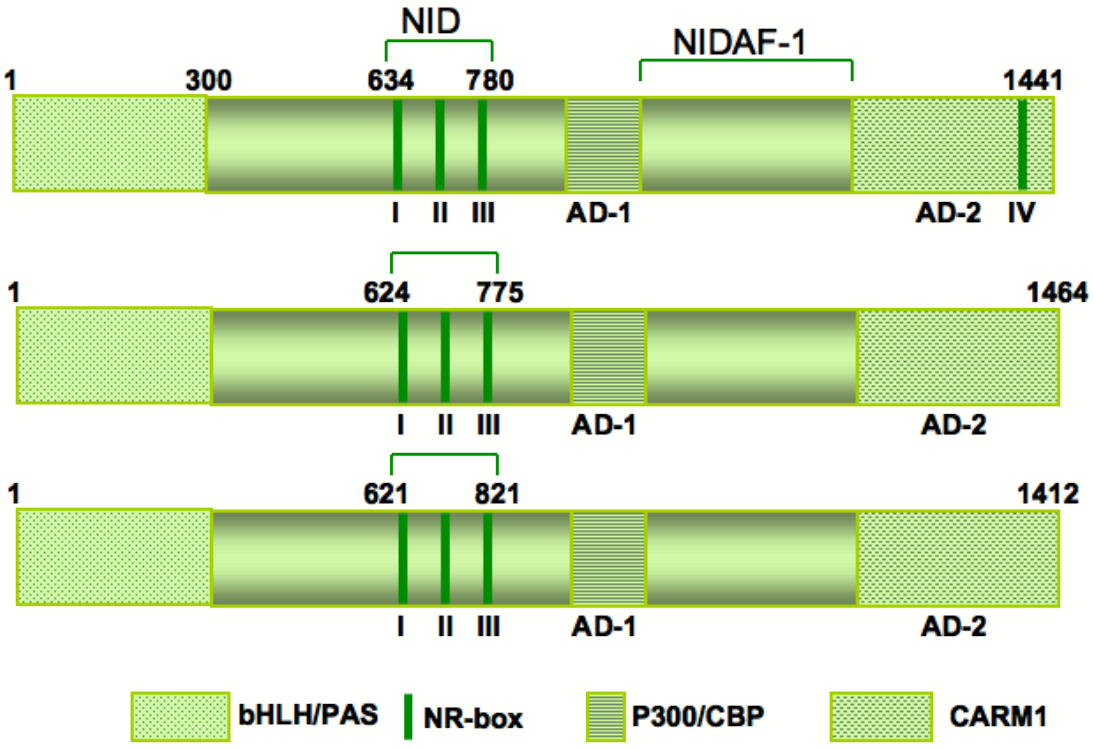

Figure 8. p160/SRC family of nuclear receptor coactivators [170-172].

SRC-1 was the first p160 coactivator shown to interact with the AF-2 domain of hormone receptors [173]. It activates ligand-dependent transactivation of a broad range of NRs, including PR, GR, ER, TR, PPARγ and RXR [173, 174]. Interactions between ERα and SRC-1 are abolished in the presence of antiestrogens, due to steric interference through orientation of ERα. SRC-1 also interacts with other transcription factors, such as TATA box binding protein (TBP) and transcription factor IIB (TFIIB), indicating that SRC-1 may act as a bridging factor between ERα and RNA polymerase II [175].

SRC-3 was cloned independently by several groups and named p/CIP [176], RAC3 [177], ACTR [178], TRAM-1 [179] or AIB1 [180], which are the same as SRC-3. SRC-3 selectively activates ERα but not ERβ transcriptional activity [181]. Higher expression of SRC-3 is detected in many hormone-sensitive tumors, such as breast, prostate and ovarian cancer [180], and overexpression

of SRC-3 may promote initiation and/or progression of carcinogenesis by affecting important signal pathways. Both SRC-1 and SRC-3 have histone/acetyltransferase (HAT) activity which results in an open chromatin structure that facilitates transcription initiation. However, the HAT activity of SRC-1 and SRC-2 is much weaker than p300/CBP and p/CAF and inactivation of the HAT activity in SRC-1 does not significantly affect its coactivation function, suggesting that the intrinsic HAT activity of SRC may not be essential for NR-directed initiation of transcription [182].

SRC-2 was first identified as glucocorticoid receptor interacting protein 1 (GRIP1) and shares homology with the human transcription intermediary factor 2 (TIF2) and SRC-1 [183, 184]. Unlike SRC-1 and SRC-3, SRC-2 does not have intrinsic HAT activity, but can recruit other HATs.

In contrast to the conserved structure among NRs, the coactivators are structurally diverse (Fig. 8). p160/SRC proteins only share 40% overall sequence homology. The NR-box is the most conserved domain which forms an amphiphathic α helix with the H12 in AF-2 domain of NRs. The NR boxes of SRCs are essential for mediating AF-2-dependent activity of NRs but are not sufficient for high affinity interactions of SRCs with NRs. The proximal sequence of the NR box is also responsible for efficient p160/SRC coactivator interaction with NRs.

SRCs recruit the general transcription factors that associate with the RNA polymerase II (RNAPII) holoenzyme and facilitate initiation of transcription. SRCs also interact with other transcription factors, including AP-1 [185], NFκB [186], signal transducer and activator of transcription (STAT) [187], E2F1 [188], interferon α and cAMP regulatory element-binding protein (CREB) [176]. In addition to post-translational modifications of chromatin, such as acetylation and methylation, coactivators also undergo modifications that regulate their transcriptional activity. Phosphorylation of different sites on SRC-3 can

determine which transcription factor it can activate, suggesting there is a "phosphorylation code" for coactivators [189]. Therefore, coactivator modification codes (acetylation, methylation, phosphorylation) may determine the transcriptional state of broad groups of functionally related genes and may control coactivator preferences among NRs and other transcription factors.

SRCs possess many common features and studies on SRC knockout mice show that they share redundant functions and may be able to partially compensate for each other (Table 2). For example, SRC-2 expression level is increased in several regions of the brain and some other tissues in SRC-$1^{-/-}$ mice. SRC-$1^{-/-}$ mice have developmental problems in the cerebellar Purkinje cells where only SRC-1 is expressed at high levels and SRC-2 at low levels in wild-type mice [190], but not in hippocampus where all SRCs are expressed in wild type mice. Therefore SRC-2 and SRC-3 can compensate for the loss of SRC-1 in hippocampus [191].

Table 2. The major phenotypes observed in SRC knockout mice [192].

Knockout Mice	Phenotypes
SRC-1	Partial resistance to steroid and thyroid hormones; delay in cerebellar PC development; no effect on PPARγ-regulated genes in liver [190, 193-195].
SRC-2	Defective spermatogenesis; testicular degeneration; placental hypoplasia; male and female hypofertility; higher lipolysis in white fat; higher energy expenditure in brown fat; resistance to obesity [196, 197].
SRC-3	Somatic growth retardation; delay in puberty and mammary gland growth; lower IGF-1 and estrogen; lower female fertility; reduced ER-dependent vascular protection [198-200].

Although SRCs were originally identified as AF-2 interacting proteins, there is increasing evidence that they can also interact with and enhance the AF-1 activity of NRs, which is a possible mechanism for AF-1 and AF-2 synergism [201]. SRCs interact with the AF-1 of ERα and PR with much weaker affinity

compared to AF-2. The interacting region for binding to AF-1 is outside of NR-box and between AD-1 and AD-2 of SRCs (Fig. 8). This region has been named as nuclear receptor interacting domain AF-1 (NIDAF-1) [202, 203] (Fig. 8). NR box deletion mutants do not enhance AF-2 function but their activity to interact and enhance the AF-1 activity is intact, suggesting that two different regions of SRC simultaneously interact with AF-1 and AF-2. Therefore, coactivators can serve as a bridge or adaptor for communication between two activation functions within ERα and other NRs.

1.1.5.2. Corepressors

Nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) interact with and repress transcriptional activity of hormone receptors [204, 205]. NCoR and SMRT are structurally-related proteins (Fig. 9) that share many similar characteristics. Both corepressors interact with the non-ligand bound receptor and upon ligand binding, corepressors are released and coactivators are recruited. NCoR and SMRT harbor autonomous, transferable repression domains, and SMRT corepresses the activity of ERα only in the presence of its partial agonists, such as tamoxifen [206]. This suggests that NRs may contain cryptic repressor-binding sites that are exposed in the antagonist-binding receptor conformation. Mutational analysis of ERα shows that H12 is not required for binding of NR box peptides derived from NCoR [91], whereas disruption of H3 and H5 has a marked effect on peptide binding. Leucine 372 may be part of an interaction surface on ERα that is responsible for corepressor binding. Histone deacetylase (HDAC) complexes specifically interact with SMRT and NCoR, and this correlates with their capacity to repress transcription [207-209]. NCoR and SMRT are not NR specific corepressors, since they can function as corepressors for many other transcription factors [210], such as pituitary-specific factor Pit-1, which is a member of the homeodomain family of proteins. The

NCoR knock-out is embryonic lethal and the majority of $NCoR^{-/-}$ embryos died by day 15.5 of gestation, indicating that the role of NCoR cannot be compensated for by SMRT [211].

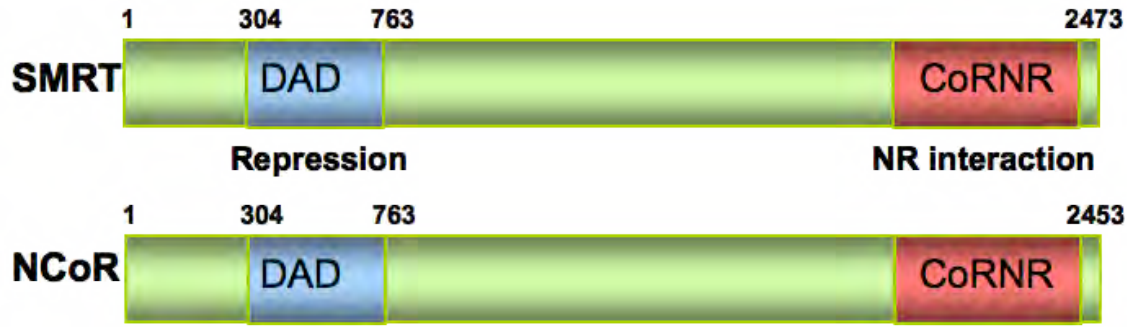

Figure 9. Schematic structure of corepressors.

Short heterodimer partner (SHP) is an orphan nuclear receptor that contains the dimerization and ligand-binding domain found in other NR family members but lacks the conserved DNA binding domain [7]. Although no conventional receptor function has yet been identified, SHP acts as a negative regulator of nuclear receptor signaling pathways, because it inhibits DNA binding and transcriptional activity of various receptors, including TR and RXR [212]. It displays a ligand-dependent interaction with ERα and ERβ that results in repression of transcription. SHP interacts with ERs via two NR-box related motifs that may antogonize ER binding of p160 coactivators [213-215]. SHP may represent a new category of corepressors for ligand-activated nuclear receptors. The orphan receptor DAX-1 is another atypical member of the NR family [216-218]. It has a putative ligand-binding domain in the C-terminus and no identified ligands. DAX-1 does not have a typical zinc finger DNA binding domain, and its N-terminus consists of putative single-stranded DNA and RNA binding motifs. Its receptor function remains enigmatic, but it acts by competing with the p160 coactivators for binding agonist-bound ERα, AR and the orphan receptor Nur77 [216-218].

1.1.5.3. Cointegrators

p300/CREB-binding protein (p300/CBP) interacts weakly with ERα and PR, but can synergize with SRC-1 in coactivation of these receptors [219]. p300/CBP was first identified as a protein that specifically bound cAMP response element binding (CREB), which is a transcription factor that binds cAMP response elements (CREs). p300/CBP can also bind the LBD of RAR, independent of ligands. p300/CBP associated factor (pCAF) was initially identified as a factor that interacted with p300/CBP. Both p300/CBP and p/CAF are general transcriptional coactivators that associate with a variety of transcription factors, functioning in part as molecular scaffold, while the p160 family is relatively specific for the activation of NRs. The CBP/SRC-1 complex is very unstable, but can form a stable complex in the presence of NRs [220, 221]. p300/CBP exhibits much stronger HAT activity compared with that of SRCs and it is tightly associated with RNAPII. P/CAF is also a HAT that associated to p300/CBP, SRC-1 and SRC-3. Unlike the p160 coactivators whose HAT activity is not essential for their activity as coactivator, the HAT activities of p300/CBP and pCAF are essential for their integration activity. Point mutations in the HAT catalytic domain of CBP or pCAF abolish their function as an enhancer of NR-dependent gene expression. p300/CBP also acetylates p160 proteins after prolonged hormone treatment [175, 176, 178, 222, 223].

Both estrogen agonists and antagonists can recruit CBP/p300 and this facilitates recruitment of coactivators. The antiestrogen ICI 182,780 promotes the interaction of ERα with CBP/p300 through a different interaction surface of CBP [224]. However, ICI-dependent recruitment of CBP does not recruit SRC-1 on the pS2 promoter and does not induce pS2 gene expression. Therefore, recruitment of the CBP/p300 without SRC-1 to ERα is insufficient to induce ERα mediated gene transcription [224].

1.1.5.4. Receptor-Specific Coregulators

Some coregulators are reported to be receptor-selective. p68 RNA helicase binds to the AF-1 region of ERα both *in vitro* and *in vivo*. It does not interact with AF-2 of ERα or any other NRs, and overexpression of p68 helicase only enhances AF-1 activity. p68 displays enhanced affinity for the phosphorylated AF-1 domain of ERα at Ser 118, suggesting that p68 maybe a target involved in mediating the phosphorylation dependent activity of AF-1 and this may be a link between phosphorylation and transactivation. This also explains why p68 more strongly activates ERα than ERβ, since ERβ has a much weaker AF-1. The RNA helicase activity of p68 is dispensable for its coactivation of ERα suggesting p68 is a dual function protein. A novel 37 kDa ER-selective repressor, termed repressor of estrogen receptor activity (REA), was also identified and shown to interact preferentially with the antiestrogen-ERα complex and potentiate the inhibitory activity of antiestrogens. Another AF-1 dependent coactivator is steroid RNA activator (SRA), which acts as an RNA transcript that mediates transcriptional activity of ERα. SRA exists in a large complex with SRC-1 and may serve as a scaffold protein to facilitate SRC-1 recruitment [225].

1.1.6. Role in Cancer

1.1.6.1. Breast Cancer

Some breast tumors are ER positive, others are ER negative. Since estrogen treatment increases ER-dependent proliferation of breast tumors/cells, endocrine therapy uses ER antagonists, either antiestrogens or SERMs to inhibit ER-dependent growth. The ideal SERMs would serve as estrogen agonists in bone, liver, the cardiovascular system and brain, but as ER antagonists in the reproductive system.

ERβ is also expressed in breast tumors, but it is unclear whether it plays a role in cancer development, progression, or treatment of breast cancer. From studies on ERKO mice, ERα is indispensable for uterus and glandmammary development, while βERKO mice have normal glandmammary development with normal sexual and lactating behavior. However, the possible role of ERβ in breast cancer cannot be excluded, since both ERα and ERβ are expressed in the breast and can form heterodimers with ERα. Overall, the majority of studies suggest that the presence of ERβ is a good prognostic marker for breast cancer, even though this is still debatable. More studies with both normal and malignant breast samples would facilitate the evaluation of the role of ERβ in breast cancer disease progression and its correlation with antiestrogen treatment and the response. The ratio of ERα/ERβ may change and impact tumorigenesis.

1.1.6.2. Prostate Cancer

ERβ is highly expressed in the prostate and may be a susceptible target for environmental estrogens. Some xenoestrogens exhibit higher binding affinities to ERβ than ERα and these include BPA. ERα and ERβ are expressed in different cell types in prostate; ERα is mainly expressed in stroma, and ERβ in epithelium. βERKO but not αERKO males exhibited hyperplasia in the prostate, suggesting that ERβ may play a protective antiproliferative role in prostate. If this is true, the ERβ selective ligands could potentially be used for prostate cancer therapy.

1.1.6.3. Colon Cancer

ERβ is expressed in the human colon cancer cells, such as HCT116, HT29 and SW380, whereas ERα was not detected in these cells [226, 227]. Studies with normal and malignant human colon samples show that ERα is expressed at

extremely low levels compared to ERβ. One study shows that there is no difference in ERβ mRNA levels in normal and malignant colon tissue. However, there is a loss of ERβ protein during malignant transformation [228], indicating that ERβ is primarily regulated at the post-transcriptional level. Another study reported that ERβ mRNA levels were decreased in colon cancer cells compared to normal tissue, whereas the ERα level remained unchanged [74], implying that the ERβ may play a protective role in colon cancer.

1.1.6.4. Ovarian Cancer

ERβ is highly expressed in granulosa cells of the ovary, whereas ERα is expressed at relative low levels in thecal and interstitial cells. αERKO mice are completely infertile, but βERKO mice are fertile with fewer pups. The amount of ERα relative to ERβ may play an important role in ovarian cancer. One study has reported an increase in ERα mRNA levels in ovarian cancer compared to normal tissue [73]. There is more ERα than ERβ in human ovarian tumors [229]. Even though it is still controversial, most of studies imply that ERα is the dominant ER isoform and ERα is overexpressed and promotes cell proliferation in ovarian cancer. Further studies are needed to fully determine the roles of ERα and ERβ in ovarian cancer.

1.2. Xenoestrogens

Many widely used synthetic chemicals and natural plant compounds can alter or interfere with the endocrine system. These foreign substances have been called as endocrine disrupting chemicals (EDCs) or endocrine disruptors (EDs); compounds exhibiting estrogenic activities have been most extensively studied. They are also known as synthetic environmental estrogens or xenoestrogens.

While most xenoestrogens bind the ER with greater than 1000-fold lower affinity than E2, these compounds induce diverse tissue-specific estrogenic responses and may exhibit both ER agonist and antagonist activities. Their activities cannot be determined from receptor binding or rodent uterus bioassays. Xenoestrogens can also interfere with ERα-dependent transcriptional activity through other mechanisms such as modulation of growth factors or activating crosstalk with other NRs [230]. They can also activate ERα-dependent kinase pathways through membrane ERα [231] or the novel seven-transmembrane estrogen receptor GPR30 [232]. BPA has approximately 8-50 times higher binding affinity for GPR30 than ERα [233]. The estrogenic activity of xenoestrogens may be indirect due to altered expression or activity of steroidgenic enzymes [234, 235]. Studies on characterization of tissue-specific receptor agonist/antagonist activities of these compounds are limited, and this compromises development of methods for hazard and risk assessment of xenoestrogens.

Studies reported in this thesis will investigate the structure-dependent activation of ERα by xenoestrogens in breast cancer cell lines and the overall hypothesis is that these compounds are SERMs and will differentially activate ERα and ERα/Sp-dependent transactivation. Figure 10 summarizes the compounds that will be used in this study and previous reports indicate that they are all weakly estrogenic and bind ERα [236].

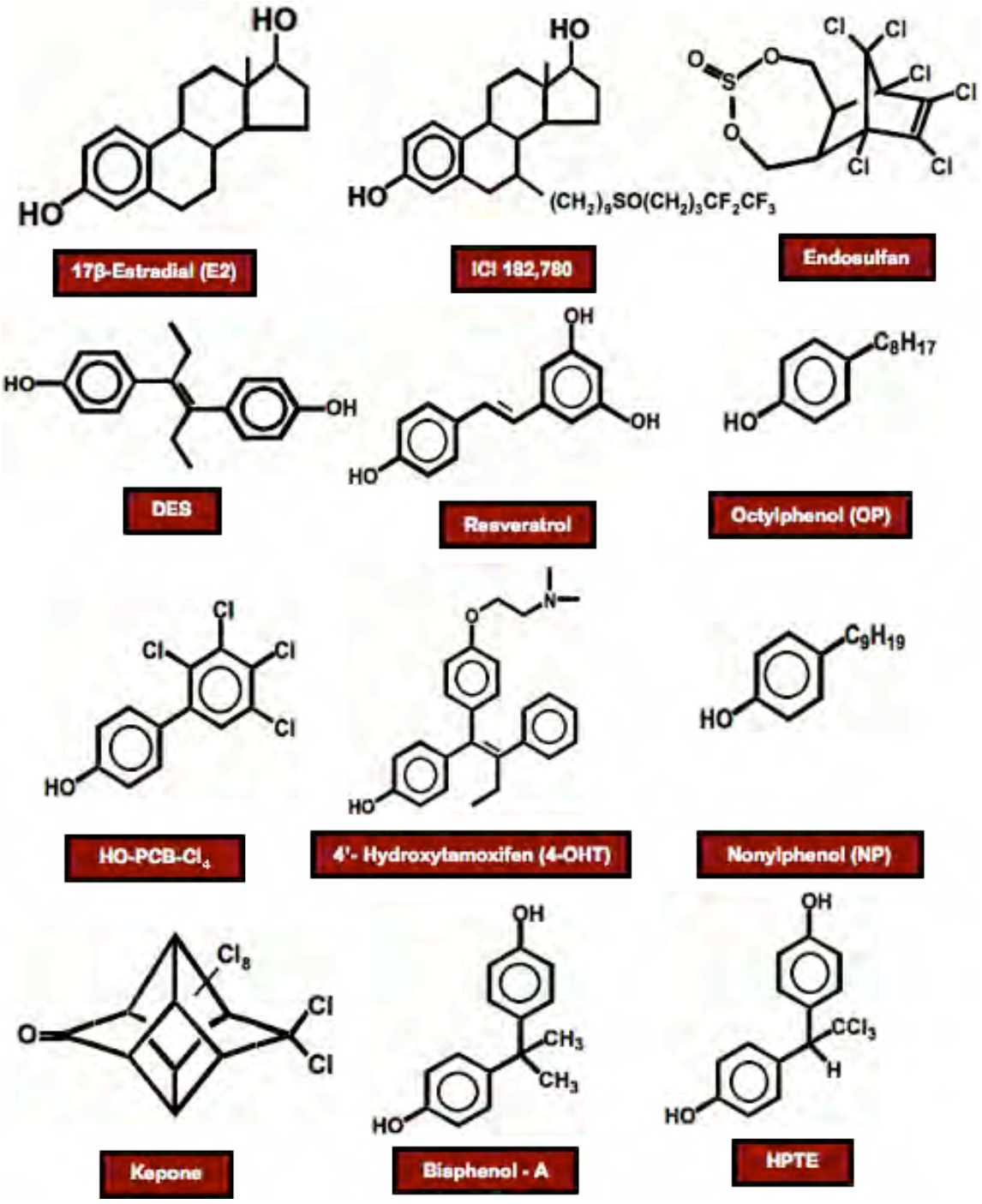

Figure 10. Structure of model estrogenic compounds, including the phytoestrogen resveratrol and the xenoestrogens, octylphenol, nonylphenol, HO-PCB-Cl_4, kepone, bisphenol-A, HPTE and endosulfan.

1.2.1. Sources of Xenoestrogen Exposure

Superfund chemical wastes are major sources of exposure to EDs and some of these chemicals include PAHs, halogenated aromatic industrial compounds/by-products (PCBs, PCDDs, and PCDFs), chlorinated pesticides, hydroxy chlorinated aromatics, phenolics, some phthalates, alkylphenols, and heavy metals. Some of the properties and endocrine disrupting effects mediated by these compounds will be discussed in the following sections.

1.2.1.1. Pesticides and Herbicides

1.2.1.1.1. Chlorinated Hydrocarbon Pesticides

Dichlorodiphenyltrichloroethane (DDT) is the first modern pesticide which was developed to kill mosquitoes and later used extensively as an agricultural insecticide. The Swiss chemist Paul Hermann Muller was awarded the Nobel Prize in Physiology or Medicine in 1948 for the invention of DDT. DDT and its metabolites are toxic to aquatic life. The major metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) inhibits prostaglandin synthesis in birds and this results in eggshell thinning. The use of DDT was banned in many countries in the 1970s. DDT and DDE are extremely stable compounds that persist in the environment and resist metabolism and exposure is mainly through food consumption, whereas occupational exposures are by inhalation and dermal contact.

In MCF-7 breast cancer cells, DDT binds ERα, induces transcriptional activity and promotes proliferation at a 1 μM concentration and similar results were observed for ERβ binding [237]. DDT interferes with endocrine processes by modulating ERα activity in either reproductive or nonreproductive tissues, such as prostate, uterus, liver and brain. DDT activates ERs in the brain and the liver of adult mice after acute administration [238] and causes liver tumors. DDT

induced luciferase activity in the prostate and testis of ERE-TK-Luc transgenic animals, and the effects can be inhibited by the antiestrogen ICI 182,780 [239]. DDT and DDE also interfere with estrogen biosynthesis, since DDE enhances aromatase activity [240], the key enzyme for catalyzing the rate-limiting step in the conversion of androgens into estrogens [241]. Various studies have examined the effects of DDT and DDE on human reproductive tissues, but the strong causal links have not been confirmed and the effects on human health are still highly controversial [242].

DDE can activate multiple receptors and transcription factors including ERα, AR, PR and NFκB [243-245]. A disruption in progesterone signaling can also affect the frequency of spontaneous miscarriages. Immune tissues are also estrogen targets. The thymus expresses both ERα and ERβ and thymic development is dependent on the ERα signaling pathway, as shown by reduced thymic size in neonatal mice lacking ERα [246, 247]. DDT can disrupt the rodent immune system by dramatically decreasing IL-2 production in activated T cells, and this is associated with decreased expression of the IL-2 activator NFκB [245].

Adipose tissue is an important storage depot for DDT and conjugation of fatty acids to DDT may be a mechanism for retention of this compound in adipose tissue [248]. DDT and DDE can be transferred from the placenta to fetuses and breast-milk to infants. Concentrations of DDT are much higher in adipose tissues than in breast-milk, and higher in breast milk than in blood or serum. High DDE concentration in breast milk was associated with a shortened duration of lactation [249, 250], due to modulation of PR activity since progesterone plays a major role in gestation maintenance.

1.2.1.1.2. Methoxychlor (MXC) and its Metabolite HPTE

1,1,1-trichloro-2,2-bis(4-methoxyphenol)ethane (Methoxychlor, MXC) is a broad spectrum organochlorine pesticide developed as a substitute for DDT. Although DDT and MXC have similar structures, MXC is more readily metabolized and excreted by mammalian systems and does not bioaccumulate or concentrate in fatty tissue [251]. MXC itself has weak intrinsic estrogenic activity and binds both ERα and ERβ. Like E2, MXC can increase uterine wet weight in the ovariectomized rat and can cause adverse developmental and reproductive effects in laboratory animals [252-257]. However, there are some differences in the *in vivo* activities of MXC and E2. Unlike E2, MXC does not increase FSH and LH levels in ovariectomized rats [258], Moreover, MXC acts as an ER agonist in the uterus and an antagonist in the ovary [257], possibly because of the high expression of ERβ relative to ERα in the ovary [259].

After *in vivo* administration, MXC is metabolized in liver via O-demethylation to form polar mono- and bis-phenolic metabolites. The bisphenolic MXC metabolite HPTE is considered as the primary metabolite responsible for the estrogenic effects of MXC and has higher ERα binding affinity which is approximately 100-times more active than that observed in MXC. HPTE is a potent ERα agonist, but primarily a weak ERβ and AR antagonist [260-262]. HPTE also inhibits testosterone biosynthesis both *in vitro* [263] [264] and *in vivo* [265] due to its weak AR antagonist activity. Exposure to HPTE can cause hematological, neurological and hormonal symptoms, which include abnormal morphology of reproductive organ [266]. Dechlorination and dehydrochlorination of MXC and HPTE have been observed and the resulting metabolites are weakly estrogenic and antiandrogenic [262, 267].

The estimated human intake of MXC is about 0.8 μg/day based on a FDA food basket survey. Due to rapid metabolism, it is unlikely that HPTE levels would reach levels in humans that would affect ER- or AR-dependent activities.

1.2.1.1.3. Endosulfan

Endosulfan is a broad spectrum chlorinated hydrocarbon insecticide used to control insects in agriculture and as a wood preservative. It is also released into the environment during its manufacture and use. Residues on crops usually break down within a few weeks, but it may take years to degrade endsulfan bound to soil. Human exposure to endosulfan is usually from eating contaminated food, occupational exposure, inhalation, and skin contact with contaminated soil. Endosulfan is rapidly metabolized and does not bioaccumulate. Animal studies show that long-term exposure can damage the kidney, liver, testis, central nervous and immune system. An unintentional exposure to endosulfan shows neurological manifestations, liver toxicity, and required mechanical ventilation and emergent hemodialysis in human [268].

Endosulfan is also a xenoestrogen that causes reproductive and developmental damage. Studies in a village in northern Kerala, India where endosulfan is widely used for insect control shows that high exposure delayed sexual maturity, lowered testosterone levels, decreased penile length in young males and caused birth defects in males [269]. However, a three-day rat uterotrophic assay with endosulfan does not change uterine weight [270, 271]. Endosulfan is weakly estrogenic in transactivation assays, but is a potent activator of the MAPK pathway in a rat prolactinoma cell line [272]. At relatively low concentrations (1~100 nM), endosulfan enhances ERK1/2 phosphorylation via multiple ERα nongenomic pathways and promotes cell proliferation [272, 273].

1.2.1.1.4. Kepone

Kepone, also known as Chlordecone, is a chlorinated polycyclic insecticide and fungicide used between 1966-1975 in America for ant and roach baits. The illegal discharge of kepone from a manufacture facility caused extensive contamination of the James River.

Kepone causes both toxic endocrine and reproductive effects in wildlife and humans. Kepone treated birds exhibit impaired reproductive function and even a small amount of kepone can decrease bird number and reduce the size of eggs in bird species [274]. In male alligators, kepone induces *de novo* synthesis of vitellogenin, which is naturally synthesized in the liver of females following exposure to elevated plasma levels of estrogen. Kepone exposure induces constant estrus in mice and increases immature rat uterine wet weight [275]. Maternal exposure to kepone in rodents causes lower birth weights, reduced postpartum survival, impaired reproductive capacity and developmental abnormalities in the offspring [276]. Kepone competitively binds to ERα, even though its binding affinity is 10,000 fold lower than E2. Kepone induces nuclear uptake of ERα and stimulates uterine PR expression [277] and competitively binds to PRa in frozen oviductal extracts from female American alligators [278]. The data indicate that kepone is estrogenic. Workers exposed to kepone suffered neurologic symptoms such as tremors and slurred speech and they also showed decreased sperm counts due to sperm maturation arrest [279].

1.2.1.2. Bisphenol A (BPA)

BPA is primarily used as a plasticizer used for production of epoxy resins and polycarbonate plastics for canned food products, CDs and eyeglass lenses. Other minor applications include its use in dental sealants, adhesives, flame retardants, varnishes, antioxidants and automotive components. The United

States produces over 1.6 billion pounds of BPA annually and this is about one third of global production.

In addition to the occupational exposure of workers, human exposure to BPA can occur when this compound leaches from plastic materials such as food cans with epoxy resin linings, polycarbonate plastics wares and dental fillings/ sealants. BPA is rapidly metabolized through glucuronidation and the major glucuronide metabolite formed in the liver is excreted in the urine [280]. Therefore, in humans there is low body burden of BPA following oral administration [281-287]. Other minor BPA metabolites identified include sulfate conjugates and glucuronide/ sulfate diconjugates [280]. All the metabolites are biologically inactive and devoid of estrogenic activity. However, recent studies in the United States shows that 95% of urine samples have measurable level of BPA [288], suggesting that humans are constantly exposed to BPA from multiple sources.

The binding affinity of BPA for the ERα and ERβ is approximately 1000 fold lower than E2 and 2000 fold lower than DES [289]. BPA exhibits ERα and ERβ agonist activity [261], but has a higher binding affinity for ERβ than ERα. BPA promotes proliferation of MCF-7 breast cancer cells [290], increases PR expression in human endometrial carcinoma and MCF-7 cells [291], and activates ERE promoter constructs [292], which can be blocked by cotreatment with the antiestrogen ICI 182,780. In rodents, BPA induces vaginal cornification [293], promotes growth and differentiation of the mammary gland [294], decreases serum cholesterol levels [295] and increases prolactin levels [296]. The ability of BPA to increase uterine wet weight in the rodent is controversial. Submaximal induction was observed in the rodent uterotrophic assay at high doses of BPA, and this may be ER-independent due to the failure of ICI 182,780 (20 µg/day) to block these responses [297]. Even though BPA binds to the ER with low affinity and has weak hormonal activity, numerous studies have

reported that low doses of BPA can induce potentially adverse effects. Low doses of BPA induce early vaginal opening [298], advanced onset of puberty [299], disruption of estrous cyclicity [300, 301], decreased serum levels of luteinizing hormone following ovariectomy [301], and altered development of the fetal mammary gland in rodent [302]. BPA can be transferred to the fetuses from pregnant mice and alter postnatal development and sexual maturity at environmentally relevant exposure doses [299]. Prenatal exposure to low doses of BPA affects both early embryonic [303], and postnatal development in mice [304]. Perinatal or pubertal exposure of mice to BPA increases ERα and ERβ expression in the mouse forebrain [305] and hypothalamus in rats [306]. BPA is a potent ligand of human estrogen-related receptor γ (ERRγ) with an EC50 value of 13.1 nM [307], whereas E2 binds ERRγ with a 5-fold lower affinity. ERRγ is highly expressed in the mammalian brain during development and it is possible that BPA-dependent estrogenic effects in brain are mediated through ERα or ERRγ.

Although most studies have focused on the estrogenic effects of BPA, this compound also disrupts thyroid hormone action [308-310], acts as an AR antagonist [311, 312] and disrupts testosterone synthesis [313]. Low doses of BPA can promote proliferation of LNCaP human prostate cancer cells and this may be mediated through the mutant AR expressed in LNCaP cells [314].

1.2.1.3. Alkylphenols (AP)

Alkylphenol polyethoxylates (APEOs) are widely used as surfactants, insecticides and chemical stabilizers in industry, and also exist in household detergents and personal care products.

Nonylphenol and octylphenol polyethoxylates are the two most widely used APEOs and approximately 60% of APEOs end up in the aquatic environment,

most entering via sewage treatment works. During anaerobic wastewater treatment, the polyethoxylate side chain is cleaved and degraded to form more stable metabolites, such as hydrophobic nonylphenol (NP) and octylphenol (OP). Male fish living below sewage outfalls in Europe, Great Britain, North America and Japan exhibit elevated plasma concentrations of the yolk protein vitellogenin, which is synthesized in the female liver following stimulation by estrogens. Many male vertebrates, such as fish, amphibians and reptiles also synthesize vitellogenin after treated with estrogens. Intensive chemical fractionation of sewage identified two major classes of estrogenic compounds namely the pharmaceutical estrogen ethinyl estradiol and industrial chemicals APs, and these compounds cause male fish feminization and the increased number of eggs in females in some contaminated rivers.

APs bind ERα, induce vitellogenin gene expression, stimulate ERα transactivation and promote proliferation of MCF-7 breast cancer cells [315]. *In vitro* studies show that OP and NP are the most potent estrogenic APs, and the potency of OP is approximately 1000-fold less than E2 [315-317].

1.2.1.3.1. Nonylphenol (NP)

In the environment, NP adsorbs to sewage sludge in anaerobic conditions, whereas in rivers, NP tends to concentrate in bottom sediments and bioaccumulates within the internal organs of fish and birds, reaching a concentration 10–100 times higher than that found in the environment based on the stability and lipophilicity of this compound.

NP binds to ERα and stimulates ligand-dependent gene expression through ERE promoter constructs in both yeast and mammalian cells, and induces cell proliferation and PR expression in MCF-7 cells [316]. NP is estrogenic in a mouse uterotrophic bioassay and high doses accelerate vaginal opening [318]. In a longterm three generation study in rats, high chronic doses of NP accelerate

vaginal opening in all three generations, increase estrous cycle length, and both of these responses are markers for estrogenicity. A single high dose injection (600 mg/kg b.w.) of NP induces calbindin-D9k (CaBP-9k) mRNA and protein via an ERα-dependent pathway in the uterus of immature rats [319, 320]. CaBP-9k is a member of a large family of intracellular calcium binding proteins that have high affinities for calcium and this is a newly developed biomarker for exposure to environmental estrogens [321, 322].

NP can also impair steroidgenesis and disrupt endocrine systems through ER indirect pathways in juvenile female Atlantic cod [323]. NP modulates brain steroidgenic and biotransformation enzymes, such as CYP1A1, CYP3A [324] and sulphotransferase [325].

NP induced apoptosis in rat primary germ [326] and Sertoli cells [327]. Exposure to NP caused severe testicular abnormalities, including poor germ cell differentiation and reduced sperm counts in rats [328, 329] and trout [330]. The mechanism for decreased testis growth may be due to the effects of NP on cell death. NP inhibits testicular Ca^{2+} ATPase in low micromolar concentrations and inhibition of endoplasmic reticulum Ca^{2+} pumps can disturb testicular Ca^{2+} homeostasis [330]. In human embryonic cells, NP induces Fas-FasL interactions which also activate apoptosis in human embryonic stem cells [331].

1.2.1.3.2. Octylphenol (OP)

OP and NP have very similar activities. In trout hepatocytes, OP is considerably more potent than NP [315] and OP also stimulates estrogen-dependent vitellogenin production.

1.2.1.4. Polychorinated Biphenyls (PCBs)

PCB mixtures are industrial pollutants containing over 200 congeners and consist of two connected benzene rings and 1–10 chlorine atoms. These compounds are non-flammable and they exhibit chemical stability, a high boiling point and electrical insulating properties which lead to their widespread use as insulators and coolants in transformers and other electrical equipment. However, the lipophilicity and stability of PCBs also leads to widespread environmental contamination by these compounds. The production of PCBs has been banned due to numerous adverse health effects associated with PCB exposure on wildlife [332-334]. PCBs cause a number of health problems in wildlife, including effects on the immune, reproductive, nervous and endocrine systems. PCBs have been associated with adverse health effects in laboratory animals, but typically at very high dose levels relative to human environmental exposures. Acute treatment of laboratory animals with PCBs causes mild liver damage, or even death. Chronic feeding with low concentrations of PCBs cause various health effects, including anemia, liver, stomach and thyroid gland injuries. High-dose lifetime treatment to PCBs cause tumors in laboratory animals [335, 336]. Tumorigenic effect of PCBs decreases with decreasing chlorination and the position of the chlorines in the PCB molecule is a key determinant for their carcinogenicity [337].

However, effects of PCBs on human health are minimal. In human epidemiological studies, newborn babies exposed to PCBs in utero show slightly decreased body weights [338], delayed cognition development [339, 340], and problems with motor skills and difficulty in short term memory. Maternal exposure to PCBs also delays implantation and increases abortion rates. Workers in PCB production facilities have an increased incidence in rare liver cancers and malignant melanoma. It has also been linked to a high incidence of Parkinson’s disease [341]. However, studies of workers exposed to high doses

of PCBs over long periods of time by inhalation and/or skin contact have not demonstrated an increased risk of cancer [342]. Most of these human studies are controversial and there are many inconsistencies between studies suggesting that compounds other than PCBs may be involved. EPA has defined PCBs as probable human carcinogens [343].

PCBs have been found in many tissues of human and animal reproductive tracts, including follicular fluids, uterus and placenta. PCBs also have antithyroidal activity, decrease thyroid hormone levels in humans and animals [344] and interfere with normal growth. PCBs accumulate in the adrenals, where they interfere with the biosynthesis of three major classes of steroid hormones, cortisol, aldosterone, and androgen. PCBs interfere with sex steroid hormone production and this may be due to upregulation of CYP11B1 and CYP11B2 [345].

Some PCBs and hydroxy PCBs are weakly estrogenic [346], whereas some PCB mixtures exhibit antiestrogenic activity [347]. For example, 3,3',4,4',5-pentachlorobiphenyl (Penta Cl_3-PCB), a prototypical AhR agonist is also estrogenic in MCF-7 cells and the mouse uterus [348]. Results from a fluorescence resonance energy transfer (FRET) assay showed Penta-Cl_3-PCB induces ERα homodimerization, directly activates ERα, recruits ERα to the pS2 promoter and AhR/ARNT to the CYP1A1 promoter, indicating that this compound exhibits both estrogenic and AhR agonist activities [348].

The ban on PCB production with restricted use of PCB-containing equipment has decreased PCB levels dramatically in the environment, fish and human blood. No conclusive evidence supports the carcinogenic or other chronic adverse effects on human health with background environmental levels of PCBs, or even with much higher occupational exposure.

1.2.1.5. Phytoestrogens

Phytoestrogens are polyphenolic non-steroidal substances found in some plants but act as estrogens in animals and cancer cells. Based on their chemical structure, phytoestrogens can be divided into four main groups, namely isofavonoids, flavonoids, stilbenes and lignans. Consumption of phytoestrogen-rich diets may have protective effects on estrogen-related conditions, such as menopausal symptoms, and estrogen-related disease, such as prostate and breast cancers, although it is not clear if the anticancer effects are due to the phytoestrogens or overall eating habits.

1.2.1.5.1. Resveratrol

3,5,4'-Trihydroxystilbene (resveratrol) can be a *cis*- or *trans*-isomer and the *trans*-isomer exhibits higher estrogenic activity in estrogen-dependent human breast cancer cell lines [349]. In this dissertation, resveratrol refers to *trans*-resveratrol which is structurally similar to DES and binds to both ERα and ERβ [350]. Resveratrol is a phytoalexin that is produced by plants in response to fungal infection and exposure to ultraviolet light. It is not widely distributed in nature and is found mainly in grapes, peanuts, pine nuts and traditional oriental medicine plants [351]. It is quite abundant in grape skin, therefore red wine contains high levels of resveratrol which may account for the cardioprotective effects of red wine. Resveratrol also antagonizes E2 stimulated cell growth and inhibits PR transcription [352]. Therefore, it is a SERM which has a mixed agonist/antogonist activity [350, 353, 354]. However, the antagonist activity of resveratrol is only for ERα but not for ERβ [350].

Resveratrol has a short half-life (~8-14 min) and is rapidly metabolized resulting in low bioavailability. *In vivo* studies in mice show that resveratrol exhibited ER antagonist activity [355, 356] and decreased uterine wet weight, shortened time

for vaginal opening and blocked cholesterol-lowering effects of estrogen. Maternal exposure to resveratrol has minimal affects on rat vaginal opening, decreases female sexual behavior and elongates the estrous cycle [357, 358]. In conclusion, resveratrol has minimal estrogenic activity *in vivo* and may even act as an ER antagonist [356].

Resveratrol exerts a wide variety of biological effects. It prevents or slows progression of a variety of illnesses, including cancer [359] and cardiovascular disease [360], as well as enhancing stress resistance and extending the lifespan of various organisms from yeast to vertebrates [361]. Resveratrol also exhibits antioxidant effects [362]. However it is unclear if this is a direct effect or the result of the activation of pathways that upregulate cellular antioxidant defenses. The weak estrogenic activity of resveratrol may contribute to its cardioprotective effects [363], since estrogen replacement therapy has been shown to reduce the risk of cardiovascular disease and osteoporosis in postmenopausal women [364]. However, a firm connection between the estrogenic activity and cardioprotective effects of resveratrol is not known.

1.2.1.5.2. Genistein

Isoflavonoids are the most studied group of phytoestrogens. Genistein and other isoflavonoids are frequently present in the human diet and in serum with much higher quantities than endogenous estrogens. Soybeans are a rich source of isoflavones and they contain approximately 2 grams of isoflavones per kilogram fresh weight [365]. In plants genistein is in the form of a glycoside [366] and the 4'-methyl ether derivative of genistein is a less potent ER agonist. Genistein competes for ER binding because of its structural similarity, resulting in agonistic or antagonistic activity; genistein is 10^5 times less active than E2 and binds to ERβ with higher affinity than to ERα. Genistein inhibits cell growth in breast and prostate cancers *in vivo* and *in vitro* and regulates genes that are critical for the

control of cell proliferation, cell cycle, apoptosis, carcinogenesis, transcription regulation, and signal transduction pathways [367, 368]. Genistein induces apoptosis and inhibits activation of NF-κB and Akt signaling pathways, both of which are known to maintain a balance between cell survival and apoptosis [369]. Cotreatment of genistein with tamoxifen strongly inhibits proliferation of BT-474 breast cancer [370] and inhibits tumor growth in a mouse tumor xenograft model bearing MCF-7 breast cancer cells [371]. Cotreatment also downregulates survivin, EGFR, human epidermal growth factor 2 (HER2) proteins which are overexpressed in breast cancer and associated with tamoxifen resistance [372]. Therefore, genistein may sensitize the effects of tamoxifen and may have beneficial effects on tamoxifen-resistant cancer [371].

Genistein inhibits the activity of the key enzyme in androgen biosynthesis [373] and also stimulates the synthesis of sex hormone-binding globulins (SHBG) [374]. Furthermore, genistein exhibits many other non-hormonal effects *in vitro*, including inhibition of tyrosine kinases [375], DNA topoisomerase [376] and antiangiogenic [377] and antioxidant activities [378]. All these non-hormonal effects may contribute to the potential preventive effects of genestein against certain types of cancer, even though many of these responses require very high concentrations that are not reached *in vivo* [379].

1.2.1.6. Pharmaceutical Estrogens

Pharmaceutical estrogens were synthesized for treatment of menopausal symptoms, pregnancy complications and breast cancer and it was reported that British rivers were contaminated by estrogenic pharmaceutical wastes along with endogenous estrogens [380].

1.2.1.6.1. Diethylstilbestrol (DES)

DES was widely used to prevent miscarriages and other complications of pregnancy. However, its clinical use was banned due to its adverse effects on reproductive tract of offspring whose mothers had taken the drug during pregnancy [381]. DES is a transplacental carcinogen in humans [382]. Numerous studies have reported that in utero exposure to DES during critical developmental periods permanently alters gene programming in estrogen responsive tissues, and this results in benign and malignant reproductive tract abnormalities later in life. DES-exposed daughters, exhibit a high incidence of abnormal pregnancies, reduced fertility, rare vaginal tumors and disorders of the immune system. Similarly, DES-exposed sons exhibit structural, functional and cellular reproductive tract abnormalities, including hypospadias, microphallus, retained testes, inflammation and decreased fertility [383-387]. DES effects on the exposed mother are controversial [388], and exposure to DES during pregnancy only has a moderate increased risk of breast cancer [389, 390].

DES is a potent non-steroidal estrogen. It binds ER with a 2-fold higher affinity than E2 and mimics E2 actions. ERα mediates the detrimental effects of neonatal DES exposure in the murine reproductive tract [391]. DES is a potent mitogen *in vivo* both in uterine and pituitary tissues. Perinatal DES exposure delays expression of HOX and Wnt7a genes which are involved in the structural differentiation of the reproductive tract and regulated by estrogen [392]. Transgenic mice overexpressing ERα are more susceptible to tumors after neonatal DES treatment and these transgenic mice develop uterine carcinoma earlier than wild-type DES treated mice [393]. Both female and male αERKO mice exhibit complete resistance to the chronic effects of neonatal DES exposure in uterus and prostate respectively [37]. Therefore, ERα plays an important role in the induction of abnormalities and tumors after neonatal DES exposure.

In addition to the detrimental effects of DES that are mediated via the estrogen signaling system, animal studies indicate that DES exposure can also cause permanent changes of gene expression. Perinatal DES treatment permanently decreases ER levels in female rodent, resulting in an attenuated uterine response to E2 during adulthood [394, 395]. Similarly in males, neonatal exposure to DES decreases AR levels in rodent prostate, leading to a failure to fully respond to endogenous androgens [396]. Perinatal DES exposure leads to expression of the lactoferrin (Ltf) gene in the seminal vesicle [397] and the urethroprostatic complex of adult male mouse [398]. Ltf is not normally expressed in the male reproductive tissues, indicating that perinatal DES exposure leads to “feminization” of these male tissues in terms of Ltf expression.

1.2.1.6.2. Tamoxifen (Nolvadex)

Tamoxifen has been widely used for endocrine therapy in treatment for all stages of ER-positive breast cancer, including advanced breast cancer. Approximately 70% of patients with advanced breast cancer with tumors expressing both ER and PR have a good response to tamoxifen treatment, whereas less than 10% of the patients with ER and PR negative tumors respond to tamoxifen [399]. It was approved for the prevention of breast cancer in high-risk population, since it reduced the incidence of ER-positive breast cancer by about 50% [400] [401].

Tamoxifen is a SERM which exhibits ER antagonist activity in the mammary gland [402, 403], but partial ER agonist activity in the bone and endometrium [404, 405]. Because of its partial ER agonist activities in endometrium, longterm administration of this drug results in two- to three-fold increase in the incidence of endometrial carcinoma. The molecular mechanism for the selective effects of tamoxifen is unclear. It may involve in differential recruitment of nuclear coregulatory proteins in different tissues. Tamoxifen acts as an agonist while

recruiting SRC-1 to the responsive gene promoters in endometrial cells possibly through activation of the AF-1 of ERα [406]. However, tamoxifen exhibits antagonist activity while recruiting SMRT and NCoR in MCF-7 breast cancer cells [407]. High SRC-1 levels may correlate with a better response to tamoxifen treatment in women with recurrent breast cancer. The agonist activity of tamoxifen is dependent on the AF-1 of ERα, and tamoxifen shows strong agonist activity in cells where AF-1 of ERα is activated, but AF-2 is inactive. Glaros et al. showed that different phosphorylation sites in AF-1 of ERα regulate the agonistic and antagonistic action of tamoxifen [408]. Phosphorylation on Ser118 of ERα is important for the antagonist activity of tamoxifen. The activity of tamoxifen also depends on the circulating level of E2, which is high in premenopausal women, but low in postmenopausal women. For instance, tamoxifen decreases bone density in premenopausal women, but increases bone density in postmenopausal women [404].

Despite the efficacy of tamoxifen for treatment of breast cancer, prolonged use of this drug leads to formation of tamoxifen resistant tumors [409]. *De novo* resistance may be caused by loss of ER/PR [410] or ER mutations resulting in estrogen independent growth [411]. Loss of ER expression is the most important mechanism of de novo resistance, since >90% of ER-negative tumors will not respond to tamoxifen. However, this cannot account for all de novo resistance. Many ER+ and/or PR+ breast tumors are already resistant at the time of diagnosis and the resistance mechanism in these tumors is unknown. The mechanism for acquired resistance is more complex. Modification of ER function by growth factor pathways may be an important mechanism for acquired tamoxifen resistance [20, 412]. In tamoxifen acquired resistant cells, phosphorylation on Ser167 of ERα by increased expression of EGFR or activated AKT-2 enhances interaction of ERα with SRC-3, resulting in a conformational change that enhances the transcriptional activity and this results

in tamoxifen resistance [408]. Phosphorylation on Ser118 by activated MAPK kinases inhibits interactions of ERα with SRC-3 and maintains tamoxifen sensitivity [408]. Recent data suggest that during breast cancer initiation and development of tamoxifen resistance, the nuclear/cytoplasmic distributional pattern of ERα can be altered. Cooperation of ERα with growth factor pathways in the cytosol may contribute to breast cancer progression and resistance to endocrine therapy [413]. Tamoxifen treatment causes translocation of ERα out of the nucleus and enhances its interactions with epidermal growth factor (EGFR).

Tamoxifen sensitivity was restored in acquired resistance breast cancer cells after disruption of DBD of ERα and intramolecular communication with disulfide benzamide (DIBA) treatment [414], which quench target gene expression and blocking cell growth of tamoxifen resistant breast cancer cells. DIBA is a zinc finger inhibitor of ERα that disrupts the DBD of ERα and inhibits its communication with other domains, thereby facilitating dissociation of ERα from coactivators and association with corepressors. This suggests a possible new approach in decreasing tamoxifen resistance.

1.2.1.6.3. ICI 182, 780 (Fulvestrant, Faslodex)

ICI 182,780 is a potent ERα antagonist and clinical data show that ICI 182,780 lacks partial ER agonist activity. ICI 182,780 has a high binding affinity for ERα which is 90% of that observed in estrogen [415]. ICI 182,780 inhibits ERα dimerization [416], nuclear translocation, enhances ERα degradation and blocks ERα-mediated gene transcription. Antiestrogen-induced downregulation of ERα is mediated through several pathways, including increased turnover rate [417], decreased half-life time and induced ERα degradation [418]. H12 is important for ubiquitin-proteasome mediated ERα protein degradation and interaction with CK8 and CK18 which draw the receptor into close proximity of nuclear matrix-

associated proteasomes that facilitate ERα degradation [419]. Upon binding ICI 182,780, ERα undergoes a conformational change and preferentially recruits corepressors [92].

ICI 182,780 also inhibits nongenomic ERα signaling pathways. *In vitro* studies show that ICI 182,780 blocks activation of the MAPK pathway via E2 activation of membrane ERα. However, activation of MAPK via EGF is unaffected [420]. ICI 182,780 abrogates activation of ERα by other signaling factors, such as EGFR and IGFR [421, 422]. Both ICI 182,780 and tamoxifen can activate GPR30 and stimulate adenylyl cyclase, leading to cAMP-mediated inhibition of Erk1/2 and the attenuation of the EGFR-MAPK signaling cascade [423, 424].

ICI 182,780 is also involved in biological processes mediated by other hormone receptors, such as GR [425], AR [425] and PR [426]. ICI 182,780 can attenuate the antiflammatory effects of dexamethasone (DEX), a synthetic glucocorticoid in ovariectomized rats [427] and effectively suppress AR expression in human prostate cancer cells [425]. ICI 182,780 attenuates androgen-induced prostate-specific mRNA and protein expression as a result of AR downregulation. ICI 182,780 can also suppress AR-dependent transcription by acting through ERβ via interaction with EREs present in the AR promoter or through ERβ/NFκB crosstalk [428].

SERMS such as tamoxifen are the first generation, aromatase inhibitors (anastrozole, letrozole and exemestane) are the second and ICI 182,780 has now been approved for treatment of postmenopausal women with ER positive, locally advanced or metastatic breast cancer following tamoxifen therapy and is now a third generation drug for endocrine therapy. Because of a different mode of action, ICI 182,780 is generally well tolerated and effective in the treatment of tamoxifen-resistant disease.

1.2.2. Exposures to Estrogenic Chemical Mixtures

Xenoestrogens are strictly regulated and present at low levels in the environment. It was reported that exposure to binary mixtures of estrogenic chemicals resulted in synergistic interactions and this provoked scientific interest and public concern [429]. However, no other studies have observed the similar synergistic effects of xenoestrogen mixtures. Exposure to most estrogenic chemical mixtures primarily results in additive responses [430], when the mixture components are near or above their individual response thresholds [430]. However, this does not exclude the possibility of synergistic interaction mediated through different pathways or different receptors activated by chemical mixtures.

1.2.3. Risk of Xenoestrogens on Reproductive Health

Xenoestrogens have been associated with reproductive problems in wildlife and laboratory animals, whereas human health risks associated with low-level exposures of these compounds are still largely unknown and highly controversial. In the early 1990s, several publications heightened concerns about the potential adverse human health effects associated with background environmental exposures to chemicals that disrupt endocrine signaling pathways [431]. It was reported that breast cancer was four times higher in women who exhibited higher levels of the pesticide metabolite DDE compared to women without this disease [383, 385, 432-434]. However, it was subsequently concluded that there was no link between the serum levels of DDE and the risk of breast cancer. A number of studies also reported an apparent global decrease in human sperm counts [435]. However, the evidence for decreased sperm counts and male reproductive capacity is still limited [436-438]. The incidence of male reproductive tract problems appears to be geographically determined with different countries having variable levels of sperm counts and related problems.

Recently human epidemiological and experimental animal studies have suggested that the consumption of foods rich in phytoestrogens may have protective effects on menopausal symptoms, prostate and breast cancer [439]. The incidence and mortality of breast cancer in the Western world is much higher compared to that in Asian countries. First-generation immigrants from Asia have a lower risk of breast cancer, but the protection is lost in the second generation and this is probably related to the Western diet [440]. It has been suggested that certain phytochemicals present in Asian diets have beneficial effects for cancer prevention and this may be combined with genetic factors.

The structural similarities between DES and phytoestrogens are also concerns. Ingestion of high levels of phytoestrogen-rich clover caused an infertility syndrome in sheep in the 1940's [441]. Infants fed on soymilk formulas have higher plasma isoflavone levels than those of infants fed human or cow's milk [442]. However, there is no direct evidence to show that soy formula-fed infants display a greater risk for development of breast or prostate cancer later in life.

1.3. Sp/KLF protein

Specificity proteins (Sp) are a family of transcription factors that are characterized by their three conserved Cys2His2 zinc fingers that act as the DNA binding domain. Kruppel-like factors (KLF) named after the Drosophila segmentation gene Kruppel have has similar zinc finger motifs are also part of an extended Sp/KLF family [443]. Due to the conserved DNA-binding motifs, Sp/KLF proteins recognize the same GC- (GGGGCGGGG) and GT- (GGTGTGGGG) boxes, albeit with different affinities [444-447]. GC and GT boxes are important for the expression of many housekeeping genes and tissue-specific cellular and viral genes [448]. In addition, they are also involved in the maintenance of the methylation-free status of the CpG islands in many housekeeping genes [449, 450]. Besides their highly conserved DNA-binding domain, this protein family has highly variable N-terminal and glutamine-rich

regions which are essential for transcriptional activation or repression [451], whereas the C-termini are involved in synergistic activation and interaction with other transcription factors [452].

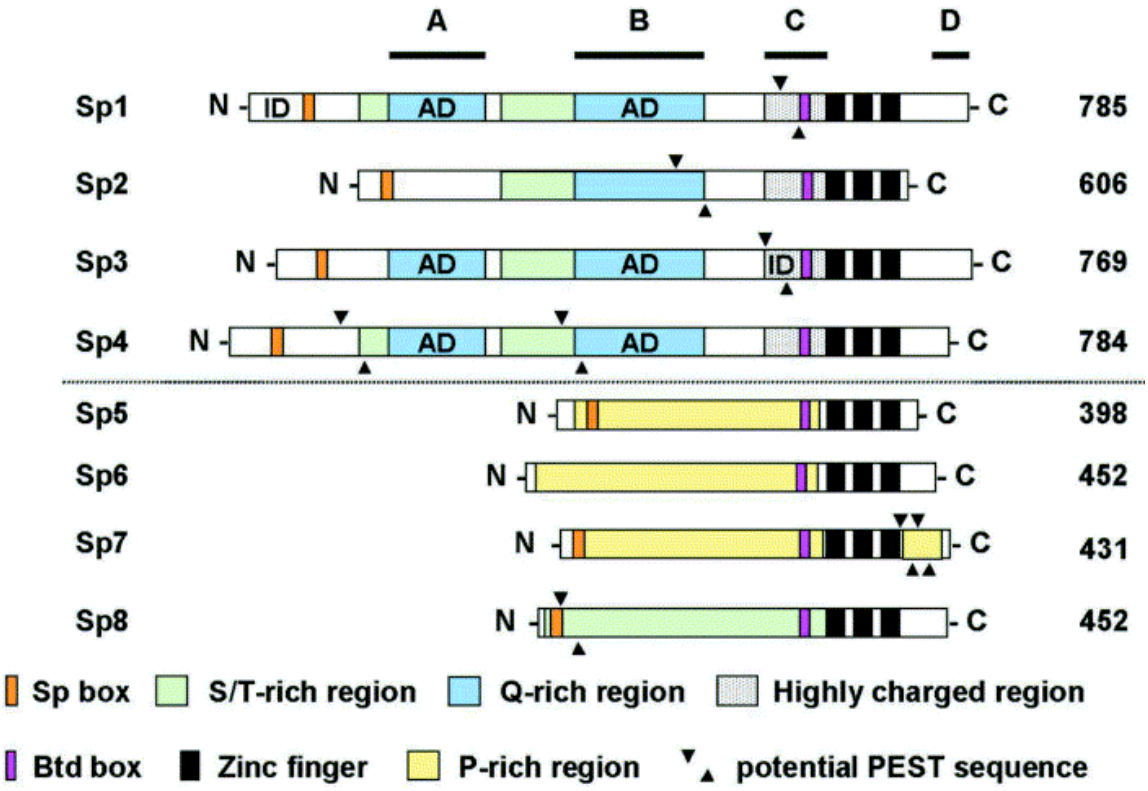

Figure 11. Structural motifs in Sp factors (Sp1-8). Sp and Btd boxes, serine/threonine-rich, glutamine-rich and highly charged regions, and zinc fingers are indicated, as well as activation (AD) and inhibitory (ID) domains. Each pair of arrow heads points at a PEST domain. Adapted from [453].

The Sp/XKLF family comprises a large number of homologous transcription factors. Currently 25 Sp/KLF proteins have been identified based on a search of the human genome database [453]. Sp1-8 are all located adjacent to a HOX gene cluster [448]. And Sp1-Sp4 form a subgroup based on their similar modular structure. Sp5-Sp8 are truncated forms that do not contain Q-rich activation domains. Sp1, Sp3 and Sp4 contain two major glutamine-rich transactivation domains A and B that are essential for transcriptional activation (Fig. 11). A serine/threonine-rich sequence is next to these A and B domains and this

sequence may be a target for post-translational modifications [451]. There is a conserved region of 11 amino acids called buttonhead box (Btd box) located next to the zinc finger motifs in all Sp proteins [454]. The Btd box was first identified in the Drosophila Sp1 homologue Btd. This region may contribute to the transactivation potential of Sp proteins, since deletion of Btd box reduces Sp1 activity *in vitro* [451]. The Btb box may also synergisticlly activate Sp1 and Sp3 of sterol-regulatory element-binding proteins (SREBP) [455]. Another conserved region namely the Sp box was identified at the N-terminus of the Sp proteins [454] and this region contains an endoproteolytic cleavage site [456], implying that it may have a function in regulation of Sp protein proteolysis. From the sequence alignment studies, Sp1, Sp3 and Sp4 are more closely related than Sp2. While Sp2 has only one glutamine-rich domain, it does share a highly charged domain C and a serine/threonine-rich region. Sp2 has a different DNA-binding preference from Sp1, Sp3 and Sp4, preferentially recognizing a GT box rather than a GC box, due to the substitution of a critical histidine residue by a leucine residue in zinc finger 1 [447].

Sp protein expression may be a critical factor in tumor initiation, development and metastasis. Sp1 protein is highly expressed in gastric [457], pancreatic [458], thyroid [459] and colon tumors [460] compared with normal tissues. The clinical results also showed that high expression of Sp1 in gastric tumors correlates with lower survival rate [457]. Sp1, Sp3 and Sp4 proteins were all overexpressed in human breast cancer cell lines, including MCF-7, MDA-MB-231, MDA-MB-453 and T47D cells compared with normal breast cells (unpublished data).

1.3.1. Sp1

Sp1 was the first transcription factor identified and Sp1 binds to multiple GC/GT boxes through three zinc finger motifs and activates transcription in the simian virus 40 (SV40) early promoter [461]. Sp1 is ubiquitously expressed and is

involved in numerous cellular processes such as cell cycle regulation [462-464], chromatin remodeling [465], methylation-free island propagation [450] and hormone activation [450, 466, 467].

It was assumed that inactivation of Sp1 would be lethal for cellular growth and differentiation and result in failure in preimplantation; however, $Sp1^{-/-}$ embryos survive until day 10 of embryonic development [468]. $Sp1^{-/-}$ ES cells show normal growth characteristics. However, after day 10 of development, all Sp1 null embryos are severely retarded in growth and die. This suggests that Sp1 is not essential for the early embryonic differentiation but is required for the maintenance of differentiated cells. Many putative Sp1 target genes are expressed at normal levels in Sp1 null mice, suggesting that Sp3 or other members of Sp family may compensate for inactivation of Sp1. Sp1 may play a role in the maintenance of methylation-free islands in the CpG island of the APRT gene [450]. However, the methylation pattern in the Sp1 null mice is not affected [468]. Further studies show that the expression of the methyl-CpG-binding protein MeCP2 is 20-fold lower in Sp1 null mice than wild type, indicating that Sp1 is important for maintaining cell methylation status by regulating MeCP2 expression. This is supported by the observation that MeCP2 knockout animals exhibit a similar phenotype as Sp1 knockout animal. However, whether Sp1 acts as a direct regulator of MeCP2 is not yet known.

Sp1 can stimulate transcription from either proximal promoters or distal enhancers [451]. Via distal enhancers, Sp1 tetramers are involved in looping out the intervening DNA through inter-protein interactions of domain A and B between adjacent Sp1 proteins (Fig. 11) [445, 451, 469]. Activation domain B appears to play a critical role for Sp1 polymer formation [469], and domain D interactions with both A and B are required for synergistic activation associated with binding to multiple sites [469].

Sp1 protein can be post-translationally modified by glycosylation [470], acetylation [223, 471] and phosphorylation [472]. Reduced glycosylation of Sp1 increases the susceptibility of Sp1 to proteasome-dependent degradation [470, 473]. Acetylation of Sp1 also increases Sp1-dependent transactivation. Sp1 can be phosphorylated by different kinases, such as PKA, casein kinase II, ERK, cyclin-dependent kinase (cdk) [474]. Its phosphorylation status is associated with the affinity of Sp1 for binding DNA and activation [474] and it is important for Sp1-dependent activation of some genes. Increased phosphorylation of Sp1 by PI3K enhanced Sp1 binding to VEGF promoter and upreguated VEGF expression in prostate cancer cell lines [475].

1.3.2. Sp3

Sp3 is highly homologous to Sp1 with similar GC/GT binding affinity. Both proteins are widely expressed. However, *in vitro* and *in vivo* studies reveal that Sp1 and Sp3 exhibit different functions. Sp3-deficient embryos are growth retarded and invariably die at birth of respiratory failure [453]. The cause for this breathing defect is unknown. Only minor morphological alterations were observed in the lung. Therefore, Sp3 is essential for immediate post-natal survival. Comparison of the phenotype of $Sp1^{-/-}$ and $Sp3^{-/-}$ mice demonstrates their redundant function in early developmental stages and their unique functions in the later developmental stages [350, 476].

Sp3 can act as a positive or negative regulator of Sp1, depending on promoter and cell context [477]. Upon cotransfection of Sp3 with Sp1, both additive and synergistic effects were observed [451, 478, 479]. It was suggested that the relative expression of Sp1 and Sp3 in cells determines the regulation of some target genes, since they compete for the same binding sites. Three key amino acids KEE define the Sp3 inhibitory domain which is located between the second glutamine-rich activation domain and the first zinc finger motif (Fig. 11) [480]. The inhibitory domain of Sp3 functions autonomously, independent of its

context or position [480]. Because of its dual nature, Sp3 can repress Sp1 or other transcription factor mediated gene expression, especially when there are multiple adjacent GC boxes [445, 481]. However, Sp3 can also act as an activator for promoters containing multiple GC-boxes. For example, Sp3 is a stronger transactivator of the p21 promoter, which contains six GC-boxes than Sp1 [482].

Sp3 has multiple isoforms, which makes the transcriptional regulation by Sp3 more complex [447, 483-485]. It has four isoforms expressed *in vivo*, two long forms and two short forms which are derived from different translational initiation sites [484]. The short isoforms are expressed in all mammalian cells along with the full length Sp3 and they express transactivation domain B and the inhibitory domain, but not domain A. Therefore, increased levels of the short Sp3 isoforms might be responsible for the Sp3-mediated transcriptional repression [483].

Sp3 can also be post-translationally regulated. Unlike Sp1, none of Sp3 isoforms are glycosylated but are post-translationaly modified by the small ubiquitin-like modifier (SUMO) specifically and exclusively at lysine 551. SUMO modification may silence the transcriptional activity of Sp3 [484]. Acetylation of the lysine residue in the inhibitory domain contributes to the low transcriptional activity of Sp3 [480, 486].

1.3.3. Sp4

Expression patterns of Sp/KLP are highly variable among different family members and this is important for determining their specificity. In contrast to the ubiquitous expression of Sp1 and Sp3, Sp4 shows a more restricted expression pattern. During development, Sp4 is highly expressed in the central nervous system, and is also abundant in epithelial tissues, developing teeth and testes. $Sp4^{-/-}$ mice develop until birth without obvious abnormalities. About two-thirds of $Sp4^{-/-}$ mice die within a few days after birth [487]. They exhibit reduced body

weight, a slightly stunted appearance and a higher incidence of sudden cardiac arrest. These phenotypes are all linked to the higher expression of Sp4 in brain and conduction system of the heart [488]. Surviving mice exhibit a striking sex-specific abnormality. $Sp4^{-/-}$ females are fertile and males do not exhibit mating behavior, even with intact spermatogenesis [489]. The most likely cause of this abnormal behavior is a neurological defect. In addition $Sp4^{-/-}$ mice have smaller thymus, spleen, uterus and testis compared to wildtype mice. In $Sp4^{-/-}$ mice, Sp1 expression is unchanged while Sp3 is induced 2-fold. Thus the absence of Sp4 might be partially compensated by enhanced transcription of the Sp3 gene.

The transactivation function of Sp4 resides in the Q-rich domains. Unlike Sp1, Sp4 additively but not synergistically transactivates promoters containing multiple GC- or GT boxes [490].

1.3.4. Cooperative Interactions with Other Proteins

Sp proteins regulate gene expression by cooperating with other proteins, such as transcription factors, the basal transcription apparatus and chromatin remodeling complexes. At its binding sites, Sp1 first forms a tetramer and then assembles multiple stacked tetramers at the DNA binding sites [491]. Therefore, it presents multiple docking sites for interactions with other proteins and multiple protein binding sites of Sp1 have been identified. While Sp3 usually only exists as a monomer [476], it does not form the same higher-order protein complex as Sp1. For other Sp proteins, most interactions are mediated by their C-terminal domains (C/D) (reviewed in [492]).

1.3.4.1. Sequence-Specific Transcription Factor

Sp1 and other Sp proteins can functionally and physically interact with other sequence-specific transcription factors, such as E2F1 [493-495], NF-Y, AhR [496], SMADs, GATA protein, jun, NFκB [497] and Oct-1 (reviewed in [108]).

Both E2F1 and Sp1 proteins physically interact and cooperatively activate the TK promoter that contains a GC-rich and an E2F1 binding site separated by 6 bp. Mutation of either site results in more than 90% loss of basal activity in Swiss 3T3 cells [465]. Sp1 also interacts with NFκB through the transactivation domain A in the HIV-1 promoter [497]. The interaction of Smad3 with Sp1 but not with Sp3 demonstrates that distinct Sp proteins can specifically co-operate with other transcription factors [498]. Other sequence-specific factors that interact with Sp proteins include AhR/Arnt, GATA1/2/3, NF-YA, MyoD, MDM2, c-jun, AP-2, SMAD2/3/4 (reviewed as [108]).

1.3.4.2. Basal Transcription Machinery

Sp1 and Sp3 can directly interact with TBP [499] and dTAF(II)110/hTA-F(II)130 via the glutamine-rich activation domains A and B, and Sp1 also interacts with hTAFII55 [500] and hTAFII250 [501-504] via its C-terminal domain. TAF(II)250 plays an important role in stimulation of Sp1 transcriptional activity through direct interactions with Rb protein [505]. And Sp1 also interacts with the human mediator or coactivator complex called cofactors required for Sp1 coactivation (CRSP) [506]. CRSP is a 700 kDa multiprotein complex which functions in conjunction with TBP-associated factors to facilitate Sp1-mediated gene transcription [24, 507, 508]. The interactions of other Sp/KLF proteins with the basal transcription machinery have not been determined.

1.3.4.3. Chromatin Remodeling Complex

Sp1 and Sp3 interact directly or indirectly with histone deacetylase 1 (HDAC1) and p300/CBP. Sp1 is also a direct target for HDAC1-mediated transcriptional repression [465]. Upon recruiting HDAC1 and p300/CBP simultaneously, the resulting Sp1 complex establishes dynamic histone acetylation/deacetylation of promoter-bound histones to regulate gene expression, acting as either a

repressor or an activator. Vitamin D receptor interacting proteins (DRIPs) are coactivators of NR-mediated transactivation [507, 509] and they form complexes with ERα and Sp1 and interact with both proteins to activate gene transcription [510]. Coactivation of ERα/Sp1 is NR-box-independent and requires the 23 amino acid sequence (789–811) containing an α-helical amino acid 795–804 region, which is also required for coactivation of ERα [511].

1.3.4.4. Steroid Hormone Receptors

Sp1 binds directly to several ligand-activated and orphan nuclear receptors and these include the ER, PR, AR, RAR, RXR, PPARγ, VDR, SF-1, COUPTFII and HNF-4 (reviewed in [108]). The C-terminal C/D domain of Sp1 (Fig. 11) is the major interaction surface for its interactions with steroid hormone receptors. However, the interaction domain of steroid hormone receptors are diverse. For example, the DBD of AR is required for AR/Sp1 complex formation on the rat luteinizing hormone β promoter [512, 513]. However, multiple regions of ERα are required for ERα/Sp1 interaction.

Sp proteins play an important role in regulating gene expression through interactions with ER in breast cancer cells associated with nucleotide metabolism (thymidylate synthase, adenosine deaminase, Cad) [117, 119, 121], cell cycle progression (cyclin D1, E2F1, c-fos, TGFα) [113, 115, 116, 122], DNA synthesis (DNAPα) [120], angiogenesis (VEGF, VEGFR1, VEGFR2) [124-127, 514] and anti-apotosis (bcl-2) [114]. ER binds Sp1 independent of estrogen, and ligand-induced transactivation requires ERα/Sp1 interactions with specific GC-boxes on gene promoters. The response does not require the DBD of ERα, and the zinc finger motifs of Sp1 are necessary for DNA binding [111]. Sp1 is important for E2-induced and basal proliferation of MCF-7 cells [515]. Transcriptional activation of genes by E2 through ER/Sp interactions is dependent on cell and promoter context, ER subtypes and ligand structure. For

example, E2 activates ERα/Sp1 in breast and prostate cancer cells but not in HeLa cells, whereas hormone-dependent activation of ERβ/Sp1 was not observed in any of the cell lines and decreased activity was observed in HeLa cells [111]. In addition, Sp proteins differentially function in mediating E2-dependent responses. For example, in ZR-75 breast cancer cells, E2-induced activation of the GC-rich region of the VEGF promoter is dependent on Sp1 and Sp3 [124], Sp3 and Sp4 play important roles for E2 responsiveness of VEGFR2 [127]. However, in pancreatic cancer cells, all three Sp proteins are important for VEGFR1 [514] and VEGFR2 [126] mRNA expression. Currently there is no evidence to show direct interaction between ERα and Sp3, Sp4.

Overexpression of a C-terminal F domain peptide (a.a. 575-595) specifically blocked E2-mediated ERα/Sp1 transactivation, suggesting that other nuclear cofactors interacting with the F domain of ERα may be important for ERα/Sp1 action [128].

1.4. Transgenic Animals

ER-binding affinities and transactivation assays in mammalian cell lines and yeast have been extensively used for determining estrogenic activities of compounds. However, these assays do not necessarily account for the *in vivo* activity of a compound, which is dependent on uptake, distribution, bioavailability and metabolism. Uterotrophic and vaginal cornification assays have been traditionally used as *in vivo* bioassays for estrogenic compounds. After treatment with the test compounds, either the uterine wet weight or the extent of vaginal cornification is examined. However, uterotropic activity does not always correlate with the estrogenic activity of chemicals. Some xenoestrogens, such as BPA, have weak uterotropic activity and fail to increase rodent uterine wet weight, but are potent activators of ER-induced genes in the uterus [516]. Tamoxifen is a SERM that has ER agonist activity in the uterus and promotes proliferation of

endometrial cells. Tamoxifen causes uterine wet weight gain but fails to enhance reporter gene activity in the uterus in a transgenic mouse model expressing an ERE promoter in the absence of the endogenous E2 [517].

Reporter genes, such as luciferase and green fluorescence protein (GFP), are readily detected. Gene targeting techniques allow for the introduction of reporter genes into mice which can be used to study the expression of specific receptors *in vivo* [518]. In the transgenic animals model with reporter gene, the expression of reporter gene does not interfere with normal physiology nor induce compensatory metabolic effects. Therefore, these transgenic mice can be generated to investigate the *in vivo* effects of xenoestrogens.

Reporter gene constructs can be introduced into the mouse genome in three ways (reviewed in [518-520]): direct pronuclear injection of exogenous DNA into fertilized zygotes; injection of genetically modified embryonic stem (ES) cells into a blastocyst; or retrovirus-mediated gene transfer. During the pronuclear injection, a chosen linear gene construct is direly microinjected into the male pronucleus. The introduced DNA may lead to the over- or under-expression of certain genes or the expression of exogenous genes. The injected DNA is randomly integrated into the mouse genome. Injected zygotes are implanted into recipient dams that act as a recipient by mating with a vasectomized male. Later genotyping is applied to select pups expressing the transgene. A major advantage of this method is its applicability to a wide variety of species. The disadvantage is the high possibility that the introduced gene will be inserted into a silent site. In contrast, embryonic stem cell-mediated gene transfer involves prior insertion of the desired DNA sequence by homologous recombination into an *in vitro* culture of ES cells. ES cells are undifferentiated cells that have the potential to differentiate into any cell type (somatic and germ cells) and therefore give rise to a complete organism. These cells are then incorporated into an embryo at the blastocyst stage of development, resulting in a chimeric animal.

ES cell-mediated gene transfer is the choice for gene inactivation (gene knock-out) or site specific knock-in of genes. A retrovirus is a virus that carries its genetic materials in the form of RNA rather than DNA. Retrovirus-mediated gene transfer is mediated by means of a carrier or vector which is generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell. Offspring derived from this method are chimeric, since not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

Numerous successful mouse models have been generated to study the effects of ligands on activating receptors. The first transgenic animal model indicating ER activity was generated in 2001 with insertion of three copies of ERE with a minimal thymidine kinase (TK) promoter linked to the reporter gene β-galactosidase (LacZ) [516]. LacZ is not a particularly good reporter for *in vivo* dynamic studies on ER activity, since LacZ has a long turnover rate and accumulates in mammalian cells. However, LacZ is easily detected in situ by a colorimetric assay. In these animals ER-dependent activity was detected in a small range of tissues, including pituitary, uterus, kidney, liver, hypothalamus, adrenal, thyroid, adipose, muscle and mammary gland in mature ovariectomized female mice. In all the tissues DES induced ER-dependent LacZ activity. Another ER transgenic animal was generated, using luciferase gene as a reporter and regulated by a TK promoter via ERE [521]. The advantages of using luciferase as reporter include the high signal to noise ratio in mammals; the short turnover rate of luciferase enables a dynamic view of ER activity; moreover the assay is highly sensitive and can be quantified. In this model, insulators were used to facilitate ubiquitous expression of transgene. Insulators are DNA sequence elements that prevent inappropriate interactions between adjacent chromatin domains [522]. Insulators not only create a locus that is permissively expressed in all tissues [523], but also facilitate the creation of an open domain for gene specific regulation. The ERE-Luc transgene was widely

expressed in many tissues and treatment with 50 μg/kg b.w. of E2 induced luciferase activity within 6 hours in liver and bone and this induction response was blocked after cotreatment with the antiestrogen ICI 182,780 [521, 524]. Immunohistochemical staining also showed that ERα and luciferase are colocalized in E2-treated ovariectomized mouse uterus. Both studies used ovariectomized mature females to minimize the endogenous estrogen level, since the ovary is the primary source for estrogen production. However, it is difficult to completely abolish endogenous estrogen as shown with the ERE-LacZ reporter mouse, the antiestrogen ICI 182,780 inhibited basal ER-dependent activity in the intestine, fat, liver, thyroid and uterus by over 50% [516]. This is due to the shortcomings of ovariectomy in which all the ovarian cells must be removed, since single ovary cell can produce estrogen. A new transgenic mouse model with insertion of ERE-Hsp68-Luc reporter and knock out of aromatase gene ($Ar^{-/-}$) was generated [525]. Therefore, no endogenous estrogens are produced. $Ar^{-/-}$ female are completely infertile and the infertility can be reversed by transplantation of wild type ovaries into ovariectomized $Ar^{-/-}$ female mice, indicating that the reproductive failures in $Ar^{-/-}$ females are primarily due to a defect in ovarian estrogen synthesis. All these models do not exclude-ERE-independent activation because of the presence of other promoter sequences, including either a minimal TK or Hsp68 promoter. Therefore, another transgenic animal model carrying a luciferase reporter gene under the control of three consensus EREs coupled to a minimal TATA-box was generated [526]. Two copies of the chick β-globin insulator were also used to improve expression and minimize effects of surrounding DNA sequences. The use of a minimal TATA-box in the transgene avoided activation of the transgene via other promoters.

The differences in the expression patterns of the reporter genes *in vivo* among transgenic mice might be attributable to different experimental conditions, the structures of the transgenes and the strains of mice. Both copy numbers of the

transgene and the integration sites also modify reporter gene expression patterns *in vivo*.

All the current transgenic models to indicate *in vivo* activity of ER are dependent on classical EREs. Therefore, studies reported in this thesis will generate a novel transgenic model which can be used to investigate the *in vivo* estrogenic activity of xenoestrogens through interactions with Sp proteins, a nonclassical ER pathway.

CHAPTER II

DIFFERENTIAL ACTIVATION OF WILD-TYPE ESTROGEN RECEPTOR α AND C-TERMINAL DELETION MUTANTS BY ESTROGENS, ANTIESTROGENS AND XENOESTROGENS IN BREAST CANCER CELLS*

2.1 Overview

E2, DES and several synthetic (or xenoestrogenic) compounds induced transactivation in MCF-7 or MDA-MB-231 cells transfected with wild-type ERα and a construct ($pERE_3$) containing three tandem EREs linked to a luciferase gene. In contrast, the antiestrogens ICI 182,780 and 4-OHT were inactive in this assay. We have investigated the effects of these compounds and several structurally-diverse estrogenic compounds on transactivation in cells transfected with $pERE_3$ and wild-type ERα, mutant ERα(1-553), and ERα(1-537) containing deletions of amino acids 595-554 and 595-538, respectively. These constructs were used to develop an *in vitro* assay to distinguish between different structural classes of estrogenic compounds. The results obtained using these constructs were highly cell context- and structure-dependent. Neither E2 nor DES induced transactivation in MCF-7 (or MDA-MB-231) cells transfected with $pERE_3$/ERα(1-537) due to partial deletion of helix 12; however, OP and NP, resveratrol (a phytoestrogen), kepone and 2,3,4,5-tetrachloro-4-biphenylol were "estrogenic" in MCF-7 cells transfected with $pERE_3$/ERα(1-537). Moreover, the structure-dependent estrogenic activities of several synthetic estrogens (xenoestrogens) in MDA-MB-231 cells were different than those observed in MCF-7 cells. These

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results demonstrate that the estrogenic activity of many synthetic compounds do not require activation function 2 (AF-2) of ERα and are mechanistically different from E2. These data suggest that xenoestrogens are SERMs.

2.2 Introduction

The ER is a ligand-activated nuclear transcription factor and a member of the NR superfamily [527-531]. The ER and other NRs regulate multiple physiological responses, many of which are dependent on endogenous hormonal or biochemical stimuli, whereas endogenous or exogenous ligands for other receptors such as orphan receptors are unknown or not well defined [532, 533]. Despite the differences among NRs, they all exhibit a similar structural organization in which the N-terminal A/B domain contains activation function 1 (AF-1), whereas the C-terminal E/F domain contains both the ligand-binding domain and activation function 2 (AF-2), which is ligand-dependent. The highest structure homology among NRs is observed in the DNA binding domain (DBD), which contains two zinc fingers that are critical for recognition and binding to cognate response elements. The hinge region or D domain is highly variable among NRs, and this region is responsible for several functions that include facilitating interactions between AF-1 and AF-2 for some NRs.

Different classes of NRs are also activated by exogenous compounds, and for the ER there has been concerns regarding the potential adverse human health effects of dietary phytoestrogens and synthetic estrogenic environmental contaminants (xenoestrogens) [534-536]. Evaluation of the estrogenic effects of these compounds is complicated by the complex pharmacology of estrogens in which estrogenic compounds exhibit tissue/cell-specific ER agonist and antagonist activities due, in part, to tissue-specific, expression of critical coactivators and other coregulatory proteins [537, 538]. Several *in vitro* assays have been developed to distinguish between different classes of SERMs, including antiestrogens, such as tamoxifen and ICI 182,780 used for treatment

of early stage breast cancer, phytoestrogens and xenoestrogens [406, 539-548]. This paper describes an assay in ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells which distinguishes between E2, antiestrogens and xenoestrogens. In this study, cells are cotransfected with $pERE_3$ containing three tandem estrogen responsive elements (EREs) linked to luciferase and wild-type ERα or E/F domain variants ERα(1-553) or ERα(1-537) containing deletions of amino acids 595-554 and 595-538, respectively. The loss of amino acid 595-538 abrogates coactivator interactions with helix 12 of ERα and loss of E2-induced transactivation in cells transfected with ERα(1-537); however, several xenoestrogens including kepone retain their estrogenic activity.

2.3. Materials and Methods

2.3.1. Chemicals, Biochemicals and Plasmids

Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Antibiotic antimycotic solution (AAS) (100X) was obtained from Sigma Chemical (St. Louis, MO). The following test chemicals (and purities) were purchased from Aldrich Chemical (Milwaukee, WI): p–t-octylphenol (97%), p-nonylphenol (98%) and bisphenol A (BPA, >99%). 2,3,4,5 -Tetrachloro-4-biphenylol (HO-PCB-Cl_4) was >98% pure as previously described [549]; 2,2- bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE, >98%) was synthesized as previously reported [67]. Kepone (98%) and endosulfan were purchased from Chem-Service (West Chester, PA). 4-OHT, E2, and resveratrol (99%) were purchased from Sigma, and ICI 182,780 was provided by Dr. Alan Wakeling (Astra-Zeneca, Macclesfield, UK). Plasmid preparation kits were purchased from Sigma. All other chemicals were obtained from commercial sources at the highest quality available. Human ER expression plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX); ERα(1-537) and ERα(1-553) were made as previously reported [550].

2.3.2. Cells and Transient Transfection Assays

Human breast cancer cell lines MCF-7 and MDAMB-231 were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture Ham's F-12 (DME/F-12) (Sigma) supplemented with 2.2 g/l sodium bicarbonate, 5% FBS, and 5 ml/l AAS. Cells were cultured and grown in a 37°C incubator with humidified 5% CO_2, 95% air.

For transient transfection studies, MCF-7 or MDA-MB-231 cells were seeded in 12-well plates in DMEM/F-12 medium without phenol red supplemented with 2.2 g/l sodium bicarbonate, and 2.5% charcoal-stripped FBS. After 24 h, cells were transfected using the calcium phosphate with 350 ng of luciferase reporter construct ($pERE_3$), 100 ng pcDNA3.0/*His*/*lacZ* (Invitrogen, Carlsbad, CA) as a standard reference for transfection efficiency, and 200 ng of the appropriate ERα expression plasmid. Six hours after transfection, cells were shocked with 25% glycerol/PBS for 1 min, washed with PBS, and then treated with dimethylsulfoxide (DMSO, solvent) or different concentrations of estrogens, antiestrogens or xenoestrogens in DMSO for another 30–48 h. Cells were then washed twice in PBS and harvested with 100 μl of reporter lysis buffer (Promega Corp., Madison, MI). After one freeze-thaw cycle, cell lysates were centrifuged for 1 min at 16,000*g*, and the supernatant was used for determination of protein activity. Luciferase (Promega Corporation, Madison, MI) and β-galactosidase activities were determined using the Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA). Light emission was detected on a lumicount micro-well plate reader (Packard Instruments, Meriden, CT), and luciferase activity was calculated by normalizing against β-galactosidase activity obtained from the same sample and compared with the DMSO control group (set at 100%) for each set of experiments.

2.3.3. Western Blot Analysis

MCF-7 cells were treated with the maximally-inducing concentrations of E2, 4-OHT, ICI 182,780, resveratrol, and the xenoestrogens for 3 h. Cells were then washed once with PBS and collected by scraping in 150 μl of high salt lysis buffer [50mM HEPES, 0.5M NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 5 μl/ml Protease Inhibitor Cocktail (Sigma–Aldrich, St. Louis, MO)]. Lysates from the cells were incubated on ice for 1 h with intermittent vortexing, followed by centrifugation at 40,000*g* for 10 min at 4 °C Equal amounts of protein (60 μg) from each treatment group were diluted with loading buffer, boiled, and loaded onto 10% SDSpolyacrylamide gel. Samples were electrophoresed and proteins were detected by incubation with polyclonal primary antibody ERα (G-20) from Santa Cruz (Santa Cruz, CA) and -actin from Sigma–Aldrich (St. Louis, MO), followed by blotting with the appropriate horseradish peroxidaseconjugated secondary antibody. After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics Corp., Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corp., Billerica, MA).

2.3.4. Statistical Analysis

For transient transfection studies, results are expressed as mean±S.D. for at least three separate experiments for each treatment group. Statistical differences ($p < 0.05$) between control (DMSO) and treatment groups were determined by ANOVA test.

2.4. Results

MCF-7 cells used in this study exhibit minimal induction of luciferase activity after transfection with $pERE_3$, however, after cotransfection with ERα, E2 induces a concentration dependent increase in luciferase activity, which is

maximal using 10 nM E2 (Fig. 12A). Similar results were observed for DES, a potent phenyl-substituted transtilbene-derived estrogenic drug where 10 nM gave the maximal response. In contrast, the antiestrogenic drugs 4-hydroxytamoxifen and ICI 182,780 decreased basal activity in MCF-7 cells transfected with $pERE_3$ and wild-type ERα (Fig. 12B). These results are consistent with previous reports in breast cancer cell lines [67, 551] although 4-hydroxytamoxifen exhibits partial ER agonist activities in other cancer cell lines [406, 544].

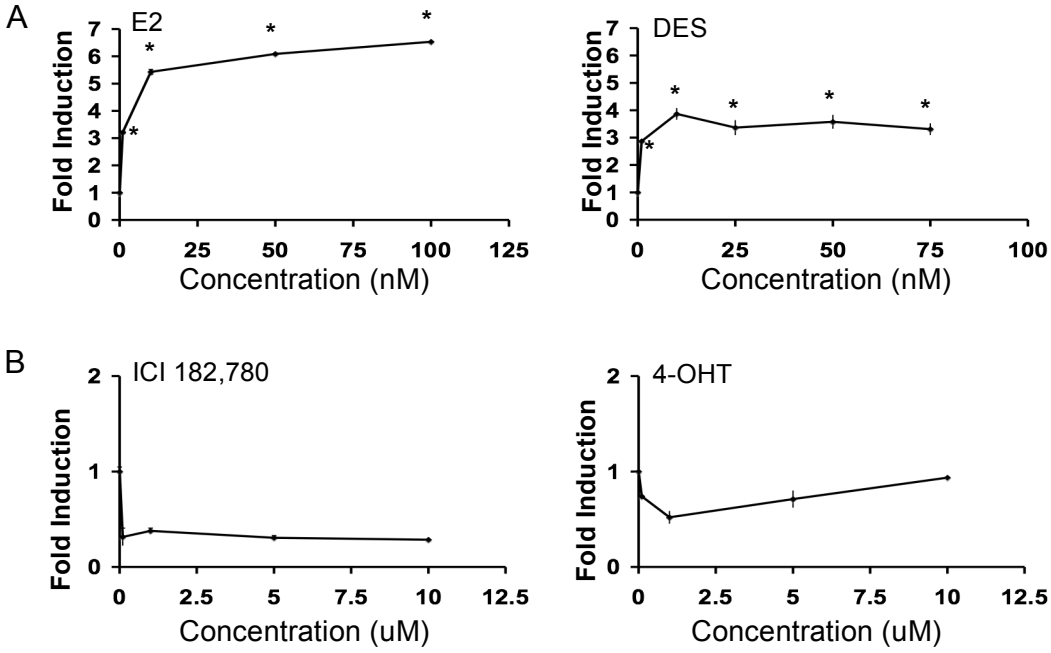


Figure 12. Estrogen- and antiestrogen-induced transactivation. MCF-7 cells were transfected with $pERE_3$ and treated with different concentrations of E2/DES (A) and ICI 182,780/4-OHT (B) and luciferase activity was determined as described in the Materials and Methods. ICI 182,780/4-OHT did not induce activity and >1 nM E2/DES significantly ($p<0.05$) induced activity. Results of all transactivation studies (Fig. 12-15) are expressed as means ±SD for three replicate determinations for each concentration. Significant ($p<0.05$) induction is indicated by an asterisk.

The concentration-dependent activities of two bisphenolic xenoestrogens are illustrated in Figure 13A and HPTE was more potent of these compounds with a

4- to 6- fold induction of luciferase activity (25-75 μM). In contrast, less than a 3.5-fold induction was observed for BPA and these differences in potency are comparable to those observed in other cell lines [67, 551]. The phenolic xenoestrogens octylphenol and nonylphenol induced a 2- to 4-fold increase in luciferase activity using 25 μM (Fig. 13B) and this concentration was used in subsequent studies due to some cytotoxicity observed at higher concentrations. A structurally unrelated phenolic estrogen HO-PCB-Cl_4 induced a 4- to 5-fold increase in activity whereas up to 75 μM resveratrol was weakly active to have a less than 2-fold induction on luciferase activity in this assay (Fig. 13C). The cyclodiene-derived endosulfan and kepone differentially induce luciferase activity with 25 μM kepone and 75 μM endosulfan inducing a >20-fold and approximately 2-fold increase in luciferase activity (Fig.13D). These results demonstrate that xenoestrogens were less potent than E2 or DES in MCF-7 cells transfected with $pERE_3$ and wild-type ERα and the fold-induction by these compounds was less than observed for E2.

Previous studies show that xenoestrogens and antiestrogens differentially activate wild-type and variant ERα in cells transfected with ERE promoters [18, 406, 418, 544, 552]. Ligand-dependent differences in ERE-dependent transactivation were further investigated in MCF-7 cells transfected with wild-type ERα and two C-terminal deletion mutants ERα(1-553) and ERα(1-537) in which the F domain, and F domain plus part of helix 12 of ERα have been deleted respectively. The concentrations of individual compounds in this study

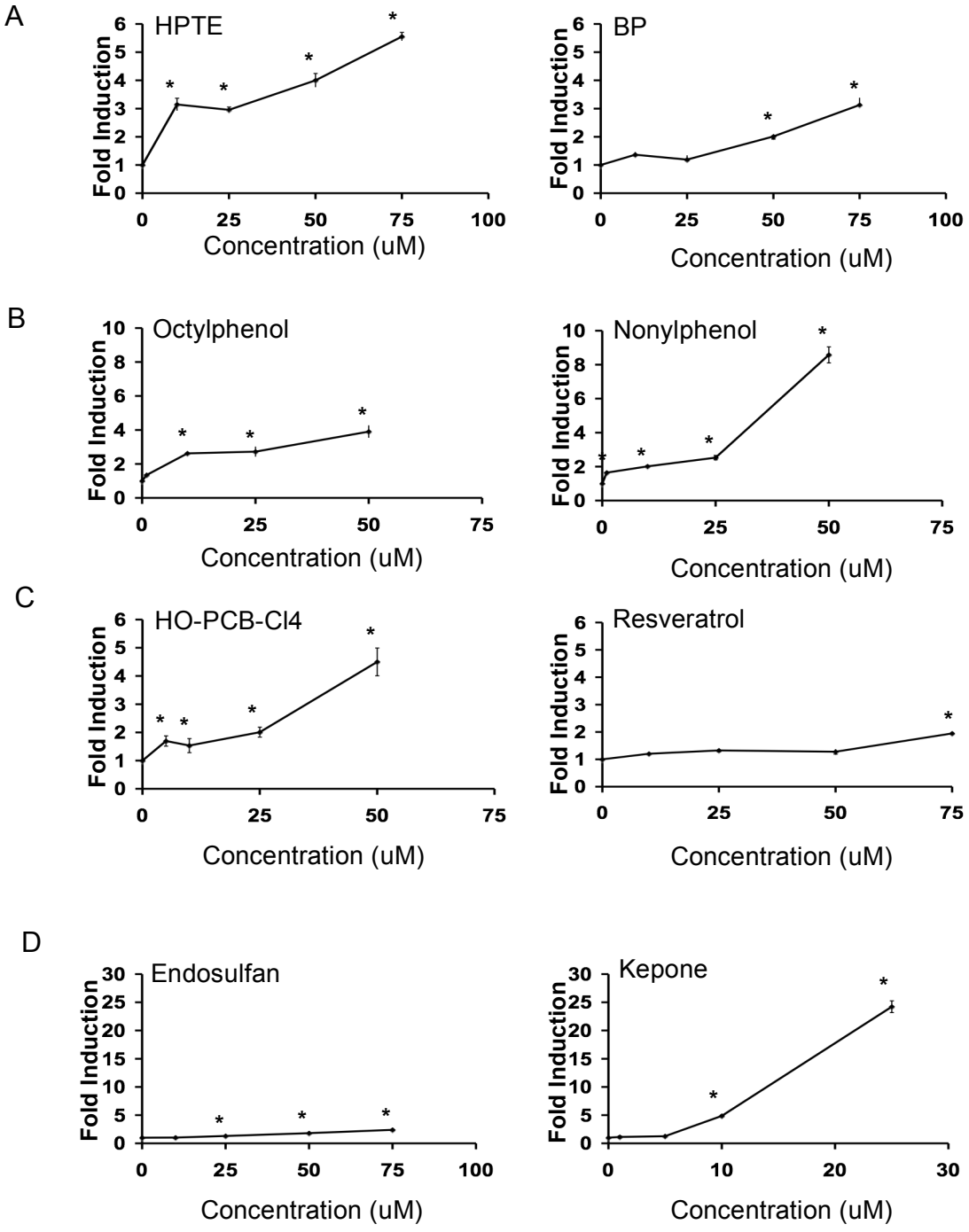


Figure 13. Xenoestrogen induced transactivation. MCF-7 cells were transfected with $pERE_3$ and treated with different concentrations of xenoestrogens and luciferase activity was determined as described in the Materials and Methods. Resveratrol did not induce activity; significant ($p<0.05$). induction by the xenoestrogens is indicated by an asterisk.

were those that induced maximal transactivation (Fig. 12 & 13) and did not cause any cytotoxicity. In cells transfected with $pERE_3$ and pcDNA3.0 (empty vector) (Fig. 14A), neither E2 nor DES induced transactivation, demonstrating that levels of endogenous ERα in these cells were insufficient to activate transfected $pERE_3$ which is highly overexpressed. Kepone induced luciferase activity (2.3-fold), whereas ICI, 4-OHT and several xenoestrogens decreased activity, and this may be due to their antiestrogenic activity through inactivation of endogenous ERα-dependent transactivation. Results in Fig. 14B are consistent with the results in Figure 12 & 13 and show that 20 μM kepone and 10 nM E2 induced comparable responses in cells transfected with wild-type ERα. Then MCF-7 cells were transfected with two C-terminus deletion mutants of ERα. Previou study has shown that these two mutants were expressed at the similar levels compared with wtERα [129]. In cells transfected with ERα(1-553), deletion of the F domain resulted in the loss of estrogenic activity of both bisphenolic compounds (BPA and HPTE) and endosulfan (Fig. 14C). Moreover, the potency of kepone and HO-PCB-Cl_4 relative to that of E2 was decreased. In contrast, E2 did not induce activity in MCF-7 cells transfected with ERα(1-537) demonstrating the importance of the helix 12 region of ERα for mediating transactivation (Fig. 14D). However, kepone (but not endosulfan) and the phenolic xenoestrogens HO-PCB-Cl_4, octyl- and nonylphenol induced transactivation in MCF-7 cells transfected with ERα(1-537), even though the potency relative to E2 was decreased, whereas the bisphenolics BPA and HPTE were inactive. These results show that in MCF-7 cells, the estrogenic activity of some xenoestrogens is AF-2 independent and the activity of antiestrogens ICI 182,780 and 4-OHT was not affected by ERα C-terminal deletions.

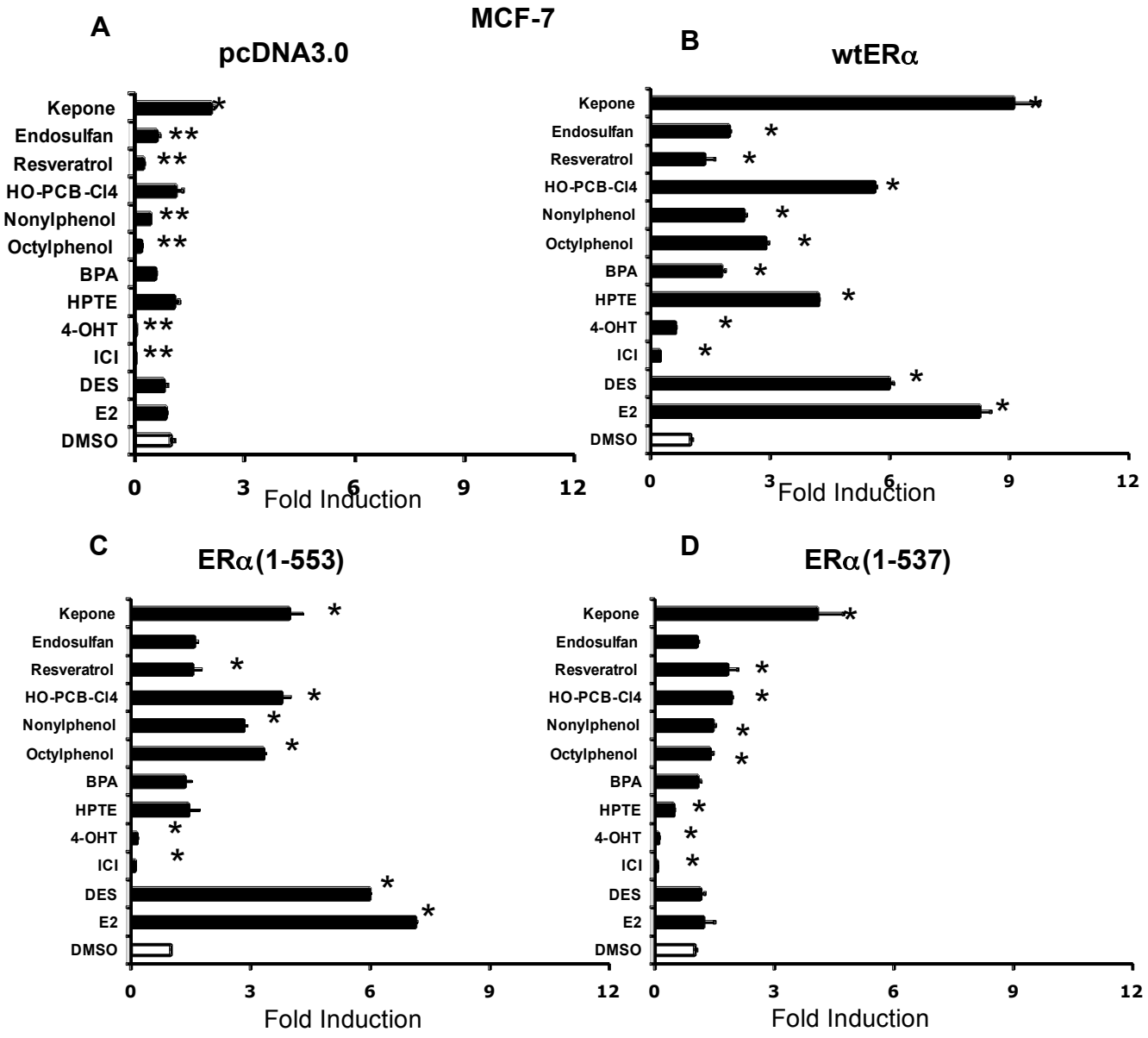


Figure 14. Estrogen, antiestrogen and xenoestrogen induced transactivation in MCF-7 cells transfected with wild-type or mutant ERα. MCF-7 cells were transfected with $pERE_3$ and pcDNA3.0 empty vector (A), wild-type ERα (B), ERα(1-553) (C) and ERα(1-537) (D), treated with various compounds and luciferase activity determined as described in the Materials and Methods. Significant ($p<0.05$) induction is indicated by an asterisk.

Structure-dependent activation of ERα is also dependent on the recruitment of different coregulators to the responsive gene promoter, therefore these compounds exhibit a cell-dependent activity. We also investigated the role of cell

context in the estrogenic activity of E2, DES, antiestrogens, xenoestrogens and resveratrol by determining their effects on transactivation in ER-negative MDA-MB-231 cells cotransfected with $pERE_3$ and empty vector (pcDNA3.0) (Fig. 15A), wild-type ERα (Fig. 15B), ERα(1-553) (Fig. 15C) or ERα(1-537) (Fig. 15D). Based on results of preliminary dose-response studies, the concentrations inducing the maximal response without any cytotoxicity were used. In the absence of cotransfected ERα, only kepone, BPA and HPTE induced luciferase activity (<3-fold) and the remaining compounds were inactive (Fig. 15A). With the exception of ICI 182,780, all the compounds induced a >3-fold increase in transactivation with a >10-fold increase observed for several xenoestrogens (kepone, BPA, nonylphenol and octylphenol) and 4-OHT in cells transfected with wild-type ERα (Fig. 15B). Similar results were observed in MDA-MB-231 cells transfected with ERα(1-553), although the overall fold induction was lower for all the compounds (Fig. 15C). In cells transfected with ERα(1-537), E2, DES, and the alkylphenols did not induce transactivation, whereas the antiestrogen 4-OHT, the remaining xenoestrogens and resveratrol induced luciferase activity (Fig. 15D).

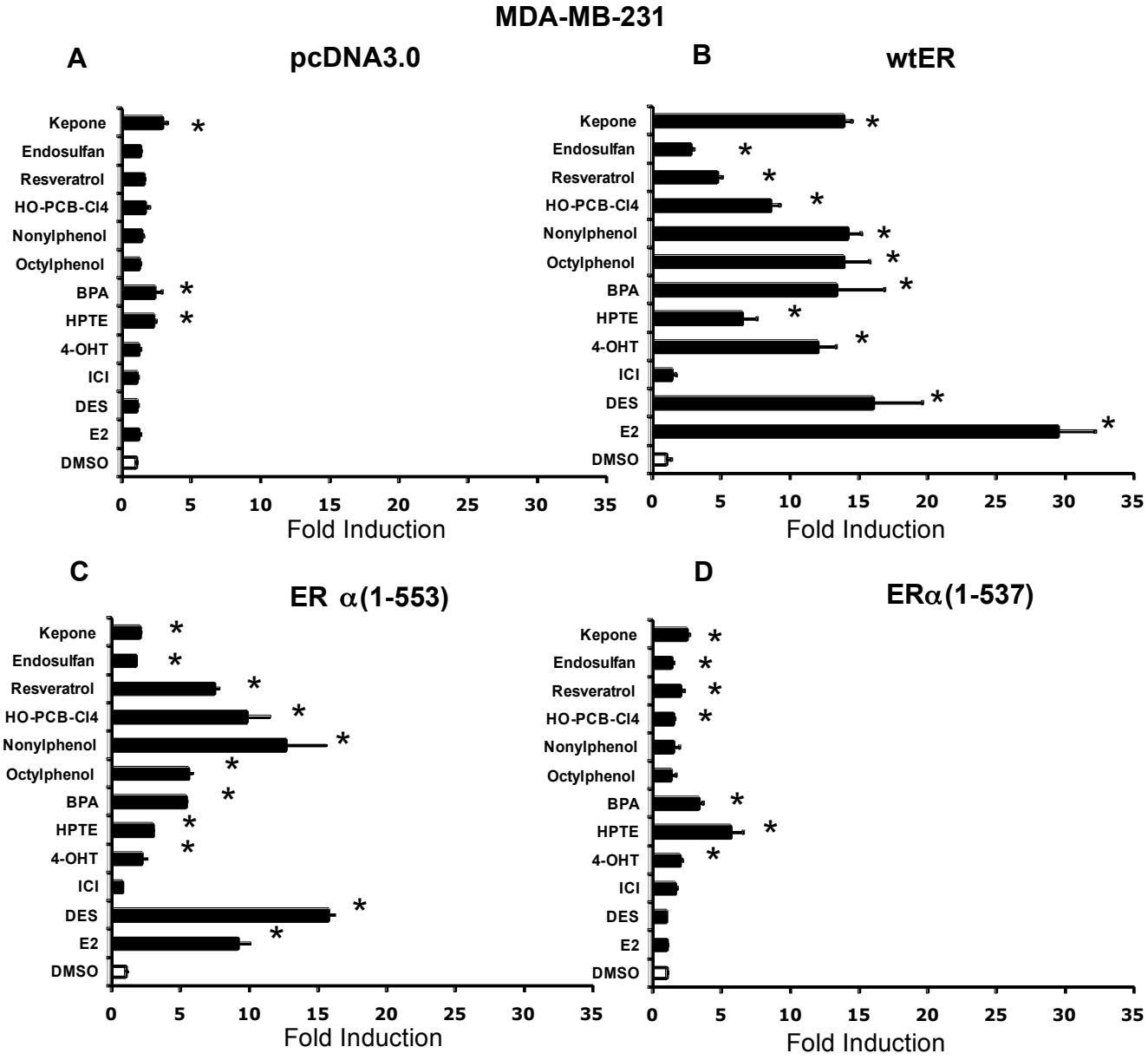


Figure 15. Estrogen, antiestrogen and xenoestrogen induced transactivation in MDA-MB-231 cells transfected with wild-type or mutant ERα. MDA-MB-231 cells were transfected with $pERE_3$ and pcDNA3.0 empty vector (A), wild-type ERα (B), ERα(1-553) (C) and ERα(1-537) (D), treated with various compounds and luciferase activity determined as described in the Materials and Methods. Significant ($p<0.05$) induction is indicated by an asterisk.

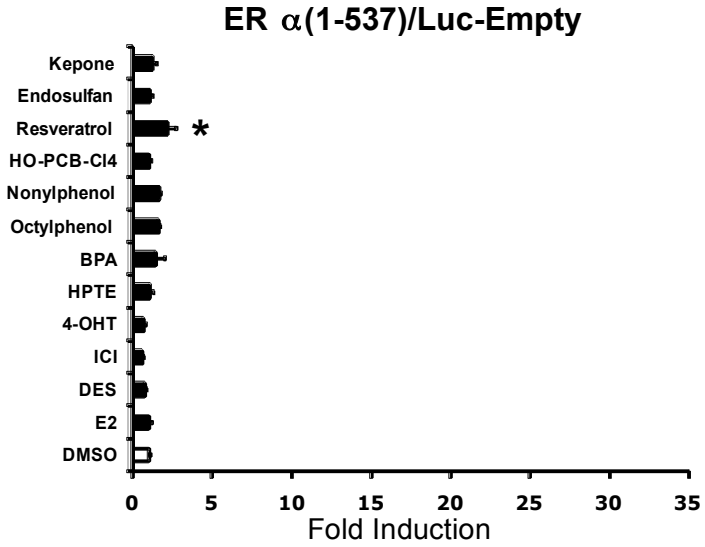


Figure 16. Estrogen, antiestrogen and xenoestrogen induced transactivation in MDA-MB-231 cells transfected with ERα(1-537) were transfected with luciferase empty reporter without ERE insert, treated with various compounds and luciferase activity determined as described in the Materials and Methods. Significant ($p<0.05$) induction is indicated by an asterisk.

In order to confirm that the activation of luciferase expression is mediated through ERE site and ERα(1-537) is not a dominant negative construct, in MDA-MB-231 cells transfected with ERα(1-537) and the vector expressing luciferase but without the 3 EREs insert, treatment with E2, 4-OHT, ICI and the xenoestrogens did not affect luciferase activity. Only resveratrol induced a response (<2.5-fold) (Fig. 16), suggesting that the activity for resveratrol was not due to activation of the ERE. These results demonstrate the complexity of xenoestrogen-induced transactivation, which is dependent on ligand structure, different domains of ERα and cell context. The results are consistent with previous studies using other ERα variants [552-554] and support the concept that the structurally-diverse compounds are selective ER modulators.

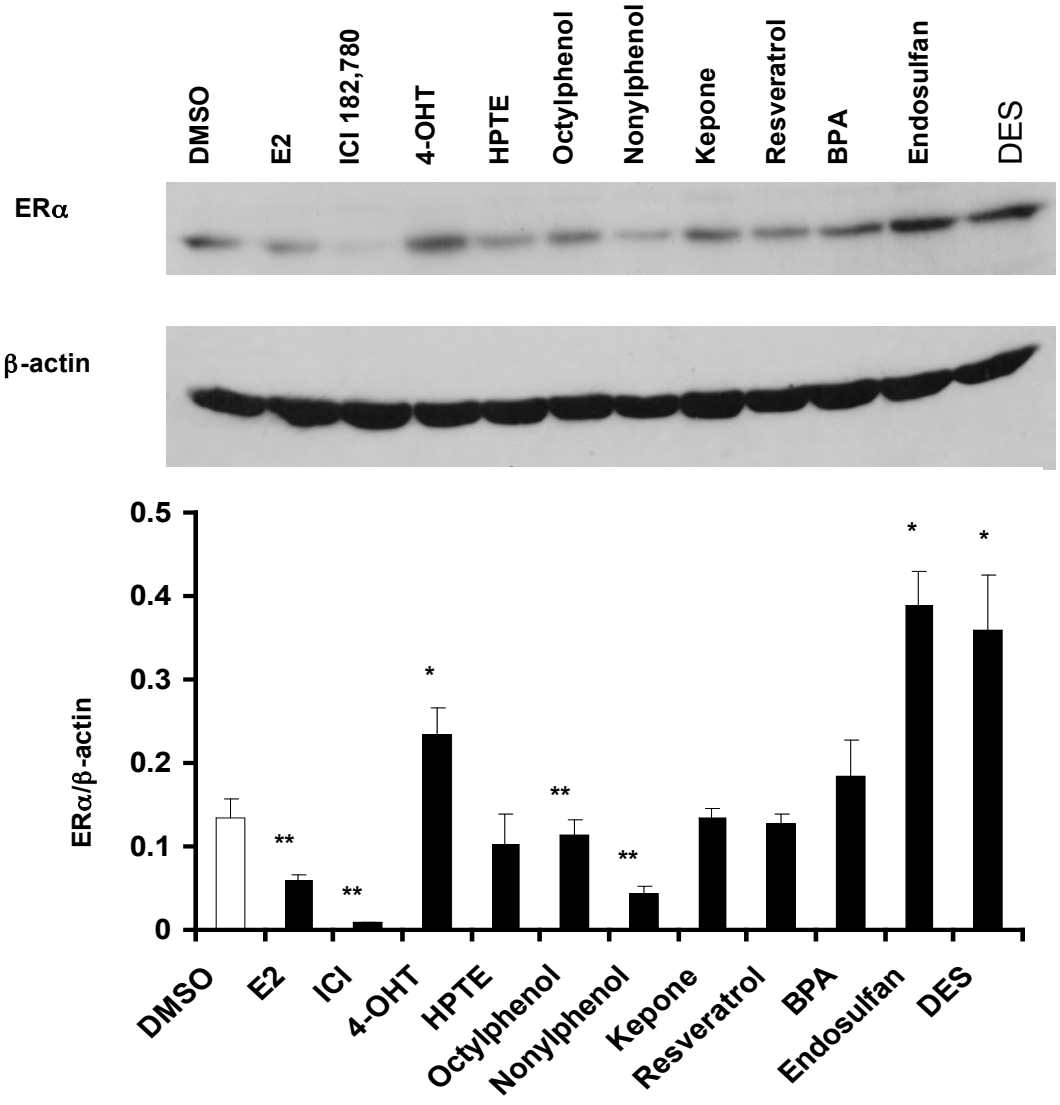


Figure 17. Effects of estrogens, antiestrogens and xenoestrogens on endogenous ERα stability in MCF-7 cells. MCF-7 cells were treated with estrogens, antiestrogens and xenoestrogens with the same concentrations used in Fig.3 and 4 for 3h. Western blot analysis of whole cell lysates for expression of ERα proteins was determined as described in Methods and Materials. Loading control bands (β-actin) indicate the comparable levels of protein loading for each treatment group. Quantification of ER protein levels relative to the loading control is shown in the adjacent bar graphs and represents three separate analyses for each treatment group. Significant ($p<0.05$) increases (*) or decreases (**) in ERα protein levels are indicated.

Previous studies show that E2 and the antiestrogen ICI induce proteasome-dependent degradation of ERα, whereas tamoxifen and other selective ER

modulators may increase or decrease ERα in breast cancer cells [555]. Results in Fig. 17 also show that E2 and ICI decrease and 4-OHT increases ERα protein levels in MCF-7 cells after treatment for 3 hours. Treatment with the synthetic estrogenic compounds and resveratrol gave responses which did not correlate with their estrogenic activity observed in the transactivation assays. Octylphenol and nonylphenol significantly decreased ERα protein, endosulfan and DES increased ERα protein, whereas HPTE, kepone, resveratrol and BPA did not affect ERα protein expression. Thus, the effects of estrogenic compounds on ERα protein levels in MCF-7 cells are variable and structure-independent, and this is consistent with results of a previous study with other ERα ligands [555].

2.5. Discussion

Xenoestrogens and phytoestrogens competitively bind ERα and ERβ, and activate ER-dependent gene expression in different cell lines [67, 551]. Most of these compounds exhibit lower ER-dependent affinity or transactivation potency than E2 or DES, and this was also observed in the present study where structurally-diverse xenoestrogens and the phytoestrogen resveratrol were >1000 times less active than E2 or DES in transactivation assays in MCF-7 or MDA-MB-231 cells. It is difficult to assess the physiological significance of exposure to these less potent estrogenic compounds; however, it is possible that the combined effects of dietary phytoestrogens and synthetic estrogens may be relevant in some tissues or during a critical exposure period. It should also be pointed out that binding and transactivation assays are insufficient to demonstrate the potential complex ligand structure-dependent pharmacology of xenoestrogens and resveratrol. Previous studies show that differences among SERMs, including antiestrogens, xenoestrogens and phytoestrogens can be observed in cells transfected with variant forms of ERα which lack the A/B (AF-1) domain or contain mutations in helix 12 of the E domain (D538N, E542Q, and D545N) [406, 544, 552-554]. ERα null contains both the AF-1 deletion and helix

12 mutations; however, in transient transfection assays, we have observed that E2 also induced transactivation in cells transfected with ERα null. In contrast, we observed that E2 did not activate gene expression in MCF-7 cells transfected with pERE$_3$ and ERα(1-537), and similar results were obtained for activation of ER /Sp1 by E2 in MCF-7 cells transfected with a GC-rich construct [129]. It was also observed that antiestrogens ICI 182,780 and 4-OHT (but not E2) activated ER /Sp1 in cells transfected with ERα(1-537) and ERα(1-553) (F domain deletion), suggesting that transactivation mediated by C-terminal deletion variants of ERα may be sensitive to structural differences among estrogenic compounds.

Therefore, we investigated the estrogenic activity of E2, DES, ICI 182,780, 4-OHT, resveratrol and seven different xenoestrogens in MCF-7 and MDA-MB-231 cells transfected with pERE$_3$ and wild-type ERα or the C-terminal mutants ERα (1-553) or ERα (1-537). BPA was a very weak ERα agonist in MCF-7 cells, but was much more potent in MDA-MB-231 cells. Therefore, the potency of the same ligand was dependent on the cell context. In MCF-7 cells transfected with wild-type ERα or ERα(1-553), deletion of the F domain did not change the pattern of responses induced by the estrogens (E2, DES) and antiestogens (ICI 182,780 and 4-OHT) (Fig. 14). This indicated that the F domain (554-595) of ERα was not important for the estrogen and antiestrogen action in MCF-7 cells which contrasted to results of previous studies [550]. However, the F domain was critical for two bisphenolic xenoestrogens (BPA and HPTE) and endosulfan. The loss of ERα (554-595) abolished their E2-responsiveness and induction of luciferase activity in MCF-7 cells. In contrast, deletion of the F domain resulted in the decrease of 4-OHT-induced transactivation in MDA-MB-231 cells, and both cyclodienederived xenoestrogens (kepone/endosulfan) exhibited low estrogenic activity (≤2-fold induction) compared to E2 (>9-fold induction). The role of the F domain in mediating the estrogenic and antiestrogenic activity of 4-OHT and

other antiestrogens has previously been reported [550, 556, 557]. This suggests that among the xenoestrogens, kepone and to a lesser extent endosulfan resemble, in part 4-OHT in their differential effects on transactivation in MDA-MB-231 cells transfected with wild-type ERα or ERα(1-553).

Deletion of amino acid 595-538 in ERα includes not only the loss of the F domain but also critical sequences from helix 12 required for AF-2-dependent interactions with coactivators. Not surprisingly, neither E2- nor DES-induced luciferase activity in MCF-7 or MDA-MB-231 cells transfected with ERα (1-537), and similar results were observed for endosulfan and both bisphenolics, HPTE and BPA in MCF-7 cells, whereas activity was still significantly induced by octylphenol and nonylphenol, kepone, resveratrol and HO-PCB-Cl_4. HPTE decreased activity in MCF-7 cells transfected with ERα(1-537) and this unexpected decrease was due to the induction of β-galactosidase which was used to normalize luciferase activity. The reason for this unusual response is unknown. In contrast, both BPA- and HPTE-induced luciferase activity in MDA-MB-231 cells transfected with ERα (1-537), and a ≥2.0-fold induction response was also observed for kepone, resveratrol, and 4-OHT and even ICI 182,780 significantly induced activity. Thus, among these structurally-diverse estrogenic compounds, an intact helix 12 was not necessarily required for induced transactivation by the antiestrogens or xenoestrogens in one or both cells lines, whereas, this region of ERα was required for E2- and DES-induced transactivation. The effects observed for resveratrol may be estrogen-independent since this compound activated luciferase in MCF-7 cells transfected with the luciferase vector that did not contain an ERE insert (data not shown).

The potential role of compound-induced ERα degradation in modulating transactivation was also investigated (Fig. 17). The results were highly variable among these compounds as previously reported for other ERα agonists and

antagonists [555] and did not correlate with estrogenic activity in the *in vitro* transfection assays.

These results further demonstrate the importance of both ERα agonist structure and cell context on activation of $pERE_3$ in breast cancer cells transfected with wild-type ERα, ERα(1-537) and ERα(1-553). The structure-dependent activation of luciferase activity in these assays confirms that xenoestrogens and resveratrol are SERMs and their estrogenic activities cannot necessarily be predicted by simple receptor binding and transactivation assays. This also implies that risk assessment of these compounds requires a more in-depth study of their tissue-specific ER agonist and/or antagonist activities.

CHAPTER III

ACTIVATION OF ESTROGEN RECEPTOR α/Sp-DEPENDENT TRANSACTIVATION BY ESTROGENIC COMPOUNDS

3.1. Overview

This study investigated the effects of the estrogens E2, DES, the antiestrogens ICI 182,780, 4-OHT, the phytoestrogen resveratrol, and the xenoestrogens OP, NP, endosulfan, kepone, HO-PCB-Cl_4, BPA, and HPTE on induction of luciferase activity in breast cancer cells transfected with a construct ($pSp1_3$) containing three tandem GC-rich Sp binding sites linked to luciferase and wild-type or variant ERα. The results showed that induction of luciferase activity was highly structure-dependent in both MCF-7 and MDA-MB-231 cells. Moreover, RNA interference assays using small inhibitory RNAs for Sp1, Sp3 and Sp4 also demonstrated structure-dependent differences in activation of ERα/Sp1, ERα/Sp3 and ERα/Sp4. These results demonstrate for the first time that various structural classes of ER ligands differentially activate wild-type and variant ERα/Sp-dependent transactivation, selectively use different Sp proteins, and exhibit SERM-like activity.

3.2. Introduction

ERα and ERβ are the two major ER sub-types, and the classical mechanism of estrogen action involves ligand-induced dimerization of ER which interacts with EREs in target gene promoters and results in transcriptional activation [528, 530]. This latter process is complex and involves interactions of the ligand-bound receptor with nuclear coactivators and other coregulatory proteins and components of the basal transcription machinery [537, 538].

Ligand-dependent activation or inhibition of ER-dependent transactivation depends on several factors including ligand structure, cell/tissue-specific expression coactivators/coregulatory proteins, gene promoter and cell context [537]. The development of selective ER modulators (SERMs) such as tamoxifen and raloxifene for treatment of breast cancer and other hormone-related problems is due to this complex pharmacology in which individual SERMs exhibit tissue-specific ER agonist or antagonist activities [558-560]. Several *in vitro* assays for estrogenic activity using wild-type and variant forms of ERα and ERE-promoter-reporter constructs can distinguish between 17β-estradiol (E2) and different SERMs such as tamoxifen, raloxifene and "pure" antiestrogens such as ICI 164,384 and ICI 182,780 [406, 544]. Moreover, studies in this laboratory have shown that structurally-diverse synthetic industrial estrogenic compounds (xenoestrogens) differentially activate ERE-promoters in cells transfected with wild-type and variant ERα expression plasmids suggesting that these compounds also exhibit SERM-like activity [552-554, 561, 562].

E2-dependent transactivation through nuclear pathways also involves non-classical mechanisms where the liganded ER interacts with other DNA-bound transcription factors including Sp, AP-1, NFκB, and globin transcription factor (GATA) [108, 563-565]. ERα/Sp-dependent transactivation is responsible for activation of several genes in breast cancer cells required for cell proliferation, cell signaling, and nucleotide metabolism [108]. Ligand-dependent activation of ERα/Sp has been observed for both estrogens and antiestrogens such as 4-OHT and ICI 182,780; however, in studies using a construct ($pSp1_3$) containing three GC-rich Sp protein binding sites, activation by estrogens and antiestrogens requires different domains of ERα [12, 108, 111, 129]. For example, E2 activates $pSp1_3$ in cells transfected with wild-type ERα or DNA binding domain (DBD) mutants of ERα containing deletions of zinc finger 1 (ERαΔZF1) or zinc

finger 2 (ERαΔZF2), whereas ICI 182,780 or 4-OHT activate ERα but not the DBD mutants [111, 129].

In this study, we investigated the structure-dependent activation of ERα/Sp1 by a series of xenoestrogens including OP, NP, endosulfan, kepone, HO-PCB-Cl_4, BPA, and HPTE. E2, DES, 4-OHT, resveratrol (a phytoestrogen), and ICI 182,780 were also used as reference compounds for the study. With the exception of resveratrol, all compounds induced transactivation in breast cancer cells transfected with ERα and $pSp1_3$; however, activation of $pSp1_3$ in cells transfected with variant forms of ERα was structure-dependent. Moreover, using RNA interference that selectively decreases Sp1, Sp3 or Sp4 protein expression, we showed that xenoestrogens, E2 and antiestrogen selectively activate ERα/Sp1, ERα/Sp3 and ERα/Sp4.

3.3. Materials and Methods

3.3.1. Chemicals, Biochemicals and Plasmids

Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Antibiotic antimycotic solution (AAS) (x100) was obtained from Sigma-Aldrich (St Louis, MO). The following test chemicals (and purities) were purchased from Sigma-Aldrich: E2 (≥ 98%), 4-OHT (≥ 98%), resveratrol (> 99%), *p*-*t*-octylphenol (97%), *p*-nonylphenol (98%) and BPA (>99%). HO-PCB-Cl_4 was >98% pure as previously described [566]; HPTE (>98%) was synthesized as previously reported [262]. Kepone (98%) and endosulfan were purchased from Chem-Service (West Chester, PA). ICI 182,780 was provided by Dr. Alan Wakeling (Astra-Zeneca, Macclesfield, UK). Plasmid preparation kits were purchased from Sigma-Aldrich. All other chemicals were obtained from commercial sources at the highest quality available. Human ERα expression plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX);

ERαΔZF1, ERαΔZF2, ERα(1-537), ERα(1-553), CFP-Sp1, YFP-ERα and CFP-YPF chimeras were made as previously reported [128, 129].

3.3.2. Cells and Transient Transfection Assays

MCF-7 and MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture Ham's F-12 (DME/F-12) (Sigma-Aldrich) supplemented with 2.2 g/liter sodium bicarbonate, 5% FBS, and 5 ml/liter AAS. Cells were cultured and grown in a 37°C incubator with humidified 5% CO_2, 95% air. For transient transfection studies, MCF-7 or MDA-MB-231 cells were seeded in 12-well plates in DME/F-12 medium without phenol red supplemented with 2.2 g/liter sodium bicarbonate, and 2.5% charcoal-stripped FBS. After 24 hr, cells were transfected using the calcium phosphate transfection method with 350 ng of luciferase reporter construct ($pSp1_3$), 100 ng pcDNA3/His/lacZ (Invitrogen, Carlsbad, CA) as a standard reference for transfection efficiency, and 200 ng of the appropriate ER expression plasmid. The $pSp1_3$ construct and other plasmids containing E2-responsive GC-rich promoter inserts are not responsive to E2 even in MCF-7 cells and this is due to minimal TATA promoter and overexpression of the transfected construct which results in limiting levels of ERα [12, 108, 111, 128, 129, 554]. E2- responsiveness requires cotransfection with ERα. Six hr after transfection, cells were shocked with 25% glycerol/PBS for 1 min, washed with PBS, and then treated with dimethylsulfoxide (DMSO, solvent) or different concentrations of estrogens, antiestrogens or xenoestrogens in DMSO for another 20 - 24 hr. Cells were then washed twice in PBS and harvested with 100 μl of reporter lysis buffer (Promega Corp., Madison, MI). After two freeze-thaw cycles, cell lysates were centrifuged for 1 min at 16,000 *g*, and the supernatant was used for determination of protein activity. Luciferase (Promega Corporation, Madison, MI) and β-galactosidase activity was determined using

the Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA). Light emission was detected on a Lumicount micro-well plate reader (Packard Instruments, Meriden, CT), and luciferase activity was calculated by normalizing against β-galactosidase activity obtained from the same sample and compared with the DMSO control group (set at 100%) for each set of experiments.

3.3.3. RNA Interference Assay

MCF-7 cells ($5x10^4$) were cultured in phenol red-free DME/F12 supplemented with 2.5% charcoal-stripped FBS without antibiotic in 12-well plates for overnight. siRNA (25 nM) was transfected by Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. After 6 hr, the transfection medium was changed with fresh DME/F-12 and 2.5% serum without phenol red. The next day, following the manufacturer's instructions, Genejuice transfection reagent (EMD Biosciences Inc., San Diego, CA) was used to transfect cells with 200 ng of luciferase reporter construct ($pSp1_3$), 50 ng pcDNA3/His/lacZ (Invitrogen) as a standard reference for transfection efficiency, and 100 ng of the human ERα expression plasmid. Six hr later, cells were treated with DMSO or estrogens, antiestrogens, xenoestrogens in antibiotic-free, phenol red-free DMEM/F12 with 2.5% serum. Cells were harvested 48 - 54 hr after siRNA transfection. Cell lysates were assayed for luciferase and β-galactosidase activity as described above.

The siRNA duplexes used in this study are indicated as follows. Silencer® Negative Control #1 siRNA purchased from Ambion (Austin, TX) was used as the non-specific control (iNS). The luciferase GL2 duplex (target sequence, 5' - CGT ACG CGG AAT ACT TCG A - 3') RNA from Dharmacon (Lafayette, CO) was used as the positive control in siRNA transfections. The siRNA oligonucleotides for Sp1, Sp3, and Sp4 were also obtained from Dharmacon as follows: Sp1, 5' - AUC ACU CCA UGG AUG AAA UGA dTdT - 3'; Sp3, 5' - GCG

GCA GGU GGA GCC UUC ACU dTdT - 3'; and Sp4, 5' - GCA GUG ACA CAU UAG UGA GCdT dT - 3'.

3.3.4. Western Blot Analysis

MCF-7 cells were seeded into six-well plates in DMEM/F12 supplemented with 2.5% charcoal-stripped FBS. The next day, cells were transfected with siRNA as described above. Forty-eight hr after transfection, protein was extracted from the cells by harvesting in a high-salt lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, protease inhibitor cocktail (Sigma)] on ice for 45 - 60 min with frequent vortex and centrifugation at 20,000 *g* for 10 min at 4°C. Protein concentrations were determined using a Bio-rad (Hercules, CA) protein assay reagent. Protein (60 μg) was diluted with Laemmli's loading buffer, boiled, and loaded onto 7.5% SDS-PAGE. Samples were resolved using electrophoresis at 150V for 3 - 4 hr and transferred (transfer buffer, 48 mM Tris-HCl, 29 mM glycine, and 0.025% sodium dodecyl sulfate) to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electrophoresis at 0.2 A for approximately 12 - 16 hr. Membranes were blocked in 5% TBS-Tween 20-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, 5% nonfat dry milk] with gentle shaking for 30 min and incubated in fresh 5% TBS-Tween 20-Blotto with 1:1,000 (for Sp1 and Sp3), 1:500 (for Sp4), and 1:5,000 (for α-actin) primary antibody overnight with gentle shaking at 4°C. The primary antibodies for Sp1 (PEP2), Sp3 (D-20), and Sp4 (V-20) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and antibody for α-actin was purchased from Sigma. Membranes were probed with a horseradish peroxidase-conjugated secondary antibody (1:5000) (Santa Cruz) for 3 - 6 hr at 4°C. Blots were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences) and exposure on Image Tek-H x-ray film (American X-ray Supply). Band quantitation was performed by ImageJ (NIH).

3.3.5. Fluorescence Resonance Energy Transfer (FRET)

FRET was performed with the Stallion DDI workstation equipped with a xenon fluorescent light source (300 W) with rapid switching (<2 msec) between excitation wavelengths and two CoolSnap HQ cameras for simultaneous detection of two emission signals. Three types of images were collected for the FRET experiments. The first type was the CFP control (donor only) which was used to calculate the spectral bleed-through of CFP emission visible through the FRET filter set. This type of image was collected using the CFP filter set and the FRET filter set. The second type of image, the YFP control (acceptor only), was used to calculate the spectral bleed-through of normal YFP emission through the FRET filter set. This image was collected using the YFP filter set and the FRET filter set. The third type of image is the raw FRET image (FRETraw) representing the sample under analysis. This type of image was collected using the FRET, CFP and YFP filter sets. Corrected FRET (FRETc), was calculated with consideration of all three images [567] using the following equation:

$$FRETc = FRETraw – Df/Dd[CFP] – Df/Da[YFP]$$

where FRETraw, [CFP], and [YFP] are the signals visualized through the FRET, CFP, and YFP filter sets, respectively. The constants Df/Dd and Df/Da are the bleed-through constants describing donor emission visible in FRET channel and direct excitation of acceptor, respectively, calculated from the first and second type images. FRET efficiency was then calculated by comparing the constructs under investigation to the CFP-YFP chimera (positive control) [128].

3.3.6. Statistical Analysis

For transient transfection studies, results are expressed as means ± SD for at least three separate experiments for each treatment group. Statistical differences ($p < 0.05$) between control (DMSO) and treatment groups were

determined by single factor ANOVA test. For FRET efficiency analysis, data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test at $p < 0.05$.

3.4. Results

$pSp1_3$ is a plasmid containing three consensus GC-rich sites with a minimal TATA promoter linked to the luciferase gene. This construct is not inducible by E2 in ER-positive MCF-7 or ZR-75 cells and E2-responsiveness is only observed after cotransfection with ERα or variant forms of ERα [12, 108, 111, 127-130]. This is due to overexpression of the construct where endogenous ERα in MCF-7 cells becomes limiting. The lack of responsiveness without cotransfection of ERα is also due to the minimal TATA promoter. This has proven to be an excellent model system for determining ligand structure-dependent requirements for different domains of ERα in transactivation studies in a breast cancer cell (ER-positive or -negative) context [12, 108, 129, 552-554]. ERE-promoter-reporter constructs have been extensively used as models for understanding the classical ERα-mediated transactivation pathways, and $pSp1_3$ is a model for investigating mechanisms of ERα/Sp-mediated gene expression [108]. The effect of ligand structure on activation of ERα/Sp-dependent transactivation was investigated in MCF-7 cells transfected with $pSp1_3$ and treated with different concentrations of E2, DES, ICI 182,780 and 4-OHT (Figs. 18A and 18B). Both E2 and DES induced luciferase activity and similar results were observed for the antiestrogens 4-OHT and ICI 182,780. The induced transactivation for the antiestrogens was similar to previous studies using this same construct ($pSp1_3$) in both ER-positive and -negative cancer cell lines [129]. Several reports show that xenoestrogens and phytoestrogens activate gene expression in MCF-7 cells transfected with ERE constructs [534, 552-554]; however, their activation of $pSp1_3$ has not previously been reported and is investigated in this study.

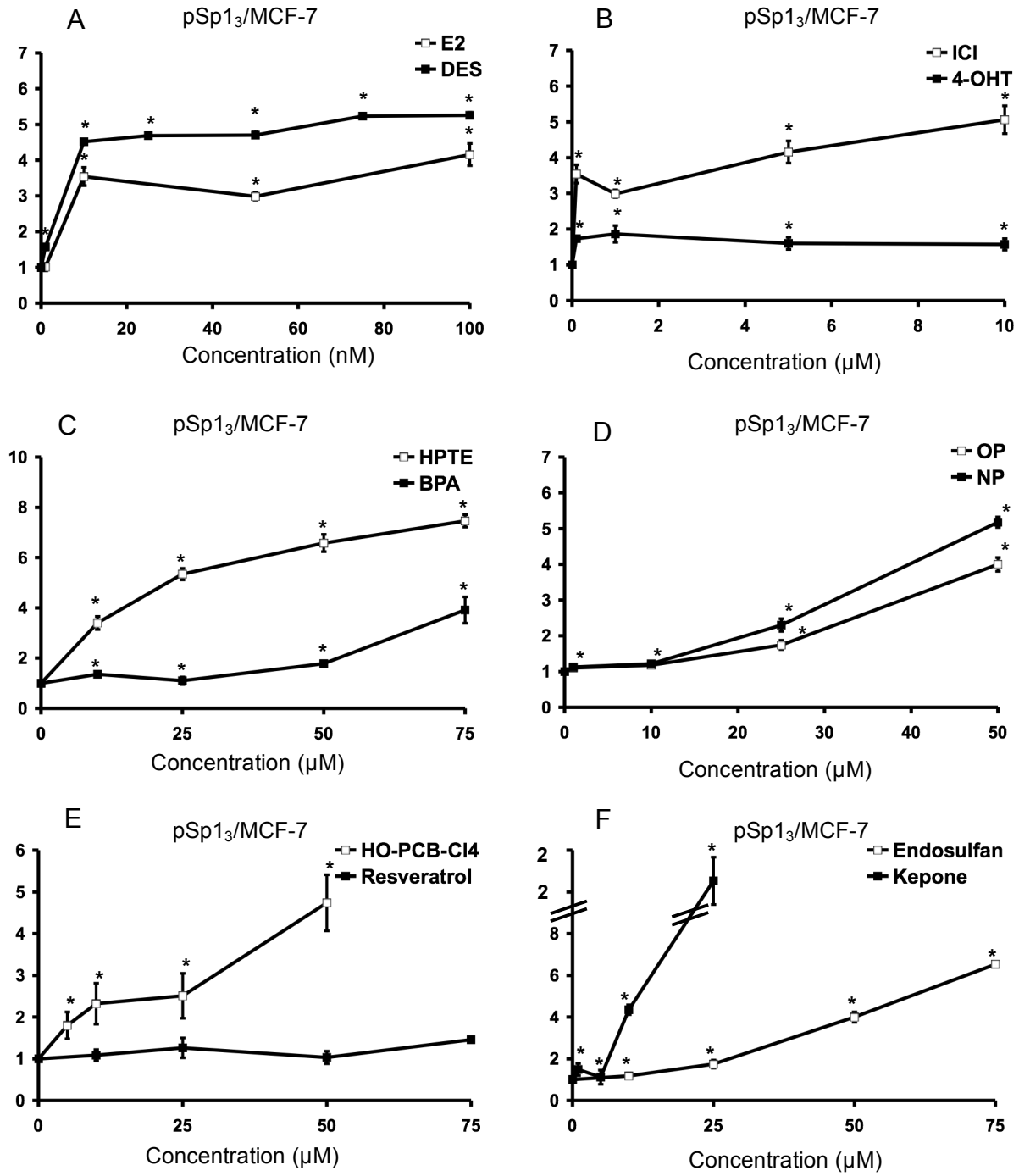


Figure 18. Activation of ERα/Sp by estrogens, antiestrogens, xenoestrogens and resveratrol in MCF-7 cells. Induction by E2/DES (A), ICI 182,780/4-OHT (B), HPTE/BPA (C), octylphenol/nonylphenol (D), HO-PCB-Cl4/resveratrol (E), kepone/endosulfan (F) in MCF-7 cells transfected with pSp1$_3$ and ERα, treated with different concentrations of the various ERα agonists and luciferase activity determined as described in the Materials and Methods. Each point is the mean ± SD for three replicate determinations and significant ($p < 0.05$) induction is indicated (*).

The results in Fig. 18C summarize the concentration-dependent activation of $pSp1_3$ by two bisphenolic estrogenic compounds HPTE and BPA; both compounds induced luciferase activity, with HPTE being the more potent of the two bisphenols. Octyl- and nonylphenol (Fig. 18D) also induced transactivation in cells transfected with $pSp1_3$ and, among the bisphenolic and phenolics xenoestrogens, their induction response varied from 4- to 8-fold. A third phenolic compound HO-PCB-Cl_4 also induced approximately a 4-fold increase in luciferase activity, whereas resveratrol, a trihydroxystilbene analog was inactive in this assay (Fig. 18E). Both endosulfan and kepone are chlorinated caged xenoestrogenic compounds and the potency of endosulfan was similar to that observed for the phenolic/bisphenolic compounds (6.5-fold induction); however, a > 25-fold increase in luciferase activity was observed in MCF-7 cells transfected with $pSp1_3$ and treated with kepone which induced the highest activity among all the 12 compounds tested in this assay (Fig. 18F). All compounds induced a concentration-dependent induction of luciferase activity and, in subsequent studies, we used concentrations that induced maximal activity with no cytotoxicity.

Previous studies show that xenoestrogens and antiestrogens differentially activate wild-type and variant ERα in cells transfected with ERE promoters [18, 406, 418, 544, 552]. E2 and antiestrogens activate wild-type ERα/Sp-dependent transactivation in MCF-7 cells transfected with $pSp1_3$; however, activation of deletion and point mutant forms of ERα (transfected) is ligand structure-dependen [111, 129]. We therefore compared the effects of different structural classes of xenoestrogens and resveratrol on induction of luciferase activity in MCF-7 cells transfected with $pSp1_3$ and wild-type ERα (Fig. 19A), ERαΔZF1 (Fig. 19B), ERαΔZF2 (Fig. 19C), ERα(1-553) (Fig. 19D), and ERα(1-537) (Fig. 19E). These ERα mutants contained deletions of zinc finger 1 (amino acids 185-205), zinc finger 2 (amino acids 218-243), the F domain (amino acids 554-595), and the F domain plus amino acids in helix 12 of the E domain (amino

acids 538-595), respectively. With the exception of resveratrol, all compounds induce transactivation in MCF-7 cells transfected with pSp1$_3$/ERα (Fig. 19A) as observed in Fig. 18. In order to decrease cytotoxocicity at the minimal level, 20 μM kepone was used instead of 25 μM in Fig. 18. The results in Figs. 19B and 19C show that the antiestrogens ICI 182,780 and 4-OHT did not induce transactivation in MCF-7 cells transfected with pSp1$_3$ and ER$\alpha\Delta$ZF1 or ER$\alpha\Delta$ZF2 as previously reported [111, 129]. In contrast, both E2 and DES significantly activated luciferase activity, and the xenoestrogens also induced transactivation and resembled E2. Resveratrol was also significantly active, and maximally induced responses were observed for HPTE, kepone and HO-PCB-Cl$_4$ in cells transfected with both zinc finger deletion mutants. The fold-induction responses were decreased in cells transfected with ERα(1-553) in which the F domain has been deleted; however, in contrast to a previous report [129], E2 and the remaining compounds all significantly induced transactivation (Fig. 19C), and HPTE, HO-PCB-Cl$_4$ and kepone were the most potent inducers. Major structure-dependent differences were observed in MCF-7 cells transfected with ERα(1-537); E2 and DES did not induce transactivation, whereas the antiestrogens, resveratrol and xenoestrogens were all inducers of luciferase activity with kepone, HO-PCB-Cl$_4$ and HPTE among the most active compounds.

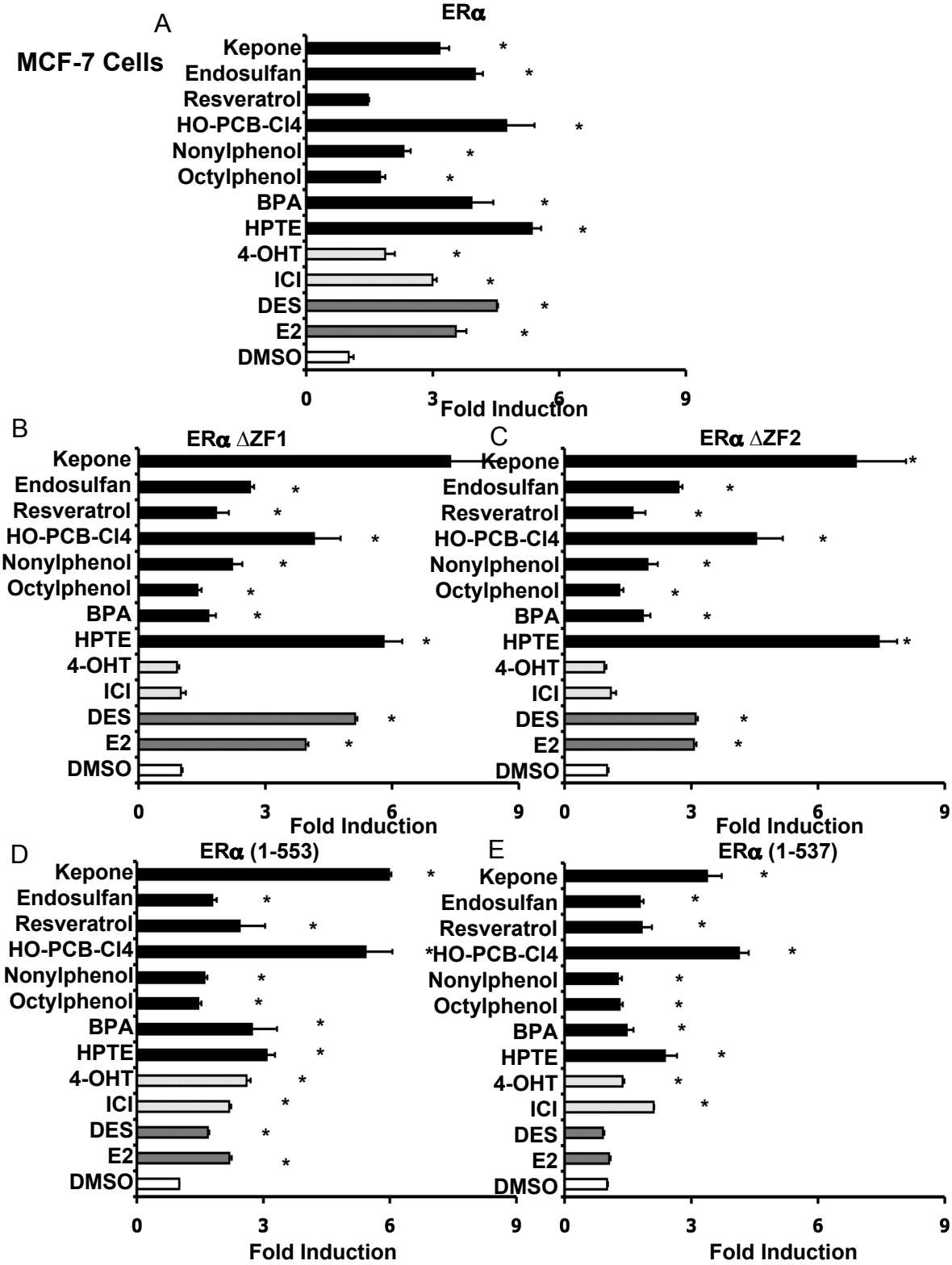


Figure 19. Structure-dependent activation of variant ERα/Sp in MCF-7 cells transfected with wild-type ERα (A), ERαΔZF1 (B), ERαΔZF2 (C), ERα(1-553) (D), and ERα(1-537) (E). Cells were transfected with $pSp1_3$ and variant forms of ERα, treated with ERα agonists and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated (*).

Differential activities of estrogens, antiestrogens and xenoestrogens are partly due to the induciton of different ERα comformation change, then recruitment of distinct coregulators to the responsive gene promoters. Therefore, they exhibit cell context-dependent activity. Next we also investigated structure-dependent activation of ERα/Sp-dependent transactivation in ER-negative MDA-MB-231 breast cancer cells transfected with $pSp1_3$, wild-type ERα or the same set of ERα mutants ERαΔZF1, ERαΔZF2, ERα(1-554) and ERα(1-537), respectively (Figs. 20A - 20E). The concentrations of xenoestrogens were initially optimized for this cell line, and the estrogens, antiestrogens and xenoestrogens all induced luciferase activity in cells cotransfected with $pSp1_3$ and wild-type ERα (Fig. 20A). The pattern of induction in MDA-MB-231 cells transfected with the zinc-finger mutants was similar to that observed in MCF-7 cells; E2 and DES but not the antiestrogens ICI 182,780 or 4-OHT induced activity, and with the exception of the alkylphenols, the xenoestrogens and resveratrol were all active with the latter compound exhibiting significantly higher activity in MDA-MB-231 than in MCF-7 breast cancer cells (Figs. 20B and 20C). In MDA-MB-231 cells transfected with the F domain deletion mutant [ERα(1-553)] (Fig. 20D), all compounds with the exception of the alkylphenols and endosulfan induced activity, and these results were similar to those obtained in MCF-7 cells (Fig. 19D) where only limited induction was observed for alkylphenols and endosulfan. Higher concentrations of these compounds may induce transactivation in MDA-MB-231 cells; however, this was not carried out due to cytotoxicity. The structure-dependent induction of luciferase activity in MDA-MB-231 cells transfected with ERα(1-537) (Fig. 20E) was also similar to that observed in MCF-7 cells (Fig. 19E) in which the xenoestrogens and resveratrol but not E2 or DES were active. These results demonstrate that xenoestrogens,

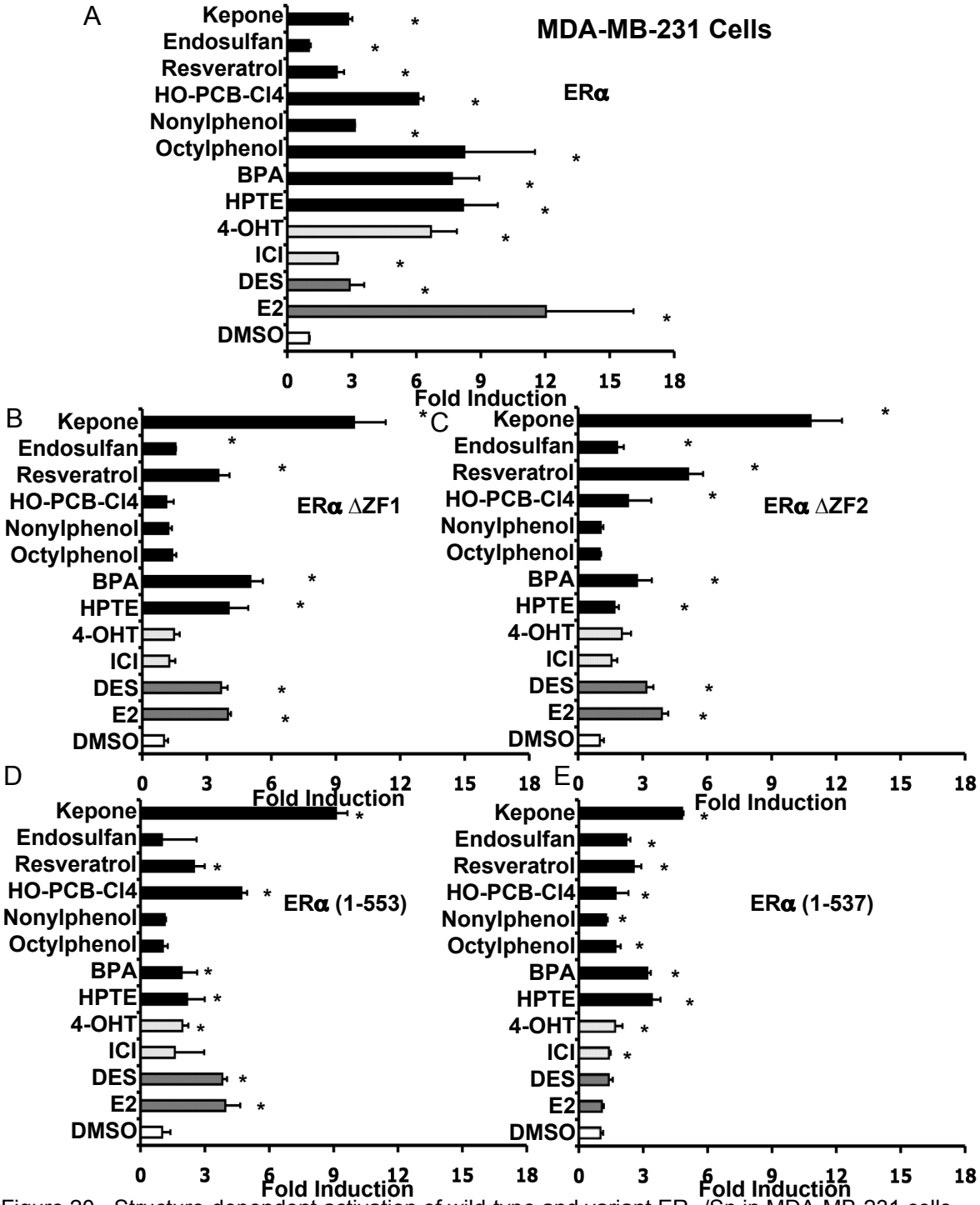


Figure 20 . Structure-dependent activation of wild-type and variant ERα/Sp in MDA-MB-231 cells transfected with wild-type ERα (A), ERαΔZF1 (B), ERαΔZF2 (C), ERα(1-553) (D), and ERα(1-537) (E). MDA-MB-231 cells were transfected with $pSp1_3$, wild-type or variant ERα, treated with ER agonists and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated (*).

resveratrol, antiestrogens and E2/DES differentially activate wild-type and variant ERα/Sp in ER-negative MDA-MB-231 cells transfected with pSp13, and the pattern of activation of wild-type and variant ERα/Sp by xenoestrogens was similar in both MCF-7 and MDA-MB-231 cells.

Previous studies show that hormonal activation of other GC-rich promoters involves ERα/Sp1, ERα/Sp3 and ERα/Sp4 [124, 127]. The role of individual Sp proteins on ligand-dependent activation of ERα/Sp in MCF-7 cells transfected with $pSp1_3$ was further investigated using RNA interference and small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4). Results illustrated in Fig. 21A summarize Western immunoblot analysis of Sp protein levels in whole cell lysates from MCF-7 cells transfected with iSp1, iSp3, iSp4 and binary combinations of these reagents. Since transfection efficiencies are in the range of 60-90%, the results demonstrate highly effective and specific Sp protein knockdown as previously reported using these same siRNAs [124, 127, 515]. The siRNAs were initially used to investigate the role of individual Sp proteins in mediating E2-, 4-OHT- and HPTE-mediated activation of $pSp1_3$ in MCF-7 cells and determine possible structure-dependent utilization of one or more Sp proteins. HPTE was selected as a prototypical xenoestrogen that induces transactivation in cells transfected with wild-type and variant ERα expression plasmids. In all experiments, iSp1, iSp3 and iSp4 decreased basal and ligand-induced activity and therefore the results are expressed as fold induction in order to directly compare the role of individual Sp proteins on ligand-dependent activation of ERα/Sp. Results in Fig. 21B demonstrate that iSp1 alone or in combination with iSp3 or iSp4 completely decreased inducibility by E2. iSp4 alone or in combination with iSp1 or iSp3 decreased induction by E2, whereas iSp3 did not affect the induction response. These results suggest that ERα/Sp1 is the major complex activated by E2, and ERα/Sp4 also contributes to this response. In contrast, iSp3 did not significantly decrease E2 inducibility,

suggesting that ERα/Sp3 does not play a role in hormonal activation of $pSp1_3$. These results were observed in several replicate experiments showing that E2-dependent activation of ERα/Sp followed the order ERα/Sp1 > ERα/Sp4 > ERα/Sp3. Using a similar approach, we also investigated HPTE- and 4-OHT-dependent activation of ERα/Sp (Figs. 21C and 21D, respectively) and the results suggest that ERα/Sp1 and ERα/Sp4 but not ERα/Sp3 play a role in 4-OHT/HPTE activation of $pSp1_3$. The major difference between E2 and 4-OHT/HPTE over several replicate experiments was the increased role of ERα/Sp4 for the latter two compounds.

The effects of ICI 182,780 and several xenoestrogens on activation of ERα/Sp-dependent transactivation were also investigated in MCF-7 cells cotransfected with $pSp1_3$ and iSp1, iSp3 or iSp4 (Figs. 22 and 23). Figure 22 summarizes the effects of various iSps on the induction of luciferase activity in MCF-7 cells transfected with $pSp1_3$ and treated with DES, HO-PCB-Cl_4, BPA, NP and endosulfan (Figs. 22A - 22E, respectively). The results show that like E2, DES and HO-PCB-Cl_4 primarily activate ERα/Sp1 and also activate ERα/Sp4 (< ERα/Sp1) but not ERα/Sp3 (Figs. 22A and 22B). On the other hand, BPA, endosulfan and NP activate both ERα/Sp1 and ERα/Sp4 (ERα/Sp4 ≈ ERα/Sp1) but not ERα/Sp3 (Figs. 22C - 22E) and resemble 4-OHT in their differential activation of ERα/Sp.

The effects of iSps on activation of $pSp1_3$ by ICI 182,780 (Figs. 23A and 23B) and kepone (Figs. 23C and 23D) were different from those observed for the other ERα agonists and antagonists. Induction of luciferase activity by ICI 182,780 was decreased 40 - 60% by individual and combined iSps (Fig. 23A); however, basal activity was decreased in the order iSp4 > iSp3 > iSp1, and the lowest basal activity was observed in cells transfected with iSp4 alone or in combination with other iSps. When the luciferase activity is plotted as fold

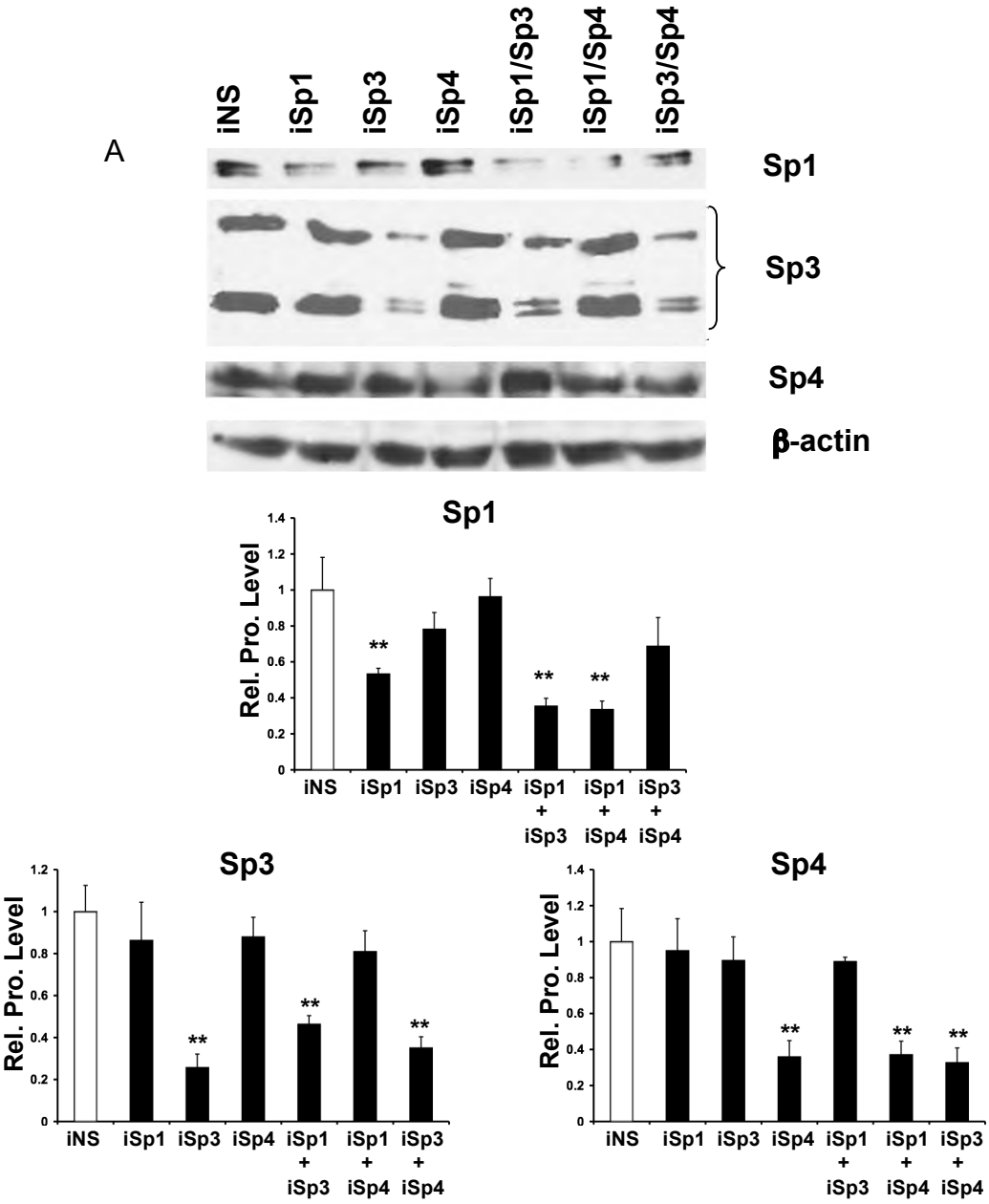


Figure 21. RNA interference studies with iSp1, iSp3 and iSp4. (A) Western immunoblot analysis. MCF-7 cells were transfected with iSp1, iSp3, iSp4, iNS (non-specific), or various combinations, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Quantitation of Sp knockdown was determined using three replicates for each treatment and levels were normalized to β-actin. Significantly ($p < 0.05$) decreased levels are indicated (*) and results are compared to those observed in the DMSO group (set at 1.0). Effects of Sp protein knockdown on activation of $pSp1_3$ by E2 (B), 4-OHT (C), and HPTE (D). MCF-7 cells were transfected with $pSp1_3$, ERα and small inhibitory RNAs for iNS or Sp proteins and treated with 10 nM E2, 1 μM 4-OHT, or 25 μM HPTE. Luciferase activity was determined as described in the Materials and Methods, and results are means ± SD for at least three replications for each treatment group. Significantly ($p < 0.05$) induced activity relative to DMSO is indicated (*) and a decrease in induced activity (compared to iNS) by iSp1, iSp3, iSp4 or their combinations is indicated by a double asterisk (**).

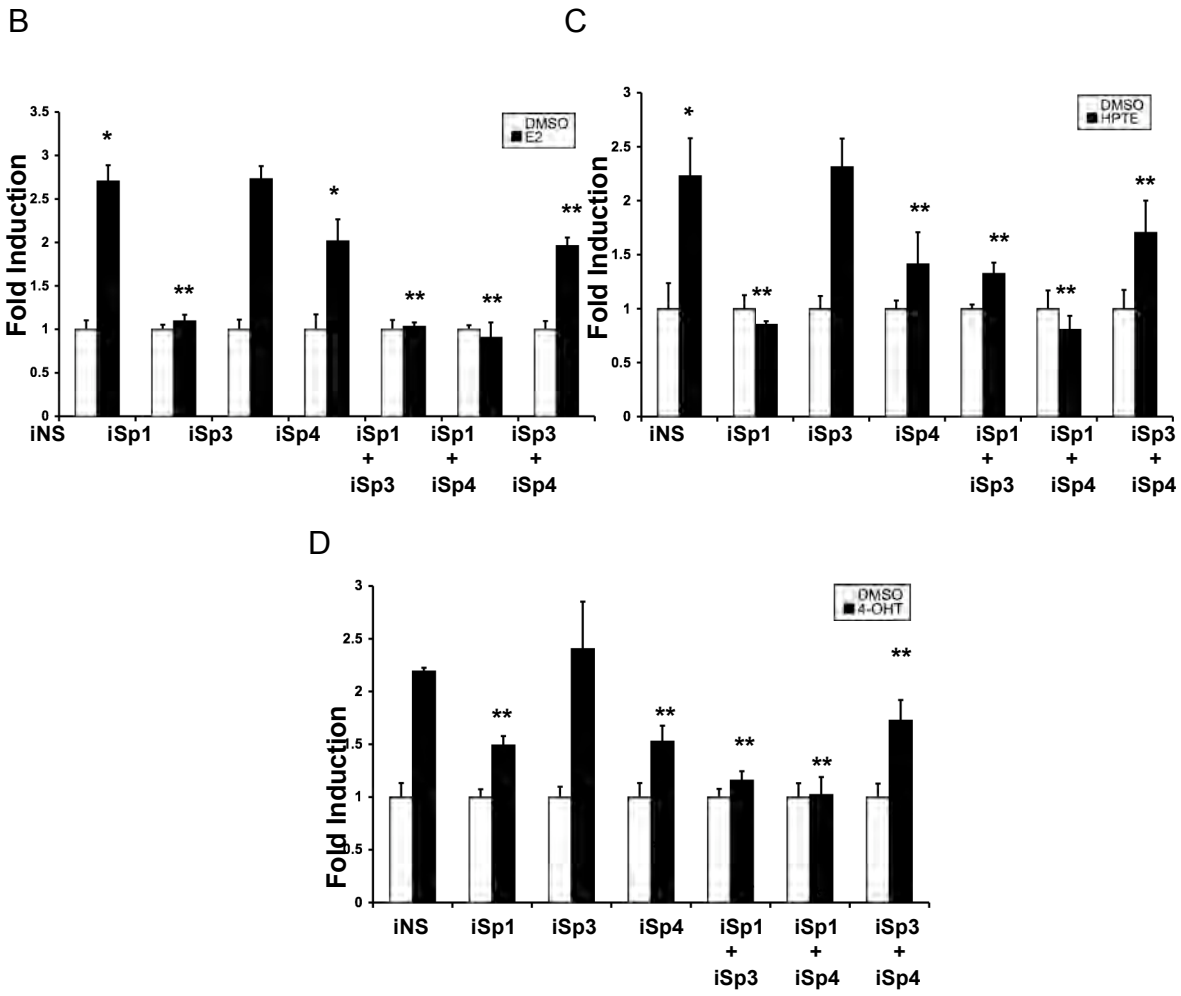


Figure 21. (continued)

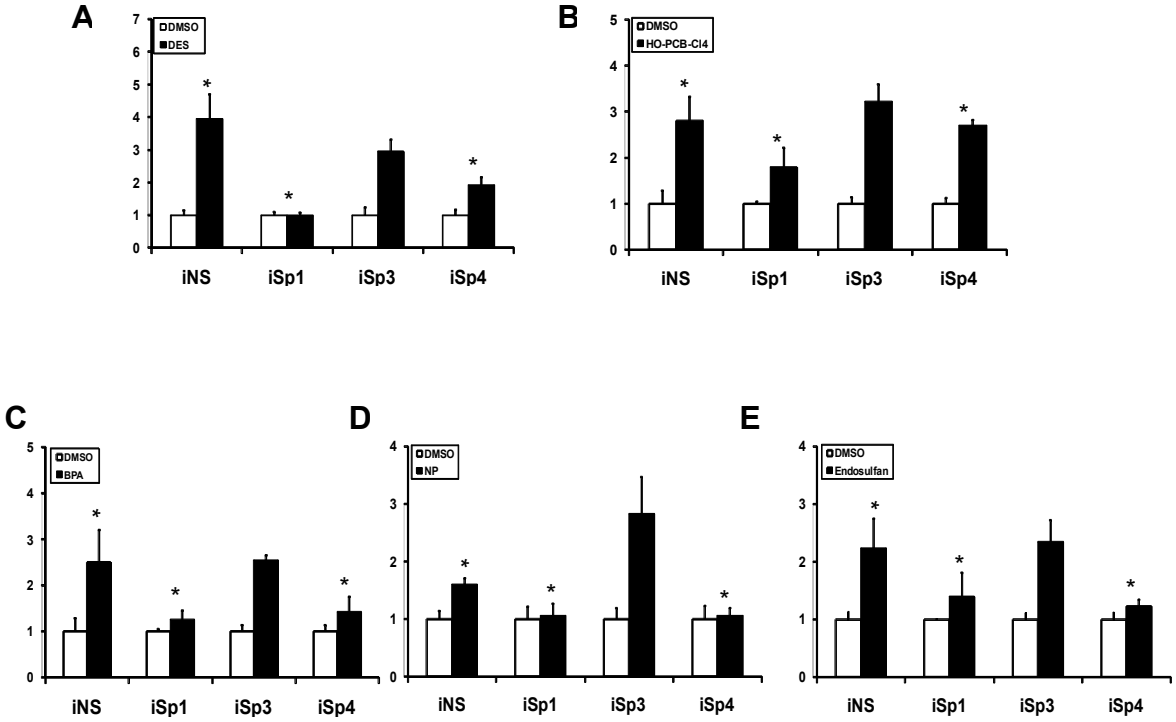


Figure 22. Differential role of Sp proteins in activation of ERα/Sp by ERα agonists. Effects of Sp protein knockdown on activation of $pSp1_3$ by DES (A), HO-PCB-Cl_4 (B), BPA (C), nonylphenol (D), and endosulfan (E). MCF-7 cells were transfected with $pSp1_3$/ERα and small inhibitory RNAs, treated with various ERα ligands, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group. Significantly ($p < 0.05$) induced activity relative to DMSO is indicated (*) and a decrease in induced activity (compared to iNS) by iSp1, iSp3 or iSp4 is indicated by a double asterisk (**).

induction, the results show that ICI 182,780 activates ERα/Sp1 and ERα/Sp3 since iSp3, iSp1 and iSp1 plus iSp3 significantly decreased transactivation (Fig. 23B). Similar results were observed for kepone (Figs. 23C and 23D), although neither iSp3 nor iSp4 were as effective in decreasing fold inducibility by kepone compared to ICI 182,780. The results demonstrate that activation of ERα/Sp-dependent transactivation by ICI 182,780 and kepone differs from the other estrogens, xenoestrogens and 4-OHT with respect to the role of Sp1, Sp3 and Sp4 in mediating this response.

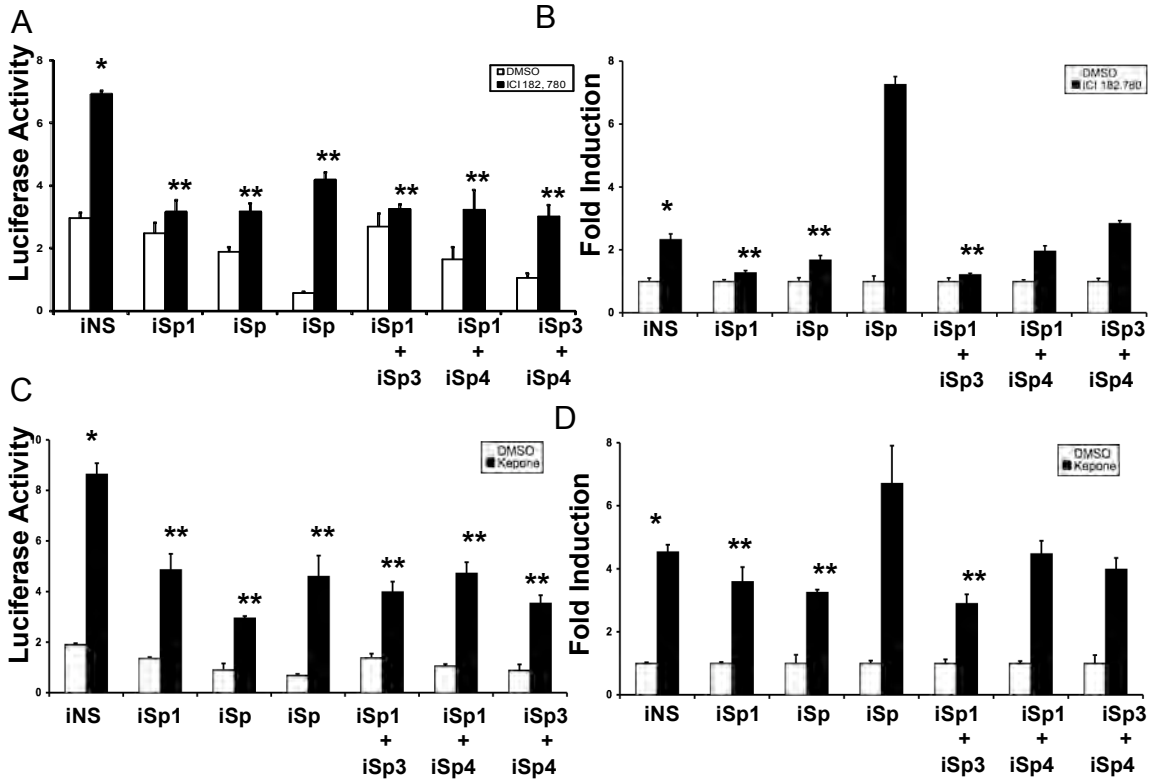


Figure 23. Differential role of Sp proteins in activation of ERα/Sp by ICI 182,780 (A and B) or kepone (C and D). MCF-7 cells were transfected with $pSp1_3$/ERα and various iSps, treated with 1 μM ICI 182,780 or 5 μM kepone, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group. Significantly ($p < 0.05$) induced activity relative to DMSO is indicated (*) and a decrease in induced activity (compared to iNS) by iSp1, iSp3 or iSp4 is indicated by a double asterisk (**).

E2 also differentially activates other GC-rich promoters, such as pCad [121] and pE2F1 [115]. Three GC-rich sequences on their proximate promoter regions were required for ERα mediated E2 transactivation. The differential activities by xenoestrogens were investigated on these two promoters (Fig. 24). MCF-7 cells were either transfected with pCad (-90~+25) or pE2F1 (-169~-54) and wtERα expression plasmid, then treated with different compounds with the same concentration used in Fig. 19. Only estrogens (E2 and DES) and xenoestrogens resveratrol, OP and Kepone can induce Cad gene expression (Fig. 24A). However, Kepone failed to induce E2F1 promoter, but BPA was able to induc it. Antiestrogen 4-OHT also induced E2F1 promoter construct (Fig. 24B), indicating that compounds differential activity on E2-responsive genes is also promoter context dependent.

Results in Fig. 25A illustrates that E2, DES and most xenoestrogens, but not ICI 182,780, induced E2F1 protein in MCF-7 cells, whereas < 2-fold induction was observed for 4-OHT and kepone. The unexpectedly low activity of kepone contrasts to transactivation studies in cells transfected with $pSp1_3$ (Fig. 18) and the induction of E2F1 by resveratrol (Fig. 25A) contrasted to the lack of induction in MCF-7 cells transfected with $pSp1_3$ and wild-type ERα and treated with resveratrol (Fig. 19A). These ligand-dependent differences could be due, in part, to promoter context (i.e. E2F1 promoter vs. $pSp1_3$) but are not entirely unexpected due to the SERM-like activity of these compounds. And the induction pattern of E2F1 protein by structure diverse compounds is not completely resembled the promoter assay (Fig. 24B), due to short sequence of E2F1 promoter used in the transient transfection or the epigenetic effects. Figure 25B shows that induction of this gene by E2 in MCF-7 cells is decreased in cells cotransfected with iSp1, iSp3 and iSp4.

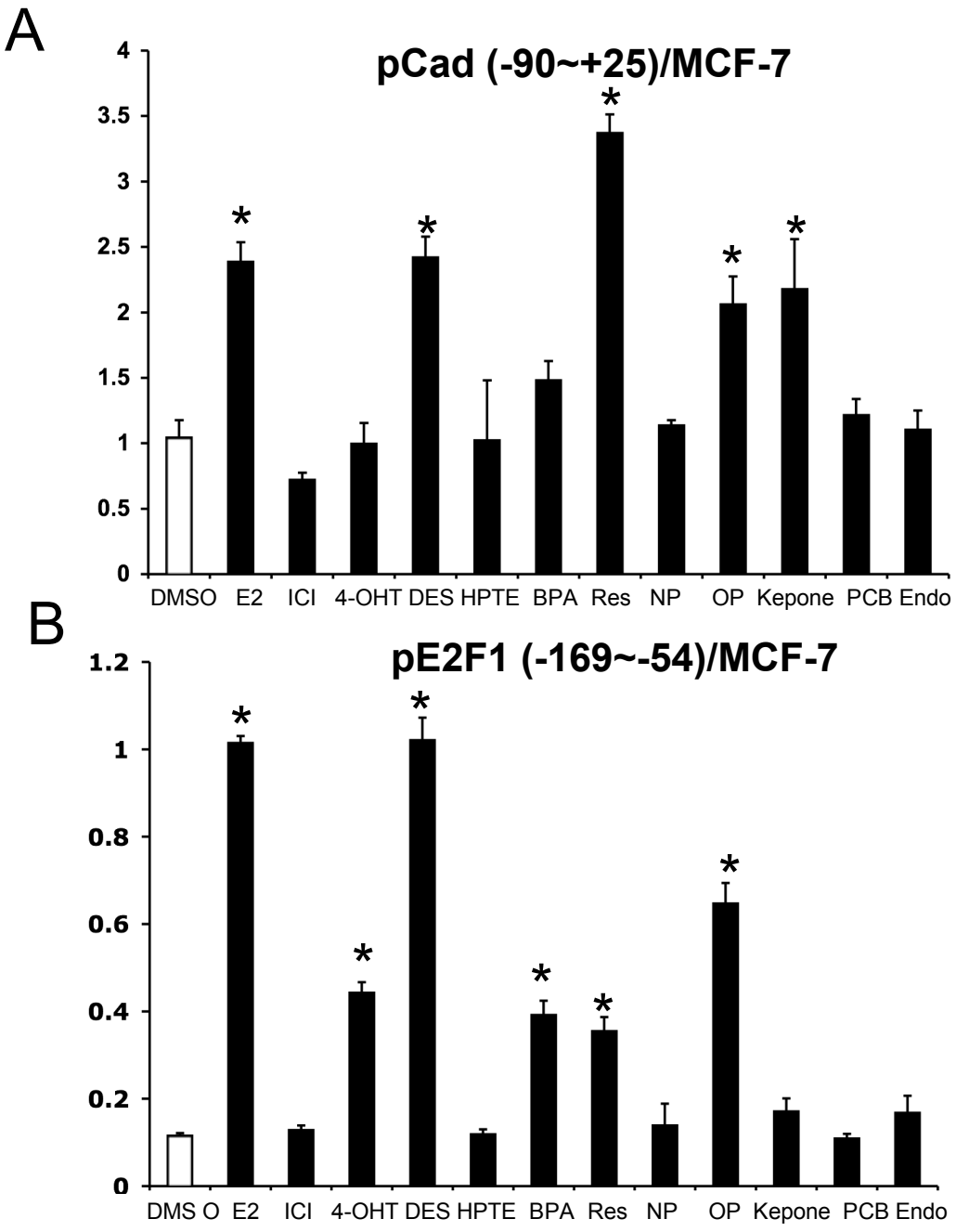


Figure 24. Structure-dependent activation of ERα mediated GC-rich promoter activation in MCF-7 cells transfected with pCad (A) and pE2F1 (B). Cells were transfected with pCad or pE2F1 with wtERα, treated with different compounds and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated (*). The concentrations used in this experiment were those which maximally induced luciferase activity using $pSp1_3$ and wild-type ERα (Figs. 18) without causing any cytotoxicity.

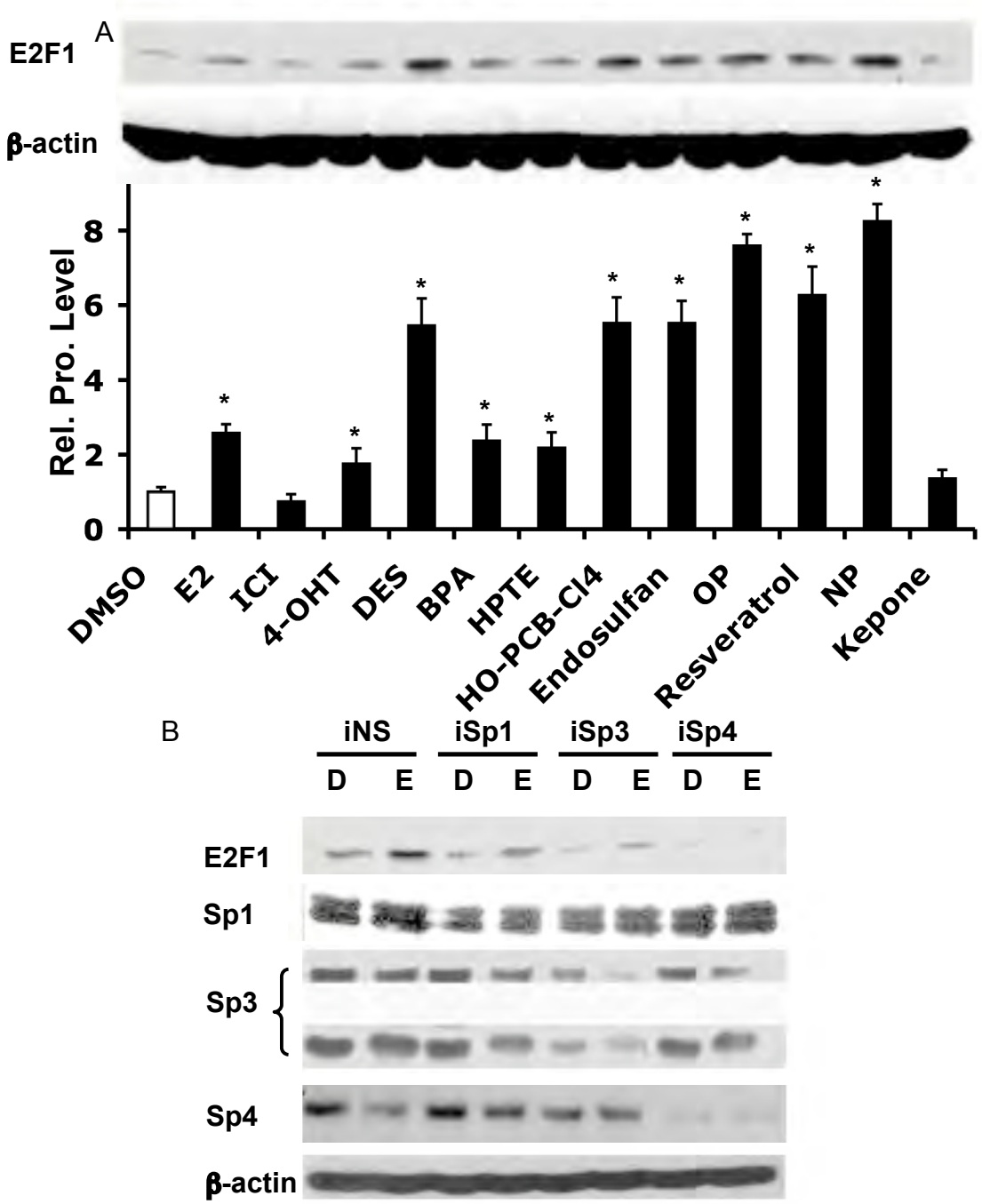


Figure 25. (A) Induction of E2F1 protein expression. Cells were treated with DMSO, 10 nM E2, 1 μM ICI 182,780, 1 μM 4-OHT, 10 nM DES, 75 μM BPA, 50 μM HPTE, 20 μM HO-PCB-Cl_4, 20 μM endosulfan, 50 μM OP, 75 μM resveratrol, 20 μM NP, and 5 μM kepone for 12 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Experiments were carried out in triplicate. (B) Role of Sp proteins in activation of E2F1 by E2. MCF-7 cells were transfected with iSp1, iSp3 or iSp4, treated with DMSO or 10 nM E2 for 12 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Results are expressed as means ± SD and the E2F1 response (normalized to β-actin) in the DMSO treatment group was set at 1.0. Significant ($p < 0.05$) induction is indicated by an asterisk.

Previous studies showed that E2, 4-OHT and ICI 182,780 induced interactions of chimeric CFP-Sp1 and YFP-ERα in MCF-7 cells transfected with expression plasmids for these chimeras and analyzed by fluorescence resonance energy transfer (FRET) [128]. FRET efficiencies could be determined by comparing results to the YFP-CFP chimera which was used as a positive control [128]. Using this approach, we compared FRET efficiencies for E2 and selected xenoestrogens in MCF-7 cells transfected with CFP-Sp1 and YFP-ERα. Figure 26A illustrates the pseudocolor CFP and YFP fluorescence intensities in MCF-7 cells treated with DMSO or 10 μM kepone. The FRET signal in the kepone-treated cells indicates an increase in FRET efficiency compared to DMSO and that kepone induces ERα-Sp1 interactions at a distance of < 10 nm and thereby allows energy transfer. The FRET efficiency values were determined 20 - 30 min after treatment with 10 nM E2, 25 μM HO-PCB-Cl_4, 10 μM kepone, and 75 μM BPA, and FRET efficiencies were significantly increased compared to solvent (DMSO) control (Fig. 26B). These results demonstrate that xenoestrogens not only activate ERα/Sp-dependent transactivation but also induce ERα-Sp1 interactions in living breast cancer cells. Currently, we are investigating ligand structure-dependent differences in the rate of ERα interactions with Sp1, Sp3 and Sp4 using the FRET technique.

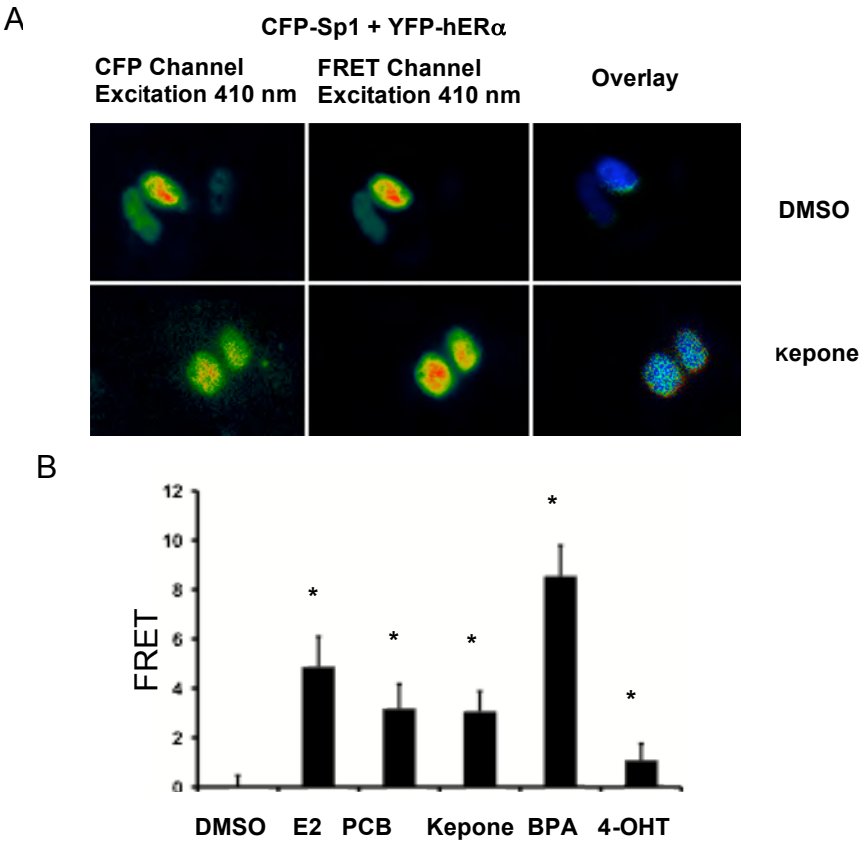


Figure 26. FRET analysis and induction of E2F1 in MCF-7 cells. (A) An example of FRET in MCF-7 cells treated with DMSO (upper panel) and 10 μM kepone (lower panel) for 20 - 30 min. Each panel represents the pseudocolor intensity of CFP, YFP and FRET signals, respectively. (B) FRET efficiencies. MCF-7 cells were treated with DMSO, 10 nM E2, 20 μM HO-PCB-Cl_4, 10 μM kepone, 75 μM BPA and 1 μM 4-OHT for 20 - 30 min, and FRET efficiencies were determined as described in the Materials and Methods. Significant differences ($p < 0.05$) in treated cells from DMSO (control) are indicated (*).

3.5. Discussion

E2 is the major endogenous hormone for both ERα and ERβ, and there is increasing evidence that activation of both receptors is complex and dependent on multiple factors including relative expression of each specific ER-subtype and their corresponding coactivators and coregulatory proteins [528, 530, 537, 538]. The complexity of estrogen signaling is also linked the multiplicity of signaling

pathways which include both genomic and non-genomic mechanisms where the latter pathway involves extranuclear interactions of ER with multiple cytosolic or membrane-associated proteins that cooperatively activate kinase signaling [568, 569]. Genomic pathways of estrogen action include not only activation through direct binding of ER (dimeric) to EREs in target gene promoter but also activation of genes through interactions of ER with other DNA bound proteins including Sps, AP1, GATA-1 and NFκB [108, 563-565]. There is also evidence showing that hormonal activation of genes through ERE promoter sequences is ligand structure-dependent and the tissue specific ER agonist and ER antagonist activities of SERMs has led to their applications for treating breast cancer and other hormone-related diseases [558-560].

Several *in vitro* assays clearly distinguish between the activities of E2 and antiestrogens such as ICI 182,780 and tamoxifen to activate ERE-dependent genes/promoter constructs [406, 534, 544, 552-554, 562]. Structurally-diverse synthetic industrial estrogenic compounds (xenoestrogens) and phytoestrogens also differentially activate various ERE-dependent constructs and related genes [539-543, 545-547, 570, 571], suggesting that these compounds also exhibit SERM-like activity. The genomic ERα/Sp1 pathway for activating E2-responsive genes containing GC-rich motifs is critical for regulating growth-promoting genes and G_0/G_1 to S phase progression in MCF-7 cells [108], and this study was carried out to determine whether structurally-diverse xenoestrogens also differentially activate ERα/Sp. Previous studies showed that E2 induced transactivation in MCF-7, ZR-75 or MDA-MB-231 cells transfected with $pSp1_3$ and cotransfection with wild-type ERα or DBD deletion mutants [111, 129], whereas ICI 182,780 or 4-OHT induced transactivation only in cells transfected with wild-type ERα. The $pSp1_3$ construct is a prototypical E2-responsive GC-rich promoter which has been used to investigate differences between E2 and the antiestrogens 4-OHT and ICI 182,780 to activate ERα/Sp in breast cancer cells. In this study, $pSp1_3$ was used to investigate the SERM-like activity of

structurally-diverse xenoestrogens and resveratrol by determining the effects of cell context (MCF-7 vs. MDA-MB-231), wild-type and variant ERα and different Sp proteins (Sp1, Sp3 and Sp4) on their estrogenic activities. With the exception of resveratrol in MCF-7 cells, all compounds including the antiestrogens ICI 182,780 and 4-OHT, E2, DES and the xenoestrogens induced wild-type ERα/Sp-dependent transactivation in MCF-7 and MDA-MB-231 cells (Figs. 18-20). E2 and DES, but not ICI 182,780 or 4-OHT, induced transactivation in MCF-7 cells transfected with ERαΔZF1 or ERαΔZF2. The pattern of xenoestrogen activation of $pSp1_3$ in cells transfected with ERα, ERαΔZF1 and ERαΔZF2 (Fig. 19) was similar to that observed for E2 and DES, and the most active compounds were kepone, HPTE, HO-PCB-Cl_4, endosulfan and nonylphenol. Activation of ERαΔZF1/Sp or ERαΔZF2/Sp by xenoestrogens distinguished between these compounds and ICI 182,780 and 4-OHT which were inactive in cells transfected with the DBD mutants.

Previous studies [129] showed that E2 and the antiestrogens ICI 182,780 and 4-OHT differentially induced activity in MCF-7 cells transfected with $pSp1_3$ and the C-terminal deletion mutants ERα(1-553) and ERα(1-537). ERα(1-537) does not express the F domain and part of helix 12 which interacts with coactivators. Not surprisingly, E2 and DES did not induce activity in MCF-7 (Fig. 19E) or MDA-MB-231 (Fig. 20E) cells transfected with ERα(1-537) and $pSp1_3$; however, significant induction was observed for both 4-OHT and ICI 182, 780. Moreover, in cells transfected with $pSp1_3$ and ERα(1-537), like the antiestrogens, the xenoestrogens and resveratrol also induced transactivation. Thus, deletion of a region of helix 12 in the C-terminal domain of ERα, which totally abrogates E2-responsiveness, does not affect xenoestrogen-induced transactivation in cells transfected with $pSp1_3$ demonstrating a fundamental mechanistic difference between activation of ERα/Sp by E2 and the xenoestrogens/resveratrol used in this study.

Thus, results of transfection studies using wild-type and variant ERα show that xenoestrogen activation of ERα/Sp required different domains of ERα than either E2/DES or ICI 182,780/4-OHT.

Recent studies in ZR-75 cells showed that ERα/Sp-dependent activation of vascular endothelial growth factor receptor 2 (VEGFR2) by E2 was primarily dependent on ERα/Sp3 and ERα/Sp4 but not ERα/Sp1 [127]. Thus, activation of VEGFR2 by E2 required interactions of ERα with only two of the Sp proteins and, in this study, we investigated structure-dependent activation of ERα/Sp1, ERα/Sp3 and ERα/Sp4 by E2, antiestrogens and xenoestrogens. RNA interference was used to individually knockdown Sp1, Sp3 and Sp4 or their combinations (Fig. 21A) in MCF-7 cells and, the effects of Sp protein knockdown on ligand-dependent activation of $pSp1_3$ was investigated. Basal activity was decreased in cells transfected with iSp1, iSp3 and iSp4; however, fold induction by estrogens (E2 and DES), xenoestrogens and antiestrogens exhibited three patterns which differentially relied upon ERα/Sp1, ERα/Sp3, ERα/Sp4 or their combinations (Figs. 21 - 23). For E2, HPTE, DES and HO-PCB-Cl_4, ERα/Sp1 > ERα/Sp4, and ERα/Sp3 had minimal to no effect on activation of ERα/Sp by these compounds. The pattern of ERα/Sp activation for BPA, endosulfan, NP and 4-OHT was ERα/Sp1 ≈ ERα/Sp4 with minimal contributions by ERα/Sp3. In contrast, both ERα/Sp1 and ERα/Sp3 play role in activation of $pSp1_3$ by ICI 182780 and kepone, but ERα/Sp4 tends to cause an inhibitory effect since iSp4 enhances the fold induction by these compounds. These results demonstrate that for E2 and other ER agonists/antagonists, activation of the GC-rich $pSp1_3$ construct by ERα/Sp involves different combinations of ERα/Sp1, ERα/Sp3 and ERα/Sp4. Since E2-induced transactivation in MCF-7 cells transfected with $pSp1_3$ required ERα/Sp1 and ERα/Sp4, these results are in contrast with the requirement of ERα/Sp3 and ERα/Sp4 but not ERα/Sp1 for activation of the GC-rich VEGFR2 promoter by E2 [127]. This suggests that both cell- and promoter-

context may also dictate which ERα/Sp complexes are required for ligand-induced transactivation. Therefore, the relative levels of Sp proteins expressed in a cell/tissue may also influence hormonal activation of specific genes, and this is currently being investigated using both cell culture and *in vivo* models.

In cells transfected with $pSp1_3$, xenoestrogens, estrogens and antiestrogens all activated ERα/Sp1. Therefore, xenoestrogen-induced interactions of ERα and Sp1 were investigated by FRET in MCF-7 cells transfected with CFP-Sp1 and YPF-ERα expression plasmids. This technique was previously used in this laboratory to show that E2 induced interactions of ERα and Sp1 in living cells [128]. We selected five compounds representative of estrogens, antiestrogens and xenoestrogens, and observed that E2, HO-PCB-Cl_4, kepone, BPA and 4-OHT all significantly induced FRET efficiencies (Figs. 26A and 26B). These data demonstrate a comparable pattern of ligand-dependent ERα-Sp1 interactions, and future studies will focus on ligand-dependent differences and similarities in the FRET efficiencies and kinetics of ERα interactions with Sp3 and Sp4.

E2F1 is induced by E2 in MCF-7 cells through ERα/Sp-dependent activation of proximal GC-rich promoter sequences that also interact with NFYA bound to nearby CCAAT sites [116]. Figure 25B illustrates that transfection of iSps decreased basal and E2-induced E2F1 expression and these observations were similar to the effects of iSp1, iSp3 and iSp4 on induction of E2F1 promoter constructs and E2F1 mRNA by E2. Figure 25A shows that with the exception of ICI 182,780 and kepone, the remaining estrogens (E2 and DES), xenoestrogens (BPA, HPTE, HO-PCB-Cl_4, endosulfan, OP and NP) and resveratrol induce E2F1. Resveratrol did not induce transactivation in MCF-7 cells transfected with $pSp1_3$ and wild-type ERα but exhibited activity with wild-type and variant ERα in MDA-MB-231 cells (Figs. 18 - 20), whereas kepone was invariably more active in these assays. Differences between resveratrol and kepone as inducers of

E2F1 protein may be gene promoter-/gene-dependent since we observed that in the same experiment, kepone but not resveratrol induced cyclin D1 expression (data not shown) which is not activated by ERα/Sp in MCF-7 cells. Kepone, E2, resveratrol and other estrogenic compounds are currently being investigated using microarrays coupled with RNA interference to further investigate the mechanisms associated with structure-dependent differences in activation or repression of hormone-responsive genes.

In summary, results of this study show that xenoestrogens activate wild-type and variant ERα/Sp-dependent transactivation in MCF-7 cells transfected with $pSp1_3$. However, the results indicate that for some variant forms of ERα (i.e. ERαΔZF2/ERαΔZF1), most of the xenoestrogens resembled E2, whereas, in cells transfected with ERα(1-537), these compounds resembled the antiestrogens ICI 182,780 and 4-OHT. We also show for the first time that ligand-induced activation of ERα/Sp in MCF-7 cells transfected with $pSp1_3$ was dependent on multiple Sp proteins (i.e. Sp1, Sp3 and Sp4) and utilization of these proteins was also ligand structure-dependent. There is evidence that Sp1 and Sp3 are differentially organized within the nucleus of MCF-7 cells [572], and this may contribute to the differences between ERα/Sp1 and ERα/Sp3 in activating $pSp1_3$ and currently we are also investigating the intranuclear distribution and organization of Sp3 and Sp4 with ERα in different breast cancer cell lines by FRET. Results of this study, coupled with other reports of *in vitro* and *in vivo* assays with xenoestrogens [539-543, 545-547, 562, 570, 571], suggest that these compounds are SERMs and their ERα agonist and antagonist activities cannot necessarily be inferred from simple ER binding or transactivation assays.

CHAPTER IV

IN VIVO PROFILING OF ESTROGEN RECEPTOR/SPECIFICITY PROTEIN-DEPENDENT TRANSACTIVATION

4.1. Overview

E2 activates the ER through multiple genomic and non-genomic pathways in various tissues/organs. ERα/Sp-dependent activation of E2-responsive genes containing GC-rich promoters has been identified in breast and other cancer cell lines and in this study we report the development of transgenic animals overexpressing a transgene containing three tandem GC-rich sites linked to a minimal TATA or thymidine kinase (TK) promoter and a luciferase transgene. Several mouse lines expressing the transgenes were characterized and in line 15, E2 induced a 9-fold increase in luciferase activity in the female mouse uterus and the synthetic estrogens bisphenol A (BPA) and nonylphenol (NP) also induced luciferase uterine activity. The “pure” antiestrogen ICI 182,780 also induced luficerase activity in the mouse uterus and similar results were observed for ICI 182,780 in breast cancer cells transfected with this construct. Differences in the ER agonist and antagonist activities of E2, NP, BPA and ICI 182,780 were investigated in the male testis and penis and the male and female stomach in line 15 transgenic mice. All of these tissues were hormone-responsive. However, the patterns of induced or repressed luciferase activity were ligand structure-, tissue-, and sex-dependent. These results demonstrate for the first time that the hormonal activation or repression of a GC-rich promoter in vivo and the results suggest that the ERα/Sp pathway may be important for ligand-dependent induction and repression of genes.

4.2. Introduction

17β-estradiol (E2) and related steroidal estrogens play a critical role in development of the male and female reproductive tract, vascular physiology, skeletal development and growth, and neuronal function [573-576]. Estrogens also play a role in hormone-dependent diseases; epidemiologic studies show that lifetime exposure to estrogen is a risk factor for development of breast cancer in women [577-581]. Since many early stage mammary tumors are estrogen receptor (ER)-positive, these tumors respond to endocrine therapy and treatment with antiestrogens such as tamoxifen and raloxifene or with aromatase inhibitors [558, 559]. Moreover, recent epidemiologic studies arising from the Women's Health Initiative demonstrate that hormone replacement therapy for postmenopausal women can also lead to adverse health effects, including decreased cognitive function, possible dementia, increased risk for venous thrombosis and strokes [582-584]. Thus, it is imperative to develop a comprehensive understanding of tissue-specific and age-dependent estrogen action in order to predict estrogen-induced responses and design appropriate selective estrogen receptor modulators (SERMs) for treatment of hormone-dependent diseases.

The classical mechanism of ER-mediated transactivation requires interaction of the ligand bound nuclear ER dimer with estrogen responsive elements (EREs) in target gene promoters, and these sequences vary from the classical palindromic ERE (GGTCANNNTGACC) to diverse nonconsensus EREs [79, 94, 585, 586]. However, it is now recognized that genomic mechanisms of ER-mediated transactivation are highly complex and involve interactions of ER with multiple ERE half-sites and with other DNA-bound transcription factors such as specificity protein 1 (Sp1). ER also indirectly activates genes through protein-protein interactions with other DNA-bound transcription factors such as NF-κB, GATA, AP-1 and Sp1 [108, 563-565]. In addition to these genomic pathways of

ER action, there is evidence that cytosolic/membrane bound ER interacts with many other proteins to mediate hormone-dependent activation of several kinase pathways which in turn modulate gene expression [568, 569, 587, 588]. The relative importance of genomic vs. non-genomic pathways of estrogen action and their interplay is complex and highly variable among tissue and cell types, and it is critical that these pathways be further investigated.

Research in this laboratory first observed that the proximal GC-rich site alone in the Hsp27 promoter was estrogen-responsive and similar results have been obtained using pSp1 and $pSp1_3$ which are constructs containing 1 or 3 consensus GC-rich Sp1 binding sites linked to luciferase [12, 104, 111]. Subsequent studies have identified a large number of hormone-responsive genes in breast cancer cells and other cell lines that are regulated by ERα/Sp1 [108, 492], [[58, 589-599]. RNA interference using a small inhibitory RNA for Sp1 (iSp1) showed that iSp1 inhibited basal and E2-induced G0/G1$\rightarrow$ S phase cell cycle progression, indicating that ERα/Sp1-dependent genes play an important role in ER-positive breast cancer cell proliferation [515].

Mechanistic studies in breast cancer cell lines show that ligand-dependent activation of GC-rich promoter constructs and their corresponding genes is complex [111, 128, 129]. For example, a recent study shows that VEGF receptor 2 (VEGFR2, KDR) is expressed in ZR-75 breast cancer cells and induced by E2 [127]. Promoter analysis showed that two proximal GC-rich sites were required for transactivation, and RNA interference with iSp1, iSp3 and iSp4 shows that Sp3 and Sp4 but not Sp1 are required for hormone-dependent activation of VEGFR2. Moreover, in MCF-7 cells E2 downregulates VEGFR2 expression and this also involves Sp proteins and recruitment of corepressors to the VEGFR2 promoter.

In this study we have generated transgenic mice expressing a construct containing three tandem GC-rich sites linked to a luciferase reporter gene and

we have used this animal model to investigate tissue-specific modulation of luciferase activity by estrogenic compounds. E2, the antiestrogen ICI 182,780, the synthetic industrial estrogens bisphenol A (BPA) and nonylphenol (NP) exhibit structure-dependent, sex- and tissue-specific effects on increased or repressed luciferase activity and confirms that the ERα/Sp pathway is functional *in vivo*.

4.3. Materials and Methods

4.3.1. Reagents and Plasmids

Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Antibiotic antimycotic solution (AAS) (x100) was obtained from Sigma-Aldrich (St Louis, MO). The following test chemicals (and purities) were purchased from Sigma-Aldrich: *p*-nonylphenol (98%) and bisphenol A (BPA>99%), 4-OHT(≥98%), E2 (≥98%), resveratrol (>99%) were purchased from Sigma, and ICI 182,780 was provided by Dr. Alan Wakeling (Astra-Zeneca, Macclesfield, UK). Plasmid preparation kits were purchased from Sigma. Human ERα expression plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX).

4.3.2. Cells and Transient Transfection Assays

ZR-75 human breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI1640 media (Sigma-Aldrich) supplemented with 1.5 g/L sodium bicarbonate, 2.38 g/L HEPES, 0.11 g/L sodium pyruvate, 4.5 g/L glucose, 10% FBS, and 5 ml/L AAS at pH 7.4. Cells were cultured and grown in a 37 °C incubator with humidified 5% CO2, 95% air. For transient transfection studies, ZR-75 cells were seeded in 12-well plates in DMEM/F-12 medium without phenol red supplemented with 2.2 g/liter sodium bicarbonate, and 2.5% charcoal-stripped FBS. After 24 h, cells

were transfected using the calcium phosphate method with 350 ng of luciferase reporter construct, 100 ng pcDNA3/His/lacZ (Invitrogen, Carlsbad, CA) as a standard reference for transfection efficiency, and increasing amounts of the appropriate ERα expression plasmid. Six hours after transfection, cells were shocked with 25% glycerol/PBS for 1 min, washed with PBS, and then treated with dimethylsulfoxide (DMSO, solvent) or 10 nM E2 in DMSO for another 30–48 h. Cells were then washed twice in PBS and harvested with 100 µl of reporter lysis buffer (Promega Corp., Madison, MI). After one freeze-thaw cycle, cell lysates were centrifuged for 1 min at 16,000×g, and the supernatant was used for determination of protein activity. Luciferase (Promega Corporation, Madison, MI) and β-galactosidase activities were determined using the Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA). Light emission was detected on a Lumicount micro-well plate reader (Packard Instruments, Meriden, CT), and luciferase activity was calculated by normalizing against β-galactosidase activity obtained from the same sample and compared with the DMSO control group (set at 100%) for each set of experiments.

4.3.3. Experiment Animals

Animal care and experiments were approved by Texas A&M University Laboratory Animal Care Committee. All animals were housed on a 12 h light/dark cycle at 22-25°C and supplied with water and rodent chow diet ad libitum. Transgenic animals were generated by microinjection into single cell-stage C57Bl/6 embryos, and the embryos were implanted into pseudopregnant mice. Transgene positive animals were used as founders to breed with wild type C57Bl/6 mice and the offspring were used as experimental animals.

4.3.4. Transgenes Construction

$(GC)_3$-TA was synthesized by IDT DNA Technology (Coralville, IA) and inserted into pGL3 Basic vector (Promega, Madison, WI), containing the firefly luciferase

gene sequence. The $(GC)_3$-TA (pGL3) plasmid was digested with *NheI* and *BamHI,* and a 2 kb DNA fragment containing a 72 bp GC rich site, a TATA box and the firefly luciferase cDNA were purified by agarose gel electrophoresis. The DNA fragment was extracted using Gel Purification Kit (Qiagen, Valencia, CA) and dissolved in the transgene buffer (10 mM Tris, 0.1 nM EDTA, pH7.4).

The TK promoter was amplified by PCR from a TK-ERE-CAT plasmid using forward primer (5'-AAT AAG ATC TCC TAG GAT CCG GCC CC-3') and reverse primer (5'-ATA CAA GCT TAT CTG CGG CAC GCT GT-3'), the TATA box of $(GC)_3$-TA-Luc was replaced by digesting with the *BglII* and *HindIII*. The construct was linearized with *NheI* and *SalI* to release the TK-$(GC)_3$-luc transgene cassette used to generate transgenic mice.

4.3.5. Genotyping

Mouse tail DNA for PCR analysis was prepared by the HotSHOT method [600]. Briefly, a 2mm tail clip is taken from 15-day old mice and is placed in a PCR tube. DNA is released from the tissue by adding 75 μl base solution (25 mM NaOH, 0.2 mM EDTA, pH12), and heated at 95°C for 1 hr, 75 μl neutralization solution (40 mM Tris-HCl pH5) was then added. PCR was used according to a universal PCR genotyping assay [601], which is sufficiently sensitive to detect a single copy of a transgene. Basically, a master mix was made and for a single reaction, the mixture contained 2.545 μl water, 2.6 μl 5 M betaine (Sigma), 1 μl 10X buffer, 0.025 μl 20 mM dNTP mix, 0.05 μl 1 mM Cresol Red (Sigma), 0.06 μl 5' Fabpi primer (20 μM) (TGG ACA GGA CTG GAC CTC TGC TTT CCT AGA), 0.06 μl 3' Fabpi primer (20 μM) (TAG AGC TTT GCC ACA TCA CAG GTC ATT CAG), 0.5 μl 5' Luciferase primer (20 μM) (5'-AGA CGC CAA AAA CAT AAA GAA AGG CCC GGC-3'), 0.5 μl 3' Luciferase primer (20 μM) (5'-TAT AAA TGT CGT TCG CGG GCG CAA CTG CAA-3'), 0.02 μl Klentaq LA (Clontech, Mountain View, CA), 2 μl genomic DNA template. The total reaction

volume is 10 μl. Primers for Fabpi were used as the loading control to monitor the quality of the genomic DNA and amplify a sequence from the intestinal fatty acid binding protein gene. PCR reactions are: Step 1=93°C for 1 min, step 2=93°C for 2 min, step 3=68°C for 3 min, steps 2 and 3 were repeated for a total of 30 cycles, step 4=4°C until samples are retrieved. After the PCR reaction is finished, PCR samples are analyzed on 1% agarose gel at 120V for 14 min in TAE buffer.

4.3.6. Uterine Wet Weight Assay

Twenty-one day-old heterozygous females were injected with 100 μl corn oil (vehicle control), or 50 μg/kg body weight E2, or 250 μg/kg ICI 182, 780, or 250 mg/kg BPA, or 250 mg/kg nonylphenol, or cotreated with ICI 182, 780 plus E2 for three consecutive days, and on day four, animals were sacrificed by CO_2 asphyxiation. After making an incision in the skin and the abdominal muscle, the uterine cervix was cut away from the vaginal fornix. Because fluid imbibition is an estrogen response, care was taken to retain all the uterine luminal fluid. The uterus was then removed by gently lifting tissue anteriorly and trimming away the mesometrium. A cut was made at the uterotubal junction, thus preserving the integrity of both uterine horns and avoiding loss of uterine fluid. The uterus was immediately weighed, and placed on absorbent cardboard in order to maintain the original *in vivo* orientation.

4.3.7. Luciferase Assay

Twenty-one-day-old heterozygous mice were treated on three consecutive days with s.c. injections of the different compounds dissolved in vehicle (corn oil). On the fourth day, animals were killed by CO_2 asphyxiation, and organs were immediately removed. Tissue extracts were homogenized and sonicated using a Pro200 micro homogenizer (Pro Scientific) in 500 μl of lysis buffer (1 mM dithiothreitol, 4 mM EGTA, 4 mM EDTA, 0.7 mM phenylmethylsulfonyl fluoride,

100 mM potassium phosphate at pH7.8). After three freeze-thaw cycles by liquid nitrogen, cell lysates were centrifuged for 30 min at 16,000×g, and the supernatant was used for determining protein activity. Luciferase activity was determined by measuring light emission using a lumicount micro-well plate reader (Packard Instruments, Meriden, CT) over 10 sec, and luciferase activity was calculated by normalizing against protein concentration obtained from the same sample measured using the Bradford reagent (Biorad, Hercules, CA).

4.3.8. Statistical Analysis

For transient transfection studies, results are expressed as means ± SD for at least three separate experiments for each treatment group. Statistical differences ($P < 0.05$) between control (DMSO) and treatment groups were determined by ANOVA and Scheffe's test.

4.4. Results

Previous reports show that E2 activates constructs containing GC-rich promoter inserts from multiple E2-responsive genes [108, 492] and the $pSp1_3$ construct containing three tandem consensus GC-rich Sp1 binding sites has been used as a prototype for mechanistic studies [128, 129]. In this study, we generated two constructs containing three tandem GC-rich sites linked to a minimal TATA box (GC_3-TATA-Luc) or TK promoter (GC_3-TK-Luc) in the pGL3 basic vector (Fig. 27A) and compared the E2-responsiveness of these plasmids to a comparable $(GC)_3$-TATA-Luc (PXP2) construct (Fig. 27B). This latter construct in a PXP2 vector has been extensively used in previous *in vitro* studies [128, 129]. In ZR-75 breast cancer cells transfected with these constructs, treatment with 10 nM E2 did not induce luciferase activity. However, after cotransfection with 5, 15 or 50 ng ERα expression plasmid significant induction by E2 was observed (Fig.25B). These results are consistent with results of previous studies with GC-rich constructs in ZR-75 or MCF-7 cells and the lack of hormonal activation in

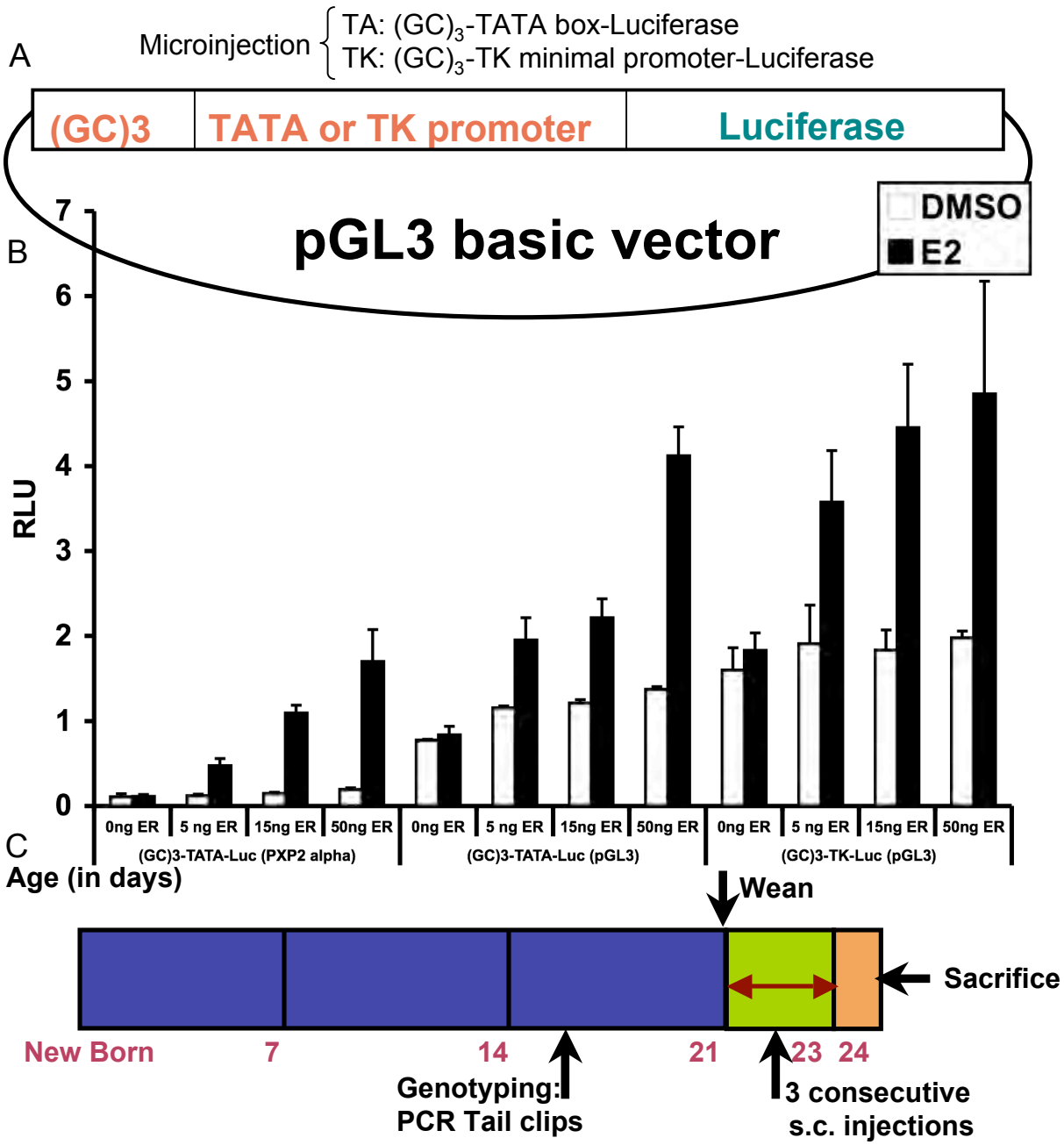


Figure 27. Transgene constructs and experimental time line. (A) Two transgene constructs were generated by inserting three tandem GC-rich sequences with a TATA box or minimal TK promoter into the pGL3 basic luciferase vector. (B) Transgene constructs are expressed and induced by E2 in breast cancer cells. ZR-75 cells were transfected with different reporter genes and increasing amount of ERα. (GC)3-TATA-Luc (PXP2) which has been extensively used in *in vitro* studies was included as a control. Cells were treated with DMSO or 10 nM E2 and luciferase activity determined as described in the Materials and Methods. Significant ($p<0.05$) induction is indicated by an asterisk. (C) Experimental time line. Twenty-one day old animals were injected with different compounds subcutaneously for three consecutive days and sacrificed 24 hr after the last injection. Tissues were separated and homogenized and luciferase activity was measured as outlined in the Materials and Methods

absence of transfected ERα is due to limiting levels of endogenous ERα in these cells and overexpression of the constructs [108, 492]. Maximal induced activities were observed with the constructs in pGL3. However, fold induction was higher using $(GC)_3$-TATA-Luc (PXP2) due to the low basal activity in the solvent (DMSO) treated group.

The linearized $(GC)_3$-TATA-Luc and $(GC)_3$-TK-Luc vectors were injected into oocytes and 8 lines were isolated and with the exception of line 17, all of the remaining lines were fertile (Table 3). Luciferase expression in various tissues/organs was determined in 24 day-old mice using the experimental protocol illustrated in Figure 27C. At 15 days of age, the mice were genotyped and then weaned at 21 days. Luciferase activity in both males and females from line 42 was not detected. Luciferase activity was only detected in male testis of line 35. The transgene is not inherited in line 41. For estrogen-responsiveness, the 21-day old female and male mice were administered corn oil [10 ml/kg body weight (b.w.)] or E2 (50 μg/kg b.w.) subcutaneously for three consecutive days and then sacrificed 24 hr after last the treatment (Fig. 27C). Table 3 summarizes results of the initial screening studies for basal and E2-inducible expression of luciferase activity in brain, thymus, heart, lung, liver, spleen, pancreas, stomach, bladder, tongue, eye, bone, uterus/ovary (females only) and testis/penis (male only). The results were highly variable not only among tissues/organs but also the various mouse lines. Figure 28A illustrates luciferase activity in line 15 female mice where the highest expression was observed in tongue and stomach and comparable responses were observed in male mice from this line (Fig. 26B). Brain and spleen luciferase activities were low in males and females. And uterine and ovarian luciferase activities were also low in females. In contrast, basal luciferase expression in line 15 penis and testis was high. Among the male mice in lines 11, 15, 16, 21, basal luciferase activity was detected and after treatment with E2 there was a decrease in activity and similar results were observed in the penis in lines 11, 15 and 21. Luciferase activity was also

observed in the brain and stomach in lines 11, 15, 16, 21 and treatment with E2 did not affect brain luciferase activity in any of the animal lines. In stomach E2 decreased luciferase activity in females (line 11, 15 and 16) or had no effects (line 21) and both increased (line 15 and 21) and decreased (line 11 and 16) luciferase activity in male mice. In female mice luciferase activity (basal and inducible) was not detected in the ovaries, whereas E2 induced activity in the uterus in lines 15, 16 and 21 but not in line 11. Based on the magnitude of the basal and E2-induced uterine luciferase activity in line 15, we further used this transgenic animal for quantitatively determining ligand-dependent activation of the $(GC)_3$-TATA-Luc construct *in vivo*.

Results in Figure 29A show that E2 (50 μg/kg b.w.) significantly induces mouse uterine luficerase activity by approximately 10-fold. The estrogenic activity of two synthetic estrogens, BPA (250 mg/kg b.w.) or NP (250 mg/kg b.w.) was also determined using the same protocol and these treatments resulted in a 7.2-fold and 38.4-fold increase in luciferase activity and this is consistent with their activation of GC-rich constructs in breast cancer cells [108, 492]. The “pure” antiestrogen ICI 182, 780 also induced mouse uterine luciferase activity and these results are consistent with the ERα agonist activity of ICI 182,780 observed in breast cancer cells transfected with the same GC-rich constructs [128, 129]. Not surprisingly, cotreatment of the animals with E2 plus ICI 182,780 also induced mouse uterine luciferase activity. We compared the activation of mouse uterine luciferase (Fig. 29A) with the well-characterized mouse uterine wet weight response in which treatment with E2 increased uterine wet weight (Fig. 29B). In addition, NP also induced a uterine wet weight increase at a dose of 250 mg/kg b.w. whereas the same dose of BPA is inactive and this is consistent with the relatively low potency of BPA for this response [602]. ICI 182,780 alone did not affect uterine wet weight but in combination with E2, the antiestrogen significantly inhibited hormone-induced uterine wet weight increase. Results obtained in the mouse uterine luciferase and uterine wet weight assays

Table 3. Luciferase expression in transgenic lines

Construct		$(GC)_3$-TATA-Luciferase										$(GC)_3$-TK-Luciferase					
Transgenic Line		Line#11		Line#15		Line#16		Line#17		Line#21		Line#35		Line#41		Line#42	
Gender		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Tissues	Brain	(+) →	(+) →	(+) →	(+) →	(+) →	(+) →	Founder female failed to successfully feed the pups		(+) →	(+) →	(-)	No protein expression in any tissue	Transgene can't be inherited to F2		No protein expression in any tissue	
	Thymus	(-)	(-)	(-)	(-)	(+) →	(+) →			(+) ↓	(+) →	(-)					
	Heart	(-)	(-)	(-)	(-)	(-)	(-)			(+) →	(+) →	(-)					
	Lung	(-)	(-)	(-)	(-)	(-)	(-)			(-)	(-)	(-)					
	Liver	(+) →	(+) →	(-)	(-)	(-)	(-)			(-)	(-)	(-)					
	Spleen	(-)	(-)	(+) →	(+) →	(-)	(-)			(-)	(-)	(-)					
	Kidney	(-)	(-)	(-)	(-)	(-)	(-)			(+) ↓	(+) ↑	(-)					
	Pancreas	(-)	(-)	(-)	(-)	(-)	(-)			(+) →	(+) ↑	(-)					
	Stomach	(+) ↓	(+) ↓	(+) ↑	(+) ↓	(+) ↓	(+) ↓			(+) ↑	(+) →	(-)					
	Bladder	(-)	(-)	(-)	(-)	(-)	(-)			(+) ↑	(+) ↑	(-)					
	Tongue	(-)	(-)	(+) →	(+) →	(+) →	(+) ↓			(+) ↑	(+) →	(-)					
	Eye	(-)	(-)	(-)	(-)	(-)	(-)			(+) ↑	(+) ↓	(-)					
	Bone	(-)	(-)	(-)	(-)	(-)	(-)			(-)	(-)	(-)					
	Uterus		(+) →		(+) ↑		(+) ↑				(+) ↑						
	Ovary		(-)		(-)		(-)				(-)						
	Testis	(+) ↓		(+) ↓		(+) ↓				(+) ↓		(+) ↓					
	Penis	(+) ↓		(+) ↓		(+)--				(+) ↓		(-)					

(+) detectable luciferase activity

(-) undetectable luciferase activity

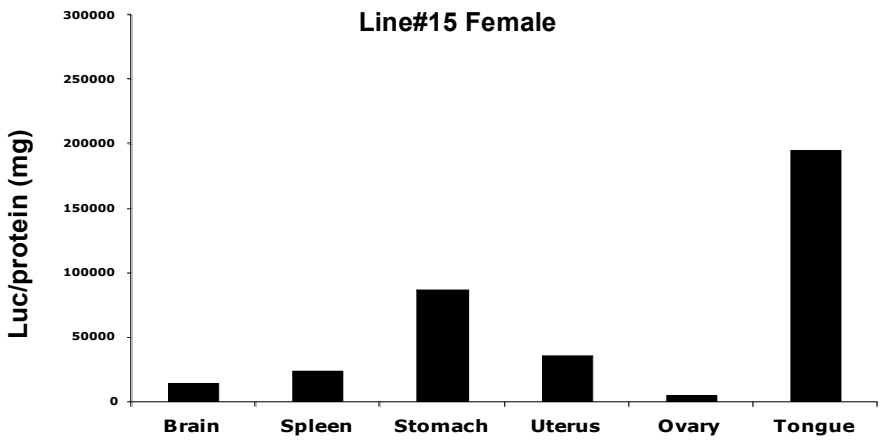
↓ downregulation by E2

↑ upreguation by E2

→ no change

? Not determined yet

A



B

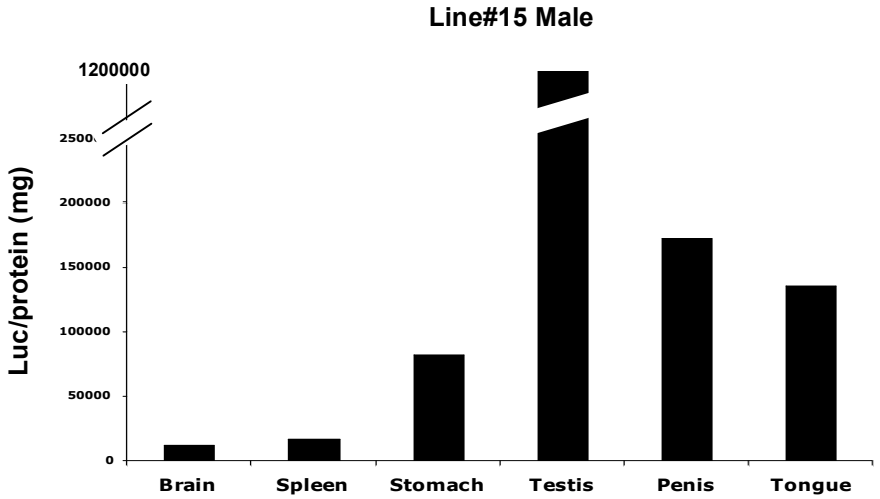
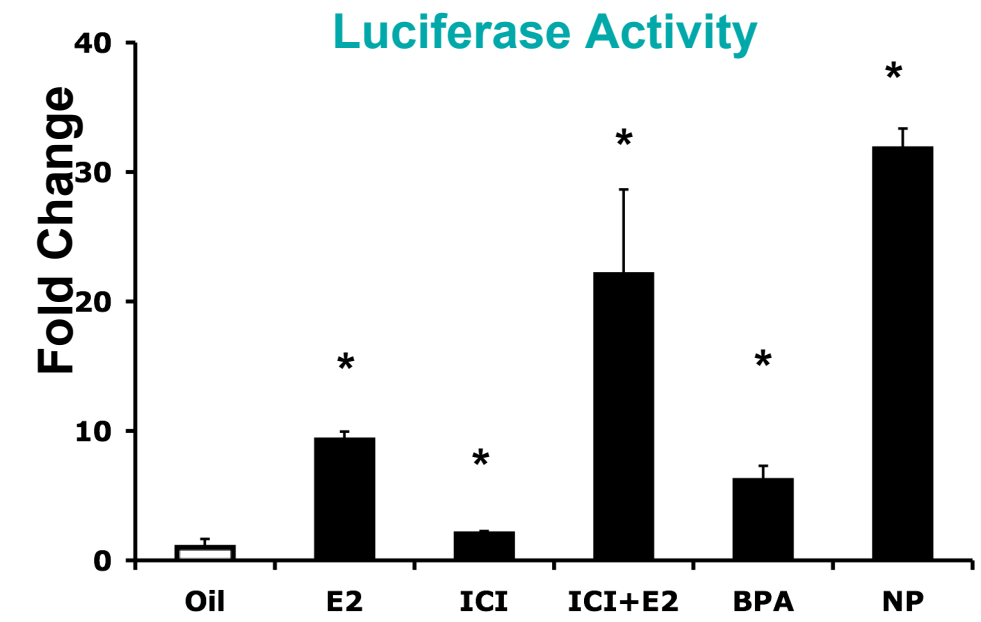


Figure 28. Basal luciferase activity is comparable in male and female mice. Twenty-one day-old animals were killed by CO_2 asphyxiation, and organs were immediately removed. Luciferase activity was determined as described in the Materials and Methods. At least 7 animals are included for male and female groups.

A



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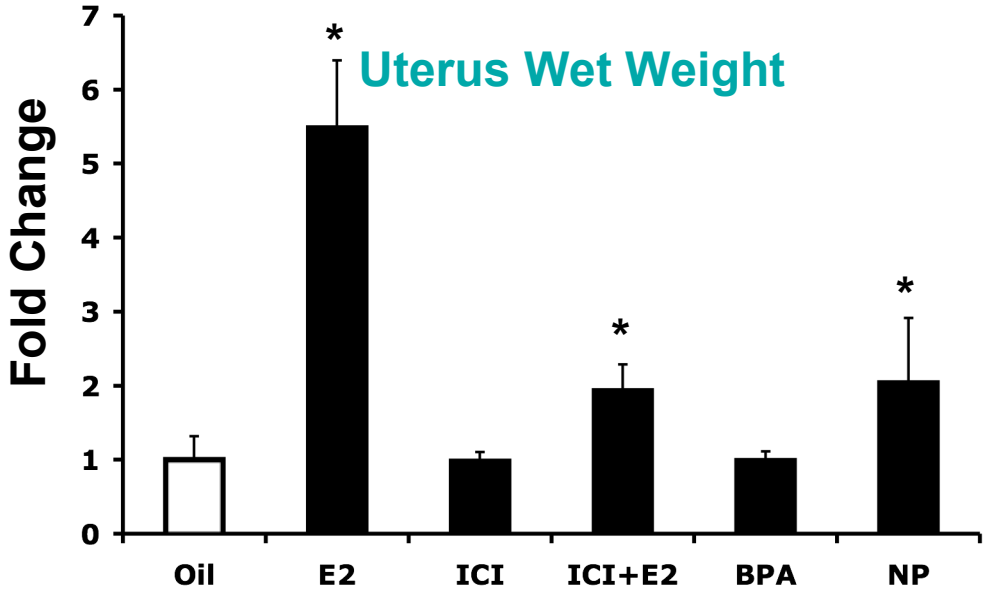

Figure 29. Uterotrophic responses in transgenic mice. Luciferase (A) and uterine wet weight responses (B) of twenty-one day-old female mice were treated by s.c. injection with corn oil, 50 ug/kg. b.w E2, 250 ug/kg b.w. ICI 182, 780 alone or in combination with E2, 250 mg/kg b.w. BPA or 250 mg/kg b.w. NP alone for three consecutive days. On the fourth day, animals were sacrificed and the uterus was obtained and weighed (B) then homogenized in the lysis buffer for determining luciferase activity. Luciferase activity (A) was determined as described in the Materials and Methods. At least 7 animals are included in each treatment group. Significant ($p<0.05$) induction is indicated by an asterisk and significant inhibition of E2-induced activity by ICI 182,780 is indicated (**).

were complementary except for the estrogenic effects of ICI 182,780 on activation of the GC-rich promoters in the mouse uterus.

We also examined activation of luciferase activity in two male-specific tissues, the penis and testis in line 15 (Fig. 30A and 30B). In the penis, treatment with E2 alone significantly decreased activity whereas ICI 182,780, BPA and NP did not induce activity. However, ICI 182,780 exhibited antiestrogenic activity in the mouse penis and inhibited E2-induced luciferase activity. We also examined ERα agonist/antagonist activities in the male testis (line 15) treated with the same compounds. The results showed that both E2 and ICI 182,780 alone and in combination decrease luciferase activity whereas the synthetic estrogens BPA and NP cause a >2.5-fold increase in activity. These results suggest that ER/Sp-mediated up- or down-regulation of luciferase activity was dependent on ligand structure. Sex-specific activation of luciferase activity was investigated in the male and female stomach of line 15 (Fig. 30C and Fig. 30D). In females, E2 decreased luciferase activity and ICI 182,780 exhibited antiestrogenic activity and blocked the effects of the hormone (Fig. 30C). ICI 182,780 alone was inactive whereas both BPA and NP induced luciferase activity. In contrast, E2, ICI 182,780 and their combination and NP induced activity in the male stomach of line 15 (Fig. 30B) and this resembled the responses observed in the female mouse uterus (Fig. 29A). BPA was inactive in the male stomach and this was in contrast to the induction response observed in females (Fig. 30A).

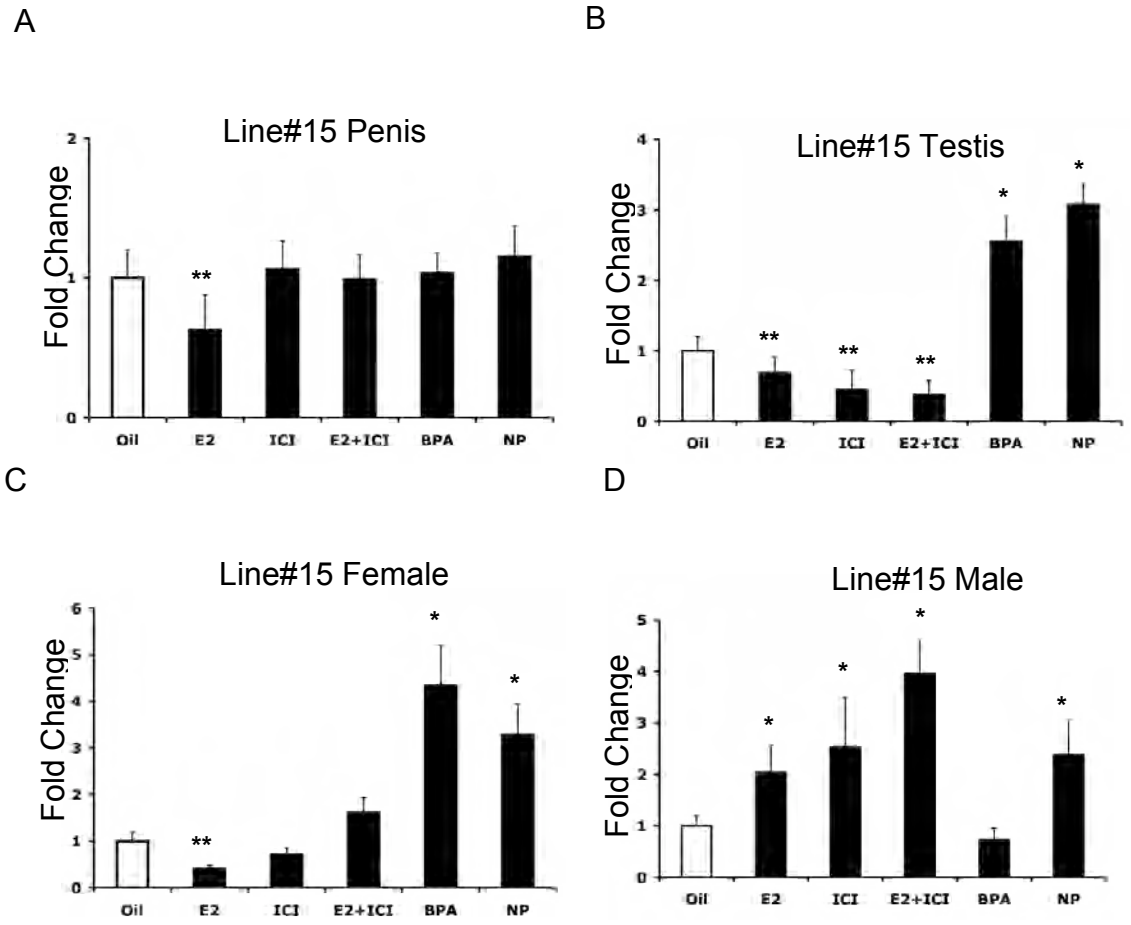

Figure 30. Effects of estrogenic compounds are tissue- and sex-specific. Effects of compounds on the male penis (A), testis (B), female stomach (C) and male stomach (D). Twenty-one day-old mice were treated by s.c. injection of corn oil, 50 ug/kg. b.w E2, 250 ug/kg b.w. ICI 182, 780 alone or in combination with E2, 250 mg/kg b.w. BPA or 250 mg/kg b.w. NP for three consecutive days. On the fourth day, animals were killed by CO2 asphyxiation, and organs were immediately removed. Luciferase activity was determined as described in the Materials and Methods. At least 7 animals are included in each treatment group. Significant ($p<0.05$) induction and inhibition are indicated by * and ** respectively.

These results demonstrate the estrogenic or antiestrogenic activity of various ligands that bind the ER in transgenic mice expressing a GC-rich promoter-luciferase construct. Activation or repression of activity was tissue/organ-, sex- and ligand structure-dependent suggesting that these compounds are selective ER modulators (SERMs).

4.5. Discussion

Estrogens induce responses in multiple tissues/organs and play a critical role in normal physiology and in hormone-dependent cancers [573-581]. The classical mechanism of estrogen action involves hormone-induced formation of ER the dimer which interacts with ERE motifs in target gene promoters and depending on the subset of nuclear proteins recruited to the promoter of the E2-responsive gene can be induced or repressed. The precise identity of ERE-dependent genes and their hormone-dependent regulation in various tissues is not well understood. However, computational and microarray approaches have identified multiple ERE sites, particularly in breast cancer cells and the rodent uterus [603-609].

An *in vivo* approach for investigating activation of ERE promoters has been reported using transgenic animals expressing ERE-luciferase constructs. While this method does not identify specific genes activated or repressed by ER-ERE interactions, luciferase activity is induced or repressed in E2-responsive tissue of ovariectomized or 21 day-old mice treated with E2 [521, 524, 526]. Tissue-specific basal and E2-dependent expression of luciferase activity in the transgenic animal models was highly variable between the different transgenic lines with the same transgene construct and variability was also observed using different transgene constructs [521, 524, 610]. In most of these studies E2 induced luciferase activity in various tissues. However, fold-inducibility was variable and dependent, in part, on basal activity in untreated control mice. For example, Ciana and coworkers observed a 5-fold or higher induction of luciferase activity in liver, lung, bone marrow, spleen, brain and thymus, a 2.5- to 4.9-fold induction in skin, bladder, uterus, eye, spinal cord and adipocytes [521]. In other organs, such as esophagus, aorta, thyroid, tail, pancreas, stomach, heart, skeletal, muscle and blood, E2 did not induce luficerase activity in mice expressing the ERE-luc transgene.

There is also evidence that E2 can act through other genomic and non-genomic pathways and research in this laboratory has focused on ER/Sp dependent activation of GC-rich promoter constructs. This pathway is important for E2-dependent activation of multiple genes in breast cancer and other cell lines associated with cell proliferation, survival, nucleotide metabolism, angiogenesis and receptors involved in nuclear and membrane associated signaling [12, 58, 104, 108, 111, 492, 589-599]. In addition, there is also evidence for ERα/Sp-dependent downregulation of VEGF in HEC1A1 endometrial cancer cells [130].

We have extensively used $(GC)_3$-TATA-Luc [128, 129] as a model for investigating ligand-dependent activation of wild-type and variant ERα/Sp-mediated transactivation and the properties of this artificial construct resemble those of a GC-rich promoter from E2-responsive genes [108, 492]. The major exception is that the antiestrogen ICI 182,780 activates $(GC)_3$-TATA-Luc [129] but acts as a typical antiestrogens in cells transfected with the E2-responsive GC-rich promoters [115, 121] and the reasons for these promoter-specific differences are unknown.

We used the consensus GC-rich constructs (Fig. 27A) to generate transgenic animals expressing luciferase activity in various mouse tissues. The expression patterns and luciferase activity observed in these animals differed from the corresponding ERE-Luc transgenic animals and this was due to differences in the constructs used. In this study, the transgene expresses a luciferase gene regulated by a $(GC)_3$-TATA or $(GC)_3$-TK promoter whereas in studies with mice expressing ERE-luc transgenes, the constructs also expressed TATA or TK promoters and insulator sequences that prevent inappropriate interactions between adjacent chromatin domains [522]. In a previous report which compared ERE constructs with or without insulators, luciferase activity was lower without the insulator but inducibility by E2 was comparable [526]. The GC-rich constructs did not contain an insulator. Table 3 illustrates differences in

basal and E2-dependent luciferase activity in the various transgenic mouse lines and we primarily focused on line 15 in which E2 significantly induced luciferase activity and the mouse uterus is a highly estrogen-responsive organ. E2 and the synthetic estrogen BPA and NP all induced uterine luciferase activity, and these same compounds induce ERα/Sp-dependent transactivation in breast cancer cells [128, 129]. Interestingly, ICI 182,780 also induces luciferase activity in the mouse uterus (Fig. 29A) as previously reported for the same transfected promoter-reporter construct in breast cancer cells [128, 129]. In contrast, using the uterine wet weight assay for estrogenicity, ICI 182,780 exhibits antiestrogenic activity. NP is also estrogenic in the uterus whereas BPA is inactive at a dose of 250 mg/kg b.w. and this is consistent with previous reports that show the relatively weak ER agonist and partial antagonist activity of this compound in the rodent uterotrophic assay [602].

We also investigated E2-responsiveness in other tissues and compared the effects of E2 with ICI 182,780 and the synthetic estrogens BPA and NP. Previous *in vitro* studies suggest that NP and BPA differentially activate wild-type and variant ERα in breast cancer cells transfected with ERE and GC-rich promoters suggesting that these compounds exhibit SERM-like activity which assumes tissue-specific ER agonist and antagonist activities. Differences in the hormonal activities of BPA/NP compared to E2 were investigated in the male penis and testis (Figs. 30A and 30B) and female and male stomach (Figs. 30C and 30D) and the results demonstrated sex- and tissue-specific differences in activation of the GC-rich promoter by various compounds. E2 decreased luciferase activity in the male penis and testis and female stomach but induced activity in the male stomach. This pattern of estrogenic activity was not observed in mice treatment with ICI 182,780, NP or BPA. For example, ICI 182,780 alone decreased luciferase activity in male penis and induced in male stomach, which acted as an E2 mimic in male testis and male stomach. But the cotreatment of ICI 182,780 with E2 antagonized estrogen's effect and acted as an antiestrogen

in male penis and female stomach (Fig. 30). NP and BPA exhibited a similar pattern of induced luciferase activity except for the male stomach where NP induced and BPA did not effect activity (Fig. 30C and 30D). BPA and NP did not affect luciferase activity in the penis. However, in two tissues where E2 significantly decreased activity (testis and female stomach), both NP and BPA induced activity. Tissue-specific differences in the estrogenic activity of o, p' and p, p'-DDT isomers and E2 have also been observed in the ERE-reporter mouse [611] confirming the SERM-like activity even among these structurally-similar isomers.

In summary our studies report for the first time that mice expressing a GC-rich luciferase construct in multiple tissues are responsive to E2 and other estrogenic/antiestrogenic compounds. The patterns of induced or repressed luciferase activity are tissue- and sex-specific and structure-dependent. These results are consistent with the SERM-like activity of estrogenic compounds which is dictated by the complex pharmacology of SERMs and is dependent on ligand structure, promoter and cell context [521, 524, 526, 558, 559, 610]. In contrast to previous studies with ERE-transgenic mice [611], we observed that in some tissues, E2 decreased luciferase activity suggesting that ERα/Sp-dependent regulation of E2-responsive genes may be an important pathway for hormone-repressed expression of genes and ongoing studies in breast cancer cells show that knockdown of Sp proteins abrogates hormone activation or repression of several genes. Current studies are focused on development and application of transgenic mice expressing other GC-rich promoters and on identifying critical E2-responsive genes regulated by ERα/Sp complexes.

CHAPTER V

CONCLUSIONS

ER can be activated by exogenous compounds, and there have been concerns regarding the potential adverse human health effects of dietary phytoestrogens and synthetic estrogenic environmental contaminants [108, 236, 436]. Activation of the ER is determined by interactions of three principal components: ligands, receptors and effectors, which together determine the magnitude and character of the transcriptional activity of ER in target tissues (Fig. 31). The conformation of the ligand bound ER complex is structure-dependent; this regulates interactions of this complex with an array of effector components to activate or inactivate gene transcription. The most critical effector components include the gene-regulatory DNA site to which the receptor binds (either directly or indirectly), as well as an array of coregulator proteins that determine the magnitude of the transcriptional response and its sensitivity to hormone regulation. The hormone-receptor complex recruits these coregulators, thereby physically linking the complex to the basal transcription machinery which in turns affects the local chromatin structure. Evaluation of the estrogenic effects of these compounds is complicated by the complex pharmacology of estrogens in which estrogenic compounds exhibit tissue/cell-specific ER agonist and antagonist activities due, in part, to tissue-specific, expression of critical coactivators and other coregulatory proteins [529, 530].

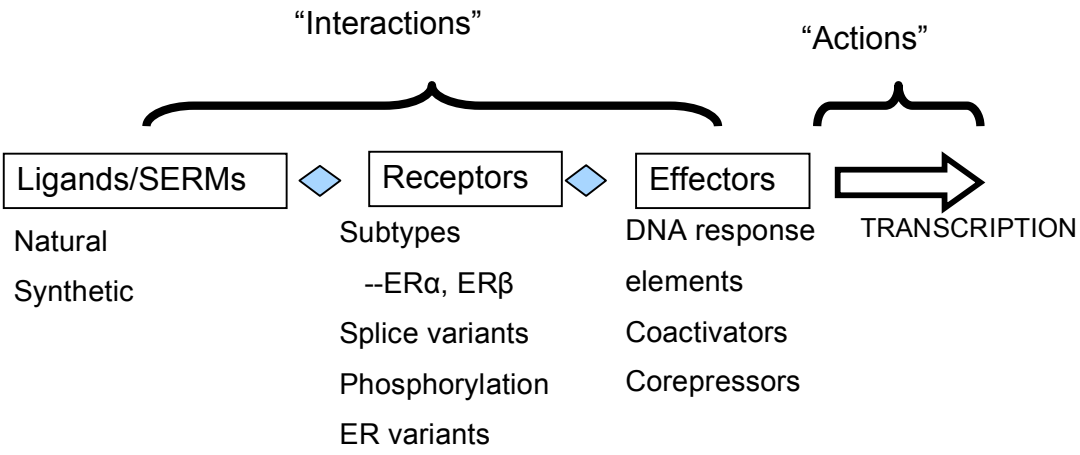

Figure 31. The complex pharmacology of ER.

This study investigated the differential activation of two ERα genomic pathways (ERα/ERE and ERα/Sp) using structurally diverse compounds as model ligands. Several *in vitro* assays have been developed to distinguish between different classes of SERMs that include antiestrogens, such as tamoxifen, ICI 182,780, phytoestrogens and xenoestrogens [12, 13, 32, 96, 97, 111, 112, 317, 410, 515]. Studies using E2-responsive constructs cotransfected with wild-type or variant forms of ERα, can distinguish between different structural classes of estrogenic chemicals. This is related to ligand structure-dependent effects on ER conformation and subsequent recruitment of coregulatory proteins (e.g. coactivators), which are necessary for ligand-induced transactivation.

In MCF-7 cells, the estrogenic activity of xenoestrogens required a different domain of ERα than either estrogens (E2/DES) or antiestrogens (ICI 182,780/4-OHT). Antiestrogen mediated inhibition of ERα/ERE did not require the C-terminal regions of ERα. However, E2 did not induce activity in MCF-7 cells transfected with the C-terminal deletion expression plasmid ERα(1-537), demonstrating the importance of the helix 12 region of ERα for mediating transactivation (Fig. 14). In contrast, the estrogenic activity of some

xenoestrogens is AF-2 independent. For example, kepone and the phenolic xenoestrogens (HO-PCB-Cl4, NP and OP) induced transactivation in MCF-7 cells transfected with a H12 deletion mutant of ERα (Fig. 14). Although both E2 and antiestrogens activate wild-type ERα/Sp-dependent transactivation in MCF-7 cells transfected with $pSp1_3$, antiestrogen-induced transactivation of $pSp1_3$ required the DBD of ERα, whereas acitivities of estrogen required H12 of ERα (Fig. 19). Even though the F domain is not required for estrogen-induced ER/Sp-dependent transactivation, it plays an important role as F domain deletion significantly abrogated estrogen inducibility (Fig. 19D). Xenoestrogens exhibited differential activities and have unique domain requirements for inducing transactivation of ERα. For example, kepone and HO-PCB-Cl4 are all active in MCF-7 cells transfected with ERα deletion mutants (ERαΔZF1, ERαΔZF2, ERα(1-537), ERα(1-553), indicating that these compounds induce a unique conformational change of ERα. Activation of ERα is ligand structure dependent.

The estrogenic activity of estrogens, antiestrogens, xenoestrogens and resveratrol is also cell context-dependent. In ER-negative MDA-MB-231 cells, with the exception of ICI 182,780, all the compounds induced a >3-fold increase in $pERE_3$ luciferase activity. In cells transfected with ERα(1-537), estrogens (E2 and DES) and the alkylphenols (NP and OP) did not induce transactivation, whereas the antiestrogen 4-OHT, the remaining xenoestrogens and resveratrol induced luciferase activity. However, in MCF-7 cells, deletion of the H12 resulted in the loss of estrogenic activity by both bisphenolic compounds (HPTE and BPA). The pattern of activation of wild type and variant ERα/Sp by xenoestrogens was similar in both MCF-7 and MDA-MB-231 cells (Figs. 19 and 20) and no cell context-dependent activities were observed.

The estrogenic activity of ligands is promoter context-dependent. The low activity of kepone to induce E2F1 protein expression (Fig. 24B and 25A) contrasts to its potent activity in transactivation studies in cells transfected with

$pSp1_3$ (Fig. 18), whereas induction of E2F1 by resveratrol (Fig. 25A) contrasts to the lack of induction by resveratrol in MCF-7 cells transfected with $pSp1_3$ and wild type ERα (Fig. 19A). Table 4 summarizes all the data for differential activity of estrogens, antiestrogens and xenoestrogens. Their activity is dependent on ERα variants, promoter context, cell context.

Ligand-dependent activation of ERα/Sp in MCF-7 cells is also determined by utilization of individual Sp proteins. E2 primarily activates ERα/Sp1 and also activates ERα/Sp4 (< ERα/Sp1) but not ERα/Sp3, which means that Sp1 played a major role and Sp4 also contributed to this response whereas Sp3 did not exert any effects. E2-dependent activation of ERα/Sp followed the order ERα/Sp1> ERα/Sp4> ERα/Sp3. The similar results were observed with several other ligands, including DES and HO-PCB-Cl4. In contrast, BPA, HPTE, endosulfan and NP activate ERα/Sp1 and ERα/Sp4 at comparable level but not ERα/Sp3 (ERα/Sp1≈ERα/Sp4>ERα/Sp3) and resembles 4-OHT in its differential activation of ERα/Sp. The effects of iSps on activation of $pSp1_3$ by ICI 182,780 and kepone were different from those observed for the other ERα agonists and antagonists. ICI 182,780 activates ERα/Sp1 and ERα/Sp3 since iSp3, iSp1 and iSp1 plus iSp3 significantly decreased transactivation (Fig. 23B). Similar results were observed for kepone, although neither iSp3 nor iSp4 were as effective as ICI 182,780 in decreasing fold inducibility by kepone. The results demonstrate that activation of ERα/Sp-dependent transactivation by estrogens, antiestrogens and xenoestrogens are different with respect to the role of Sp1, Sp3 and Sp4 in mediating the response.

Table 4. Differential activity of estrogens, antiestrogens and xenoestrogens are dependent on ERα variants, promoter context and cell context.

		E2	DES	ICI	4-OHT	BPA	HPTE	Res	OP	NP	Kepone	Endo	PCB
ER variants at $pERE_3$	wt ERα	"++"	"++"	"-"	"-"	"+"	"++"	"+"	"+"	"+"	"++"	"+"	"++"
	ERαΔ(1~537)	"-"	"-"	"-"	"-"	"0"	"0"	"+"	"+"	"+"	"++"	"0"	"+"
	ERαΔ(1~553)	"++"	"++"	"-"	"-"	"0"	"0"	"+"	"+"	"+"	"++"	"0"	"++"
ER variants at $pSp1_3$	wt ERα	"++"	"++"	"++"	"+"	"++"	"++"	"0"	"+"	"+"	"++"	"++"	"++"
	ERαΔZF1	"++"	"++"	"0"	"0"	"+"	"++"	"++"	"+"	"+"	"++"	"++"	"++"
	ERαΔZF2	"++"	"++"	"0"	"0"	"+"	"++"	"++"	"+"	"+"	"++"	"++"	"++"
	ERαΔ (1~537)	"0"	"0"	"+"	"+"	"+"	"++"	"+"	"+"	"+"	"++"	"+"	"++"
	ERαΔ(1~553)	"+"	"+"	"+"	"+"	"+"	"++"	"+"	"+"	"+"	"++"	"+"	"++"
Promoter in MCF-7	$p(ERE)_3$	"++"	"++"	"-"	"-"	"+"	"++"	"+"	"+"	"+"	"++"	"+"	"++"
	$pSp1_3$	"++"	"++"	"++"	"+"	"++"	"++"	"0"	"+"	"+"	"++"	"++"	"++"
	pCad	"++"	"++"	"0"	"0"	"0"	"0"	"++"	"++"	"0"	"++"	"0"	"0"
	pE2F1	"++"	"++"	"0"	"+"	"+"	"0"	"+"	"++"	"0"	"0"	"0"	"0"
Cell line at $pERE_3$	MCF-7	"++"	"++"	"-"	"-"	"+"	"++"	"+"	"+"	"+"	"++"	"+"	"++"
	MDA-MB-231	"++"	"++"	"0"	"++"	"++"	"++"	"++"	"++"	"++"	"++"	"+"	"++"
Cell line at $pSp1_3$	MCF-7	"++"	"++"	"++"	"+"	"++"	"++"	"0"	"+"	"+"	"++"	"++"	"++"
	MDA-MB-231	"++"	"++"	"+"	"++"	"++"	"++"	"+"	"+"	"+"	"++"	"+"	"++"

"++" High induction
"+" Low induction
"0" Inactive
"-" Inhibition

The *in vivo* activity of estrogens, antiestrogens and xenoestrogens varies in different tissues and sexes. A new transgenic mouse model was generated with insertion of a transgene containing three tandem GC-rich Sp binding sites with a minimal TATA box linked to a luciferase gene (Fig. 27A). E2 (50 μg/kg b.w.), ICI 182,780 (250 μg/kg b.w.) and xenoestrogens [BPA and NP (250 mg/kg b.w.)] all induce luciferase activity in the mouse uterus. However, in the penis, treatment with E2 alone significantly decreased luciferase activity whereas ICI 182,780, BPA and NP did not induce activity. However, ICI 182,780 exhibited antiestrogenic activity in the mouse penis and inhibited E2-induced luciferase activity. These data demonstrate that the estrogenic activity of various ligands that bind the ER in transgenic mice expressing a GC-rich promoter-luciferase construct was tissue/organ-, sex- and ligand structure-dependent.

The study demonstrates the complexity of xenoestrogen-induced transactivation, which is dependent on ligand structure, different domains of ERα, promoter, cell context and specific use of different Sp proteins. Their differential estrogenic activity suggests that these compounds are selective ER modulators (SERMs).

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VITA

Fei Wu
Texas A&M University, MS 4466
College Station, TX, 77843-4466
Phone: 979-204-5208
Email: wufei@tamu.edu

Education

Texas A&M University, College Station, TX 12/07

Ph. D. in Biochemistry

Fudan University, Shanghai, China 07/01

B.S. in Biological Science

Research Experience

Research Assistant: 05/02~12/07

Department of Veterinary Physiology & Pharmacology, Texas A&M University

Research Assistant: 10/97~07/01

Department of Genetics, Institute of Life Science, Fudan University

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