HORMONAL ACTIVATION OF GENES THROUGH NONGENOMIC PATHWAYS BY ESTROGEN AND STRUCTURALLY DIVERSE ESTROGENIC COMPOUNDS

A Dissertation

by

XIANGRONG LI

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

May 2005

Major Subject: Toxicology

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Approved as to style and content by:

Stephen H. Safe (Chair of Committee)	Weston W. Porter (Member)
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ABSTRACT

Hormonal Activation of Genes through Nongenomic Pathways by Estrogen and Structurally Diverse Estrogenic Compounds. (May 2005)
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Chair of Advisory Committee: Dr. Stephen H. Safe

Lactate dehydrogenase A (LDHA) is hormonally regulated in rodents, and increased expression of LDHA is observed during mammary gland tumorigenesis. The mechanisms of hormonal regulation of LDHA were investigated in breast cancer cells using a series of deletion and mutant reporter constructs derived from the rat LDHA gene promoter. Results of transient transfection studies showed that the -92 to -37 region of the LDHA promoter was important for basal and estrogen-induced transactivation, and mutation of the consensus CRE motif (-48/-41) within this region resulted in significant loss of basal activity and hormone-responsiveness. Gel mobility shift assays using nuclear extracts from MCF-7 cells indicated that CREB family proteins interacted with the CRE. Studies with kinase inhibitors showed that estrogen-induced activation of this CRE was dependent on protein kinase C, and these data show that LDHA is induced through a nongenomic (extranuclear) pathway of estrogen action.

Estrogen activates several nongenomic pathways in MCF-7 cells, and this study investigated the effects of structurally diverse estrogenic compounds on activation of mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), protein kinase A (PKA), and calcium/calmodulin-dependent protein kinase IV (CaMKIV). Activation of kinases was determined by specific substrate phosphorylation and transactivation assays that were diagnostic for individual kinases. The compounds investigated in this study include E2, diethylstilbestrol (DES), the phytoestrogen resveratrol, and the following synthetic xenoestrogens: bisphenol-A (BPA), nonylphenol, octylphenol, endosulfan, kepone, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), and 2',3',4',5'-tetrachloro-4-biphenylol (HO-PCB-Cl_4). With the

exception of resveratrol, all the compounds activated PI3K and MAPK whereas activation of PKC by the xenoestrogens was structure-dependent and resveratrol, kepone and HO-PCB-Cl_4 were inactive. Only minimal estrogen/xenoestrogen-dependent activation of PKA was observed. CaMKIV was activated only by E2 and DES, and HO-PCB-Cl_4 was a potent inhibitor of CaMKIV-dependent activity. These results demonstrate that activation of nongenomic pathways by estrogenic compounds in MCF-7 cells is structure-dependent.

DEDICATION

To my parents, Mingzhi Li and Jiayao Yu
and my sister Ruohong Li

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INTRODUCTION

1.1 BREAST CANCER

1.1.1 Incidence and mortality of breast cancer

Cancer is one of the leading causes of premature death in the United States, second only to cardiovascular diseases. The overall cancer death rates have been dropping at a rate of 1.1% per year from 1993-2001, but the overall cancer incidence in the United States remains stabilized from 1995-2001 (Jemal et al., 2004). For women in the United States, one of the most frequent sties for developing cancer is the breast. It is estimated that women in the United States have a one-in-eight chance to develop breast cancer in their lifetime (Feuer et al., 1993; Wingo et al., 1995). The incidence of female breast cancer increased 3.7% annually from 1980-1987 and 0.4% annually from 1987-2001(Jemal et al., 2004).

In the United States, breast cancer accounts for 17.1% of total cancer deaths in females in 1990s (Wingo et al., 2003). Fortunately, the mortality rate of breast cancer has been declining (Ghafoor et al., 2003). This decreasing trend may reflect the improvements on the diagnosis and therapy for breast cancer treatment (Peto et al., 2000).

Female breast cancer incidence and mortality rates differ greatly across racial and ethnic groups. In the United States, from 1996-2000, the breast cancer incidence is 140.8 in every 100,000 among white women, 127.1 among black women, 97.1 among Asian/Pacific islanders, 89.8 among Hispanics and 58.0 among Native Americans. In the same period, the mortality rate was 35.9 per 100, 000 women among black women, 27.2 among white women, 17.9 among Hispanics, 14.9 among Native Americans and 12.5 among Asian/Pacific Islanders (Weir et al., 2003).

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Worldwide, about 1 million new cases are diagnosed each year and 0.6 million deaths annually result from this disease. Incidences are highest in Western nations, such as the United Kingdom and the United States (Mettlin, 1999).

1.1.2 Classification of breast cancer

1.1.2.1 Structure of the mammary gland

The mammary gland is a highly specialized eccrine gland unique in mammals. Its milk-producing tissues are organized into 15-20 glandular units called lobes, each of which is connected to the nipple through ductal structure. Unlike most organs, which reach maturity before adulthood, mammary glands achieve final development and differentiation only after the first full term pregnancy (Donegan and Spratt, 2002). Before puberty, mammary glands largely consist of ductal structures. During puberty, mammary glands in females drastically increase the lobe tissue and elongate the ductal system. This is the period when mammary glands are most susceptible to carcinogens. The growing ducts end in bulbous structures termed terminal end buds (TEBs), which further divide in to smaller structures termed alveolar buds. In non-pregnant adult women, the breast tissue contains three types of lobules. Lobule type 1(Lob1), or terminal ductal lobular unit (TDLU), composes of terminal duct and several ductules (alveolar buds) that sprout from it. Additional sprouting of ductules will transform Lob1 to Lob2 or even Lob3. In nulliparous women, mammary glands consist of mainly Lob1 structures, with only a small proportion of Lob2 and minimal proportion of Lob3. In parous women, during pregnancy, mammary glands undergo massive ductal lengthening and branching, forming large ductules. This process results in the progression of Lob1 towards Lob2 and Lob3. By middle pregnancy, the ductules progress to secretory acini and Lob3 develops into well-differentiated Lob4. The secretory activities of acini continue to grow in the second half of pregnancy. During lactation, no further major morphological changes in mammary glands can be observed, but the process of growth and differentiation can still be detected. After lactation, mammary glands undergo a series of involutional changes. In this process, the secretory acinar structures collapse and Lob4 regresses to Lob3. After menopause, in both nulliparous and parous women, the lobular structures degenerate and eventually, mammary glands enter Lob1-dominated status (Russo and Russo, 2004).

Ductal carcinoma, the most frequent form of breast malignancy, occurs at terminal ductal lobular unit (TDLU) or Lob 1. The Lob2 structure has been associated with the development of lobular carcinoma *in situ* (LCIS), a benign breast lesion (Russo et al., 1992; Donegan and Spratt, 2002). Overall, Lob1 and Lob2 have greater susceptibility to carcinogenesis than more differentiated Lob3 (Russo et al., 1988). These findings help explain the protective effect of pregnancy against breast cancer because parous women have predominantly Lob3 structures in breast tissues (Russo and Russo, 2004).

1.1.2.2 Clinical classification of breast cancer

Breast cancers can be divided into the following groups: Noninvasive carcinoma *(in situ* carcinoma), microinvasive carcinoma, invasive (nonfiltrating) carcinoma and Paget's disease.

Noninvasive carcinomas are early benign lesions that can be further divided into two groups: ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). DCIS is carcinoma of ductal origin. It is confined locally to the ductal system of breast, with the basement membrane and the surrounding stroma intact. The standard treatment for DCIS is lumpectomy followed by radiation therapy. LCIS is a breast tissue lesion of lobular origin. It is considered a risk factor for breast cancer, rather than a precursor of invasive breast cancer *per se*. Women carrying LCIS have a lifetime risk of 25-30% for breast cancer. Because of its benign nature, LCIS usually only requires close monitoring. For high risk women with LCIS, prophylactic mastectomy and chemoprevention can be considered.

Invasive carcinomas can be of ductal or lobular origin. These are cancers that have penetrated the surrounding lymphatic and vascular channels. For treatment of invasive carcinomas, it is important to determine the status of metastasis. Microinvasive carcinoma is a relative new category of breast cancer introduced to describe the intermediary state between noninvasive and invasive carcinoma. Paget's disease is a special type of breast cancer which accounts for approximately 2% of total cases. It is characterized by the presence of large neoplastic cells in the epidermis of nipple-areola complex. Symptoms include erythema, rash, ulceration and discharge around nipples (Donegan and Spratt, 2002; Torosian, 2002).

1.1.2.3 TNM staging

AJCC (American Joint Committee on Cancer) adopted a cancer classification system termed TNM, which is now used internationally. In TNM system, a stage for tumor is assigned according to information about the tumor (T), the regional lymph nodes (N) and distant metastasis (M).

Table1

TNM Stage based on TNM components. Adapted from AJCC (2002).

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	Any N3	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

In T categories, Tis is assigned only to carcinomas in *situ* or noninvasive, T0 is assigned when no primary tumor can be detected. T1 stage tumor is 2 cm in diameter or smaller and a T2 tumor is 2 cm to 5 cm in diameter. A T3 tumor is more than 5 cm in diameter and a T4 tumor is of any size with direct extension to the chest wall and skin, or an inflammatory tumor. In N categories, N0 is assigned when no regional lymph node metastasis is detectable. N1 is assigned when metastasis occurs in 1 to 3 axillary lymph nodes. N2 is assigned when metastasis occurs in 4 to 9 axillary lymph nodes. N3 is assigned when the tumor spreads to 10 or more axillary lymph nodes. In M

categories, M0 is assigned if metastasis has not occurs in distant tissues, M1 is assigned if tumor has spread to distant tissues.

Once the TNM category has been determined, an overall stage number can be assigned to a particular case of breast cancer (Table 1).

The stage I and II are considered the early stage of invasive carcinoma, with a 5-year survival rate of more than 75% when properly treated. Survival rates rapidly decrease when the cancer reaches a later stage. Stage III cancer has a 5-year survival rate of approximate 50%, while the 5-year survival rate for Stage IV cancer is less than 20% (AJCC, 1997).

1.1.2.4 ER-positive and ER-negative cells

Base on the status of estrogen receptor (ER), breast cancers can be divided into ER-positive and ER-negative groups. Compared to ER-negative tumors, ER positive tumors generally are better-differentiated, respond better to antiestrogen treatment and have relatively lower recurrence rate and better prognosis for survival (Putti et al., 2004). ER is expressed in most normal terminal ductal lobular units, although the percentage of epithelial cells that expresses ER at a particular time is less than 10% (Petersen et al., 1987). The elevation of ER expression in normal epithelial cells sensitizes the cells to estrogen and increase the breast cancer risk (Khan et al., 1994). ER-positive tumors account for approximately 60-65% of total primary tumors (Putti et al., 2004). However, during the progression of cancer, some originally ER-positive breast tumors evolve into ER-negative tumors (Kuukasjarvi et al., 1996).

The loss of ER from ER-positive tumors is due to a diverse array of mechanisms. Some studies have found that breast cancer cells frequently have abnormalities on chromosome 6, in which the ERα gene is located. In some breast cancer cells, the ER coding sequence has been totally deleted (Devilee et al., 1991; Magdelenat et al., 1994). DNA methylation may also play a role since methylation of CpG sites on the ER promoter can silence ER expression and ER-negative breast cancer cell lines tend to have a higher percentage of promoter methylation (Ottaviano et al., 1994).

ER-negative cells usually express elevated activities of growth factor receptors. High levels of Erb-B2 and EGFR have been associated with ER-negative breast tumors (Allred et al., 1992; Schroeder et al., 1997; Elledge et al., 1998). Downstream targets of growth factor receptors, such as Ras/Raf/MAPK, are also elevated in ER-negative tumors. Overexpression of Raf can downregulate ER levels in ER-positive MCF-7 cells and transform them into ER-negative cells. The transformed cells lose estrogen-responsiveness and are no longer inhibited by antiestrogens (El-Ashry et al., 1997). Similar results were obtained by overexpression of Erb-B2 (growth factor receptor upstream of Raf) or MAPK (kinase downstream of Raf) in MCF-7 cells (Oh et al., 2001). Extracellular signal-regulated kinase (ERK7), a member of MAPK, enhances the degradation of ERα by targeting DNA-binding domain of ER α and increasing ubiquitination (Henrich et al., 2003).

Although ER-negative tumors may be derived from ER-positive tumors, some breast tumors are ER-negative from the inception of lesion, suggesting that ER-negative tumors may arise independently from ER-positive tumors. In most cases, primary ER-negative tumors remain ER-negative in recurrent tumors and it is rare that ER-negative tumors change to ER-positive status during the progression of cancer (Johnston et al., 1995; Kuukasjarvi et al., 1996).

1.1.3 Carcinogenesis

Cancer is the collective name for a family of diseases that differ greatly from one another but share a common symptom, i.e., uncontrolled or dysregulated cell proliferation. Cancer is a leading cause of death in industrialized countries and in 1995, cancer account for approximately 500,000 deaths in United States (Wingo et al., 1995) (Feuer et al., 1993). With the life expectancy of general population increasing, more people are expected to develop and die from cancer.

The development of a tumor (carcinogenesis) is a very complex multiple-stage process and often takes decades to occur. Although there are still many controversies regarding the origins and development of cancer, it is generally believed that carcinogenesis can be divided into three steps: initiation, promotion and progression (Fig. 1).

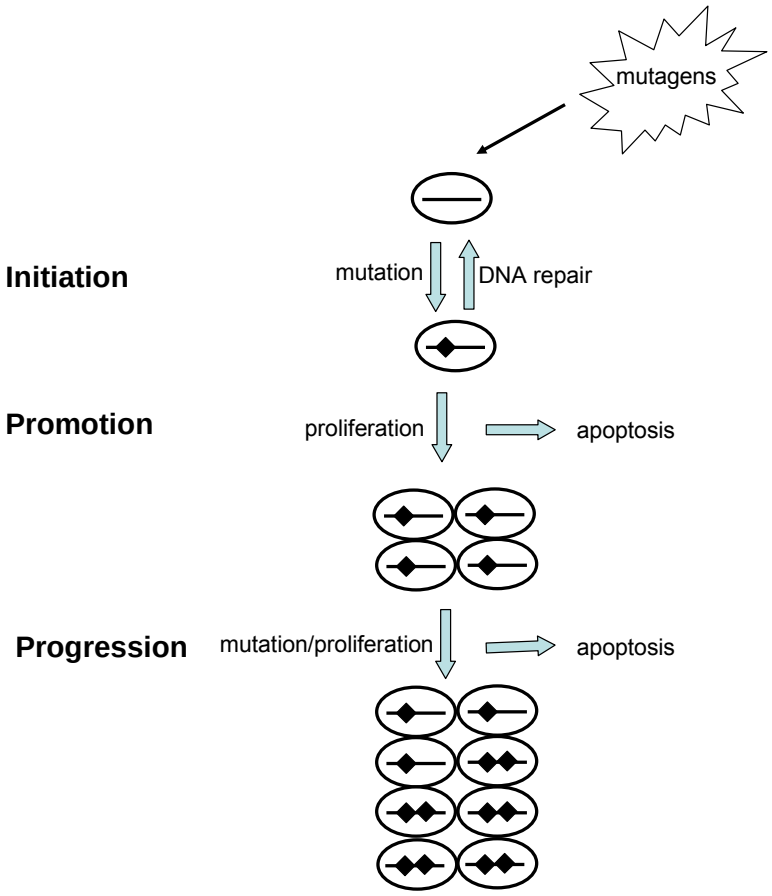

Fig.1. The steps of carcinogenesis

Initiation is the process by which cells gain abnormalities that are due primarily to DNA mutations. There is a strong correlation between carcinogenesis and mutagenesis, which lends support to the importance of mutations for cancer development (Ames et al., 1973). Virtually all cancer causing agents, such as chemical carcinogens, radiation and viruses, possess the ability to induce DNA mutations, either directly or after metabolic activation. The exact mechanisms for mutagenesis can be diverse. Ultraviolet A (UVA) irradiation produces pyrimidine dimers in epidermal cells (Applegate et al., 1999). Aflatoxin B1, after enzymatic activation, forms DNA adducts through the N7 position of guanine (Ross et al., 1992). Benzo[α]pyrene is metabolized into 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene (BPDE), which subsequently forms adducts with the amino group at the N2 position of guanine (Hsu et

al., 2004). In some cases, carcinogens induce different DNA lesions in target genes. For example, p53 tumor suppressor gene is frequently mutated in cancer cells and C to T and CC to TT mutations are usually found in UV-induced skin cancer (Brash, 1997); G to T mutations in p53 are linked with aflatoxin-induced liver cancer (Foster et al., 1983) and A to T mutations are associated with vinyl chloride-induced liver cancer (Hollstein et al., 1994). These preferences in mutations can serve as “signatures” to help elucidate the links between certain mutagens and etiology of specific cancers. Mutagens can also act synergistically. For instance, both Hepatitis B Virus (HBV) infection and aflatoxin are carcinogens in liver. Exposure to both HPV infection and aflatoxin increases the risk of liver cancer compared to exposure to the single agents (Kew, 2003).

Although in most cases, carcinogenesis requires mutations, there are some exceptions. For example, teratocarcinoma, a rare form of cancer, is of epigenetic origin and does not involve DNA mutations (Alvarez et al., 1999).

Most cancers appear to arise from only a single founding cell (Fialkow, 1976; Fearon et al., 1987). This monoclonal origin of cancer means that a single malignant cell has the ability to develop into a lethal tumor (Skipper and Perry, 1970). Usually the founding cell arises from the local tissue. Epithelia are the most common tumor formation sites and this may reflect the fact that epithelial cells bear the brunt of mutagenic insults. However, it is worth mentioning that primary cancer does not always originate *in situ*. For example, contrary to common belief, stomach cancers caused by chronic *Helicobacter pylori* arise from infiltrating bone-marrow derived cells instead of epithelial cells in the stomach lining (Houghton et al., 2004).

The initiation step of carcinogenesis is important but not sufficient for carcinogenesis. In multicellular organisms, cell proliferation is under strict control and some cells temporarily gain the ability of independent proliferation. However, unless this trait is inherited and transmitted to progeny cells, these cells will eventually revert to normal growth rate or be eliminated. The integrity of a cell is guarded by a multitude of cellular

defense pathways, which includes cell growth inhibition and DNA repair, or induction of cell death.

Multiple mutations are required for a single founding cell to develop into a full-blown cancer. This requirement reflects the multiple restraints against malignant growth. Hanahen and Weinberg (2000) proposed six new capacities necessary for malignancy: self sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Exactly how many mutations are required for transformation is still a matter of debate. Vogelstein and Kinzler (1993) estimated three to six mutations were needed for carcinogenesis. Hahn *et al* (1999) reported that ectopic expression of three genes, namely large-T antigen, oncogenic Ras and telomerase, can transform normal human epithelial and fibroblast cells into tumor cells.

Accumulation of additional mutations can be greatly accelerated by agents called promoters. The promotion stage of carcinogenesis does not directly involve changes in genetic information, but is linked to induction of genes that stimulate cell division and/or inhibit cell death. When tumor promoters are applied after initiation, they can greatly multiply the number of cells containing the initial mutation (Sisskin et al., 1982). With more mutant cells there is a higher probability for further mutations and independence, which enhances tumor formation.

Many promoters function by inducing cell proliferation. For example, one commonly used promoter is 12-O-tetradecanoylphorbol-13-acetate (TPA), a naturally occurring phorbol ester that has been extensively used to promote mouse skin tumors (Takigawa et al., 1982). The cancer-promoting ability of TPA is linked to the activation of protein kinase C (PKC) and subsequent enhanced cell proliferation (Housey et al., 1988). Some promoters act by inducing cytolethality followed by compensatory cell growth (Marks et al., 1995). One example of this type of promoter is chloroform. Chloroform increases the liver tumor rate in mice model by inducing centrilobular necrosis and subsequent regenerative proliferation (Larson et al., 1994). Unlike initiation which is

irreversible, promotion is largely reversible after withdrawal of the promoting factor (Miller and Miller, 1981)

After persistent tumor promotion, the large number of mutant cells will continue to replicate into numerous clones. Eventually, further mutations occur to induce cells into an irreversible progression stage (Fig. 1). One prominent trait of this stage is karyotypic instability, accompanied by alterations of genetic information on a large scale. Gradually, the cellular responses to environmental cues are altered or lost (Noble, 1977) and at this stage, cells are poised to develop full blown cancers.

Although progression is considered irreversible because of the large scale of genomic alterations, cells in any stage of carcinogenesis, under certain conditions, can terminally differentiate and stop development towards malignancy (Reiss et al., 1986). Several lines of evidence support the notion of “cancer stem cells”. This theory states that in some types of cancers only a very small proportion of cells are capable of infinite self-renewal. Thus, in those tumors, most cells will eventually stop proliferation. The whole tumor growth is driven by a very small group of mutated cells resembling normal stem cells (Marx, 2003).

Inherited genetic mutations can predispose carriers to certain cancers. For example, a single missense mutation (I1307K) of APC (adenomatous polyposis coli) gene among Ashkenazi Jews increases the risk of colon cancer almost by two-fold (Stern et al., 2001). Environmental factors are also important etiological factors for development of cancers. The respective contributions of genetic factors and environmental factors can be estimated by twin studies. Data from cohorts of 44,788 pairs of Scandinavian twins indicates there is an increased risk of developing cancer on the same site in twin siblings, especially for cancer of stomach, colorectum, lung, breast and prostate. However, the rates of concordance are generally less than 10%, which means that the increased risks are very moderate although twins share significant or identical genetic background. Thus, studies on twins suggest both genetic and environmental factors contribute to carcinogenesis, but environmental factors play a major role (Lichtenstein et al., 2000).

1.1.4 Breast cancer risk factors

As with other cancers, both genetic and nongenetic factors play a role in development of breast cancer. Breast cancer is the ultimate outcome of multiple genetic and environmental/lifestyle risk factors.

1.1.4.1 Age

The risk of breast cancer increases with age of the individual. The incidence is relatively low in young women but begins to rise sharply after age 45 (Hankey et al., 1994). This is consistent with the model of cancer development as a multiple-stage process. Women who develop breast cancer at a young age (<35) are more likely to carry genetic predisposing factors and the resulting cancers are often more aggressive (Winchester et al., 1996).

1.1.4.2 Life time estrogen exposure

Epidemiological studies indicate that cumulative exposure to unopposed estrogen over a lifetime is a major risk factor for breast cancer. Two well-recognized breast cancer risk factors include early menarche and late menopause, can be at least partly attributed to the increased lifetime estrogen exposure. Conversely, ovariectomy, a procedure greatly decreases endogenous estrogen levels, decreases breast cancer risk (Kelsey et al., 1993).

The extremely low incidence of male breast caner also highlights the role of estrogen in mammary carcinogenesis (Giordano et al., 2002). Only about 1500 new male breast cancer cases are diagnosed in the United States annually (Giordano et al., 2002). Risk factors for male breast cancer include elevated estrogen levels and BRCA2 mutations (Tischkowitz et al., 2002). Due to its lower incidence, little is known about male breast cancer, although some reports suggest that male breast cancer is similar to female breast cancer and should be treated using the same guidelines. However, other studies indicate that male breast cancer has distinct immunophenotypic differences from female breast cancer and different treatments should be explored (Muir et al., 2003).

Exogenous estrogen exposure also contributes to breast cancer risk. Hormone replacement therapy (HRT), an estrogen-progestogen regimen for relieving

postmenopausal symptoms, increases breast cancer risk (Chen et al., 2002). Recently, large clinic studies carried out by Women's Health Initiative (WHI) not only confirmed that the use of HRT is a breast cancer risk factor, but also found that HRT increases the risk of heart attack, stroke and blood clots. On the other hand, HRT offers some benefits, such as a modest decreased risk of colorectal cancer and hip fracture (Enserink, 2002). Another major source of exogenous estrogen is Combined Oral Contraceptive (COC). The use of COC has been associated with a weakly increased breast cancer risk. The excess risk is most significant for current or recent users and gradually declines once the use has been ceased. After 10-year COC-free period, the increased risk can no longer be detected (Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Ursin et al., 1998).

1.1.4.2.1 Estrogen promotes proliferation and suppresses apoptosis

Eukaryotic cell cycle can be divided into S, M, G1 and G2 phases, with S phase for DNA synthesis, M phase for mitosis, G1 and G2 phases for the two gaps between S phase and M phase. The non-proliferating cells remain in a quiescent, noncylcing stage termed G0 and out of cell cycle. Proliferative stimuli can drive G0 cells to enter G1 phase and resume cell proliferation. Conversely, growth inhibitory signals cause cells to leave cell cycle and enter G0 phase. One important checkpoint for cell cycle is the restriction point at late G1, after which a cell will commit itself to finishing the cycle (Sherr, 1996). Estrogen is mitogenic in a number of target tissues and regulates many components involved in cell cycle. Estrogen treatment stimulates G0 phase cells into the cell cycle and increases the rate of progression through G1 phase (Sutherland et al., 1983; Leung and Potter, 1987; Nandi et al., 1995).

Retinoblastoma protein (Rb) and E2F play central roles in cell cycle progression. E2F is a family of transcription factors that transactivate essential genes for G1/S transition, including proteins involved in DNA replication (DNA polymerase α, proliferating cell nuclear antigen), nucleotide biosynthesis (thymidylate synthase, ribonucleotide reductase), DNA repair (RAD51) and cyclin proteins (cyclin E, cdk2) (Nevins, 2001). In quiescent cells, Rb sequesters E2F and represses S phase entry. Under proliferative stimuli, Rb is phosphorylated and dissociated from E2F, relieving the inhibitory effect on E2F (Hiebert et al., 1992). In primary human fibroblast cells, Rb antisense

oligonucleotides knockdown Rb levels and increase cell proliferation (Strauss et al., 1992). Many oncogenic proteins, such as adenovirus E1A, SV40 tumor antigen and human papillomavirus E7, stimulate cell proliferation by sequestrating Rb and blocking its inhibition on E2F (Chellappan et al., 1992).

Cyclins, through periodic associations with their cognate cyclin-dependent kinases (Cdks), function as molecular clock to keep the pace of the cell cycle (Fig. 2). Phosphorylation of Rb can be achieved by cyclin D1/cdk4,6 and cyclin E/cdk2 complexes (Matsushime et al., 1992); (Sherr, 1994). Cyclin D1 is an important cyclin for G1 phase progression and essential for both mammary gland development and carcinogenesis (Sherr, 1994). Mammary glands require cyclin D1 for normal development of lobular-alveolar structure. This vital role is illustrated by the fact that mammary glands of cyclin D1 knockout mice are underdeveloped and hypotrophic (Sicinski et al., 1995). Overexpression of cyclin D1 has been associated with mammary carcinogenesis (Bartkova et al., 1995). Conversely, disruption of cyclin D1 in mice confers resistance to breast cancer-inducing effects of Erb-B2 and Ras, although other oncogenic pathways driven by c-Myc and Wnt-1 are still functional (Yu et al., 2001a). Cyclin E is another major factor governing G1/S transition (Sherr, 1994). Overexpression of cyclin E in breast cancer is associated with a higher proliferation rate and poorer prognosis (Nielsen et al., 1998). Dysregulation of cyclin E is also linked with chromosome instability in human breast epithelial cells (Spruck et al., 1999).

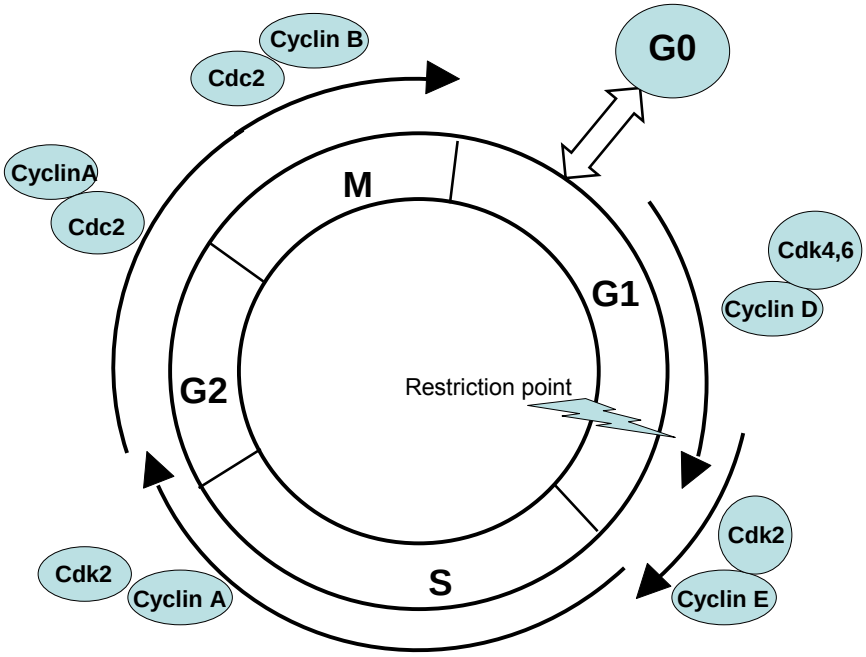

Fig. 2. The mammalian cell cycle.

Cyclin D1 forms complexes with Cdk4 and Cdk6, while cyclin E associates with Cdk2. These complexes phosphorylate Rb, causing the activation of E2F and entry into S phase. Both cyclin D1 and cyclin E are induced by estrogen. The expression of cyclin D1 is stimulated by E2 in breast cancer cells through transcriptional activation (Altucci et al., 1996; Castro-Rivera et al., 2001). The cyclin E is less E2-induible; however, estrogen increases the cyclin E/Cdk2 complex activity within 6 hours and the activation appears to be related to the redistribution of Cdk inhibitor p21 rather than elevated cyclin E level (Planas-Silva and Weinberg, 1997).

In breast cancer cells, estrogen upregulates the mitogenic gene c-Myc, which is a proto-oncogene and important regulator in cell cycle progression (Dubik et al., 1987). c-Myc gene is frequently amplified in primary breast tumor (Bieche et al., 1999) and overexpression of c-Myc in transgenic mice predisposes them to spontaneous mammary adenocarcinomas (Stewart et al., 1984). Conversely, c-Myc phosphorothioate antisense oligonucleotides abolish the E2-induced MCF-7 breast

cancer cell proliferation, suggesting that c-Myc is essential for the proliferative effect of E2 (Watson et al., 1991). Another important proto-oncogene induced by estrogen is c-fos (van der Burg et al., 1989; Duan et al., 1999). The expression of c-fos is associated with diminished endocrine responsiveness and poor survival rate for breast cancer patients (Gee et al., 1995).

Estrogen activates several kinase pathways important for cell proliferation. For example, estrogen-induced ERK mediates the G1/S transition in HepG2 cells by enhancing the cyclin D1 transcription (Marino et al., 2002). Furthermore, E2 induces the expression of bcl-2, a proto-oncogene that blocks apoptosis (Dong et al., 1999). Apoptosis is essential for normal mammary gland development and involution after pregnancy and lactation. Suppressing apoptosis can facilitate neoplastic development in breast tissues (Schedin et al., 1996).

1.1.4.2.2 Estrogen induces mutagenesis

Oxidative metabolism of certain estrogens by cytochrome P450 (CYP) can produce mutagenic metabolites and free radicals (Liehr, 1990). Two major endogenous estrogens, 17β-estradiol (E2) and estrone can be hydroxylated at multiple positions. The C4 and C16α-hydroxylated estrogens can be oxidized into electrophilic semiquinones and quinones to form DNA adducts (Osborne et al., 1993; Zhu et al., 1994; Liehr, 2000). In addition, the redox cycling between semiquinones and quinones generates superoxide and hydroxyl radicals, both of which increase oxidative stress and DNA damage (Liehr, 1990). Estrogens and their metabolites also induce other genetic injuries, including microsatellite instability, chromosome aberration and aneuploidy (Hodgson et al., 1998; Tsutsui et al., 2000).

1.1.4.2.3 Estrogen facilitates tumor invasion

Cadherins are a group of adhesion molecules that are essential for the tight junctions between cells. Cadherins associate with cytoplasmic protein catenins and other cytoskeletal components to form adhesion systems among cells. An intact adhesion system suppresses cancer cell invasion and prevents metastasis. One subclass of cadherin, E-cadherin, has been associated with metastasis suppression. Low levels of

E-cadherin expression are linked with invasiveness of mammary carcinomas (Oka et al., 1993; Siitonen et al., 1996). Estrogen downregulates E-cadherin levels in both immortalized breast epithelial cells and breast cancer cells. The inhibition can be abolished by antiestrogens and is mediated through recruiting ER and corepressors to the E-cadherin promoter (Oesterreich et al., 2003). Furthermore, estrogen induces the production of proteolytic enzymes Cathepsin D that destroys extracellular matrix (Augereau et al., 1994; Wang et al., 1997). There is a significant correlation between high cathepsin D levels in primary breast cancer and metastasis (Rochefort, 1990). Estrogen also induces vascular endothelial cell growth factor (VEGF), which facilitates angiogenesis, a process essential for solid tumor growth and metastasis (Stoner et al., 2004).

1.1.4.2.4 Estrogen induces proliferation through paracrine pathways

In beast cancer cells estrogen induces the production of growth factors such as TGFs and IGFs (Manni et al., 1991; Kenney et al., 1993). Secreted growth factors can stimulate the growth of neighboring cells. Medium conditioned by estrogen-treated ER-positive breast cancer cells induce the proliferation of ER-negative cells, suggesting estrogen can induce cell proliferation indirectly through a paracrine pathway (Clarke et al., 1992). Tissue recombination studies confirm the paracrine actions in a rodent model. In one study, breast epithelial cells from ER-knockout mice were combined with breast stromal cells from wildtype mice to obtain hybrid tissues. The hybrid tissues were cultured in athymic mice under estrogen treatment. Estrogen stimulates epithelial cell proliferation in hybrid tissues with wildtype stromal cells, regardless of the ER status of epithelium. Conversely, the epithelial tissues from wildtype or ER knockout mice combined with ER-knockout stromal cells were unresponsive to estrogen treatment (Cunha et al., 1997). Similar observations were also made in the mouse uterus (Cooke et al., 1997).

1.1.4.3 Lifestyle and environmental factors

Reproductive history plays a role in modifying breast cancer risk. Late first complete pregnancy or nulliparity is linked with elevated risk (Trichopoulos et al., 1983). Pregnancy before the age of 30 reduces the risk and the protective effect persists into old age. However, first pregnancy after the age of 30 is linked to increased risk (Rosner

et al., 1994). Lactation decreases the risk of breast cancer in premenopausal women and this protective effect increases with the duration of lactation (McTiernan and Thomas, 1986; Newcomb et al., 1994). It is suggested that the protective effects of pregnancy and lactation may be due to the terminal differentiation of mammary gland (Russo et al., 1982).

Great differences in breast cancer risks exist across different geographic regions. The risks of breast cancer in Western countries are significantly higher than those in Asia. Genetic background can not account for these differences because women who have migrated from low risk to high risk areas soon acquired the breast cancer risk of high risk areas (Stanford et al., 1995). However, it has been difficult to pinpoint the environment and lifestyle factors that are responsible for this increase in breast cancer risk.

High doses of radiation, especially received during an early age, increase breast cancer risk, as demonstrated by the increased breast cancer risk among atomic bomb survivors of Hiroshima and Nagasaki (Tokunaga et al., 1979). Radiation-associated risk is confirmed by other epidemiological studies among women treated with ionizing radiation for acute postpartum mastitis or non-neoplastic conditions in breast tissues (Baral et al., 1977; Shore et al., 1977). However, modest amounts of radiation, as received in routine diagnostic imaging, appears to pose no increased breast cancer risk (Boice et al., 1995)

Drinking alcohol is generally associated with increased risk of breast cancer (Willett et al., 1987; Longnecker et al., 1995; Feigelson et al., 2001; Singletary and Gapstur, 2001). The underlying mechanism is postulated to be that alcohol increases endogenous estrogen level by enhancing aromatase, an important enzyme for estrogen production (Purohit, 2000). Other theories suggest that induced expression of insulin-like growth factor 1(IGF1) (Yu and Berkel, 1999) or IGF1 receptor (IGF1R)(Stoll, 1999) by alcohol is the cause of increased breast cancer risk. The link between smoking and breast cancer is more controversial. Some studies reported a positive linkage (Johnson et al., 2000; Kropp and Chang-Claude, 2002) while others found protection or no effects (Nordlund et al., 1997; Lash and Aschengrau, 2002). These conflicting results may be confounded

by the fact that smokers also tend to drink. Another issue that further complicates the issue is that smoke contains PAHs (polynuclear aromatic hydrocarbon), which can be antiestrogenic through the AhR (aryl hydrocarbon receptor)-dependent pathway (Safe et al., 1991).

Animal studies show that high fat/high calorie diet predisposes laboratory animals to mammary tumors (Henderson, 1993). However, studies on human subjects are mixed. One meta-analysis study shows a positive association between breast cancer risk and saturated fat intake (Howe et al., 1990). In contrast, The Nurses' Health Study found no association between fat intake and breast cancer incidence, regardless of the types of fat (Holmes et al., 1999). Limited data favor a protective role of physical activity against breast cancer (Thune et al., 1997). Strenuous physical activity delays menarche, which in turn increases the length of menstrual cycle, decreases the frequency of ovulatory cycles and reduces circulating estrogen levels (Bernstein et al., 1987).

1.1.4.4 Xenoestrogen hypothesis

The estrogen receptor (ER) is promiscuous and binds a broad spectrum of structurally diverse compounds that induce estrogenic responses. Many of these compounds can be found in the environment and diet. Thus, there is a possibility that some of these compounds can potentially perturb the hormonal homeostasis and lead to hormone-related cancer, developmental and reproductive problems (Sonnenschein and Soto, 1998). These compounds are named 'xenoestrogen" and can be divided into two categories: industrial and pharmaceutical estrogens, which are mainly man-made chemicals, and phytoestrogens, which are plant products. Industrial and pharmaceutical estrogens include polychlorinated biphenyls (PCBs), bisphenol-A (BPA), octylphenol (OP), diethylstilbestrol (DES), 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE, the stable breakdown product of DDT). Examples of phytoestrogens are daidzein, genistein and resveratrol (Fig. 3). Recently, it was reported that some heavy metals may also possess estrogenic activity. Cadmium was proposed as an ER activator by interacting with the ligand-binding domain of ER (Stoica et al., 2000; Johnson et al., 2003). Thus the name "metalloestrogen" was given to describe the putative estrogenic heavy metals. This finding needs further confirmatory studies.

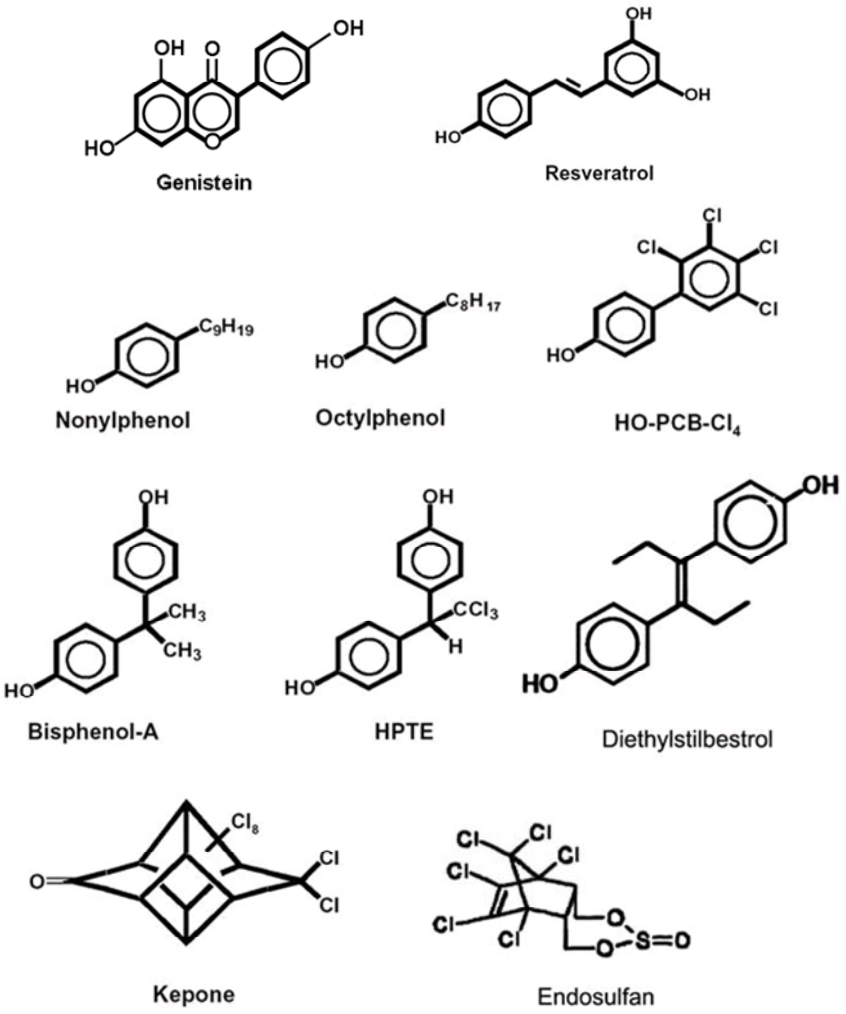

Fig. 3. Structures of some xenoestrogens.

The adverse effects of xenoestrogens have been demonstrated in animals. An accidental DDT spill has been associated with reproductive defects and reduced birthrates in alligators of Lake Apopka in Florida (Guillette et al., 1994). Bisphenol-A (BPA) and octylphenol (OP) cause feminization in male snails under laboratory conditions (Oehlmann et al., 2000). Treatment of immature rats with kepone increases their uterine weight and stimulates the ER-dependent production of progesterone receptor (PR) (Hammond et al., 1979). However, whether these findings have relevance on human health is still a hotly debated issue. In some studies, PCBs and DDE has been linked with elevated breast cancer risk (Falck et al., 1992; Wolff et al., 1993). In

other studies, no links were found between PCBs or DDE and breast cancer (Krieger et al., 1994; Hunter et al., 1997; Hunter et al., 1997; Lopez-Cervantes et al., 2004). Overall, available evidence does not favor a putative link between PCBs/DDE and breast cancer (Safe, 2004).

One pharmaceutical estrogen, DES, has been proven to slightly increase risk of breast cancer (Colton et al., 1993). However, DES is the exception rather than the rule since most industrial estrogens are several orders of magnitude weaker than DES in terms of estrogenicity. Industrial estrogens constitute only a negligible estrogen intake and the major dietary estrogen source comes from phytoestrogens (Safe, 1995).

Phytoestrogens include three main categories; namely isoflavones, coumestans and lignans (Peeters et al., 2003). Genistein (4',5,7-trihydroxyisoflavone), an intensively studied estrogenic flavone, is very rich in soybeans and is often used as a model for phytoestrogens (Dixon and Ferreira, 2002). In some animal models, genistein is estrogenic and induces mammary tumor growth (Hsieh et al., 1998; Day et al., 2001; Allred et al., 2004). However, in other systems, genistein suppresses mammary cancer development (Lamartiniere et al., 1995). Studies on humans also yield mixed results. In one study, premenopausal women on a soy-rich diet have elevated proliferation of breast lobular epithelia cells (McMichael-Phillips et al., 1998). In contrast, another study indicated that consumption of food rich in phytoestrogens confers a protective effect against breast cancer, a notion supported by the fact that breast cancer risk is low in East Asian countries, where soybean consumption is high (Ganry, 2002).

Several mechanisms have been proposed to explain this seemingly paradoxical phenomenon. One hypothesis suggests that the timing of genistein administration may be important for its protective effects. Neonatal genistein treatment induces cell differentiation in mammary glands and reduces breast cancer risk while genistein treatment in adults increases estrogen burden and elevates breast cancer risk. This hypothesis is supported by animal models. In rodents treated with genistein neonatally or prepuberally, breast cancer risk decreases and in adult ovariectomized nude mice, genistein increases proliferation of breast cancer cells (Barnes, 1997; Lamartiniere et al., 2002). Besides functioning as a weak estrogen, genistein is also a tyrosine kinase

inhibitor (Fan and Rillema, 1992), apoptosis inducer (Brown et al., 1998; Katdare et al., 2002) and inhibitor of proteasome-mediated protein degradation (Kazi et al., 2003). All these functions can modify the impact of genistein on breast cancer risk and complicate the issue.

Resveratrol (trans-3,4',5-trihydroxystilbene) is a phenolic compound found in grapes, wine and other food products (Jang et al., 1997). It is a phytoalexin that protects plants against fungal infections (Hain et al., 1990). Resveratrol shares structural similarity with ER-agonist DES and has been shown to bind to estrogen receptor and subsequently activates estrogen-responsive genes. In MCF-7 breast cancer cells, resveratrol treatment induces ERE-luciferase reporter activity. Resveratrol also stimulates the proliferation of T47D breast cancer cells and the proliferation can be abolished by cotreatment with antiestrogen ICI 182,780 (Gehm et al., 1997). Although considered a xenoestrogen, the actions of resveratrol go far beyond its estrogenic activities. It possesses antioxidant and anti-inflammatory activity and protects against cardiovascular diseases (El-Mowafy, 2002). The high concentration of resveratrol in red wine has been proposed to explain the fact of moderate consumption of read wine reduces risk of cardiovascular disease (Frankel et al., 1993). Resveratrol activates ERα-dependent PI3K at a concentration of 10 uM but concentrations higher than 50 uM inhibits PI3K in MCF-7 cells (Pozo-Guisado et al., 2004). Activation of MAPK (mitogen-activated protein kinase) by UV or phorbol ester can be blocked by resveratrol (Yu et al., 2001b). Despite its estrogenic activity, resveratrol has not been linked with breast cancer, on the contrary it may possess cancer preventive activity (Jang et al., 1997).

In conclusion, there is not sufficient evidence to support the notion that xenoestrogens in general pose a risk of breast cancer. However, this conclusion does not exclude the possibility that certain individual xenoestrogens may elevate breast cancer risk. Neither does it rule out the possibility that some xenoestrogens cause detrimental effects through mechanisms other than the disruption of endocrine systems.

1.1.4.5 Genetic factors

Genetic factors also influence breast cancer risk. A family history of breast cancer is a strong predictor of breast cancer (Colditz et al., 1993). Many mutations of tumor suppression genes predispose their carriers to increased rates of breast cancer. Together, genetic factors account for 5-10% of total cases of breast cancer (Colditz et al., 1993; Weber and Garber, 1993).

1.1.4.5.1 BRCA1

BRCA1 was mapped to chromosome 17q21 by genetic linkage analysis and subsequently cloned in 1994 (Miki et al., 1994). It is estimated that carrying BRCA1 mutations confer a 54% chance of developing breast cancer by the age of 60 (Easton et al., 1995). Other estimations on penetrance vary from 56% to 36%, depending on the populations (Struewing et al., 1996; Fodor et al., 1998). In addition, BRCA1 mutations carry increased risks for ovarian, prostate and colon cancer (Ford et al., 1994).

BRCA1 is a large protein of 1863 amino acids, containing a BRCT (BRCA1 C-terminus) domain in the C-terminus and a RING domain in N-terminus, which are both involved in interaction with DNA repair proteins (Powell and Kachnic, 2003). BRCA1 localizes in nucleus during S and G2 phases and associates with Rad51, a protein homologous to bacterial RecA and vital for homologous recombination and double-strand DNA break (DSB) repair. Both BRCA1 and Rad51 are present in the asynapsed (axial) elements of human synaptonemal complexes, suggesting a role for BRCA1 in guarding the genome integrity (Scully et al., 1997a). Upon DNA damage in S phase, BRCA1 rapidly translocates to sites of DNA synthesis and become phosphorylated. Phosphorylation is important for BRCA function and can be mediated by several DNA-damage responsive kinases, such as ATM, ATR and Chk2 (Cortez et al., 1999; Lee et al., 2000b; Tibbetts et al., 2000). Phosphorylated BRCA1 associates with Rad51 and another protein BRAD1 (BRCA1 associated Ring Domain). The complex is proposed to participate in a replication checkpoint response (Scully et al., 1997b).

BRCA1 promotes apoptosis in breast and ovarian cancer cell lines in a mechanism dependent on Ras/MEK4/JNK pathway when DNA damage signals exist. Mutations on

BRCA1 cause the loss of apoptotic activity and may contribute to tumor development (Thangaraju et al., 2000). Another role of BRCA1 is to act as an ubiquitin (Ub) protein ligase (E3). Although the exact function of BRCA1- mediated ubiquitination is still unknown, it appears to be a significant part of BRCA1 activity. Many cancer-predisposing mutations disrupt the Ub ligase activity in the BRCA1 RING domain and these mutations have been linked with sensitivity to gamma-radiation, suggesting Ub ligase activity of BRCA1 is important for DNA repair (Ruffner et al., 2001).

BRCA1 is involved in regulating gene transcription. Fusion protein of BRCA1 linked to a GAL4-DNA binding domain can activate transcription of reporters driven by GAL4 DNA-binding sites (Haile and Parvin, 1999). BRCA1 exists in the SWI/SNF-related chromatin-remodeling complex (Bochar et al., 2000) and is also associated with the RNA polymerase II (RNAPII) complex through RNA helicase A (Anderson et al., 1998). Furthermore, BRCA1 complexes with transcription factors such as p53 and c-Myc and regulates their transcriptional activities either positively (p53) or negatively(c-Myc) (Wang et al., 1998; Zhang et al., 1998). BRCA1 also transactivates Cdk-inhibitor p21/WAF1/CIP1, which in turn participates in cell cycle arrest (Somasundaram et al., 1997).

Overall, BRCA1 plays an important physiological role in cell cycling and in response to DNA-damage (Thomas et al., 1997; Scully and Livingston, 2000). Disrupting BRCA1 in transgenic mice leads to impaired cell proliferation, non-detectable mesoderm formation and eventual embryonic lethality. In knockout embryos, cyclin E is downregulated while cdk inhibitor p21 is upregulated, leading to cell growth arrest and eventual premature death of embryos (Hakem et al., 1996).

Estrogen induces BRCA1 expression in breast cancer cells (Gudas et al., 1995; Spillman and Bowcock, 1996). The BRCA1 protein in turn suppresses ER-mediated proliferative activities. Both ligand-dependent and ligand-independent transcription of ER can be inhibited by BRCA1 in breast and ovarian cancer cells (Fan et al., 1999; Zheng et al., 2001). Recently, it was found that BRCA1 inhibited ERK (extracellular response kinase) signals initiated by membrane estrogen receptor and growth factor receptor and subsequently halt cell cycle in breast cancer cells. The authors suggested

that BRCA1 induces a dual-specificity phosphatase that is capable of deactivating ERK (Razandi et al., 2004). These findings may explain why mutations on BRCA1 increase cancer risks primarily only on mammary glands and ovaries.

1.1.4.5.2 BRCA2

Mutations on BRCA1 alone cannot account for all the familial breast cancer cases, suggesting other genes may have a role in genetic susceptibility to breast cancer (Easton et al., 1993). Shortly after the identification of BRCA1, another gene whose mutations also predispose individuals to breast cancer was discovered and named BRCA2 (Wooster et al., 1994).

The lifetime breast cancer risk rendered by BRCA2 mutations is similar to BRCA1 mutations; however, BRCA2 mutation carriers have a later onset of disease (Schubert et al., 1997). Mutated BRCA2 is also associated with risk of male breast cancer, pancreatic cancer, prostate cancer, and ovarian cancer (Thorlacius et al., 1996; Tischkowitz et al., 2002). Mutations on BRCA1 and BRCA2 together are responsible for about 5-10% of all breast cancer and 40-60% of hereditary breast cancer (Paakkonen et al., 2001).

BRCA2 does not share significant homology with BRCA1; however, it plays a role very similar to BRCA1. Cultured cells expressing mutated BRCA2 quickly accumulate abnormalities on chromosome structures over rounds of cell division, suggesting that BRCA2 is essential for maintaining genome integrity (Patel et al., 1998). BRCA2 colocalizes and cooperates with Rad51 and BRCA1 in double-strand DNA break repair and homologous recombination (Sharan et al., 1997; Chen et al., 1998). Like BRCA1, BRCA2 deficiency in mice leads to embryonic lethality. The level of p21/WAF1/CIP1 is elevated and cell proliferation is reduced. The development of mesoderm is detectable but with reduced size. Overall, the symptoms closely resemble that of BRCA1 knockout but with less severity, suggesting that BRCA1 and BRCA2 modulate some of the same physiological responses (Hakem et al., 1996) (Suzuki et al., 1997).

Despite their functional similarities, BRCA1 and BRCA2 are not equivalent. BRCA1 and BRCA2-assocaited breast cancers have different characteristics. BRCA1-assocated breast cancers are frequently invasive and estrogen receptor and progesterone receptor negative, while BRCA2-assocated breast cancers largely resemble sporadic breast cancers (Lakhani et al., 2002). Another difference between BRCA1 and BRCA2 is that BRCA2 has no ubiquitin protein ligase activity.

1.1.4.5.3 p53

p53 is an important tumor suppressor gene that mediates cellular stress response pathways. Activation of p53 leads to cell growth arrest or apoptosis upon cellular stress signals, which can be either DNA damage or oncogenic stress signals (Sherr, 1998; Woods and Vousden, 2001).

The p53 protein is a nuclear transcription factor and in the inactivated form, p53 has a very quick turnover and is expressed at low levels. Rapid degradation of p53 is regulated by Mdm2 and JNK (Haupt et al., 1997; Fuchs et al., 1998b). Mdm2 is a ring-finger protein that binds to the N-terminus of p53. This interaction not only targets p53 for ubiquitination and proteasome-dependent degradation (Fuchs et al., 1998a), but also inhibits the transcriptional activity of p53 (Oliner et al., 1993). Furthermore, Mdm2 mediates the export of p53 from nucleus into cytoplasm (Tao and Levine, 1999). Upon stress signals, Mdm2 undergoes downregulation (Zeng et al., 2000), or phosphorylation, which reduces Mdm2-p53 interactions (Mayo et al., 1997), or de-sumoylation, which increases Mdm2 turnover (Buschmann et al., 2000). All these events stabilize p53, which in turn transcriptionally activates Mdm2. This negative feedback mechanism ensures that the elevation of p53 is kept in check (Wu et al., 1993). The central role of Mdm2 in p53 regulation is highlighted by Mdm2 knockout mice, whose embryos undergo massive apoptosis at post-implantation stage. This embryonic lethality can be rescued if mice are deficient of both p53 and Mdm2 (Jones et al., 1995). JNK also play a similar role in regulating p53. In the absence of stress signals, JNK associates with p53 and targets p53 for proteasome-dependent degradation (Fuchs et al., 1998b). In response to stress signals such as radiation and oxidative stress, JNK phosphorylates p53 on Thr-81. Phosphorylated p53 exhibits

increased transcriptional activity and elevated resistance to degradation mediated by Mdm2 (Fuchs et al., 1998c; Buschmann et al., 2001).

The major role of p53 is to function as a central mediator of stress signals. Under DNA damage stress, p53 quickly accumulates due to inhibition of its degradation. At the same time, p53 is phosphorylated and activated by several stress-responsive kinases, such as ATM, ATR, Chk1/2, p38 and JNK. The phosphorylation serves to stabilize p53 and enhance its transcriptional activity (Banin et al., 1998; Waterman et al., 1998; Tibbetts et al., 1999; She et al., 2000; Shieh et al., 2000; Buschmann et al., 2001). Under oncogenic signals, p53 is mainly activated through p14/ARF, an alternate transcriptional product of INK4α/ARF locus (anther product is p15/ARF). Overexpression of oncogenic Myc and E1A rapidly induces p19/ARF (the mouse homolog of human p14/ARF) in primary mouse embryo fibroblasts (de Stanchina et al., 1998; Zindy et al., 1998). ARF in turn interacts with Mdm2 and abolishes Mdm2-mediated p53 suppression (Pomerantz et al., 1998).

Activated p53 interacts with specific DNA motifs as a tetramer and transactivates an array of genes that arrest cell cycle progression and induce apoptosis. GADD45 (DNA damage-inducible gene 45), a DNA-damage responsive protein that blocks cell cycle, is transactivated by p53 (Zhan et al., 1994). p53 also induces Cdk inhibitor p21/WAF1/Cip1, which is essential for p53-mediated cell cycle arrest and apoptosis (el-Deiry et al., 1993; Xiong et al., 1993). Furthermore, some pro-apoptotic genes, such as Fas and Bax, are p53-inducible (Miyashita et al., 1994; Owen-Schaub et al., 1995). p53 may also downregulate proto-oncogene c-fos and Bcl-2 (Ginsberg et al., 1991) (White, 1996). Moreover, p53 suppresses transcription by associating with TBP (TATA-binding protein), a component of the TFIID complex (Seto et al., 1992). Some studies suggest that p53 participates in DNA repair, as demonstrsnatred by its interaction with DNA recombination component Rad51 and nucleotide excision repair factors XPB and XPD (Rad3) (Buchhop et al., 1997). In addition, p53 binds single stranded DNA ends and catalyzes DNA annealing, suggesting a direct role in repairing DNA breaks (Bakalkin et al., 1994).

Mutations on p53 compromise cell cycle arrest and attenuate apoptotic signals upon cellular stress, thus greatly increasing risks of cancer. Mice with disrupted p53 develop normally but have an elevated frequency of spontaneous tumorigenesis (Donehower et al., 1992). In humans, the congenital mutations of p53 are associated with Li-Fraumeni syndrome, a condition that renders patients susceptible to a wide range of cancers, including brain, breast, bone and lung tumors (Malkin et al., 1990). Conversely, mice expressing constitutively activated p53 have lower incidence of cancer, however, their aging process is greatly accelerated (Tyner et al., 2002). In contrast, another study indicates that in mice with multiple copies of p53 under the control of native promoter, tumor resistance is still enhanced as expected but the aging process progresses at normal pace (Garcia-Cao et al., 2002).

About 50% of human cancers contain p53 mutations (Donehower, 1996) and 20-40% breast cancer patients have p53 mutations (Faille et al., 1994; Greenblatt et al., 1996). However, germline p53 mutations are very rare in hereditary breast cancers, therefore, mutations on p53 only account for a very small portion of hereditary breast cancers (Prosser et al., 1991; Zelada-Hedman et al., 1997).

1.1.4.5.4 ATM

AT (Ataxia telangiectasia) is a hereditary recessive disorder characterized by cerebellar ataxia, telangiectasia, immune defects, chromosomal instability, radiosensitivity and cancer susceptibility. The mutation responsible for AT is identified to occur on the ATM (ataxia telangiectasia mutated) gene (Savitsky et al., 1995). There is a correlation between homozygous ATM mutations and elevated breast cancer risk (Stankovic et al., 1998). Moreover, the ATM mutation heterozygotes may also be susceptible to breast cancer (Swift et al., 1976; Morrell et al., 1990; Athma et al., 1996), although some studies argue that heterozygosity of ATM does not confer significantly increased breast cancer risk (FitzGerald et al., 1997). It is estimated that 1.4% of the general population harbor heterozygous mutation on ATM and this condition contributes to 3.8-6.6% of all breast cancers (Easton, 1994; Athma et al., 1996).

ATM protein is a kinase that contains a phosphatidylinositol 3- kinase (PI3K) domain at the C-terminus (Savitsky et al., 1995). ATM binds to DNA directly, with a preference for

double strand ends. After DNA damage by radiation exposure, ATM is activated to phosphorylate p53 on Ser15, resulting in p53 upregulation and a subsequent stress response (Banin et al., 1998; Canman et al., 1998; Smith et al., 1999). A proline-rich motif within ATM interacts with the SH3 domain in c-Abl, a non-receptor protein tyrosine kinase that is important for radiation-induced G1 arrest (Shafman et al., 1997). Phosphorylation by ATM is required for BRCA1 to respond to DNA damage, a function that may explain the predisposition of ATM mutations to enhance breast cancer (Cortez et al., 1999).

1.1.4.5.5 PTEN

PTEN is a tumor suppressor whose mutation has been linked with increased breast cancer risk. PTEN is a phosphatase that dephosphorylates D3 position of PIP_3 (phosphatidylinositol 3,4,5-trisphosphate), a crucial regulator of cell growth and survival. By increasing PIP_3 turnover, PTEN inhibits the activity of PDK1 and AKT, two pro-survival protein kinases (Alessi et al., 1997; Vanhaesebroeck and Alessi, 2000). The loss of PTEN function is linked with tumorigenesis. Homozygous deletion of PTEN in mice results in embryonic lethality. The heterozygous PTEN deletion mice are viable, but with a much higher incidence of tumor formation (Di Cristofano et al., 1998).

Germline mutations on PTEN lead to Cowden's syndrome, which is a rare autosomal dominant inherited disease characterized by high incidence of breast, endometrium, brain and thyroid cancer (Lynch et al., 1997; Eng, 2003). It is estimated that woman with Cowden's disease have 30-50% increase in the incidence of breast cancer (Starink et al., 1986). PTEN Mutations are infrequent in familial breast cancer patients except those with Cowden disease (FitzGerald et al., 1998). Since Cowden's disease is rare, PTEN mutations only have a small impact on hereditary breast cancer incidence.

1.1.4.5.6 STK11

STK11(LKB1) is a tumor suppressor gene whose mutations lead to Peutz-Jeghers syndrome, a rare autosomal dominant disorder. Peutz-Jeghers syndrome is characterized by multiple gastrointestinal hamartomatous polyps and increased risk of cancers in gastrointestinal tract, lung, breast, uterus, ovary, and pancreas (Boardman et al., 1998; Giardiello et al., 2000). GI tract cancers are the greatest risk for Peutz-

Jeghers syndrome patients, however, risk for breast cancer is also significant, as high as 29% by the age of 65 (Lim et al., 2003). However, because of the extremely low prevalence of Peutz-Jeghers syndrome in general population, STK11 mutations contribute little to familial breast cancer.

Overexpression of STK11 leads to apoptosis in a p53-dependent manner (Karuman et al., 2001). STK11 also mediates cell-cycle arrest by inducing cdk inhibitor p21/WAF1/CIP1 in the presence of p53 (Tiainen et al., 2002). Brg1 (brahma-related gene-1), an ATPase associated with SWI/SNF chromatin-remodeling complexes, interacts with STK11. This interaction appears to increase Brg1 ATPase activity and result in suppression of cell proliferation (Marignani et al., 2001).

Although mutations on single high-penetrant genes attract much attention in breast cancer research, the majority of breast cancers cannot be accounted for by a single-gene mutation. BRCA mutations together only contribute to about 5-10% of all breast cancers (Paakkonen et al., 2001), with other mutations noted above accounting for a lower percentage. It has been hypothesized that breast cancer risk can be modified by polymorphism of various genes involved in estrogen production, action and metabolism. Although each individual polymorphism alone only slightly modifies the breast cancer risk, together, the polymorphisms may have significant cumulative impact. Several candidate genes for this model have been proposed, including CYP17 (an enzyme vital for steroidogenesis), ESR(ERα), HSD17B1 (17β-hydroxysteroid dehydrogenase I, an enzyme converting estrone to more biologically active E2), COMT (catechol-O-methyltransferase, an enzyme deactivated catechol estrogen by O-methylation) and CYP1A1 (an estrogen hydroxylase) (Feigelson et al., 1996; Huang et al., 1999).

1.1.5 Breast cancer treatment

The principle of breast cancer treatment is to specifically, or at least preferentially eliminate breast cancer cells while leaving normal cells relatively intact. Given the physiological similarities between cancer cells and normal cells, this has proven to be a difficult task. However, significant progress on development of drugs with minimal side-effects has been made. At present, the predominant treatments for breast cancer are surgical removal, chemotherapy, endocrine and radiation therapy.

1.1.5.1 Surgery and radiation

In the early days of breast cancer treatment, radical mastectomy was widely used. This procedure involves removing the entire breast, the overlaying skin and pectoral muscles and axillary contents. With better understanding of the biology of breast cancer, a more moderate approach termed modified radical mastectomy (MRM) has been adopted. MRM is a collection of procedures that remove the entire breast and the auxiliary node-bearing tissues. Survival rates for women who have undergone MRM is comparable with that for women who have had a radical mastectomy (Maddox et al., 1983).

For early stages of breast cancer (DCIS or stage I and II invasive breast cancer), BCT (breast-conserving therapy) is the treatment of choice. For eligible patients, BCT is as effective as radical and modified radical mastectomy (van Dongen et al., 2000). BCT is a combination of lumpectomy and radiotherapy. Unlike mastectomy, BCT preserves the structure and cosmetic appearance of the breast. The rationale for BCT is that after surgical removal of the tumor, radiant therapy can be used to eliminate microscopic tumors that cannot be removed by surgery. Radiation therapy usually begins 4 weeks after surgery, allowing for adequate healing from the operation. A typical radiation dose is 50 Gy in 5 weeks with a boost of 10 Gy into the lumpectomy cavity. Although still controversial, the 10-Gy boost is believed to help reduce the risk of early local recurrence (Romestaing et al., 1997).

Besides its application in BCT, radiation therapy can also be used in conjunction with mastectomy. The NIH/NCI Consensus Conference recommends postmastectomy radiation therapy for patients with four or more positive axillary lymph node metastases. The benefit of postmastectomy radiation therapy for patients with one to three positive lymph nodes remains uncertain (Eifel et al., 2001). Furthermore, radiation therapy is also employed to relieve the pain from cancer metastasis. The most frequent sites for breast cancer metastasis are bone, lung, liver and brain. Among them, bone is the most common site for palliative bone radiation (Arcangeli et al., 1989). In one study, after 15-Gy radiation was given in 3 fractions over 2-week period, breast cancer patients with bone metastasis experience pain relief and mobility improvement (Rasmusson et al., 1995).

1.1.5.2 Chemotherapy

Chemotherapy has been employed either as an adjuvant therapy for breast cancer after surgery or a primary treatment for metastatic breast cancer. Multiple regimens have been developed and typically involve multiple cycles of treatment by a combination of several cytotoxic chemicals. In adjuvant chemotherapy, the goal is to eliminate microscopic tumors and prevent relapse. The commonly used regimens for adjuvant chemotherapy include CMF (cyclophosphamide, methotrexate, 5-fluorouracil), AC (Doxorubicin, cyclophosphamide), CAF (cyclophosphamide, doxorubicin, 5-fluorouracil), AC plus paclitaxel and CEF (cyclophosphamide, epirubicin, 5-flurouracil). For metastatic breast cancer, the goal of chemotherapy is to prolong the survival and alleviate the symptoms. Major chemicals used for treatment of metastatic breast cancer are taxanes and anthracyclines. These agents are used alone or in various combinations (Donegan and Spratt, 2002; Torosian, 2002).

Despite its beneficial effects for breast cancer patients, chemotherapy is well-known for its serious side effects, which are specific for individual drugs or may be shared by a broad spectrum of reagents. Proliferating cells are most vulnerable to chemotherapy and typical symptoms include myelosuppression, nausea, vomiting, diarrhea, skin rashes or photosensitivity, hair loss, kidney damage and premature menopause (Torosian, 2002).

1.1.5.3 Endocrine therapy

1.1.5.3.1 SERMs and antiestrogens

Since estrogen plays an important role in mammary carcinogenesis, the estrogen receptor pathway presents itself as an attractive chemotherapeutic target. Antiestrogens can be used to disrupt ER signaling pathway and thereby inhibit breast cancer growth. However, estrogen is a multi-function hormone and important not only for the mammary gland, but also for numerous other organs, such as ovary, bone, nervous system and cardiovascular system. Therefore, any method that disrupts ER action and effective in inhibiting breast cancer will also interfere with normal estrogen functions in other parts of the body and cause undesirable side effects. Thus the concept of SERM (selective

estrogen receptor modulator) was introduced to describe compounds that are estrogenic in some tissues and antiestrogenic in others (Miller, 2002). In contrast, some compounds, such as ICI 182,780, show almost universal antiestrogenic activity regardless of cell-context. These compounds are termed “pure antiestrogens” (Fig. 4).

Tamoxifen (Nolvadex) is a SERM that represents a great advance in breast cancer treatment. Tamoxifen is a substituted triphenylethylene derivative widely used for breast cancer treatment. In rodent models, tamoxifen can prevent the mammary carcinogenesis in DMBA (1,2-dimethylbenzanthracene) induced animals (Jordan, 1976). In clinical trials compiled by the Early Breast Cancer Trialists' Collaborative Group in 1998, tamoxifen adjuvant therapy significantly decreases the mortality of ER-positive breast cancer patients regardless of age and menopausal status. The 10-year death rate was reduced by 14% with one year of adjuvant tamoxifen treatment, by 18% with two years and by 28% with five years treatment. No additional benefits were found to prolog tamoxifen treatment beyond five years. For ER-negative patients, the benefit of tamoxifen is only minimal (Early Breast Cancer Trialists' Collaborative Group, 1998). ER-status is a strong predictor of tamoxifen response, consistent with the notion that tamoxifen inhibits breast cancer primarily by functioning as an antiestrogen in breast tissue (MacGregor and Jordan, 1998). However, there is evidence that tamoxifen may inhibit breast cancer through other mechanisms. For instance, tamoxifen inhibits tumor growth independent of ER by inducing the inhibitory growth factor TGF-β (Brandt et al., 2003).

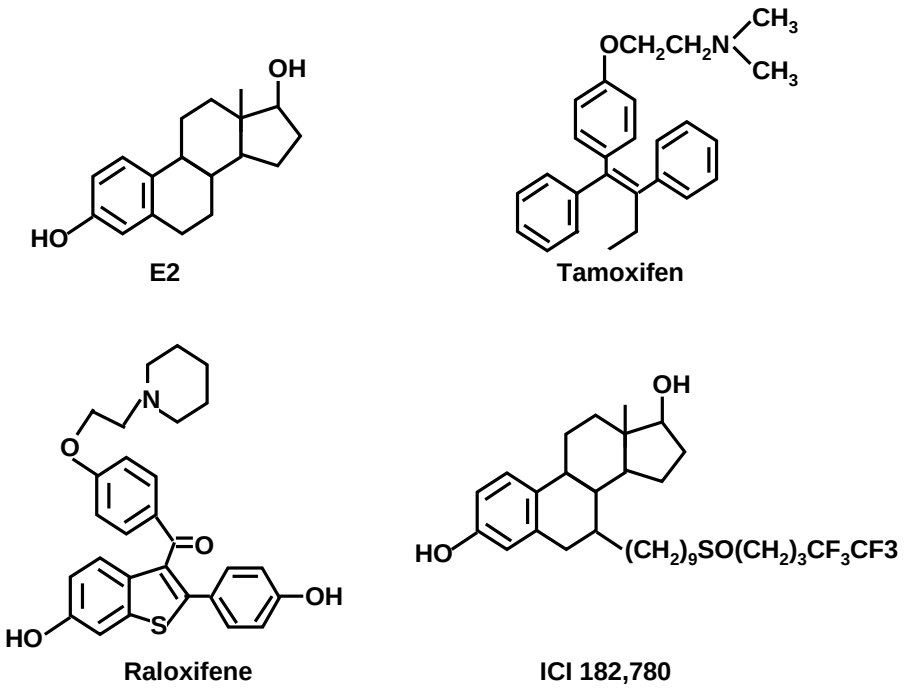

Fig. 4. Estrogen (E2), SERMs (tamoxifen and raloxifene) and the pure antiestrogen (ICI 182, 780).

Tamoxifen-responsive tumors often develop resistance during the course of treatment (Osborne et al., 1980). Frequently, the development of resistance is paralleled by the loss of ER (Vihko et al., 1986). However, loss of ER expression alone cannot account for all the resistance. In many cases, breast cancer cells remain ER-positive even after becoming tamoxifen resistant (Encarnacion et al., 1993; Johnston et al., 1995). Many hypotheses on tamoxifen-resistance have been proposed, including the increased metabolic deactivation of tamoxifen (Osborne et al., 1992; Johnston et al., 1993), elevated expression of the P-glycoprotein multidrug resistance efflux pump (Chen et al., 1986) or activation of cellular kinases (Hori et al., 2000; Kurokawa et al., 2000; Schiff et al., 2000; Osborne et al., 2003).

Tamoxifen also offers some beneficial estrogenic effects. In postmenopausal breast cancer patients, tamoxifen treatment helps maintain bone density, although in premenopausal women tamoxifen slightly accelerates bone loss (Love et al., 1992).

Postmenopausal women receiving tamoxifen for 2 years have a 12% decrease in total cholesterol levels and 20% decrease in low-density lipoprotein (LDL) cholesterol levels (Love et al., 1991). Breast cancer patients receiving tamoxifen treatment also experience a reduced incidence of fatal myocardial infarction (McDonald et al., 1995).

Tamoxifen also has some undesirable side-effects. Most symptoms are minor, including hot flashes, mood disturbances, weight gain and atrophic vaginitis. However, two conditions, endometrial carcinoma and thromboembolic phenomena, are severe enough to demand cautions (Fisher et al., 1998; Ragaz and Coldman, 1998). Tamoxifen induces the growth of endometrial tumor transplanted in athymic mice (Gottardis et al., 1988) and the incidence of endometrial cancer was significantly elevated in women receiving tamoxifen (Early Breast Cancer Trialists' Collaborative Group, 1998; Bernstein et al., 1999). However, the increased risk was only observed in postmenopausal women but not premenopausal women (Fisher et al., 1998). The incidence of stroke, pulmonary embolism, and deep-vein thrombosis are elevated with tamoxifen treatment (Fisher et al., 1998). Another condition that initially caused concerns is that tamoxifen a rodent hepatocarcinogen through an ER-independent pathway. Clinical studies found that patients taking tamoxifen have no increased risk of liver cancer, suggesting that the hepatic carcinogenic effects in rats are probably not relevant for humans (Early Breast Cancer Trialists' Collaborative Group, 1998).

Raloxifene (previously named keoxifene and LY 156758) is a newly developed SERM. It binds to ER with a high affinity and inhibits the growth of breast cancer cells in culture and reduces the incidence of NMU-induced mammary carcinoma in rodents. However, with a short biological half-life, raloxifene is less potent than tamoxifen (Gottardis and Jordan, 1987; Poulin et al., 1989). High dose (150 mg per day) raloxifene has a modest inhibitory effect against ER-positive breast cancer in postmenopausal women (Gradishar et al., 2000). Cross-resistance with tamoxifen is common and tamoxifen-resistant cancer cells are frequently not responsive to raloxifene (O'Regan et al., 2002). Unlike tamoxifen, raloxifene does not induce significant estrogenic activity in uteri, although it is not totally antiestrogenic in uterus because raloxifene supports the growth of tamoxifen-induced endometrial cancer cells in nude mice (Black et al., 1994; O'Regan et al., 2002). The low estrogenicity of raloxifene in the uterus has been

confirmed in clinical studies, demonstrating that raloxifene does not stimulate morphological changes in the endometrium of postmenopausal woman free of endometrial abnormities (Boss et al., 1997; Cauley et al., 2001).

The potential applications of raloxifene are primarily not for breast cancer treatment, but for prevention of breast cancer, osteoporosis and cardiovascular diseases. Raloxifene intake reduces the incidence of breast cancer without increasing risk of endometrial cancer, although the risk of thromboembolic disease is elevated (Cauley et al., 2001). A large clinical trial STAR (Study of TAM and Raloxifene) is underway to compare the effects of 5 years of raloxifene or tamoxifen among postmenopausal women with high risk for breast cancer. Raloxifene can prevent bone loss in ovariectomized rats (Black et al., 1994). Postmenopausal women receiving raloxifene have had significant improvements in maintaining bone mineral density of the lumbar spine, hip, and total body (Delmas et al., 1997). The results from MORE (Multiple Outcomes of Raloxifene Evaluation) trials demonstrated that in postmenopausal osteoporotic women, raloxifene increases bone mineral density in the spine and femoral neck and reduces risk of vertebral fracture (Ettinger et al., 1999). Raloxifene decreases serum LDL cholesterol levels and the MORE trials found that incidence of CHD (coronary heart disease) in women with elevated CHD risks can be reduced by 40% with raloxifene (Barrett-Connor et al., 2002). A large trial named RUTH (Raloxifene Use for The Heart) is being conduct to assess the effect of raloxifene on CHD high risk women (Mosca et al., 2001).

Arzoxifene (LY 353, 381) is a raloxifene analog with a longer biological half life. As a chemopreventive agent, arzoxifene is superior to raloxifene in rat mammary tumorigenesis model (Suh et al., 2001). Arzoxifene maintains bone density in ovariectomized rats much more potently than raloxifene, without causing uterotropic effects (Sato et al., 1998). A phase I clinical trial has shown the efficacy of arzoxifene on advanced breast cancer (Munster et al., 2001). GW 5638 is a high affinity ER-antagonist in breast tissue but functions as full estrogen in bone and cardiovascular system (Willson et al., 1997). Interestingly, GW 5638 has no cross-resistance with tamoxifen and has potential to be used for treatment of tamoxifen-resistant tumors (Connor et al., 2001).

ICI 182,780 (also known as Fulvestrant or Faslodex), a “pure” antiestrogen, have been shown effective for treating breast cancer (Howell et al., 1995). A unique feature of this pure antiestrogen is that it greatly accelerates degradation of ER (Dauvois et al., 1992; DeFriend et al., 1994). This mechanistic difference may explain why the pure antiestrogen is less cross-resistant with tamoxifen compared to other SERMs. For example, ICI 182,780 is effective against tamoxifen-resistant MCF-7 breast cancer cells (Lee et al., 2000a). ICI 182, 780 is now being used as a second-line agent after tamoxifen failure (Howell et al., 1996).

1.1.5.3.2 Ovarian ablation and aromatase inhibitors

The estrogen biosynthetic pathway is also an important target for breast cancer therapy. In premenopausal women, ovaries are the predominant sites of estrogen production, therefore ovarian ablation constitutes a therapy for premenopausal breast cancer patients. Both surgical ovariectomy and ovarian radioablation are effective for ER-positive patients (Torosian, 2002). Recently, medical ovarian ablation using a gonadotropin releasing hormone (GnRH) analog has been gaining in popularity. GnRH analog inhibits the ovarian production of estrogen and induces a status resembling menopause. The main advantage of the GnRH regimen is its reversibility and non-invasiveness. In clinical trials, the GnRH analog goserelin (Zoladex) has response rates comparable to that of ovariectomy in hormone-receptor positive premenopausal patients (Taylor et al., 1998). In the IBCSG (International Breast Cancer Study Group) trial, a comparable performance of goserelin has been shown against CMF chemotherapy in ER-positive premenopausal patients (Castiglione-Gertsch et al., 2003).

In postmenopausal women, the major estrogen-producing site is peripheral adipose tissue. The enzyme responsible for conversing androgen to estrogen is aromatase (CYP19) (Simpson et al., 1993) (Fig. 5) and inhibition of aromatase has been an important strategy for breast cancer treatment in postmenopausal women.

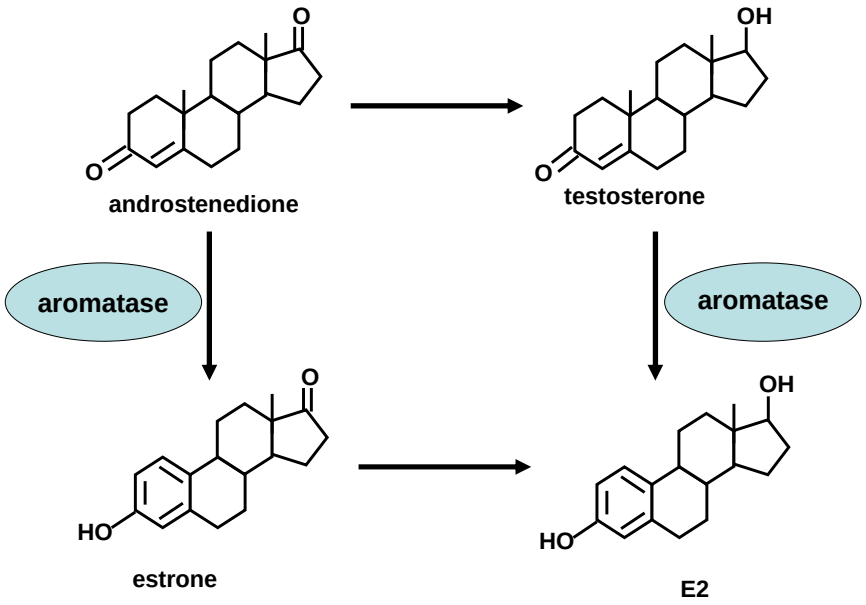

Fig. 5. The role of aromatase in estrogen synthesis.

Three generations of aromatase inhibitors have been developed, including first-generation aminoglutethimide, second-generation fadrozole and formestane, third-generation letrozole (Femara), anastrozole (Arimidex) and exemestane (Aromasin). Depending on whether they possess a steroid structure, aromatase inhibitors can be divided into two categories: steroidal and nonsteroidal. Formestane and exemestane belong to steroidal category whereas aminoglutethimide, fadrozole, letrozole and anastrozole are nonsteroidal aromatase inhibitors (Fig. 6).

Compared to tamoxifen, aromatase inhibitors are linked to lower incidences of endometrial cancer and thromboembolic phenomena. The major undesirable side-effects of aromatase inhibitors are increased osteoporosis and bone fracture (Baum et al., 2002; Coombes et al., 2004). Because of their distinct mechanisms compared to SERMs, aromatase inhibitors may also overcome resistance to tamoxifen. Aromatase inhibitors have been used as second-line therapy after tamoxifen treatment. In the IES (Intergroup Exemestane Study) trial, patients who have completed 2-3 years of tamoxifen were assigned to continue tamoxifen treatment or switch to exemestane. After a median follow-up of 30.6 months, a superior reduction in breast cancer occurrence was observed in the exemestane group compared to the tamoxifen group

(Coombes et al., 2004). In the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, postmenopausal patients were put on anastrozole, tamoxifen or combination treatment. Among hormone-receptor-positive patients, anastrozole is more effective than tamoxifen in prolonging disease-free survival (Baum et al., 2002). Another clinic trial reported that for postmenopausal women who have finished 5 years of tamoxifen treatment, letrozole caused 43% reduction in breast cancer events as compared to a placebo for a median follow-up of 2.4 years (Goss et al., 2003).

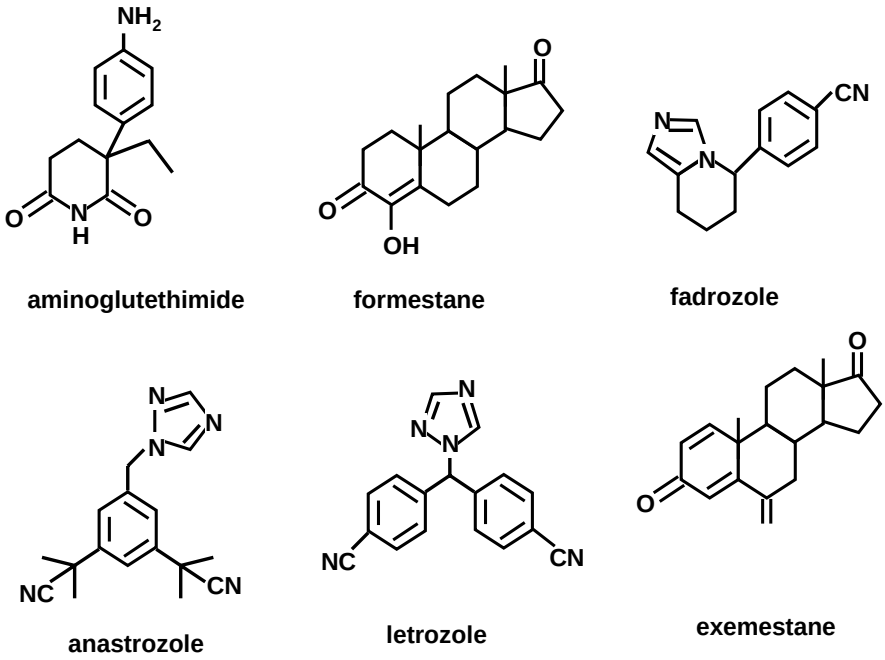

Fig. 6. Aromatase inhibitors.

Aromatase inhibitors are generally not used alone for treating premenopausal women because they cannot sufficiently downregulate estrogen levels in premenopausal women. However, the combination of aromatase inhibitors and GnRH analogs shows potential for treating premenopausal breast cancer patients (Dowsett et al., 1992; Torosian, 2002). Several large, randomized clinical trials are underway to explore this possibility (Winer et al., 2005).

1.1.5.3.3 Progesterone

Progestational agents such as megestrol acetate (Megace) and medroxyprogesterone acetate (Provera) have been used to treat hormone-receptor-positive breast cancer in postmenopausal women, with a response rate comparable to that of tamoxifen (Morgan, 1985; Ettinger et al., 1986; Sedlacek, 1988; Muss and Cruz, 1992). The major side effects are weight gain and thromboembolic phenomena, although sometimes weight gain may be beneficial for breast cancer patients who have cancer cachexia (Sedlacek, 1988).

1.1.5.3.4. Estrogen therapy

Although counterintuitive, DES, a potent synthetic estrogen, had been employed to treat advanced breast cancer in postmenopausal women before the widespread use of tamoxifen. Unlike antiestrogen therapies which were developed under the guidance of receptor-ligand theory, estrogen therapy was established on empirical data that mass doses of estrogen cause tumor regression in postmenopausal breast cancer patients. The mechanism for this estrogen-induced breast tumor regression is not clear. One theory suggests that estrogen induces apoptosis through a putative zinc-finger protein (Szelei et al., 2000). Other studies propose that high doses of estrogen reduce membrane fluidity of cancer cells in an ER-independent manner and this leads to cytotoxicity (Clarke et al., 1990).

Daily treatment of 1.5, 15, 150 and 1500 mg of DES caused a 10, 15, 17 and 21% regression of breast cancer, respectively (Carter et al., 1977). A randomized trial demonstrated that DES and tamoxifen treatment have comparable efficacy but DES is associated with more side-effects, including nausea, vomiting, anorexia, fluid retention, thromboembolic phenomena, uterine bleeding and hypercalcemic flares (Ingle et al., 1981; Donegan and Spratt, 2002). Estrogen therapy with DES has been largely superseded by tamoxifen treatment.

1.1.5.3.5 Trastuzumab

ErbB2 (HER2/neu) is a growth factor receptor with high homology to the EGFR (epidermal growth factor receptor). Low levels of ErbB2 are expressed at low levels in epithelial tissues but are often amplified in breast cancer. Overexpression of ErbB2 in breast cancer is associated with highly aggressive tumor and poor prognosis (Slamon et al., 1987; Press et al., 1990). A humanized monoclonal antibody MAb 4D5 (trastuzumab or Herceptin) has been developed to block the ErbB2 (Baselga et al., 1996) and clinical trials demonstrate that trastuzumab is effective against ErbB2-overexpressing metastatic breast cancer (Cobleigh et al., 1999). The combination of trastuzumab with chemotherapy further improved the outcome of patients (Slamon et al., 2001). Trastuzumab treatment increases the risk of cardiotoxicity, which is probably due to the disruption of ErbB2 signaling that is important for cell survival upon cardiac stress (Chien, 1999).

1.2 Estrogen receptor

1.2.1 The structure of estrogen receptor

Estrogen exerts its effects through the estrogen receptor (ER), which is a member of the nuclear receptor (NR) superfamily of transcription factors (Evans, 1988; Mangelsdorf et al., 1995). Two isoforms of ER, ERα and ERβ, have been identified.

The human ERα gene was cloned and sequenced from MCF-7 breast cancer cells. It encompasses 140 kb DNA segment, encoding a 66 KDa protein with 595 amino acids (Green et al., 1986). Like all NRs, ERα possesses a modular structure that can be divided into several independent but interacting structural and functional domains. The domains are designated A through F from the N-terminus to C-terminus (Fig. 7). The A/B domain contains activation function 1(AF1), which activates gene transcription in a ligand-independent manner. The AF1 domain is not only important for constitutive activity, but also for mediating the agonistic activity of antiestrogens (Berry et al., 1990). The C domain is a highly conserved DNA-binding domain (DBD), which recognizes a consensus DNA motif named estrogen response element (ERE). The C domain contains two regions termed CI (aa185-215) and CII (aa 216-250), and each contains a zinc finger. The zinc fingers are crucial for sequence recognition and DNA-binding and

the removal of zinc ion results in abolishment of ER-ERE binding (Green et al., 1988). Three residues Glu203, Gly204 and Ala207 form a site called P-box that is involved in ERE-recognition (Mader et al., 1989). Five amino acids between Cys221 and Cys227 are termed the “D box”, which mediates ER dimerization (Mader et al., 1993). The D domain serves as a hinge to give ER flexibility for conformational changes. In addition, the D domain is an important region for binding nuclear receptor coregualtors (Jackson et al., 1997). The E domain contains AF2, which modulates ligand-dependent transactivation activity of ERα. E domain also includes the ligand binding region (LBD) (Kumar et al., 1987; Lees et al., 1989; Tora et al., 1989), which has a pocket-like structure consisting of 12 α-helices designated helix 1 to helix 12. The conformation of the LBD upon ligand binding is important for ER-dependent activity. Estrogen binding is accompanied by repositioning of helix12, which forms a hydrophobic groove crucial for the interactions with LXXLL motifs of coactivators. The ER antagonist raloxifene binds to the LBD in a similar way as E2, however, the antagonist binding causes helix 12 to adopt a different conformation and disrupts interaction with nuclear coactivators, resulting in the gene silencing (Brzozowski et al., 1997; Heery et al., 1997). The function of the F domain is relatively unknown, but has been implicated in distinguishing between ER agonists and antagonists (Montano et al., 1995).

Estrogen-binding to the ER is accompanied by ER degradation through the ubiquitin-proteasome pathway. ERα turnover is important not only for controlling ERα levels, but also for maintaining the transcriptional activity of ERα. Inhibition of the ubiquitin-proteasome pathway causes accumulation of ERα and inhibition of E2-induced transactivation (Reid et al., 2003). The impact of ligand-binding on ERα degradation is highly ligand-dependent. E2 and the antiestrogen ICI182,780 accelerates the ERα turnover while tamoxifen attenuates the degradation (McDougal et al., 2001; Preisler-Mashek et al., 2002). Increased ER turnover is an important mechanism for the anti-proliferative activity of ICI182,780 (Fan et al., 2003). The antiestrogenic activity of SAhRMs (selective AhR modulators) can be partially accounted for by their ability to induce ER degradation through the ubiquitin-proteasome pathway (McDougal et al., 2001; Wormke et al., 2003).

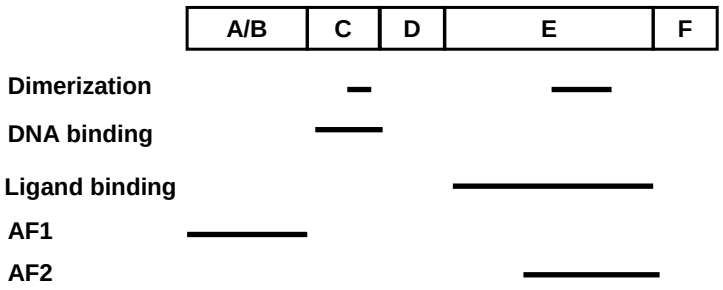

Fig. 7. The domain structure of ERα.

ERα had been considered the only estrogen receptor until ERβ was discovered in 1996 (Kuiper et al., 1996; Mosselman et al., 1996). ERβ shares significant homology with ERα in the DBD (96%) and LBD (58%), but differs greatly from ERα in the AF1 region (Mosselman et al., 1996). E2 binds to ERα and ERβ with comparable affinity (Kuiper et al., 1997) and ERβ can dimerize with itself or form heterodimers with ERα (Cowley et al., 1997; Pettersson et al., 1997). The order of DNA binding affinity is: ERα homodimer≈ERα-ERβ heterodimer>ERβ homodimers (Cowley et al., 1997)

The discovery of ERβ added another level of complexity to the effects of estrogen. ERβ mediates the transactivation of ERE-containing promoters in a way similar to ERα (Cowley et al., 1997). However, many important ERα-regulated genes, such as progesterone receptor (PR), are not regulated by ERβ but primarily by ERα (Fuqua et al., 2003). Conversely, ERβ mediates some actions in which ERα has no role. For example, E2 initiated rapid CREB phosphorylation in GnRH neurons in ERα knockout mice but not in ERβ knockout mice (Abraham et al., 2003). ERβ also exhibits different transcriptional activities than ERα depending on ligand structure and cellular context (Meyers et al., 2001). Some xenoestrogens, such as 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), show significantly higher affinity for ERβ than ERα (Kuiper et al., 1997). Furthermore, some xenoestrogens, such as HPTE, are ERα agonists but ERβ antagonists (Gaido et al., 1999). The tissue distribution of ERβ is significantly different from that of ERα. Human ERα is expressed in testis, ovary, prostate and skeletal muscle while ERβ expresses in thymus, spleen, ovary and testis (Mosselman et al., 1996). This differential expression of ERα and ERβ in various cell

types may contribute to the cell-context-dependent responses to estrogen and tissue-specific responses of various estrogenic compounds.

1.2.2 The physiological functions of estrogen

Estrogens mediate the development and function of the female reproductive system. However, estrogens are by no means only female hormones. In both males and females, estrogens play crucial physiological roles, including sex differentiation, bone development and maintenance, central nervous system and cardiovascular function (Martinez-Vargas et al., 1975; Ansar Ahmed et al., 1989; Maggi et al., 1989; Smith et al., 1994; Brosnihan et al., 1997; McEwen and Alves, 1999).

1.2.2.1 Reproductive system and estrogen

The ER is important for both mammary gland development and tumorigenesis. ERα knockout (αERKO) mice are viable but sterile and are estrogen-insensitive in several estrogen target organs, including mammary glands, reproductive tracts and gonads. The well-recognized estrogenic responses, such as uterine weight increase and vaginal cornification, are all absent in αERKO mice (Lubahn et al., 1993; Korach, 1994). In αERKO mice, mammary glands undergo normal development in the prenatal period, but fail to develop terminal end buds and are severely undertropic during adulthood (Lubahn et al., 1993; Korach, 1994). The under-developed mammary glands are resistant to DMBA (7,12-dimethylbenz[a]anthracene)-induced carcinogenesis (Day et al., 2001). Breast caner can still be induced in αERKO mice by overexpression of Wnt-1 or ErbB2 oncogenes, but with a much later onset compared to wild type mice, suggesting that ERα is facilitative but not indispensable for mammary carcinogenesis (Bocchinfuso et al., 1999; Hewitt et al., 2002).

The reproductive tract of female αERKO mice develop normally before puberty, however, sexual maturation is severely impaired. In adult αERKO mice, the uterus is hypoplasitc and weighs only half of that of wild-type mice (Lubahn et al., 1993). No significant ovarian abnormalities were detected in neonatal αERKO mice, but ovaries in the mature αERKO mice have disrupted ovulation and luteinization and develop multiple hemorrhagic cysts (Schomberg et al., 1999). Surprisingly, adult male αERKO

mice are also infertile because of impaired spermatogenesis (Eddy et al., 1996). Similar results were observed in male mice with disrupted aromatase, an enzyme essential for estrogen synthesis (Robertson et al., 1999).

In contrast to the extensive reproductive tract abnormalities in αERKO mice, ERβ knockout (βERKO) mice are fertile and have no significant abnormalities except reduced fertility (Korach, 1994; Krege et al., 1998).

1.2.2.2 Cardiovascular system and estrogen

Women have significantly lower risk of cardiovascular disease than men. This gender difference has been putatively attributed to the beneficial effects of estrogen to cardiovascular systems (Nathan and Chaudhuri, 1997). Estrogen decreases low-density lipoprotein (LDL) level and increases high-density lipoprotein (HDL) level, thus favorably altering the lipoprotein profile (Bush, 1990; Knopp et al., 1996). Estrogen has vasodilatory effects and protects against vascular injury (Spyridopoulos et al., 1997; Pare et al., 2002). Recently, estrogen was found to upregulate atheroprotective prostacylin PGI_2 by activating cyclooxygenase 2 (COX-2), thus protecting against atherogenesis (Egan et al., 2004). In epidemiological studies, estrogen has been linked with improved cardiovascular functions (PEPI, 1995; Grodstein et al., 1996). Bilateral ovariectomy before menopause elevates the risk for coronary heart disease (CHD) and the increased risk is eliminated by estrogen-replacement therapy (Colditz et al., 1987). Based on these results, it has been theorized that hormone replacement therapy (HRT) can be used as a preventive regimen in postmenopausal women against cardiovascular diseases. However, a large clinical trial conducted by Women's Health Initiative has reported contradictory results. This study found that women taking HRT have an increased risk for heart diseases and stroke (Rossouw et al., 2002).The relationships between estrogen and cardiovascular diseases require further assessment.

1.2.2.3 Bone and estrogen

Estrogen plays a key role in bone development and maintenance. Before and during puberty, estrogen stimulates bone growth in both males and females and may be the major driving force of pubertal growth spurt (Cutler, 1997). In adulthood, estrogen is crucial for maintaining bone destiny. A young adult male with homozygous disrupted ERα gene has normal masculinization, but incomplete epiphyseal closure, tall stature (204 cm, 80.3 in), and decreased bone mineral density (Sudhir et al., 1997). In premenopausal women, estrogen exposure is strongly associated with bone mass (Armamento-Villareal et al., 1992). The deficiency of estrogen in postmenopausal women leads to an increased risk of osteoporosis and estrogen intake can alleviate bone loss (Ettinger et al., 1985). The Women's Health Initiative also confirmed that HRT protect against hip fracture in postmenopausal women (Rossouw et al., 2002). The mechanism for the effects of estrogen in bone is still not entirely understood. Estrogen suppresses the bone-resorption activity of osteoclasts (Oursler et al., 1991). Furthermore, estrogen enhances calcium intake in the intestine (O'Loughlin and Morris, 1998), while reducing calcium excretion in kidney (Dick et al., 2004).

1.2.2.4 Nervous system and estrogen

As a sex hormone, estrogen plays important roles in sexual differentiation in the brain and reproductive behavior (Breedlove, 1992; McCarthy, 1994; Bakker et al., 2003) However, the impact of estrogen on the nervous system go far beyond reproductive functions. Sustained estrogen treatment improves learning ability, memory and fine motor skill in animal models (O'Neal et al., 1996; Lacreuse and Herndon, 2003). Estrogen promotes neural cell survival against a plethora of stresses, including hypoxia, excitotoxicity, oxidative stress, glucose deprivation and amyloid-β peptide (Goodman et al., 1996; Regan and Guo, 1997; Wang et al., 2001b). Brain injury can be alleviated by estrogen in an ERα-dependent manner (Dubal et al., 2001). Estrogen stimulates neurite outgrowth and increases dendritic spine density (Brinton et al., 1997; Murphy et al., 1998). Moreover, estrogen inhibits the central nervous system (CNS) inflammation by downregulating inflammatory factors and preventing the recruiting of inflammatory cells in the CNS (Ito et al., 2001; Matejuk et al., 2001).

It has been hypothesized that estrogen can prevent and alleviate the neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. However, clinic trials have reported conflicting results and further studies are required to resolve this issue (Henderson, 1997; Miller et al., 2001; Shulman, 2002). Estrogen may have a role in protecting against schizophrenia, but again, this hypothesis is controversial (Huber et al., 2001)

1.2.3 The genomic actions of estrogen receptor

1.2.3.1 General transcription

In normal eukaryotic cells, RNA polymerase II (Pol II) is responsible for the transcription of protein-encoding genes. However, numerous other factors are also required for transcription and these factors, along with Pol II, are termed "general transcriptional machinery". Core promoters are the minimal DNA sequences that are essential for basal transcriptions and they located around the transcription initiation site and frequently contain some consensus DNA motifs, such as TATA element, TFIIB-recognition element (BRE), Initiation element (Inr) and downstream promoter element (DPE) (Smale and Kadonaga, 2003). Basal transcriptional activity can be modified by the binding of site-specific regulatory proteins to cis-elements proximal to core promoter.

Among all the core promoter motifs, the TATA element is the most studied. Thus TATA-containing promoter serves as a model for transcription initiation. In TATA-containing promoters, upon the binding of transcription factors in regulatory regions, TBP (TATA-binding problem) binds to the TATA element with or without TAFs (TBP-associated factors). TBP and TAFs together form the general transcription factor TFIID. This event is followed by the sequential recruitment of TFIIB, Pol II, TFIIF, TFIIE, and TFIIH. The resulting complex is usually referred to as the preinitiation complex (PIC), which induces DNA melting in the core promoter and starts transcription. TFIIH then phosphorylates the C-terminal domain (CTD) of Rpb1, a subunit of Pol II. The phosphorylation of CTD leads to destabilization of PIC and progression into elongation phase (Nikolov and Burley, 1997) (Cosma, 2002; Hahn, 2004).

In eukaryotic cells, DNA is associated with proteins to form the tightly compact structure named chromatin. Access to DNA and subsequent transcription are only made possible by “relaxation” on this tight structure. Chromatin is organized into repeating structures of 200 bp of DNA associated with highly conserved proteins named histones, including H1, H2A, H2B, H3 and H4. The core of this structure is nucleosome, which consists of two superhelical DNA loops wrapped around an octmer composed of two of H2A, H2B, H3 and H4 each (Arents et al., 1991). H1 histone and its variant H5 associate with the DNA that links the nucleosomes and are important for higher order of packing (Carruthers et al., 1998; Widom, 1998). Electron microscopy reveals nucleosome structures as 10 nm chromatin fiber with a “beads-on-a-string” appearance. Additional folding packs the 10 nm chromatin fibers into 30 nm chromatin fiber. More folding processes can condense chromatin into more compact forms, eventually giving rise to the highly packed metaphase chromosomes (Albert et al., 1994; Russell, 1998) (Fig. 8).

The relaxation of chromatin structure is essential to grant the access of DNA to Pol II and other components of the general transcriptional machinery. Two distinct mechanisms have been employed in cells to remodel chromatin: the covalent modification of histones and the ATP-dependent histone-DNA dissociation.

Histones can undergo acetylation, methylation, phosphorylation and ubiquitination. These post-translational modifications are important in regulation of chromatin structure. The best characterized histone modification is acetylation. Histone acetyltransferases (HATs) acetylate lysine residues at N-terminus of core histones. This acetylation facilitates binding of transcription factors to their cognate DNA motifs (Vettese-Dadey et al., 1996) and disrupts higher-order chromatin structure (Tse et al., 1998). Conversely, histone deacetylases (HDACs) reverses the acetylation and repress gene expression (Grunstein, 1997; Kouzarides, 2000).

Another important histone modification is methylation. Both lysine and arginine residues of histones can be methylated (Zhang and Reinberg, 2001). Histone methylation has been linked with both transcriptional activation and repression (Nielsen et al., 2001; Wang et al., 2001a). Until recently, no histone demethylase has been identified, leading to the speculation that histone methylation may be an irreversible step. However, a

recent study identified a nuclear amine oxidase homolog LSD1 that specifically demethylates Lys4 on histone H3 (Shi et al., 2004). Chromatin structure can also been affected by other forms of histone modifications, such as phosphorylation (Wei et al., 1999) and ubiquitination (Wang et al., 2004).

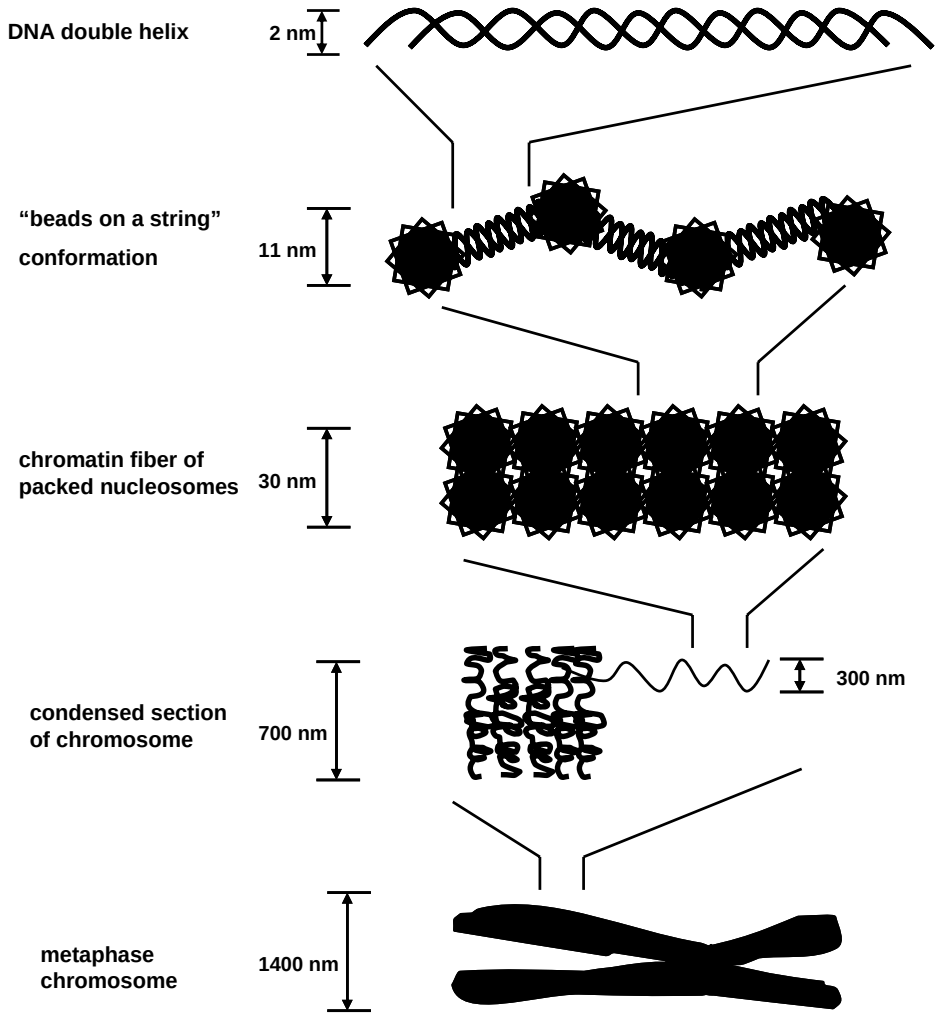

Fig. 8. The different stages of chromatin packing. Adapted from Albert et al. (1994).

ATP-dependent chromatin remodeling complexes reposition histones and alter the accessibility to DNA utilizing the energy from ATP hydrolysis (Martens and Winston, 2003). These complexes can be divided into several subfamilies, including SWI (switch)/SNF (sucrose nonfermenting), ISWI (imitation switch)(Lemon et al., 2001) and

Mi-2/NURD (nucleosome remodeling and deacetylation) (Knoepfler and Eisenman, 1999). The ATP-dependent chromatin remodeling usually elevates gene expression, but Mi-2/NURD actions are frequently associated with gene repression (Solari and Ahringer, 2000). The ATP-dependent chromatin remodeling and covalent modification of histones are not mutually exclusive. These two distinct mechanisms can cooperate in regulating gene expression (Cosma et al., 1999).

1.2.3.2 ER-mediated transcription

In the classical model of ER action, ER exists in the cell nucleus (King and Greene, 1984; Press et al., 1989) and forms a complex with heat shock protein 90 (hsp90). Hsp 90 protects unliganded ER from degradation and keeps ER in an inactive state (Chambraud et al., 1990). Most ER ligands, such as E2, are small hydrophobic molecules and can enter cells easily and bind ER. Upon ligand binding, the ER undergoes a conformational change, which results in dissociation from hsp90, dimerization and binding to regulatory regions of E2-responsive promoters (Tsai and O'Malley, 1994). The consensus ERE GGTCAnnnTGACC was identified in the in *Xenopus* vitellogenin A2 gene promoter (Klein-Hitpass et al., 1988). The specificity of ER-ERE binding is not strict because ER can also bind nonconsensus EREs or even ERE half-sites (Berry et al., 1989; Wang et al., 1997). ER-ERE binding affinities may be influenced by flanking regions and additional regulatory factors, such as heat shock protein 70 (Landel et al., 1994; Driscoll et al., 1998). ER-DNA interactions are accompanied by subsequent recruitment of coactivators, interaction with the preinitiation complex and activation of gene expression (Tsai and O'Malley, 1994) (Fig. 9). The level of ER-mediated gene expression is determined by many factors, such as the estrogenic potency of the ligands, posttranslational modifications especially phosphorylation of ER, the availability of accessory proteins and cofactors (Katzenellenbogen et al., 1996).

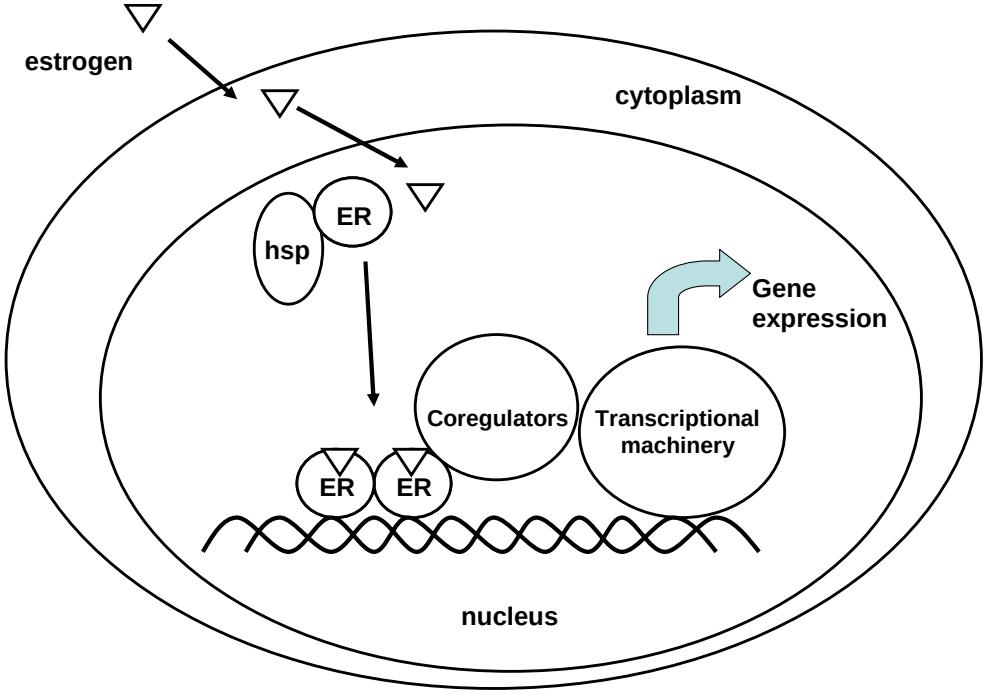

Fig. 9. The classic model of ER action

ER interacts with a number of basal transcriptional factors, including TFIIB (Ing et al., 1992), $TAF_{II}30$ (Jacq et al., 1994) and $TAF_{II}28$(May et al., 1996). Furthermore, ER recruits numerous coregulatroy proteins which function as bridging modules between nuclear receptors and the general transcription machinery. These proteins can be divided into two categories, coactivators, whose interactions with ER enhance transcription, and corepressors, whose interactions with ER repress transcription.

Numerous coactivators enhance ERα transactivation and these include CBP/p300, PCAF (p300/CBP-associated factor), SRC-1/NCoA-1/ERAP-160, SRC-2/GRIP1/NCoA-2/TIF2, SRC-3/ACTR/RAC3/pCIP/AIB1, RIP-140, SWI/SNF, p68, L7/SPA and E2-AP (Smith et al., 1996; Voegel et al., 1996; Klinge, 2000). Some coactivators, including SRC-1 and CBP/p300, require E2-bound ERα (Halachmi et al., 1994) while other coactivators, such as L7/SPA, are stimulated by tamoxifen-bound ERα (Jackson et al., 1997). Coactivators, such as CBP/p300, SRC-1 and PCAF, possess intrinsic HAT activities. The chromatin-remodeling complex SWI/SNF can also be recruited by E2-

bound ERα as coactivators (Ichinose et al., 1997). These properties help explain the ability of coactivators to enhance transcription (Bannister and Kouzarides, 1996; Spencer et al., 1997). Knocking out SRC-1 in mice can hamper the effects of estrogen on many target organs, including uterus, mammary gland, prostate and testis (Xu et al., 1998).

ERα corepressors include SMRT, NCoR, REA, SHP and BRCA1 (Chen and Evans, 1995; Horlein et al., 1995; Seol et al., 1998; Fan et al., 1999; Montano et al., 1999). Corepressors can recruit proteins with histone deacetylases (HDAC) activity and suppress gene expression (Alland et al., 1997). Levels of coactivators and corepressors differ in different cell types and this differential expression may partially account for the tissue and cell-context specific action of certain ER-ligands (Shibata et al., 1997).

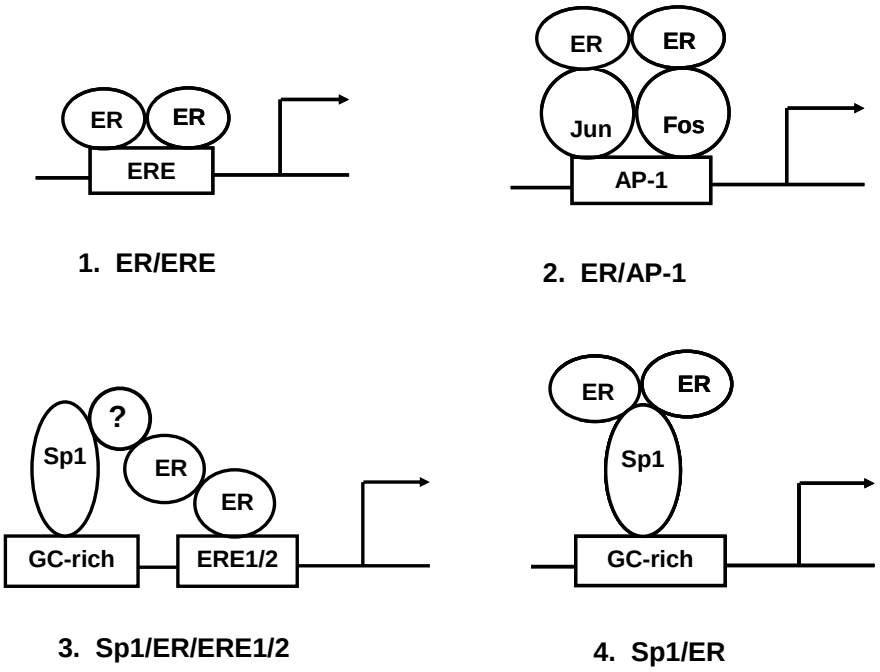

Fig. 10. Genomic models of ER actions

ER also activates target genes through protein-protein interaction. By association with certain transcription factors, such as activator protein 1 (AP1) (Tzukerman et al., 1991;

Webb et al., 1995) or Sp1 protein (Krishnan et al., 1994; Porter et al., 1996; Vyhlidal et al., 2000), ER can activate genes without directly binding DNA (Fig. 10).

The aforementioned actions of ER are all mediated by the nuclear ER. It has been found that a small percentage of ER may also reside in cytoplasm and cell membrane. Estrogen can rapidly elicit a broad spectrum of kinase cascades by activating an extracellular ER. Some studies also suggest the existence of ER inside the mitochondria. Estrogen treatment increases the localization of ER to mitochondria and induces the transcription of several mitochondrial genes, such as cytochrome oxidase subunits I, II, and III. These responses may be due to the binding of ER to the ERE in mitochondrial DNA (mtDNA) (Chen et al., 2003; Chen et al., 2004b).

Although ER is usually associated with activation of gene expression, in some cases ER can inhibit gene expression through diverse mechanisms. ER represses interleukin-6 transcription by interfering with NF-kB binding to the NF-kB site in interleukin 6 promoter (Ray et al., 1997). The inhibition of quinone reductase (QR) expression by ER is mediated by ER binding to electrophile/antioxidant response element (EpRE/ARE) in the 5'-flanking region of QR gene. Antiestrogen relieves this inhibition, confirming that the repression is ER-mediated (Montano et al., 1998). ER represses prolactin-induced β-casein expression by direct interactions with the transcription factor STAT5 (Faulds et al., 2001). ER mediates inhibition of vascular endothelial growth factor (VEGF) expression in HEC1A endometrial cancer cells by ERα-Sp3 interactions (Stoner et al., 2000).

ER frequently modulates proliferative activities, but under certain conditions, ER also mediates apoptosis. For example, long-term estrogen-deprived MCF-7 cells undergo apoptosis upon treatment with E2 and this apoptosis is mediated by induction of Fas which can be inhibited by the antiestrogen ICI 182,780 (Song et al., 2001). In the colon cancer cell line COLO205, E2 induces apoptosis through ERβ, which may be the underlying mechanism for the epidemiological finding that postmenopausal hormone replacement therapy reduces in the risk of developing colon cancer (Qiu et al., 2002).

1.2.3.3 Phosphorylation of estrogen receptor

ER contains multiple phosphorylation sites that significantly modify ER activity. The estrogen-bound ERα exhibits increased phosphorylation on Ser104, Ser106 and especially on Ser118. Phosphorylation of these sites can enhance ERα transcriptional activity, although the kinases responsible have not been conclusively identified (Denton et al., 1992; Joel et al., 1995; Rogatsky et al., 1999; Lannigan, 2003). Estrogen binding is not essential for phosphorylation of ERα. In response to growth factors, ERα is phosphorylated on multiple sites, with Ser118 as a major target. Several kinases phosphorylate Ser118 of ERα and these include MAP kinase (Ali et al., 1993; Le Goff et al., 1994; Kato et al., 1995), the complex formed by TFIIH and cyclin-dependent kinase 7 (Chen et al., 2000) and PKC (Joel et al., 1995). Interestingly, not all PKCs have the same effects on phosphorylation of ERα. Ser122 in mouse ERα, which is equivalent of Ser118 of human ERα, can be phosphorylated by PKCδ, but not PKCα or PKCε (Lahooti et al., 1998). Ser118 phosphorylation leads to enhanced ERα transcriptional activity independent of ligand-binding (Kato et al., 1995).

Ser167 on ERα is also phosphorylated, leading to increased transcriptional activity of ERα and resistance to tamoxifen-induced apoptosis. This phosphorylation can be mediated by p90 ribosomal S6 kinase 1 (pp90rsk1, downstream kinase of MAPK) (Joel et al., 1998), casein kinase II (Arnold et al., 1995) and AKT (Campbell et al., 2001).

Src family tyrosine kinases p60c-src and p56lck phosphorylate ERα at Tyr537, which is important for DNA binding and dimerization (Castoria et al., 1993; Arnold et al., 1995). Pak1 (p21-activated kinase-1) phosphorylates Ser305 of ERα and this phosphorylation is crucial for transactivation functions of ER by PAK1(Wang et al., 2002). Additional phosphorylation can be achieved at Ser104 and Ser106 by CKD2 (Rogatsky et al., 1999) and at Ser 122 by PKC δ (Lahooti et al., 1998). Not all phosphorylation increase ERα-dependent activity. For example, PKA-mediated phosphorylation of Ser236 leads to inhibition of ERα dimerization (Chen et al., 1999).

Like ERα, ERβ activity can also be modulated through phosphorylation. For example, MAPK phosphorylates Ser106 and Ser124 of ERβ and enhances the recruitment of steroid receptor coactivator-1 (SRC-1) (Tremblay et al., 1999).

1.2.4. The nongenomic actions of estrogen receptor

1.2.4.1 Mitogen activated protein kinase (MAPK)

Mitogen activated protein kinases (MAPKs) are important enzymes in signal transduction and are highly conserved among eukaryotes. Human MAPKs can substitute for their homologs in yeast in some cases (Atienza et al., 2000). In mammalian cells, MAPKs include extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), p38 kinase (CSBP) and big MAP kinase-1(ERK5/BMK-1). ERK primarily responds to mitogenic signals, while JNK and p38 are predominantly activated by stress signals and ERK5/BMK1 is activated through both pathways (Kyriakis and Avruch, 2001; Pearson et al., 2001).

Although MAPKs are a diverse group of kinases, they share an evolutionarily conserved model of activation, which consists of the sequential phosphorylation of three kinases. The MAPK kinase kinase (MAPKKK/MEKK) phosphorylates the serine/threonine residues on MAPK kinase (MAPKK/MEK). The phosphorylated MAPKK in turn activates MAPK through phosphorylation (Chang and Karin, 2001). A hallmark of MAPK is a dual-phosphorylation motif Thr-X-Tyr in the activation loop and both threonine and tyrosine phosphorylation are required for the full activity of MAPK (Kyriakis and Avruch, 2001). MAP kinase phosphatase (MKP) can dephosphorylate the threonine/tyrosine and thereby attenuate MAPK-dependent responses (Camps et al., 2000).

To date, the most studied MAPKs are ERK1 and ERK2, which serve as the prototype for understanding of MAPK signaling. For ERK1/2, the MAPKKKs are Rafs (A-Raf, B-Raf, and Raf-1) (Howe et al., 1992; Moodie et al., 1993) and Mos (Solhonne et al., 1999). Typically, membrane-associated receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs) transmit the activating signals to Ras through a guanine nucleotide exchange factor (GEF) named SOS (Son of Sevenless). Ras is a small GTP-

binding protein and in the resting state exists as a GDP-bound form. The association with SOS induces Ras to exchange GDP for GTP and the GTP-bound Ras in turn activates Rafs (Otsu et al., 1993). Other mechanisms for activation of Raf have also been identified. For example, Rap1 can mediate activation of B-Raf in a cAMP-dependent pathway (York et al., 1998; Rueda et al., 2002). G-protein-activated PKCα can directly phosphorylate and activate Raf-1(Kolch et al., 1993). Regardless of the activating mechanisms, activated Rafs phosphorylate two MAPKKs (MEK1 and MEK2), which in turn phosphorylate two MAPKs (ERK1 and ERK2). Other MAPKs share the same three-tiered kinase activation system, although the specific components can be variable. The specificities are summarized as Figure 11.

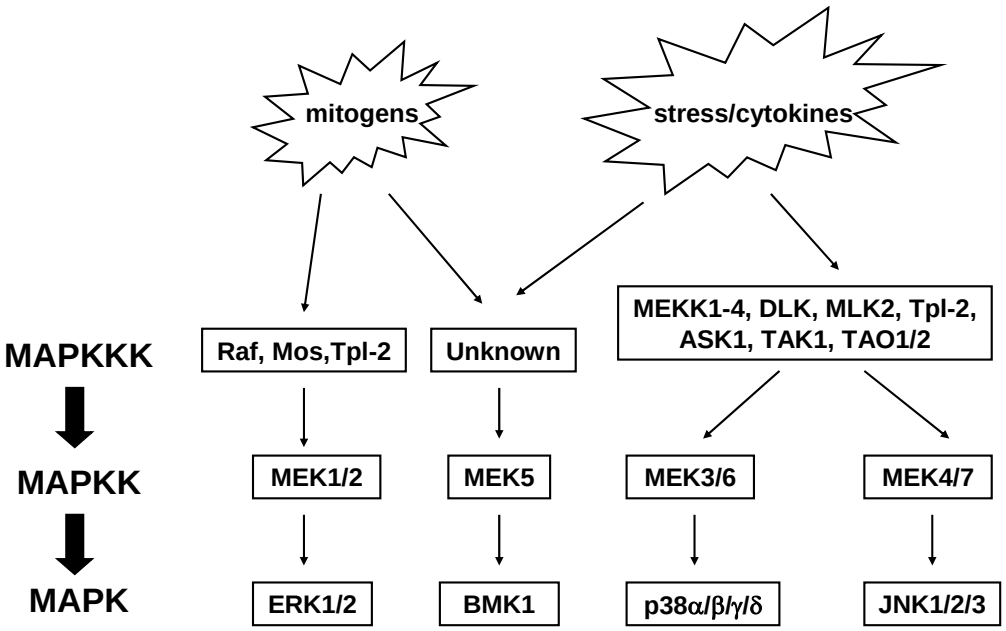

Fig. 11. MAPKs, MAPKKs and MAPKKKs. Adapted from Schaeffer and Weber(1999) and Roux and Blenis (2004).

Activated ERK1/2 phosphorylates a wide array of downstream effectors, such as transcriptional factors (Elk-1, SP1, STAT3)(Ng and Cantrell, 1997; Yang et al., 1998; Liu et al., 2001), transcriptional coregulatory proteins (CBP, SRC-1, DRIP205) (Liu et al., 1999; Rowan et al., 2000; Misra et al., 2002) and membrane proteins (CD120a, Syk,

calnexin)(Chevet et al., 1999; Xu et al., 1999; Van Linden et al., 2000). The targets of ERK1/2 also include several protein kinases that are involved cell cycle and apoptosis, such as RSK (p90 ribosomal S6 kinase or MAPK-activated protein kinase-1, MAPKAP-K1) (Dalby et al., 1998), MSK (mitogen- and stress-activated protein kinase) (Deak et al., 1998) and MNK (MAP kinase-interacting kinase) (Waskiewicz et al., 1997). ERK1/2 are important regulators of cell proliferations and are targets for development of anticancer drugs (Kohno and Pouyssegur, 2003). ERK1 knockout mice display delayed thymocyte maturation but no other major abnormalities, possibility due to the compensatory influence of ERK2 (Pages et al., 1999). In contrast, MEK1 knockout embryos failed to undergo normal angiogenesis in the placenta and died at 10.5 days after gestation (Giroux et al., 1999).

The p38 kinases have four isoforms (α,β,γ and δ) that respond to an array of stress signals, including reactive oxygen species (ROS), hypoxia, cytokines, heat shock, viral infection and ultraviolet irradiation (Chen et al., 2001b; Kyriakis and Avruch, 2001). Cell cycle progression can be arrested by p38 kinases at G1/S and G2/M checkpoints (Molnar et al., 1997; Tarn et al., 2002; Hirose et al., 2003). and p38 mediates apoptosis (Aoshiba et al., 1999; Kumar et al., 2004; Van Laethem et al., 2004) and inflammation (Dean et al., 1999). p38α knockout mice have defective angiogenesis in the placenta, yolk sack and embryo itself and this results in embryonic death (Mudgett et al., 2000).

The JNK family is also known as SAPK (stress-activated protein kinase) due to its reference to its response to stress and cytokines. To date, three members of the JNK family have been identified, namely JNK1 (SAPKγ), JNK2 (SAPKα) and JNK3 (SAPKβ) (Roux and Blenis, 2004). JNKs have been implicated in maintaining the immune system and regulating apoptosis. Mice with a single gene disruption on JNK1, JNK2, JNK3 or double gene disruptions on JNK1/JNK3 or JNK2/JNK3 all have normal phenotypes, but JNK1/JNK2 double knockout died before birth due to dysregulation in neuronal apoptosis (Kuan et al., 1999).

Estrogen activates ERK1/2 in MCF-7 breast cancer cells in an ER-dependent manner (Migliaccio et al., 1996). Estrogen-induced ERK activity has also been found in

osteoblastic and neuroblastoma cells and primary cortical neurons (Endoh et al., 1997; Watters et al., 1997; Singer et al., 1999). Activation of ERks by E2 involves Ras (Lu and Giguere, 2001), however, the upstream components of this pathway remain unknown. Other MAPKs, such as JNK and p38, can also be activated via ER upon estrogen treatment (Lee et al., 2001; Prifti et al., 2001). Activation of p38 by E2 may inhibit cell growth by inducing apoptosis in vascular smooth muscle cells (Mori-Abe et al., 2003). Tamoxifen can activate JNK in both ER-positive and negative cells and this leads to increased apoptosis (Mandlekar et al., 2000).

One model for E2-dependent activation of ERK involves an interaction between ER and Src through adaptor proteins. An adaptor protein called modulator of nongenomic activity of estrogen receptor (MNAR) has been identified and this protein bridges the interaction of ER with Src (Wong et al., 2002). MNAR contained two LXXLL motifs, which interact with ER, and one PXXP motif, which interacts with the SH3 domain of Src. By bridging Src and ER, MNAR mediates activation of Src by ligand- bound ER and subsequent activation of downstream kinase targets, including MAPK (Barletta et al., 2004). Other cellular components may also be involved in forming the ER-Src complex and these include Shc and cytoskeletal protein p130Cas (Song et al., 2002; Cabodi et al., 2004). ER coupling with G-proteins is another possible mechanism for activation of MAPK by E2 (Razandi et al., 1999).

Another study proposed an autocrine/paracrine model for MAPK activation by E2. In this model, HRG (Heregulin) is synthesized and secreted into extracellular environment upon E2 stimulation. HRG binds to ErbB2 (HER-2), a member of epidermal growth factor receptor family. ErbB2 in turn activates PKCδ, which activates Ras and initiates downstream MAPK signaling (Keshamouni et al., 2002).

A G-protein coupled receptor, GPR30, is capable of mediating estrogen-induced ERK activity independent of ER. GPR30 not only modulates activation of ERKs by estrogen, but it is also involved in subsequent deactivation of ERKs. E2-induced ERK activity was rapidly reduced to basal levels by Raf-1 inactivation through GPR30-induced cAMP signaling (Filardo et al., 2000; Filardo et al., 2002). In ER-negative SKBR3 breast cancer cell line, GPR30 responds not only to E2, but also to antiestrogens (ICI 182780

and tamoxifen) and xenoestrogen (o, p'-DDE) (Filardo et al., 2000; Thomas et al., 2004).

ERβ also activates MAPK. By transfecting ERα or ERβ into ER-negative Rat-2 fibroblast cells, MAPK can be activated upon E2 or E2-BSA treatment. Interestingly, the antiestrogen ICI 182,780 inhibits ERα-initiated MAPK activity but has no effects on MAPK activation through ERβ (Wade et al., 2001)

1.2.4.2 Phosphatidylinositol 3- kinase (PI3K)

Phosphatidylinositol 3- kinase (PI3K) phosphorylates the 3' hydroxyl group of inositol ring on inositol phospholipids, especially phosphatidylinositols (PtdIns) (Fig. 12). PI3Ks include three distinct classes: Class I, II and III. Class I PI3Ks serve as prototypes for PI3Ks and can be further divided into two subclasses, Class IA and IB. Both classes are heterodimers consisting of a 110 kDa catalytic subunit and a regulatory subunit.

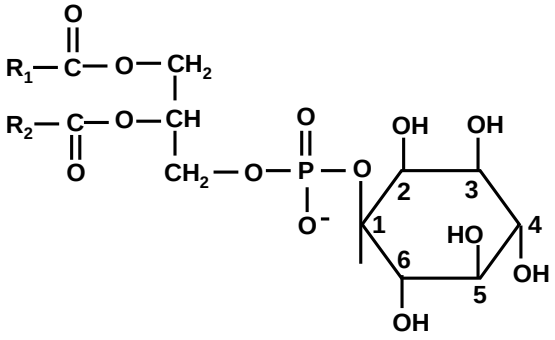

Fig.12. Phosphatidylinositol (PtdIn).

Class IA PI3Ks are predominantly activated by tyrosine kinases and have three catalytic (p110α, p110β and p110δ) (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997) and three regulatory isoforms (p85α, p85β and p55γ) (Otsu et al., 1991; Pons et al., 1995). The p85α isoforms have two alternative splicing variants p50α and p55α (Inukai et al., 1996; Inukai et al., 1997). Class IB PI3Ks, which are activated by G-proteins, have only one catalytic subunit (p110γ) (Stoyanov et al., 1995) and one regulatory subunit (p101) (Stephens et al., 1997). Despite their similarity, the Class I PI3Ks are not functionally redundant. For instance, p110α knockout mouse embryos

experience a proliferative defect and developmental delay, leading to embryonic lethality (Bi et al., 1999). In contrast, p110γ knockout mice are viable but have a defective immune response (Hirsch et al., 2000).

Unlike heterodimeric Class I PI3Ks, Class II PI3Ks are single molecules. The most noticeable feature of Class II PI3Ks is their C-terminus C2 domain, which can bind phospholipids. However, the C2 domain of Class II PI3Ks lacks the Ca^{2+}-binding capacity, which is a common function of C2 domains in other proteins (MacDougall et al., 1995; Arcaro et al., 1998). The precise functions of Class II PI3Ks are still unclear.

Class III PI3K have only one identified catalytic subunit in mammals and share homology with the yeast kinase Vps34p, which is involved in vesicular protein sorting. The main function of Class III PI3K is to modulate intracellular trafficking (Volinia et al., 1995).

The major cellular substrate of Class I PI3K is PtdIns (4,5)P_2 (PIP_2), which is a phosphatidylinositol (PtdIn) phosphorylated at 4D and 5D positions of the inositol ring. PI3K phosphorylates PIP_2 at the 3D position of the inositol ring, converting it into PtdIns(3,4,5)P_3 (PIP_3). PIP_3 locates on the cytosolic side of the cell membrane and recruits AKT (also called PKB, protein kinase B) from the cytosol to the plasma membrane by interacting with the Pleckstrin homology (PH) domain of AKT (Salim et al., 1996). The translocation of AKT brings it into close proximity to another membrane-bound kinase named 3-phosphoinostitide-dependent protein kinase -1 (PDK1), which activates AKT by phosphorylation of Thr308 (Alessi et al., 1997; Vanhaesebroeck and Alessi, 2000). There is evidence that another kinase, PDK2, may further phosphorylate AKT at Ser473 and enhance its activity, however, the exact nature of PDK2 remains unclear (Toker and Newton, 2000; Hresko et al., 2003).

AKT is a cellular homolog of akt retrovirus oncogene (Bellacosa et al., 1991). In vertebrates, there are three AKT family members: AKT1, AKT2 and AKT3. These there isoforms share similar structures and functions but are regulated differently (Okano et al., 2000). Knockout models suggest that AKT isoforms are not functionally equivalent. Akt1 knockout mice have smaller size, impaired growth and increased apoptosis upon

chemical insults (Chen et al., 2001a). In contrast, AKT2 knockout mice develop insulin resistance (Cho et al., 2001)

AKT is an important effector of PI3K and mediates many important functions of PI3K. Many downstream targets of AKT regulate cell survival. AKT phosphorylates BAD and prevents its inhibitory association with anti-apoptotic factor BCL-X_L (Datta et al., 1997). AKT also inhibits the apoptotic transcription factor FKHR (Brunet et al., 1999) and the apoptotic protease caspase-9 (Cardone et al., 1998). Survival factor NFκB is activated by AKT through activating IκB Kinase (IKK), which induces the degradation of NFκB inhibitor IκB (Romashkova and Makarov, 1999). AKT negatively regulates the tumor suppressor p53 by activating MDM2 and enhancing p53 degradation (Mayo and Donner, 2001). Furthermore, AKT inhibits glycogen synthase kinase-3 (GSK-3), an apoptosis-inducing kinase (Cross et al., 1995) and activates the anti-apoptotic p70S6 kinase (RSK) (Burgering and Coffer, 1995). Another important target activated by AKT is mTOR (the mammalian target of rapamycin), which is a kinase that integrates the signals of nutrient availability and enhances protein synthesis upon activation (Nave et al., 1999) (Fig. 13).

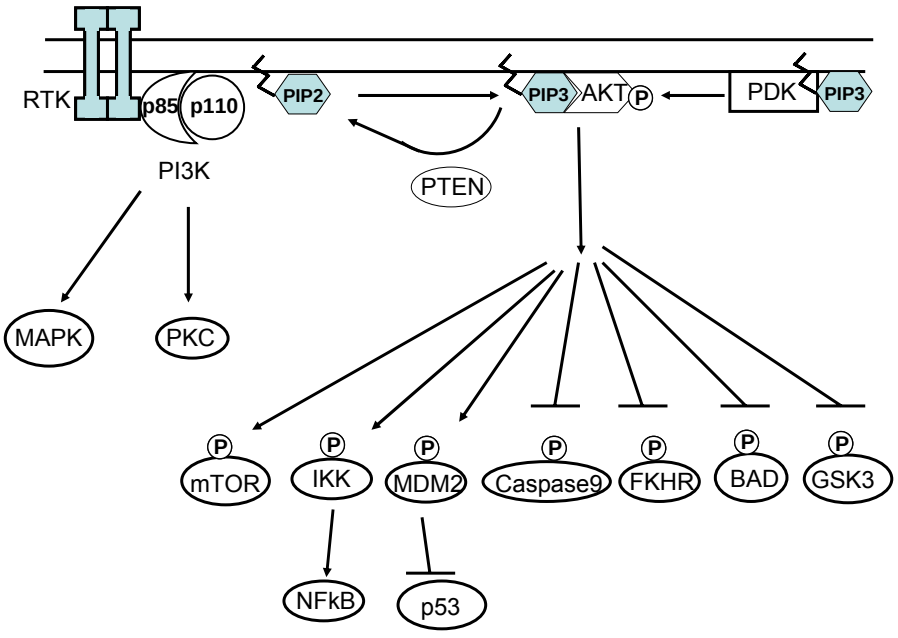

Fig. 13. Roles of PI3K in cellular signaling. Adapted from Vivanco and Sawyers (2002).

Although AKT is a major downstream target of PI3K, it can not account for all the actions of PI3K. AKT and PI3K knockout mice exhibit significantly different phenotypes as indicated above, supporting the notion that other factors also play roles in mediating PI3K function (Bi et al., 1999; Hirsch et al., 2000; Chen et al., 2001a; Cho et al., 2001). This is corroborated by the finding that PI3K can regulate the Ras/Raf/ERK pathway as a serine-threonine protein kinase, in contrast to its usual role as a lipid kinase (Bondeva et al., 1998). Protein kinase C (PKC) can be activated by the PI3K product PIP_3 or the PI3K downstream kinase PDK1 (Le Good et al., 1998; Wymann and Pirola, 1998). Furthermore, PI3K can phosphorylate insulin receptor substrate-1(IRS-1) and activate insulin pathways (Lam et al., 1994).

The activity of PI3K is modulated by a series of phosphatases that dephosphorylate PIP_3 and these include the phosphatase and tensin homolog (PTEN), which is mutated in multiple advanced cancers and also called MMAC1 or TGFβ-regulated and epithelial cell-enriched phosphatase (TEP1). The central role of PTEN is to dephosphorylate the 3D position of inositol ring in PIP_3 to give PIP_2 and thereby functioning as a negative regulator of PI3K activity. Overexpression of PTEN results in significantly decreased levels of PIP_3 and decreased AKT activity (Inukai et al., 1997; Maehama and Dixon, 1998; Cantley and Neel, 1999). In fact, PTEN was originally identified as a tumor suppression gene even before its function as phosphatase was determined. The tumor suppressing function of PTEN is attributed to its phosphatase activity (Myers et al., 1998) and PIP_3 appears to be the most important target of PTEN in terms of inhibiting mitogenesis (Tamura et al., 1998). Another phosphatase named Src-homology-2-containing inositol 5'-phosphatase (SHIP) can also dephosphorylate PIP_3, however, the dephosphorylation occurs on the 5D position of inositol ring instead of the 3D position (Ware et al., 1996).

To date, the most studied model for PI3K activation is the Class IA PI3K, which consists of regulatory a subunit p110 and a catalytic subunit p85. The p85 subunit contains several important domains, including one Src-Homology-3 domain (SH3), two proline rich domains (PRDs), two Src-Homology-2 domains (SH2s) and one breakpoint cluster

region homology domain (BCR). The SH2 domain functions as a docking point that binds phosphorylated tyrosine residues in both receptor tyrosine kinases and adaptor proteins. In the inactive state, p85 constitutively binds to p110 via a region named iSH2 domain (inter SH2) because it is located between the two SH2 domains. In this manner, p85 recruits and anchors p110 to cell membrane. This association inhibits the catalytic activity of p110 until the SH2 domains of p85 interact with phosphorylated tyrosine residues and release p110 (Yu et al., 1998). The PRD motif has an affinity for SH3 domains and can mediate the association of p85 with several Src family kinases, such as src, lck, lyn and fyn (Liu et al., 1993; Vogel and Fujita, 1993; Kapeller et al., 1994; Pleiman et al., 1994). The association between p85 and Src family kinases is also an important pathway of PI3K activation (Pleiman et al., 1994). p85 also contains a SH3, which can interact with the PRD domain prevent activation of PI3K without proper stimulation (Kapeller et al., 1994). Another pathway for PI3K activation is by activating p110 catalytic subunit through Ras binding (Rodriguez-Viciana et al., 1994). The activated p110 in turn phosphorylates p85 and inhibits the PI3K activity. This negative feedback helps maintaining basal PI3K activity (Dhand et al., 1994). The p85 subunit binds to small G-proteins Rac1 and Cdc42 though the BCR domain (also named rho-GAP homology region) and thereby functions a direct activator of G-proteins (Zheng et al., 1994; Tolias et al., 1995)..

In contrast to Class IA PI3Ks that are usually activated by tyrosine kinase receptors, Class IB PI3K is generally activated through stimulation of its catalytic subunit p110γ by the β and γ subunits of heterotrimeric G-proteins (Stoyanov et al., 1995). It has also been suggested that Class IA PI3Ks can also be activated by β and γ subunits and this requires further investigation (Kurosu et al., 1997).

PI3K is a downstream target of estrogen in MCF-7 breast cancer cells and cultured hippocampal neurons (Yokomaku et al., 2003). Moreover, in vascular endothelial cells, E2 induces eNOS in an ERα-dependent manner via the AKT pathway (Hisamoto et al., 2001; Simoncini et al., 2002). Activation of PI3K by estrogen may be mediated by the association between ERα and p85, the PI3K regulatory subunit. The ligand-binding domain of estrogen-bound ERα binds to p85, relieving the inhibitory effects of p85 and

activating p110. Src may also be a component of this ERα-p85 complex by functioning as an enhancer of ERα-p85 action (Castoria et al., 2001; Sun et al., 2001).

There is evidence that PI3K activation by estrogen can also occur in the absence of ER. For example, estrogen activates PI3K in ER-negative MDA-MB-435 and MDA-MB-231 breast cancer cell lines and this activation can be inhibited by Src kinase inhibitor PP2 but not by antiestrogen ICI 182,780, suggesting that ER-independent pathway exists for PI3K activation (Tsai et al., 2001)

1.2.4.3 Protein Kinase A (PKA)

PKA (protein kinase A) is an important component in cell signaling and involved in apoptosis, differentiation, neuronal transmission and cell proliferation (Ghirardi et al., 1992; Indolfi et al., 1997; Yao et al., 1998; Saavedra et al., 2002). Activation of PKA is mediated through the second messenger cyclic adenosine 3',5'-monophosphate (cAMP) upon environmental stimuli. In the absence of cAMP, PKA is a tetramer consisting of two catalytic (C) subunits and two regulatory (R) subunits. There are three isoforms of C subunits (Cα, Cβ and Cγ) and four isoforms of R subunits (RIα, RIβ, RIIα and RIIβ). The R subunits repress C subunits and inhibit kinase activity. Stimulating signals activate membrane-associated G protein coupled receptor (GPCR), which in turn activates adenylate cyclase. Second messenger cAMP is produced by adenylate cyclase from ATP and binds to R subunits, causing their dissociation from C subunits to give the active kinase (Chin et al., 2002) (Fig. 14). Phosphodiesterases (PDEs) can degrade cAMP and function as negative regulators of PKA (Conti et al., 1995). The downstream targets of C subunits include CREB family transcription factors, NFkB and nuclear receptors (Daniel et al., 1998). At least two mechanisms contribute to the specificity of PKA pathways. One is the differential C and R isoform distribution in various cell types (Skalhegg and Tasken, 2000) and the other is the association of PKA with a scaffold protein called A kinase anchoring protein (AKAP)(Coghlan et al., 1995).

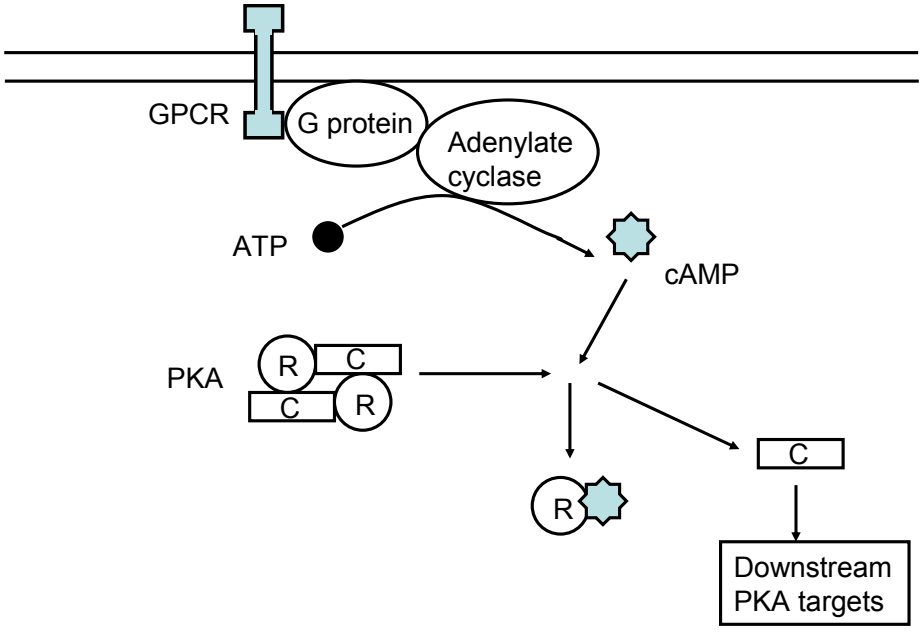

Fig. 14. Mechanism of PKA activation.

PKA is the major mediator of cAMP signals; however, not all cAMP actions are PKA-dependent. A guanine exchanging factor Epac can directly associate with cAMP and activate small G-protein Rap1 independent of PKA (de Rooij et al., 1998). Conversely, not all the effects of PKA involve cAMP. The C subunit of PKA can associate with NFkB-IkB complex and regulate NFkB activity independent of cAMP. In response to cytokines or inflammatory signals, IkB undergoes degradation and activates the C subunit to phosphorylate p65 subunit of NFkB (Zhong et al., 1997). Although predominantly an intracellular kinase, PKA can exist outside of cells either as proteins anchored to outer cell membranes or as a soluble form (Schlaeger and Kohler, 1976; Korc-Grodzicki et al., 1988; Hatmi et al., 1996). Interestingly, cancer cells have elevated secretion of PKA C subunits and extracellular PKA may serve as a biomarker for carcinogenesis (Cho et al., 2000a; Cho et al., 2000b).

PKA RIα-deficient mice demonstrate severe developmental defects and embryonic lethality (Amieux et al., 2002). In contrast, PKA RIIβ knockout mice are viable and have markedly diminished white adipose tissue and develop resistance against diet-induced obesity, fatty livers and diabetes (Cummings et al., 1996; Schreyer et al., 2001). Furthermore, PKA RIIβ knockout shows high voluntary ethanol consumption and low sensitivity to ethanol-induced sedation (Thiele et al., 2000). These differences in knockout phenotypes highlight the fact that the R isoforms are not functionally exchangeable.

PKA plays a regulatory role in mammary tumorigenesis. Cholera toxin (CT), a PKA activator, is capable of inducing breast cancer cell growth *in vitro* and *in vivo* (Sheffield and Welsch, 1985). The activation of PKA is associated with increased tamoxifen resistance in breast cancer cells (Michalides et al., 2004). PKA can prevent ubiquitin-proteasome-dependent ERα degradation induced by the ligand-binding (Tsai et al., 2004).

Estrogen rapidly induces cAMP levels and subsequently activates PKA in breast cancer and uterine cells through activation of adenylate cyclase (Aronica et al., 1994), however, the mechanism of estrogen-induced adenylate cyclase is still unknown. In rat pulmonary vascular smooth muscle cells, calcium removal blocked induction of cAMP by E2, suggesting that the intracellular calcium may have an important role. In contrast, the G-protein inhibitor, pertussis toxin, had no impact on E2-induced cAMP level (Farhat et al., 1996).

1.2.4.4 Protein kinase C (PKC)

Protein kinase C (PKC) was first described as a protein kinase requiring calcium, phospholipids (especially phosphatidylserine), and diacylglycerol (DAG) for complete activation (Inoue et al., 1977; Takai et al., 1979). At least 12 different isoforms of PKCs have been identified and divided into three classes: conventional PKCs (α, β and γ), novel PKCs (δ, ε, η and θ) and atypical PKCs (ζ, ι and λ). Conventional PKCs are activated by diacylglycerol, phospholipid, and Ca^{2+}. Novel PKCs are activated by diacylglycerol and phospholipid, but not by Ca^{2+}. Atypical PKCs are activated only by

phospholipid, not by diacylglycerol and Ca^{2+}. Protein kinase C μ (PKCμ) and its mouse homolog protein kinase D (PKD) that share a certain degree of homology with PKCs (Johannes et al., 1994; Valverde et al., 1994) and they are usually termed PKC-related kinases.

PKCs have a modular structure, with an N-terminus regulatory domain and a C-terminus catalytic domain. Both domains consist of several relatively conserved regions (C) interspersed by several variable regions (V). In conventional PKCs, the N-terminus locates a sequence that suppresses the catalytic domain in the absence of cofactors and activators. This sequence mimics the PKC substrate but lacks the phosphorylation site, and is also called pseudosubstrate site or autoinhibitory domain (House and Kemp, 1987). The C terminus side of the pseudosubstrate site are two copies of conserved C1 region, which are cysteine-rich motifs and responsible for phorbol ester binding (Kaibuchi et al., 1989). Next to the C-terminus of double-C1 region is another conserved region C2, which binds to Ca^{2+} and phospholipid (Shao et al., 1996). The catalytic domain at the C-terminus includes another two conserved domain C3 and C4. C3 is the ATP binding site and essential for kinase activity. C4 is the substrate binding site and is blocked by pseudosubstrate site when PKC is inactive (Newton, 1997).

In general, the structure of novel PKCs are very similar to conventional PKC. The major difference is that novel PKCs do not have a C2 region. Instead, they have a C2-like region on the N-terminus side of C1. This C2-like region shares significant homology with C2 but cannot bind to Ca^{2+}. The lack of Ca^{2+}-binding C2 regions explains why novel PKCs are insensitive to Ca^{2+} (Sossin and Schwartz, 1993). The atypical PKCs have only one copy of the C1 region instead of the two copies in conventional and novel PKCs. Like novel PKCs, atypical PKCs also possess a C2-like region to the N-terminus of C1 (Mellor and Parker, 1998) (Fig. 15).

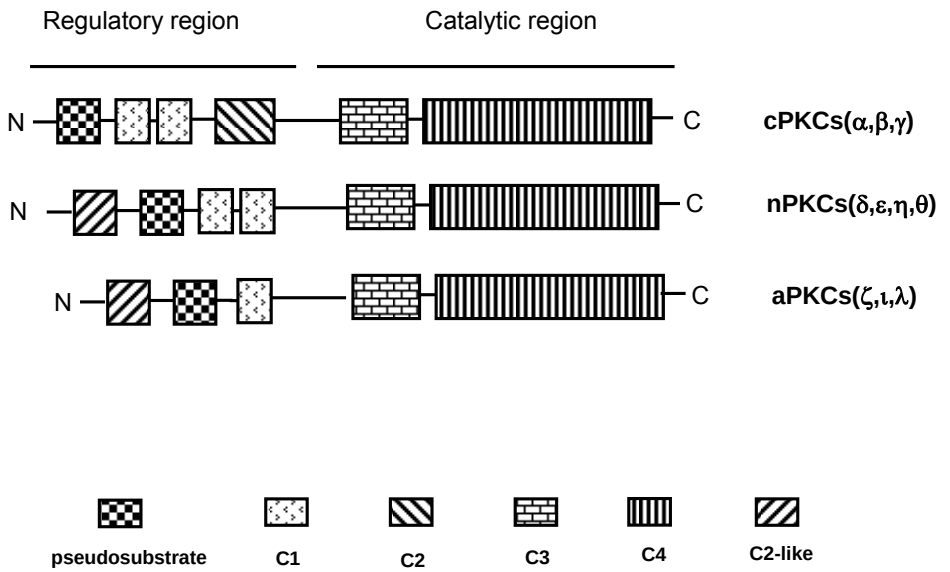

Fig. 15. Structure of PKCs. Adapted from Webb et al. (2000).

The activation of conventional PKCs is usually initiated by stimulation of phospholipase C (PLC) through G-protein coupled receptors, receptor tyrosine kinases or non-receptor tyrosine kinases. The phospholipase C cleaves the PtdIns(4,5)P_2 (PIP_2) into diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP3). Diacylglycerol interacts with PKC by displacing its pseudosubstrate and activating PKC. IP3 elevates the intracellular calcium levels by inducing release of calcium from intracellular calcium stores. By binding to calcium, DAG and PS (phosphatidylserine), conventional PKCs are able to achieve full activity (Nishizuka, 1984). The activation of PKC increases the calcium flux from extracellular sources and the increase in calcium enhances PKC activity (Nishizuka, 1992).

Some PKCs, such as PKCε, PKCη, PKCζ and PKC-related kinase PRK1, can be activated by phosphoinositides PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Nakanishi et al., 1993; Toker et al., 1994; Palmer et al., 1995). However, the biological relevance of this response is still unknown.

PKC modulates estrogen responsiveness and estrogen receptor levels in osteoblast-like cells (Migliaccio et al., 1993; Migliaccio et al., 1998). PKC can be activated by PDK1, the downstream target of PI3K (Chou et al., 1998; Le Good et al., 1998). Apoptosis is inhibited by PKCs in thymocytes and breast cancer cells (McConkey et al., 1989; Rajotte et al., 1992; Lu et al., 2004). The elevation of PKC activity was detected in breast cancer tissues compared to the surrounding normal tissues (O'Brian et al., 1989) and PKCα levels tends to be higher in breast tumors with low or no ERα expression (Lahn et al., 2004). Furthermore, stimulation of PKC greatly enhances the invasiveness of MCF-7 breast cancer cells (Johnson et al., 1999).

Although PKC has been identified as a target of E2 nongenomic actions, little is known about the mechanism (Kelly et al., 1999; Sylvia et al., 2001). Both G-protein inhibitor GDP beta S and phospholipase C inhibitor U73122 can block E2-induced PKC activity, suggesting that this process is dependent on G proteins as well as phospholipase C (Sylvia et al., 2001). Another study showed that E2 stimulates PKC in both ER-positive MCF-7 and ER-negative HCC38 cells through an ER-independent mechanism. This report also shows that phosphatidylinositol-dependent phospholipase C and G proteins are both involved. Interestingly, in this study, the antiestrogen ICI 182,780 did not block activation of PKC by E2 whereas tamoxifen inhibited activation (Boyan et al., 2003). In contrast, treatment of HepG2 cells with E2 rapidly increased intracellular IP_3 levels and activated PKCα and inhibition by antiestrogens suggests that ER is required in this response (Marino et al., 1998)

1.2.4.5 Calcium/calmodulin-dependent protein kinase IV (CaMKIV)

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) is a member of a group of kinase regulated by calmodulin and calcium. Calcium regulates many different aspects of cellular functions while still maintaining remarkable specificity. The compartmentalization of localized calcium signals is an important way to distinguish between diverse calcium signals and the different amplitudes and frequencies of calcium level oscillations also convey differential signaling information (Thomas et al., 1996).

For some calcium responsive proteins, such as conventional PKCs, the calcium signal can be directly detected by calcium binding. For others, calcium responsiveness requires the assistance of a calcium-binding protein. Calmodulin (CaM) is one of such proteins that can transduce the signal of increasing intracellular calcium to target molecules. In vertebrates, CaM is a highly conserved small protein of 148 amino acids that contains four helix-loop-helix motifs named EF hands, which are capable of calcium ion binding. The binding process is cooperative and this type of binding can confer tremendous calcium sensitivity to CaM (Chin and Means, 2000).

CaMKIV (CaMKIV Gr) functions as a monomer (Miyano et al., 1992) and is located mainly in the nucleus but also exist in granular endoplasmic reticulum in neuronal somata, dendritic processes and axons (Jensen et al., 1991; Nakamura et al., 1995). CaMKIV contains an autoinhibitory domain in the C-terminus, whose inhibitory effect can be relieved by Ca^{2+}/calmodulin binding (Tokumitsu et al., 1994). However, Ca^{2+}/calmodulin binding only has a limited activating effect for CaMKIV. For full activation, CaMKIV requires phosphorylation by CaM kinase kinases (CaMKKs). There are two CaM kinase kinases, namely CaM kinase I kinase-α (CaMKIKα) and CaM Kinase I kinase-β (CaMKIKβ). Upon stimulation by Ca^{2+} and calmodulin, both kinases activate CaMKI and CaMKIV through phosphorylation (Tokumitsu et al., 1995; Edelman et al., 1996). On rat CaMKIV, phosphorylation by CaMKIK occurs at Thr196 (Selbert et al., 1995) whereas human CaMKIV is phosphorylated at the homologous Thr200 (Chatila et al., 1996) (Fig. 16).

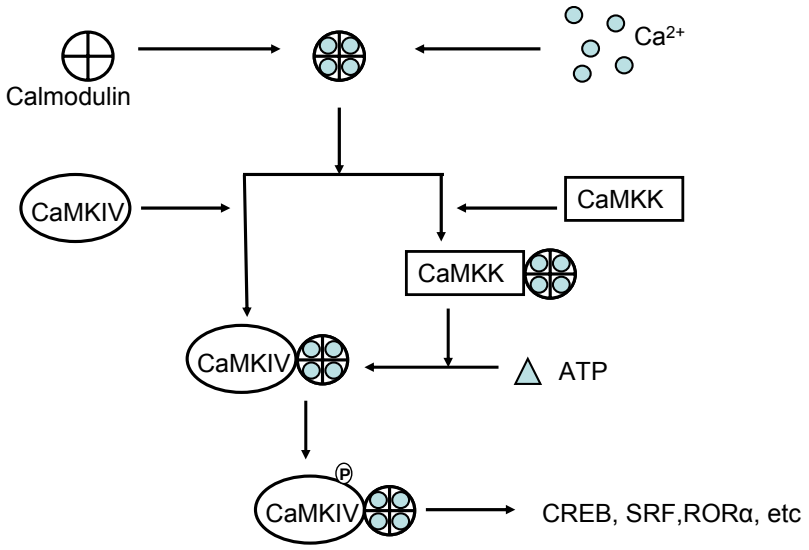

Fig. 16. Mechanism of CaMKIV activation.

Ser12 and Ser13 in the N-terminus of CaMKIV may also be involved in the autoinhibition of kinase activity and the inhibitory effect can be abolished by autophosphorylation on Ser12 and Ser13 once CaMKIV is activated by Ca^{2+} and calmodulin (Chatila et al., 1996) (Fig. 17). CaMKIV can also gain its Ca^{2+}/calmodulin-independent activity through self-phosphorylation within the putative calmodulin-binding domain (Watanabe et al., 1996). Activated CaMKIV can be attenuated by dephosphorylation by protein phosphatase 1 (PP1), protein serine-threonine phosphatase 2A (PP2A) and Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin) (Westphal et al., 1998; Kasahara et al., 1999).

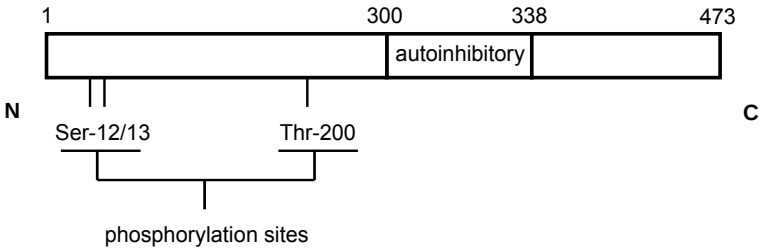

Fig. 17. Structure of CaMKIV.

CaMKIV is highly conserved and its homologs have been found in species ranging from mammals to *Aspergillus nidulans* (Joseph and Means, 2000). This degree of conservation suggests important functions for this protein. CaMKIV is involved in regulation of many transcription factors, including cAMP response element binding protein (CREB) (Matthews et al., 1994), activation protein 1(AP1) (Chatila et al., 1997) and serum-response factor (SRF) (Miranti et al., 1995). CaMKIV knockout mice have ataxia, impaired viability, defective Purkinje cell maturation and aberrant lymphocyte differentiation. The males are infertile due to impaired spermiogenesis and females have reduced fertility because of abnormal follicular development and ovulation (Wu et al., 2000a; Wu et al., 2000b). Furthermore, CaMKIV knockout mice have reduced memory for unpleasant events compared to wildtype (Wei et al., 2002). Overexpression of CaMKIV in transgenic mice induces mitochondrial biogenesis in muscle cells and enhances the endurance of muscles (Wu et al., 2002). CaMKIV protects granule neurons and T-lymphocytes from apoptosis (Anderson et al., 1997; See et al., 2001). CaMKIV has also been implicated in carcinogenesis and there is significant correlation of CaMKIV expression with malignancy in endometrial carcinoma (Shang et al., 2003).

Estrogen is known to cause a rapid increase of intracellular calcium levels (Morley et al., 1992). Presumably this process leads to the activation of CaM kinase. Qin and coworkers reported that estrogen can stimulate CaMKIV activity in MCF-7 breast cancer cells (Qin et al., 2002) and only CaMKIV but not CaMKII was activated by estrogen. This suggests a certain specificity in E2-dependnet calcium signaling.

Although estrogen increases intracellular calcium levels in many cell types, the mechanisms appear to be different. For example, in primary rabbit kidney proximal tubule cells, BSA-conjugated E2 stimulates calcium uptake from medium in a cAMP- and PKC-dependent manner. This stimulation can be abolished by a Ca^{2+} blocker, suggesting the uptake of extracellular calcium through membrane channel is the cause of increased calcium (Han et al., 2000). In contrast, another study showed that in MCF-7 cells, the E2-stimulated calcium increase is caused by mobilization of intracellular calcium stores, not by the influx of extracellular calcium (Improta-Brears et al., 1999).

1.2.4.6 The nature of membrane associated estrogen-binding factor

Nongenomic actions induced by estrogens were initially observed in endometrial cells as a rapid increase in intracellular calcium (Pietras and Szego, 1975; Pietras and Szego, 1977). Similar observations were made in other cells, suggesting that estrogen is capable of initiating various kinase cascades in breast cancer cells and many other cell types. Kinases activated by estrogen include MAPK (Migliaccio et al., 1996; Endoh et al., 1997; Duan et al., 2001; Kousteni et al., 2001), PI3K (Simoncini et al., 2000; Hisamoto et al., 2001; Duan et al., 2002), PKA (Aronica et al., 1994; Farhat et al., 1996), PKC (Kelly et al., 1999; Sylvia et al., 2001) and CaMKIV (Qin et al., 2002). There is evidence that E2 may also induce tyrosine phosphatase activity through nongenomic pathway (Chen et al., 2001c)

Nongenomic pathways induced by estrogen share several common traits. They occur within minutes and are insensitive to the transcription inhibitor actinomycin D and translation inhibitor cycloheximide since no transcription or translation is required. Furthermore, these responses can be triggered by estrogen conjugated with bovine serum albumin (BSA), a complex too bulky for cellular entry (Coleman and Smith, 2001). It is generally believed that the nongenomic actions of estrogen are mediated by a membrane associated estrogen binding factor, however, the exact nature of this factor is has not been established.

One hotly debated issue is whether the putative membrane estrogen-binding factor is identical to the intracellular ER. There is evidence supporting a role for membrane ERα and ERβ as mediators of the nongenomic actions of estrogen (Razandi et al., 1999). One study proposed that the putative membrane estrogen- binding factor may be identical or at least similar in structure to nuclear ER, because the membrane form can be recognized by specific ER antibodies (Pappas et al., 1995). Razandi *et al* (1999) found that transfecting ER-negative Chinese hamster ovary (CHO) cells with ER expression vector resulted in ER expression in both the cell membrane and nuclear compartments. Furthermore, the nuclear and membrane types of ERs were identical in term of estrogen affinity and it was suggested that the cell membrane and intracellular ERs share the same transcript. The putative membrane ER was also found to cluster

with many signal transduction components, such as Src and Shc, in specialized membrane compartments called caveolae. One major protein of the caveolae, caveolin-1, is known to physically interact with ER and overexpression of caveolin-1 enhances ER mediated kinase activation by facilitating the translocation of ER from nucleus to membrane (Schlegel et al., 1999; Razandi et al., 2002). The Ser 522 in mouse ERα is important for translocation of ERα to membrane. The S522A mutant is much less effective in membrane localization, caveolin-binding and kinase activation but is equally efficient as wildtype ERα in transactivating ERE-luciferase reporter through a genomic pathway (Razandi et al., 2003).

The hypothesis that the membrane associated estrogen-binding factor is ER poses an interesting question with regard to the conformation of membrane ER. ER lacks any apparent transmembrane domain so presumably cannot adopt a transmembrane conformation. The association of ER with the cell membrane is supposedly either through posttranslational modification or formation of complexes with other membrane-associated factors. Norfleet and coworkers (1999) employed an enzyme-linked immunocytochemistry method to detect membrane ER in nonpermeabilized rat pituitary cells. The results showed that a variety of ER-antibodies targeting different regions of ER all recognized membrane ER, suggesting the all the membrane ER domains (AF1, AF2 and DBD) were accessible to extracellular environment. The putative membrane ER appears to be very dynamic and transitory, a trait that further complicates this perplexing issue (Watson et al., 2002).

There are several lines of evidence supporting that certain nongenomic actions of estrogen are independent of ER. For example, estrogen can activate PI3K in ER-negative breast cancer cells (Tsai et al., 2001). In hippocampal neurons, E2 induces membrane potentiation through PKA and the antiestrogen ICI 182,780 cannot block this PKA activation, suggesting a process that is ER-independent (Gu et al., 1999). Another study proposed the existence of a novel estrogen receptor because estrogen can induce MAPK in cerebral cortex in ERα knockout mice and this action cannot be inhibited by antiestrogen ICI 182,780 (Singh et al., 2000).

Luconi and coworkers (1999) identified in sperm cell membrane a 29-KDa novel receptor that binds estrogen and mediates increased intracellular calcium. Nadal and coworkers (2000) suggested that E2 and certain xenoestrogens can elicit nongenomic actions through a receptor not related to ER but a receptor that shares the pharmacological profile of γ-adrenergic receptor. GPR30, a G-protein coupled receptor, has been shown to bind E2, antiestrogens (ICI 182780 and tamoxifen) and a xenoestrogen (o, p'-DDE). Through recruiting a stimulatory G-protein (G_s), the ligand-bound GPR30 mediates various kinase pathways in an ER-independent manner (Thomas et al., 2004).

It is quite possible that the nongenomic actions of estrogen and estrogenic compounds are the combinational results of membrane ER and several other membrane associated estrogen binding factors. The exact contributions of each factor are determined by the cell context and the nature of the ligands. These nongenomic actions of estrogen are by no means only curious but biologically insignificant events. Many important physiological functions of estrogen are believed to be mediated through nongenomic pathways. For example, nongenomic actions play an important role in estrogen-stimulated cell proliferation (Marino et al., 2002) and activation of eNOS (endothelial nitric oxide synthase) by E2 is carried out through AKT pathway via ERα (Hisamoto et al., 2001; Simoncini et al., 2002).

1.3 Lactate dehydrogenase A (LDHA)

Normal cells favor aerobic metabolism over glycolysis as the main avenue for energy production when the oxygen supply is sufficient. Anaerobic metabolism predominates only under hypoxic conditions (Racker, 1974) and solid tumors also usually develop in conditions with a limited supply of oxygen. This strong selection pressure forces cancer cells to adopt anaerobic metabolism. However, unlike normal cells that will revert to aerobic metabolic pathways when oxygen becomes available, cancer cells maintain anaerobic metabolism even under aerobic conditions. This observation led to the hypothesis that cancer was caused by aberrant metabolism (Warburg, 1956). Although this hypothesis was proven incorrect, the observation itself, now termed "Warburg effect", has been validated.

The underlying cause for Warburg effect is still unknown, but proto-oncogenes R*as*, *Src*, and M*yc* enhance glycolysis under aerobic conditions by upregulating proteins involved in glucose transportation and glycolysis, suggesting some underlying links between carcinogenesis and glycolysis (Dang and Semenza, 1999). At first glance, glycolysis appears to be an unsuitable choice and puts cancer cells at a disadvantage because it is a much less efficient way to extract energy from glucose. However, closer examination reveals that glycolysis can confer some growth advantage to cancer cells. Lactate, the byproduct of anaerobic metabolism, significantly decreases pH in the surrounding microenvironment and induces apoptosis in normal cells. In contrast, with disrupted apoptotic pathways, cancer cells are more tolerant of the acidic environment. Thus, lactate can help cancer cells to decrease competitions from neighboring normal cells (Williams et al., 1999; Gatenby and Gillies, 2004). Some studies also suggest that lactate, or acidic conditions in general, facilitate breakdown of the extracellular matrix and metastasis (Schlappack et al., 1991; Rozhin et al., 1994; Stern et al., 2002). High levels of glycolysis in cancer cells are associated with poor prognosis (Kunkel et al., 2003) Based on these studies, anaerobic metabolism is not only just the result of hypoxic conditions during carcinogenesis, but also an active player in cancer development. The relationship between glycolysis and carcinogenesis can be viewed as a mutual positive feedback, on one hand with proto-oncogenes upregulating glycolytic enzymes and, one the other hand with the glycolytic product lactate promoting metastasis.

Lactate dehydrogenase (LDH) plays a crucial role in glycolysis, catalyzing the conversion between lactate and pyruvate. LDH is a tetramer formed by random association between two type of subunits, lactate dehydrogenase A (LDHA) and lactate dehydrogenase B (LDHB) (Boyer et al., 1963). Lactate dehydrogenase A is also called lactate dehydrogenase M because it predominates in muscle and liver, while lactate dehydrogenase B is also called lactate dehydrogenase H because it predominates in heart. Kinetically, LDHA is more efficient in catalyzing the conversion of pyruvate to lactate and LDHB is more efficient in catalyzing the conversion of lactate to pyruvate. The ratio between LDHA and LDHB is an indicator of cellular metabolic patterns and an increase on LDHA portion suggests a metabolism shift toward glycolysis. Besides its role in glycolysis, LDHA has also been implicated in regulating gene transcription and

DNA replication (Williams et al., 1985; Zhong and Howard, 1990). There is a third type of LDH subunit called LDHC, which only exists in mammalian testes and spermatozoa (Goldberg, 1963).

In mouse models heterozygous LDHA disruption resulted in reduced LDHA activity but apparently had no detrimental effects, however, homozygous LDHA deletion led to embryonic lethality (Merkle et al., 1992). In contrast, humans with congenital LDHA disruption are viable and largely normal, but susceptible to exertional myoglobinuria and easy fatigue after strenuous activity (Kanno et al., 1980).

LDHA and its product lactate are often elevated during tumorigenesis and are linked with high malignancy. Thus, they are used as biomarkers for cancer prognosis (Goldman et al., 1964; Farron et al., 1972; Walenta et al., 1997). Breast cancer cells also tend to express higher levels of LDHA (Farron et al., 1972; Hilf et al., 1976). Interestingly, LDHA can be upregulated by estrogen, a mitogen for mammary carcinogenesis (Richards and Hilf, 1972; Burke et al., 1978; Li and Hou, 1989). Thus, it is not unreasonable to hypothesize that E2-induced LDHA facilitates mammary carcinogenesis. Mechanistic studies on the E2-responsiveness of LDHA may give new insight into the development of breast cancer.

1.4 Objectives

1.4.1 Identifying the E2-responsvie region of LDHA promoter

LDHA is an E2-responsive gene (Richards and Hilf, 1972; Li and Hou, 1989) and its promoter contains multiple *cis*-elements, including TRE (TPA-responsive element)(Huang and Jungmann, 1995), CRE (cAMP-responsive element) (Short et al., 1994) and Sp1(Specificity protein 1)(Short et al., 1994) motifs. Thus the first goal of this study is to identify the E2-responsive region. The promoter region of LDHA was inserted into the luciferase reporter vector pGL2 and a series of LDHA promoter deletion and mutant constructs were made to determine the E2-responsiveness through transient transfection assays in MCF-7 cells.

1.4.2 Identifying the mechanism of activation of CREB by estrogen

Preliminary results indicated that a CRE (cAMP response element) located in –58/+9 region of LDHA promoter is responsible for the E2-dependent transactivation. CRE is recognized and induced by CREB family transcription factors (Comb et al., 1986; Montminy et al., 1986; Hoeffler et al., 1988; Yamamoto et al., 1988; Gonzalez and Montminy, 1989). Thus the second goal of this research is to investigate the mechanism of hormonal activation of CREB in MCF-7 cells. The binding of CREB family proteins to this CRE site was confirmed with gel mobility shift and supershift assays. A constitutively active PKA expression vector, a cAMP inducer cholera toxin and a dominant negative CREB expression vector were used to investigate the role of CREB in mediating E2-responsiness of LDHA promoter.

CREB requires phosphorylation on Ser133 to be transcriptionally active (Gonzalez and Montminy, 1989) and Ser133 can be phosphorylated by a plethora of kinase cascades, including PKA (Gonzalez and Montminy, 1989), Calcium-calmodulin-dependent Kinases (Matthews et al., 1994; Sun et al., 1994) and PKC (Xie and Rothstein, 1995) (Fig. 18). A series of kinase inhibitors were used to identify the kinase cascades responsible for the hormonal activation of the LDHA promoter.

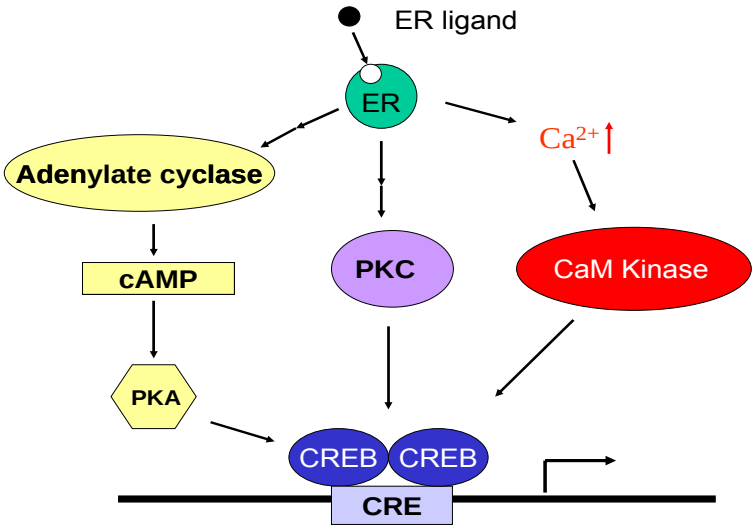

Fig. 18. The potential mechanisms of activation of CREB by estrogen.

1.4.3 Investigating the roles of ERα domains in kinase activation

The third goal of this research is to further characterize the nature of membrane ER and the roles of ERα domains (AF1, AF2 and DBD) in the activation of kinase cascades. The activation of kinase pathways by E2 was investigated in a mammalian one-hybrid system. A luciferase reporter vector driven by five tandem GAL4 sites was cotransfected with GAL4-Elk-1, GAL4-SRF, GAL4-CREB and GAL4-p65 fusion proteins to assess the E2-induced MAPK, PI3K, PKA/PKC and CaMKIV respectively (Fig. 19). ER mutation variants HE11, HE15, HE19 and TAF1 were used to investigate the domain requirements of ERα in activation of these kinases. The assay was carried out in ER positive MCF-7 cells and ER-negative cell line C4 derived from MCF-7 cells (Oesterreich et al., 2001) .

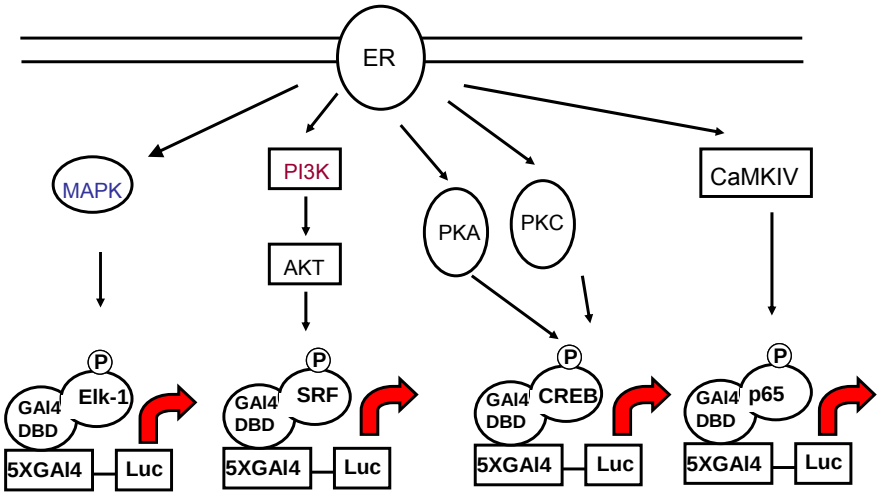

Fig. 19. GAL4-fusion protein system for measuring E2-indcued kinase activities.

1.4.4 Investigating the roles of xenoestrogens in kinase activation

Although the nongenomic activities of estrogen on have been extensively studied, little is known about the nongenomic activities of synthetic industrial estrogenic compounds. Since xenoestrogens include a broad spectrum of structurally diverse chemicals, their profiles on kinase activation may differ from estrogen and from one another. These differences may be important for elucidating the mechanisms of nongenomic pathways

of estrogen and for evaluating the possible adverse impacts of xenoestrogens on wildlife and human health. The GAL4-fusion protein system (Fig. 19) and the downstream targets of various kinases were used as end points to explore this problem.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals, enzymes and antibodies

Antibiotic/antimycotic solution, trypsin, Tris base, acetic acid, disodium EDTA, sodium bicarbonate, sodium chloride, hydrochloric acid, sodium hydroxide, magnesium chloride, urea, calcium chloride, HEPES, glycerol, dimethylsulfoxide (DMSO) , methanol, ethanol and acetone were purchased from Sigma (St. Louis, MO). LB medium was purchased from Life Technologies (Gaithersbrug, MD, USA). Cholera toxin, bisindolylmaleimide I, Ro-31-8425, W7, SQ22536, H89, PD98059, LY294002, KN93, wortmannin, 2',5'-dideoxyadenosine and TPA were obtained from Calbiochem (San Diego, CA). ICI 182, 780 was kindly provided by Dr. Alan Wakeling (AstraZeneca, Macclesfield, UK). MEM medium and DME/F-12 medium were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY). Nonylphenol, *p*-*t*-octylphenol, and bisphenol A were purchased from Aldrich Chemical (Milwaukee, WI). 2′,3′,4′,5′-Tetrachloro-4-biphenylol (HO-PCB-Cl_4) and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) were prepared in this laboratory with purity>98% as determined by gas chromatographic analysis. E2, resveratrol and diethylstilbestrol (DES) were purchased from Sigma Chemical (St Louis, MO). Endosulfan and kepone were purchased from Chem-Service (West Chester, PA). Bgl II, Hind III, Kpn I and Xho I restriction enzymes and T4 polynucleotide kinase were purchased from Promega (Madison, MI). The antibodies for p-AKT (sc-7985-R), p-ERK (sc-7383), p-CREB (sc-7978-R), ATF-1 (sc-241X), ATF-1 (sc-270X), CREB-1 (sc-186X), C/EBPβ (sc-746X), CREB-2 (sc-200X), CERM-1 (sc-440X) and p53 (sc-126) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The gylceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (4300) was purchased from Ambion (Austin, TX).

2.2 Oligonucleotides and constructs

Oligonucleotides were purchased from Genosys/Sigma (Woodlands, TX) or IDTDNA (Coralville, IA). The construct containing the 5' flanking region –1173/+25 of rat LDHA gene was kindly provided by Dr. Richard A. Jungmann (Northwestern University,

Chicago, IL). TpGL2 luciferase reporter plasmid was made by inserting the TATA sequence between the Bgl II and Hind III sites of pGL2 vector (Promega). A series of fragments of LDHA promoter were PCR amplified (95°C 1 min, 50°C 30 sec, 72°C 1 min for 24 cycles) using GeneAMP PCR kit from Roche (Branchburg, NJ). The amplification were performed in 2 mM $MgCl_2$, 1 uM each primer, 1 mM dNTPs and 2.5 U Taq DNA polymerase using a PTC-200 Peltier Thermal Cycler from MJ Research (Waltham, MA). PCR products were cut with KpnI/XhoI enzymes and inserted between KpnI and XhoI sites of TpGL2. Ligation products were transformed into DH5α competent *E. coli* cells, and clones were verified by sequencing. All the promoter fragments shared the same reverse primer: 5' aac tcg agt cat gac gca gag cag 3'. The forward primers are (5' to 3'): pLDH-1(ggg gta ccg gga aca gca atg ta), pLDH-2(ggg gta cca ttt cgg gct act g), pLDH-3(tcg gta ccg tgt cgc agc aca), pLDH-4(acg gta cct gga cgc ccg ccc ccg gcc cag cct ac), pLDH-5(acg gta ccc cag cct aca cgt ggg ttc), pLDH-7(acg gta ccg ctc cca ctc tga cgt cag c), pLDH-6 (acg gta ccg gag ctt cca ttt aag), pLDH-7m(acg gta ccg ctc cca ctc aga aga tcg cgc gga gct, mutated region is underlined).

The wildtype human ERα expression vector was supplied by Dr. Ming-Jer Tai (Baylor College of Medicine, Houston, TX). Expression plasmids for ERα deletion mutants HE11, HE15 and HE19 were provided by Dr. Pierre Chambon (Institute de Genetique et Biologie Moleculaire et Cellulaire, Illkirch, France) and inserted in pcDNA3 (Invitrogen, Carlsbad, CA) vector in this laboratory. TAF1 ERα mutant was obtained from Dr. D. McDonnell (Duke University, Durham, NC). The chimeric protein expression vectors GAL4-Elk-1, GAL4–SRF and GAL4-CERB were provided by Dr. Roger Treisman (Imperial Cancer Research Center, London, England), Dr. Linda Sealy (Vanderbilt University, Nashville, TN) and Dr. Richard Goodman (Oregon Health Science Center, Portland, OR), respectively. The GAL4-p65 expression vector and GAL4-Luc vector were provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). The mutant CREB inhibitory expression plasmid (KCREB) was provided by Dr. Richard Goodman (Oregon Health Science Center, Portland, OR). Constitutively active Protein Kinase A expression plasmid (PKAc) was supplied by Dr. Richard Maurer (Oregon Health Science Center, Portland, OR). All the plasmids were prepared using DNA maxprep kit (BioRad, Hercules, CA).

2.3 Cell maintenance

MCF-7 and ZR-75 human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in MEM medium with phenol red and supplemented with 10% fetal bovine serum (Intergen, Newark, NJ or Sigma, St. Louis, MO) , 2.2 g/l sodium bicarbonate plus 1% antibiotic-antimyotic solution in an air/carbon dioxide (95:5) atmosphere at 37°C. MCF-7-C4 cells were kindly provided by Dr. W. Welshons (University of Missouri-Columbia, Columbia, MO) and maintained in DME/F-12 medium without phenol red and supplemented with 2.5% dextran/charcoal-stripped fetal bovine serum, 2.2 g/l sodium bicarbonate plus 1% antibiotic-antimyotic. All cells were maintained in 150cm^2 tissue culture dishes from Falcon (Lincoln Park, NJ) or Corning (Corning, NY). Cells were passaged by trypsinizing, centrifuging at 500Xg for 1 min, resuspending in fresh medium and seeding in new culture dishes.

2.4 Transient transfection assay

Cells were seeded in DME/F-12 medium without phenol red supplemented with 2.5% dextran/charcoal-stripped fetal bovine serum, 2.2 g/l sodium bicarbonate plus 1% antibiotic-antimyotic solution. After 24 hr, cells were transfected by the calcium phosphate-DNA coprecipitation method. After 8 hr, the transfection mixture was withdrawn and the cells were treated with fresh DME/F-12 medium containing chemical treatments, or DMSO alone as a solvent control. Alternatively, cells were transfected with Oligofectamine (Invitrogen, Carlsbad, CA) according to manufacturer's recommendation. After 24-48 hr, cells were harvested in 1 x Lysis buffer (Promega) by manual scraping. Cell lysates were prepared by freezing cells in liquid nitrogen for 30 sec, vortexing for 30 sec, and centrifuging at 12,000 x g for 1 min. Luciferase activity was determined using a luciferase assay reagent (Promega) by Lumicount micro-well plate reader (Packard Instrument Co.).The activities of β-galactosidase was determined by luminescent Galacto-Light plus assay system from Tropix (Bedford, MA) and used to normalize for transfection efficiency.

2.5 DNA probe labeling

γ-P^{32}-ATP was purchased from NEN Research Products B (Boston, MA). Oligonucleotides were diluted to 5 uM and 5'-end-labelled by incubating with T4 polynucleotide kinase and γ-P^{32}-ATP in 37°C for 1 hr. The labeled product were purified by TE-10 column (Clontech, Palo Alto, CA) and count on Beckman LS liquid scintillation counter to ensure the radioactivity exceed $1X10^5$ CPM.

2.6 Northern analysis

MCF-7 cells were plated in 100 mm culture dishes with 80% confluence and maintained in serum-free DME/F-12 medium supplemented with 2.2 g/l sodium bicarbonate plus 1% antibiotic-antimyotic solution for 48 hr before treated with DMSO or E2. Total RNA was extracted using RNAzol B (Tel-test Inc., Friendswood, TX) according to manufacturer's recommendation. Total RNA was quantified by measuring the 260/280 absorption ratio with a Beckman DU640 spectrometer. Equal amounts of RNA for each treatment were loaded on 1.2% agarose gel containing 3% formaldehyde in 1XSPC (20 mM NaH_2PO_4, 2 mM EDTA, pH 6.8) buffer, electrophoresised for 3 hr in SPC and transferred to Hybond-N^+ nucleic acid transfer nylon membrane (Amersham Pharmacia Biotech) using a capillary blotting method. The membrane was exposed to UV light for 5 min and baked at 80°C for 2 hr to crosslink RNA to membrane. The membrane was incubated in a pre-hybridization buffer (0.1% BSA, 0.1% Ficoll, 0.1 % polyvinylpyrollidone, 10% dextran sulfate, 1% SDS, 0.15 M sodium chloride, 10 nM sodium dihydrogen phosphate, 1 mM EDTA) for 18 hr at 65°C and hybridized in the same buffer for 24 hr with the ^{32}P-labeled LDHA probe (5' to 3': agg tct gag att cca ttc tgt ccc aaa atg caa gga aca c). After hybridization, the membrane was washed twice at room temperature in 1XSSPE buffer (0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA). The resulting blots were visualized and quantitated on Storm imager system (Molecular Dynamics Inc., Sunnyvale, CA). The LDHA mRNA levels were standardized against β-tubulin levels.

2.7 Nuclear extract preparation

MCF-7 cells were cultured in 150 mm cell culture dish in DME/F-12 medium supplemented with 2.5% FBS, 2.2 g/l sodium bicarbonate plus 1% antibiotic-antimyotic

solution for 24 hr. Treatment were added to medium 1 hr before harvesting if applicable. Medium was aspirated and cells were washed in ice-cold HE buffer (25 mM HEPES, 1.5 mM EDTA, pH 7.6) and harvested in ice-cold HEG buffer (25 mM HEPES, 1.5 mM EDTA, 10% glycerol (v/v), pH 7.6) supplemented with protease inhibitor cocktail (Sigma) by manual scraping. Cells were homogenized with a Type-B Dounce homogenizer (Kontes Glass Co., Vineland, NJ) on ice for 40 strokes to disrupt the cells while leaving the nucleus intact. The cellular homogenate was centrifuged at 800g for 10 min at 4^{o}C to collect the nuclear pellet. The supernatant containing cytoplasmic proteins was discarded and the nuclear pellet was incubated in HEGK (25 mM HEPES, 1.5 mM EDTA, 0.5 M KCl,10% glycerol(v/v)pH 7.6) buffer for 20 min on ice with frequent vortex to dissolve the nuclear proteins. The sample was centrifuged at 12,000Xg for 10 min at 4^{o}C and the supernatant containing nuclear proteins was quantified by Bradford method and aliquoted to store at -80^{o}C for future use.

2.8 Gel electrophoretic mobility shift assay (EMSA)

Specific oligonucleotide probes (5 pmol) were ^{32}P-labeled at the 5'-end using T4 polynucleotide kinase (Promega) and γ-^{32}P-ATP. Gel mobility shift and supershift assays were performed on ice. For each sample, appropriate amount of HEGK buffer (25 mM HEPES, 1.5 mM EDTA, 0.5 M KCl, 10% (v/v) glycerol, pH 7.6) was added to 5 ug MCF-7 nuclear extract protein to bring the total volume to 5 ul. HEG buffer (25 mM HEPES, 1.5 mM EDTA, 10% (v/v) glycerol, pH 7.6) of 15 ul was added to dilute the salt concentration and 1ug poly(dI-dC) (Roche) was added to block the nonspecific binding. After incubation for 5 min, 0.01 pmol labeled DNA probe was added and incubated for 10 min. Antibodies or IgG control were added to the mixture and incubated for 5 min if applicable. The mixture was resolved in 5% non-denaturing PAGE in TBE buffer at 120V for 3-4 hr. Gels were dried and the protein-DNA complexes were visualized using Storm Imager system (Molecular Dynamics Inc., Sunnyvale, CA). The sequences of oligonucleotide probes (5' to 3') are: CRE (aga gat tgc ctg acg tca gag agc tag), LDHA (gct ccc act ctg acg tca gcg cgg agc ttc cat), mLDHA (gct ccc act cag aag atc gcg cgg agc ttc cat, mutated region was underlined).

2.9 Western analysis

Cells were seeded into 35-mm six-well tissue culture plates in phenol red-free DME/F-12 medium (Sigma) supplemented with 2.5% dextran/charcoal-stripped fetal bovine serum, 2.2 g/l sodium bicarbonate plus 1% antibiotic-antimyotic solution. After 24 hr, cells were treated and harvested in ice-cold high salt lysis buffer (50 mM HEPES, 500 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA. PH 7.5) supplemented with protease inhibitor cocktail (Sigma). After 30 min incubation with frequent vortexing, the samples were centrifuged at 14000xg for 10 min at 4^{o}C. The supernatant was collected and the protein concentrations were quantified by Bradford method. Equal amounts of protein from each treatment group were boiled for 5 min in 1 x Laemmli buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromphenol blue, 175 mM ß-mercaptoethenol), separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Membranes were blocked in PBS containing 5% nonfat milk and 0.05%Tween-20 for 1 hr, probed with 1:1000 primary antibodies for 2 hr and incubated with peroxidase-conjugated secondary antibodies for 1 hr. The membranes were washed in 0.05%Tween-20 for 10 min and the blots were visualized using Western Lightning kit (PerkinElmer, Boston, MA) on Kodak (Rochester, NY) X-Omat AR autoradiography film. The images were scanned with a Sharp JX-330 scanner and quantified with Zero-D Scanalytics software.

2.10 Statistics

All data were analyzed by ANOVA and Scheffe's post-hoc test and were presented as means ± SE for at least three replicate experiments for each treatment group. Statistical analyses were performed using SuperANOVA software (Abacus Concepts, Berkeley, CA) and were considered significant if $p<0.05$.

CHAPTER III

RESULTS*

3.1 The E2-responsiveness of LDHA promoter requires the CRE (-48/-41)

In MCF-7 cells treated with E2, LDHA mRNA levels were determined by Northern blot analysis. The results (Fig. 20) showed that significant induction by E2 was observed within one hour and persisted for 48 hours. This observation is in agreement with previous reports showing that E2 induced LDHA activity in breast cancer cells (Richards and Hilf, 1972; Burke et al., 1978; Li and Hou, 1989).

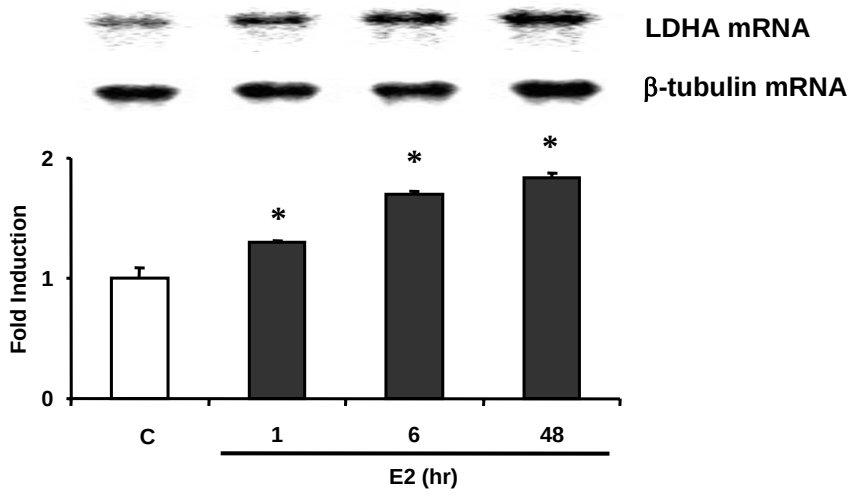

Fig. 20. Induction of LDHA mRNA levels by E2 in MCF-7 cells. Cells were treated with 10 nM E2 in serum free DME/F-12 medium for 1, 6 or 48 hr. The mRNA was isolated and analyzed by Northern blot as described in *Materials and Methods*. β-Tubulin mRNA was used as a loading control. Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

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*Part of the data reported in this chapter is reprinted with permission from Li, X., Qin, C., Burghardt, R., Safe, S., 2004. Hormonal regulation of lactate dehydrogenase-A through activation of protein kinase C pathways in MCF-7 breast cancer cells. Biochem. Biophys. Res. Commun. 320, 625-634 by Elsevier (doi:10.1016/j.bbrc.2004.05.205).

The 5' promoter region of rat LDHA gene contains multiple cis-elements that may contribute the regulation of LDHA expression; these include an ERE half site, GC rich site (Sp1), NF1 site, USF site and CRE site (Fig. 21). The rat LDHA promoter shares significant homology with human LDHA promoter and has been extensively used a model to understand the molecular mechanisms of LDHA regulation (Fukasawa and Li, 1986; Jungmann et al., 1998).

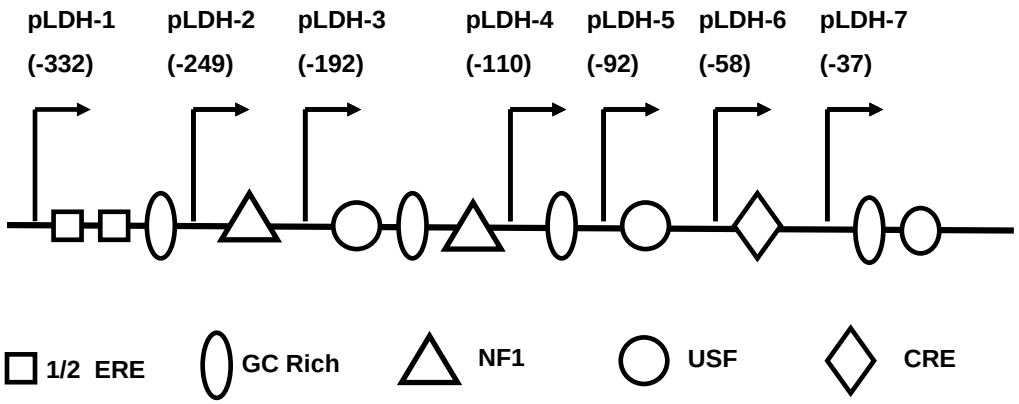

Fig. 21. The schematic map for the major cis-elements on LDHA promoter region.

In order to investigate the mechanism of LDHA regulation by estrogen, we constructed a series of luciferase reporter vectors containing the various fragments of LDHA promoter by PCR amplification as described in the *Materials and Methods*. They were named pLDH-1(-332 to +9), pLDH-2(-249 to +9), pLDH-3(-192 to +9), pLDH-4(-110 to +9), pLDH5 (-92 to +9), pLDH6 (-37 to +9) and pLDH7 (-58 to +9), respectively (Fig. 21).

In MCF-7 cells transfected with pLDH-1 but without ERα, no estrogen-induced activities were observed. In contrast, E2 induced up to 7-fold of increase in luciferase activity in MCF-7-cells after cotransfection with pLDH-1 and wildtype ERα (Fig. 22). Although MCF-7 cells are ERα-positive, endogenous ERα is apparently a limiting factor in the transfected cells due to the overexpression of promoter-reporter constructs and cotransfection of additional ERα is necessary for induction of transactivation by E2. This phenomenon has been commonly observed in many other studies (Berry et al., 1989;

Savouret et al., 1991; Wang et al., 1999). E2 does not induce luciferase activity when cotransfected with pLDH-1 and ERα mutants containing deletions of AF1 (A/B domain) (HE19), AF2 (E/F domain) (HE15) or the DNA binding (C) domain (HE11) (Fig. 22). Previous studies have shown that HE11 can mediate induction of HE11/Sp1 by E2 through interaction with GC-rich Sp1 binding sites (Duan et al., 1998; Sun et al., 1998). Since HE11 did not mediate transactivation by E2 in cells transfected with pLDH-1, it is unlikely that the GC-rich sites in LDHA promoter are E2-responsive.

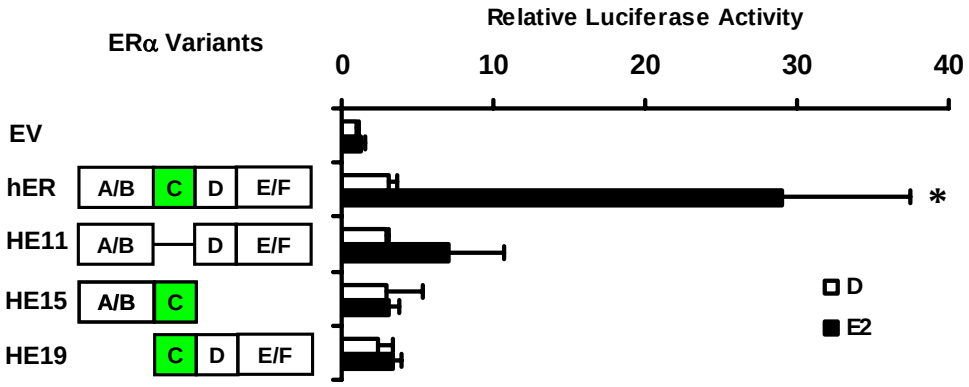

Fig. 22. E2-responsiveness of pLDH-1 when cotransfected with wildtype/variants ERα expression plasmids. MCF-7 cells were transfected with pLDH-1 reporter plus empty vector (EV), wild type hER or ERα mutants HE11, HE15 and HE19. Cells were treated with 10 nM E2 or D (DMSO control) for 48 hr. Luciferase activities were determined for three separate determinations for each treatment group as described in the *Materials and Methods*. Significant induction ($p<0.05$) compared to DMSO is indicated by an asterisk.

To identify E2-responsive DNA motifs in the LDHA promoter, a series of LDHA promoter constructs (pLDH-1 to pLDH-7) were transfected along with hER into MCF-7 breast cancer cells. E2 induced the luciferase activity in cells transfected with pLDH-1(-332 to +9), pLDH-2 (-249 to +9) and pLDH-3 (-192 to +9) in MCF-7 cells (Fig. 23). The response in cells transfected with pLDH-1, pLDH-2 and pLDH-3 was comparable, suggesting that the DNA motifs within the -332 to -249 region of LDHA promoter are not

required for the E2-responsiveness. Further deletion analysis showed that pLDH-4 (-110 to +9), pLDH-5 (-92 to +9) were still E2-responsive but E2 did not induce luciferase activity in cells transfected with pLDH-6 (-37 to +9) (Fig. 24), indicating that -92 to -37 region of the promoter is essential for E2-responsiveness.

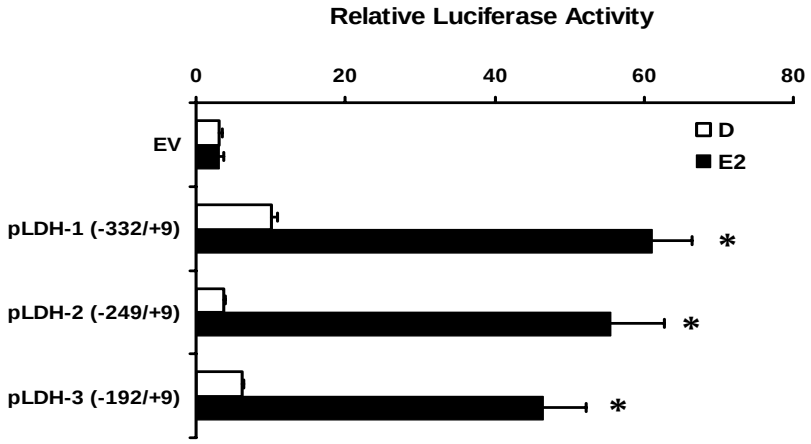

Fig. 23. E2-induced transactivation in MCF-7 cells transfected with pLDH-1, pLDH-2 and pLDH-3. Luciferase promoter-reporter constructs pLDH-1, pLDH-2 and pLDH-3 were cotransfected with hER into MCF-7 cells. Cells were treated with 10 nM E2 or D (DMSO control) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods*. Significant induction ($p<0.05$) compared to DMSO is indicated by an asterisk.

A prominent feature within the -92 to -37 region of LDHA promoter is a CRE site from -48 to -41. The construct containing a mutation of this CRE (pLDH-7m) was not E2-responsive, suggesting that the CRE at -48 to -41 is required for the estrogen-induced transactivation of LDHA in MCF-7 cells. There was a notable decrease in basal luciferase activity for mutant construct pLDH-7m compared to wildtype pLDH-7 (Fig. 25). These results suggest that the CRE (-48/-41) is an important regulatory motif in LDHA promoter, an observation in agreement with findings from other laboratories (Short et al., 1994; Firth et al., 1995). Moreover, the estrogen-induced activity of pLDH-7 was inhibited by cotreatment of antiestrogens ICI 182, 780 or 4'-hydroxytamoxifen, indicating that the induction is mediated by ER (Fig. 26).

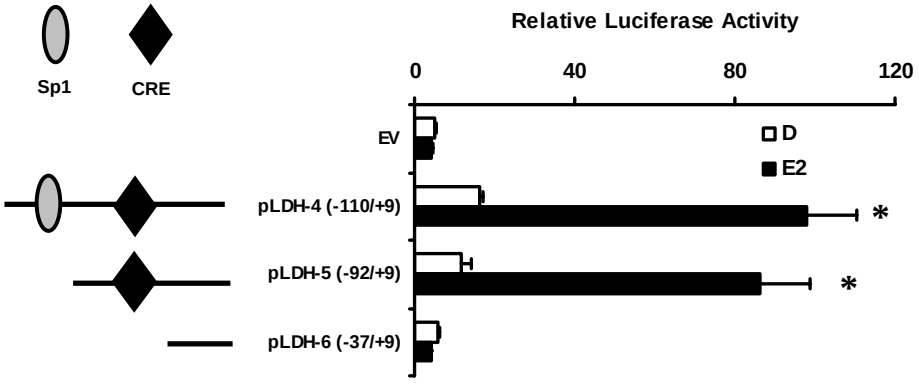

Fig. 24. E2-induced transactivation in MCF-7 cells transfected with pLDH-4, pLDH-5 and pLDH-6. The pLDH-4, pLDH-5 and pLDH-6 constructs and hER were cotransfected into MCF-7 cells. Cells were treated with 10 nM E2 or D (DMSO control) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods*. Significant induction ($p<0.05$) compared to DMSO is indicated by an asterisk.

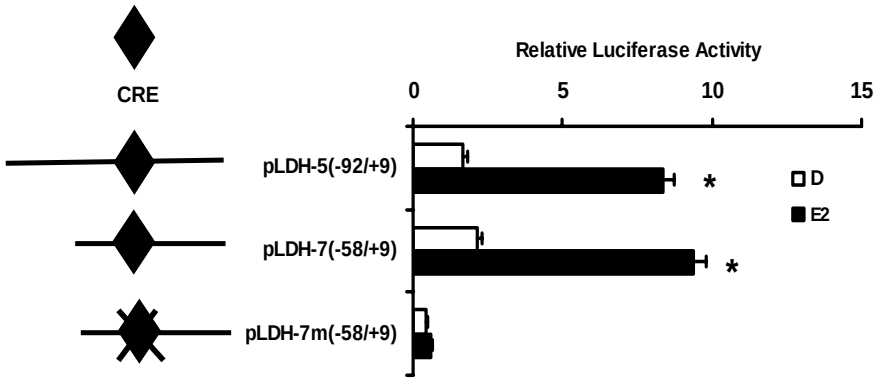

Fig. 25. E2-induced transactivation in MCF-7 cells transfected with pLDH-5, pLDH-7 and pLDH-7m. pLDH-5, pLDH-7 and pLDH-7m constructs and hER were cotransfected into MCF-7 cells. Cells were treated with 10 nM E2 or D (DMSO control) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods*. Significant induction ($p<0.05$) compared to DMSO is indicated by an asterisk.

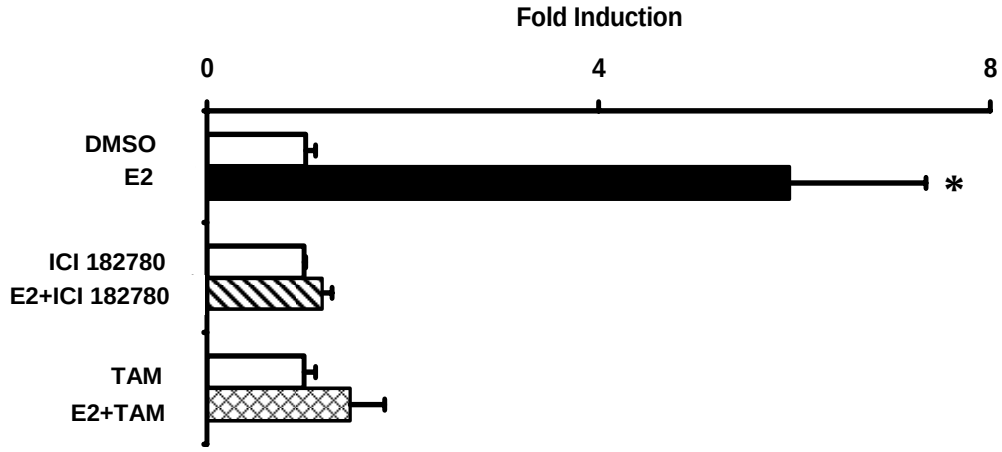

Fig. 26. Effects of antiestrogens on the E2-responsiveness of pLDH-7. MCF-7 cells were transfected with pLDH7 and hER and treated with DMSO (control) or 10 nM E2 ± antiestrogen ICI 182780 (1uM) or tamoxifen (1uM) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods*. Significant induction by E2 ($p<0.05$) is indicated by an asterisk.

3.2 CREB family proteins bind to the CRE (-48/-41) site

To investigate the binding of nuclear proteins to the CRE (-48/-41), a fragment of the LDHA promoter (-58/-36) which contains the CRE motif was used as DNA probe in gel mobility shift assays (Fig. 27). One major retarded band was observed using nuclear extracts prepared from either DMSO- or E2-treated MCF-7 cells (lane 8 and 9). The E2 treatment increased the band density, suggesting that E2 enhances the binding of nuclear protein to the radiolabeled DNA probe. Competition with 200-fold excess of unlabled consensus CRE or LDHA/CRE probe decreased the retarded band densities (lane 10 and 11). In contrast, mLDHA, the LDHA promoter fragment (-58 to -36) with a mutation on the CRE site, did not competitively decrease the retarded band density (lane 12). These results show that specific nuclear proteins bind the CRE. As a positive control, a radiolabeled consensus CRE DNA probe was also used to perform the same experiment and comparable results were observed (lane 1-6), confirming the specificity of nuclear protein-CRE interactions.

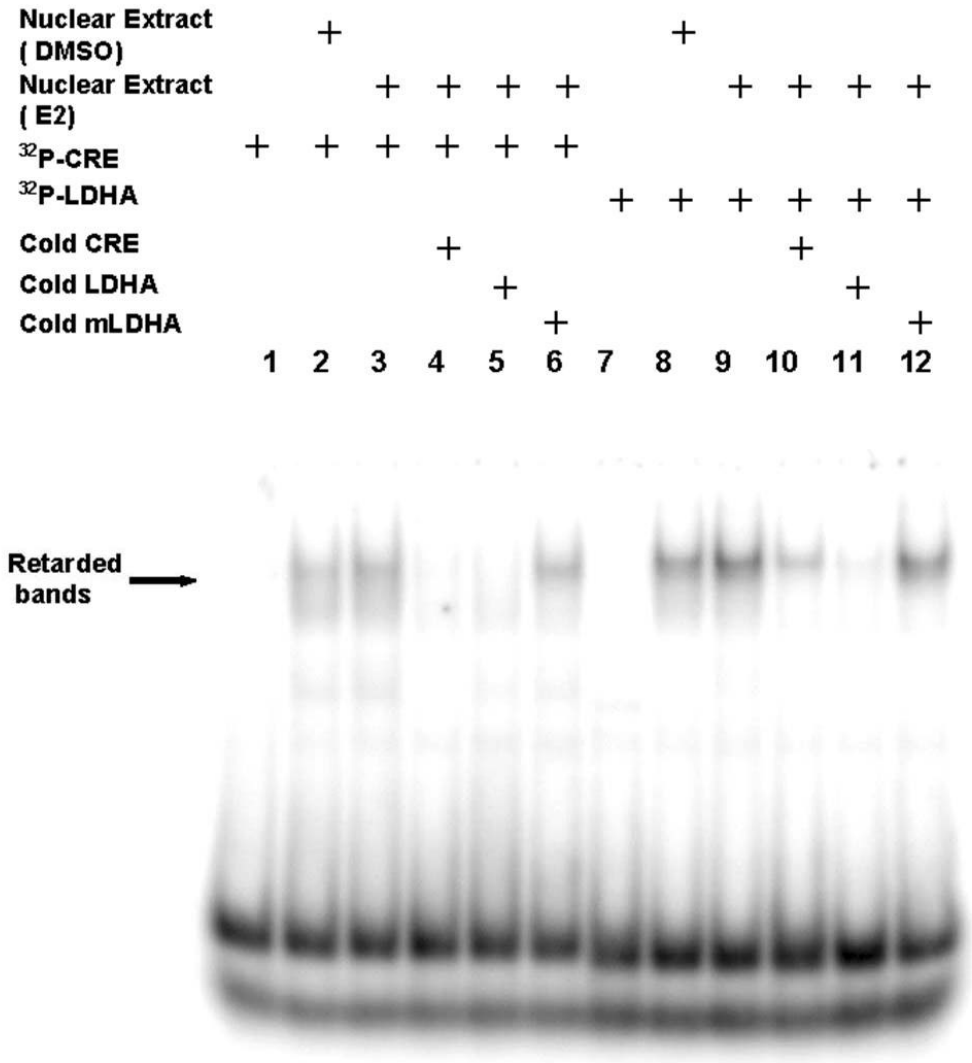

Fig. 27. Gel mobility shift assay. Nuclear extracts were prepared from MCF-7 cells treated with DMSO or E2 (10 nM) for 2 hr. Radiolabled consensus CRE or -58 to -36 region of LDHA promoter were used as probes. Retarded bands are indicated with an arrow.

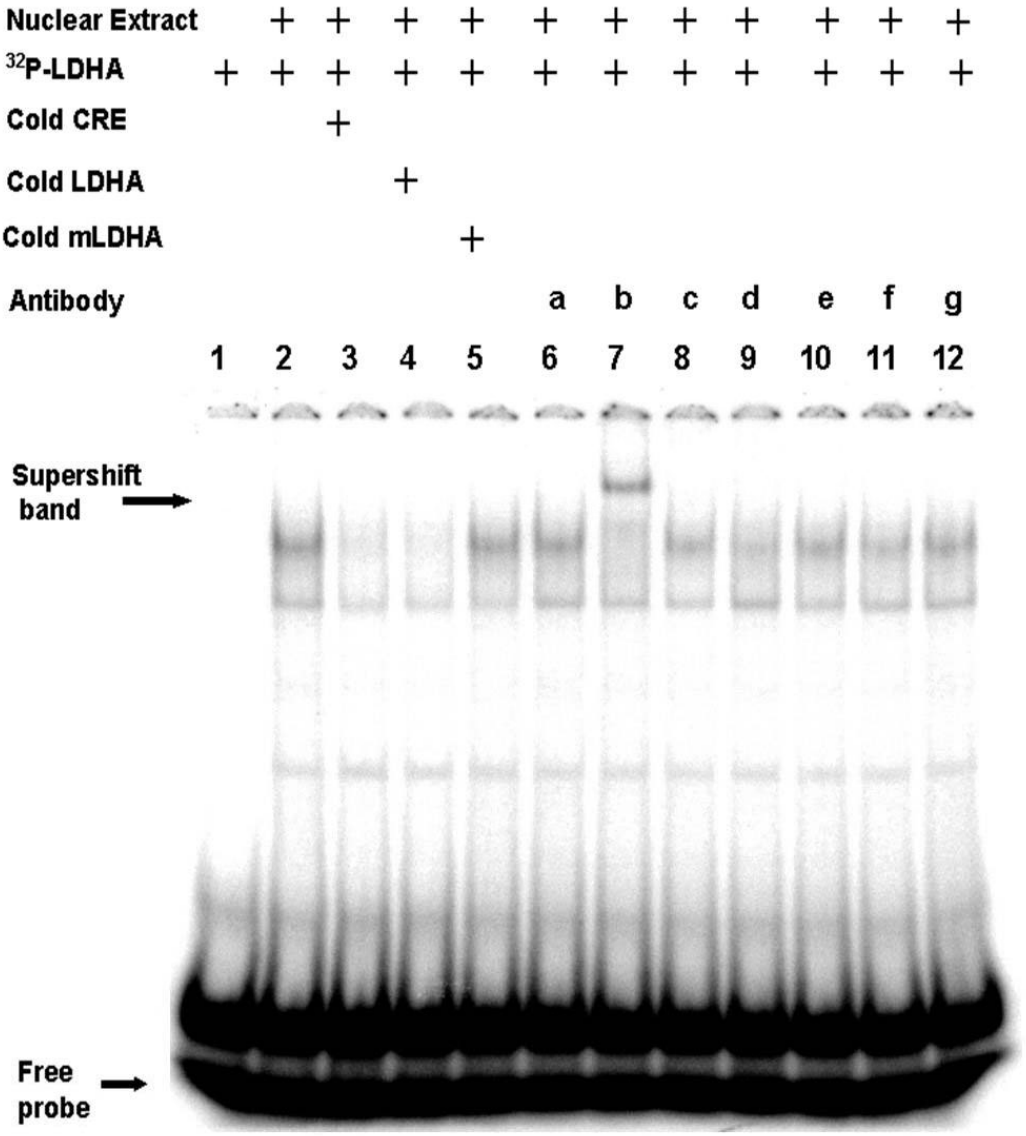

Fig. 28. Supershift assay. Nuclear extracts were prepared from MCF-7 cells treated with E2 (10 nM) for 2 hr. Radiolabled LDHA promoter (-58 to -36) DNA was used as a probe. Supershifted band is indicated with an arrow. Antibodies used for supershift experiments were: ATF-1(a), ATF-1(b), C/EBPβ(c), CREB-1(d), CREB-2(e), CREM-1(f) and IgG (g).

The nuclear proteins bound to the CRE were investigated using protein antibodies in a supershift assay (Fig. 28). The effects of various antibodies on the nuclear protein-DNA

probe retarded band are indicated in lanes 6-12. Of all the antibodies, only ATF-1 antibody gave a supershifted ternary complex (lane 7/antibody b). Some weak protein immunodepletions were also observed for antibodies against CREB-1 and CREM-1 (lane 9/antibody d and lane 11/antibody f). These results confirm that the CREB family proteins bind to the CRE (-48/-41) of LDHA promoter.

The roles of CREB family proteins in mediating the E2-responsiveness of LDHA were further investigated by transfection of a dominant negative form of CREB, KCREB (Coleman et al., 1996). In MCF-7 cells transfected with pLDH-5, E2-induced luciferase activity was gradually diminished after cotransfection of increasing amounts of KCREB (Fig. 29). This result demonstrated the important role of CREB in mediating the E2-responsiveness of LDHA.

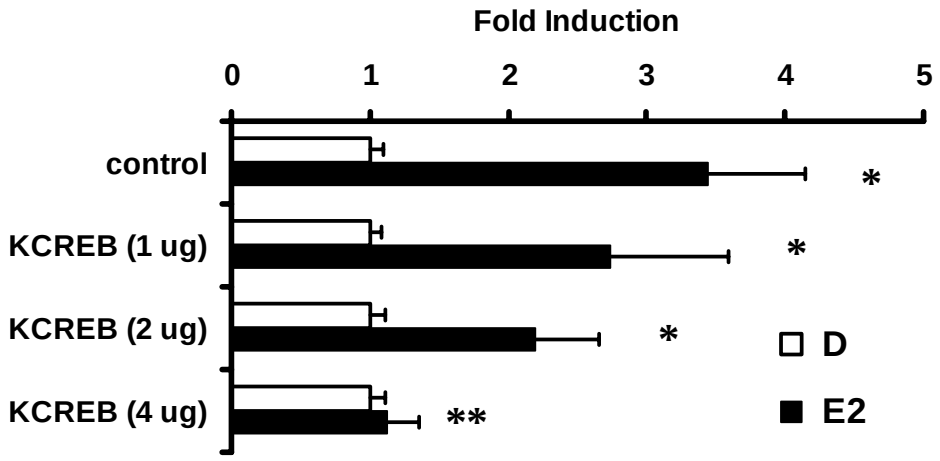

Fig. 29. Inhibitory effects of dominant negative CREB (KCREB) on the E2-responsiveness of pLDH-5. MCF-7 cells were transfected with pLDH-5, hER and different amounts of KCREB. Cells were treated with DMSO or E2 (10 nM) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods*. Significant induction ($p<0.05$) by E2 is indicated by an asterisk and significant inhibition ($p<0.05$) of this response by KCREB is indicated by double asterisks.

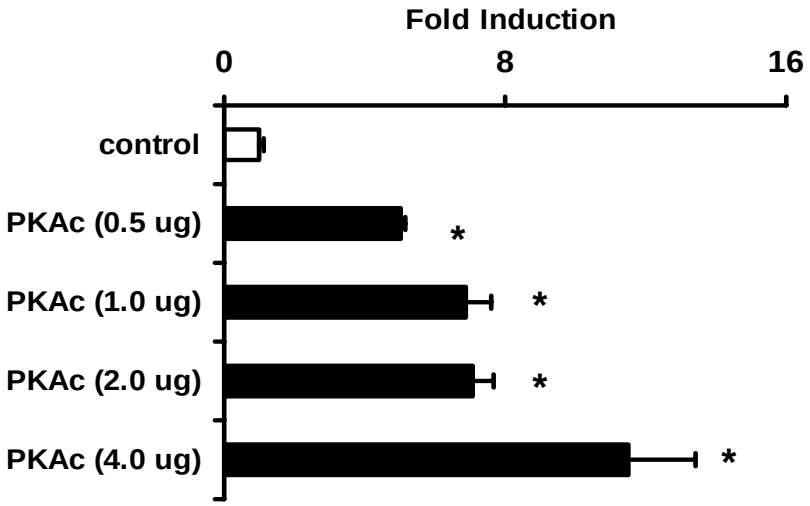

Fig. 30. Activation of pLDH-5 by constitutively active PKA (PKAc). MCF-7 cells were transfected with pLDH-5 and different amounts of PKAc for 48 hr. Luciferase activities were determined for three separate determinations as described in the *Materials and Methods.* Significant induction ($p<0.05$) by PKAc is indicated by an asterisk.

A constitutively active PKA (PKAc) expression vector induced luciferase activity in cells transfected with pLDH-5 in a dose-dependent manner (Fig. 30). PKA is a well known inducer of CREB transcriptional activity (Rehfuss et al., 1991). In agreement with this finding, a PKA activator, cholera toxin, also induced luciferase activity in cells transfected with pLDH-7 (Fig. 31). These results confirm that the CREB mediates LDHA gene expression through the CRE site.

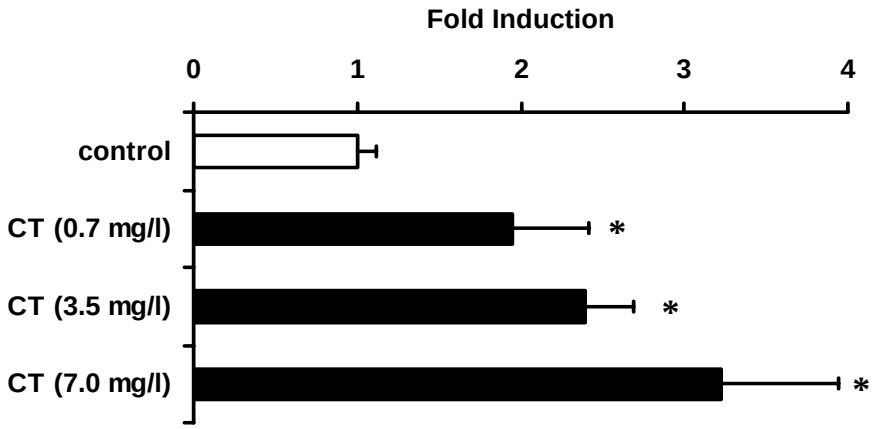

Fig. 31. Activation of pLDH-7 by cholera toxin. MCF-7 cells were transfected with pLDH-7 and treated with different doses of cholera toxin (CT) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by CT is indicated by an asterisk.

Hormonal activation of CREB in MCF-7 cells was also investigated using a GAL4-CREB fusion protein and a luciferase reporter gene containing five tandem GAL4 response elements (GAL4-Luc). In MCF-7 cells cotransfected with GAL4-CREB, GAL4-Luc and hER, E2 induced the luciferase activity, indicating that E2 activates CREB. In contrast, hormone-induced transactivation was not observed without cotransfection of hER (Fig. 32). These observations suggest that induction of LDHA constructs by E2 is mediated by ER through activation of CREB.

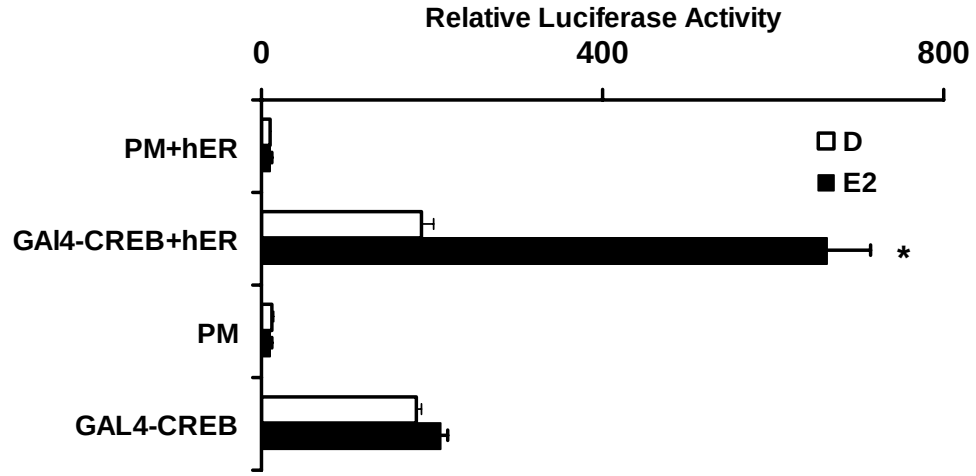

Fig. 32. Activation of GAL4-CREB fusion protein by E2 in MCF-7 cells. MCF-7 cells were transfected with hER, GAL4-Luc, GAL4-CREB or GAL4 DNA binding domain (PM) as a negative control. Cells were treated with DMSO or E2 (10 nM) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated with an asterisk.

3.3 Activation of CREB by E2 is primarily mediated through PKC pathway in MCF-7 cells

CREB is a transcription factor regulated through multiple pathways, including kinase cascades PKA, PKC and CaMKIV (Gonzalez and Montminy, 1989; Matthews et al., 1994; Sun et al., 1994; Xie and Rothstein, 1995). These three kinases are also activated by E2 through nongenomic pathways (Aronica et al., 1994; Sylvia et al., 2001; Qin et al., 2002). A number of kinase inhibitors were used to identify the kinase pathways responsible for mediating activation of LDHA and CREB by E2. The CaM kinase inhibitor (W7), MAPK inhibitor (PD98059) and PI3K inhibitor (wortmannin) did not affect transactivation in MCF-7 cells transfected with pLDH-7 and treated with E2 (Fig. 33), suggesting that CaM kinase, MAPK and PI3K are not involved in mediating hormonal activation of LDHA. PKA is a major kinase responsible for activating CREB and previous studies have indicated that estrogen can activate CREB through the PKA pathway (Dong et al., 1999). Three cAMP-PKA pathway inhibitors SQ22536, 2',5'-dideoxyadenosine (ddA) and H89 were used to analyze the involvement of PKA in activation of LDHA by E2. None of these inhibitors had a major impact on E2-dependent

transactivation, although a small inhibitory effect was observed (Fig. 34). Therefore, the PKA pathway does not play a predominant role in estrogen-dependent activation of LDHA.

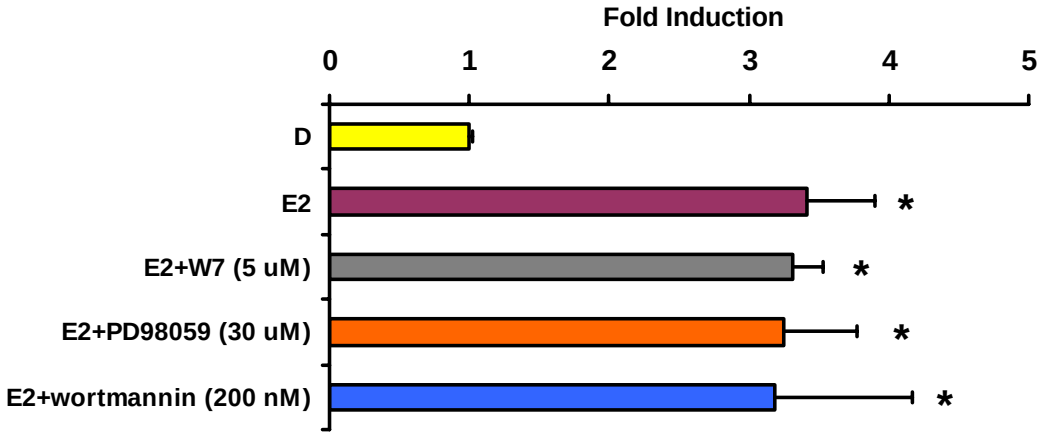

Fig. 33. Effects of W7, PD98059 and wortmannin on activation of pLDH-7 by E2. MCF-7 cells were transfected with pLDH7 and hER and treated with DMSO, E2 (10 nM) alone, or E2 plus W7, PD98059 and wortmannin for 24 hr. Cells were treated with DMSO or E2 (10 nM) for 48 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

In contrast to PKA and CaM Kinase inhibitors, the PKC inhibitor bisindolylmaleimide I (Bi) inhibited the E2-induced transactivation in MCF-7 cells transfected with pLDH-7 and significant inhibition was observed with 5 and 10 uM Bi (Fig. 35).

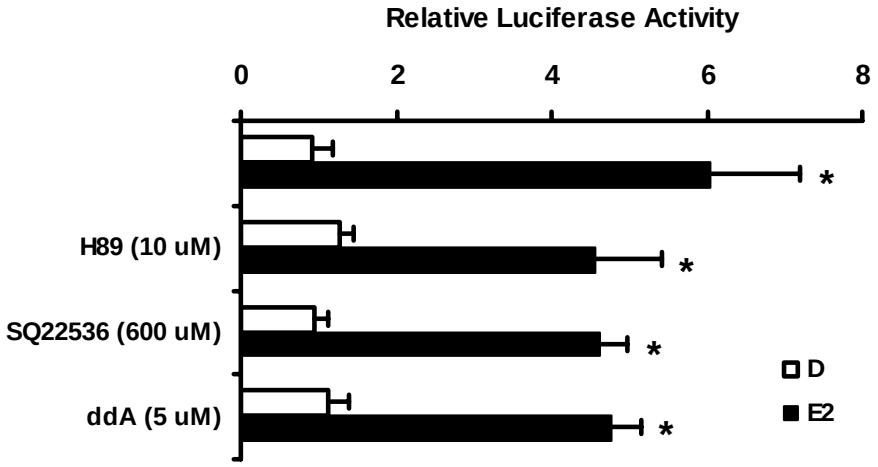

Fig. 34. Effects of PKA inhibitors H89, SQ22536 and 2'5'-dideoxyadenosine (ddA) on activation of pLDH-7 by E2. MCF-7 cells were transfected with pLDH-7 and treated with DMSO, E2 (10 nM) alone, or E2 plus W7, PD98059 and wortmannin for 24 hr. Luciferase activities were determined for three separate determinations as described in the *Materials and Methods.* Significant induction by E2 is indicated by an asterisk ($p<0.05$).

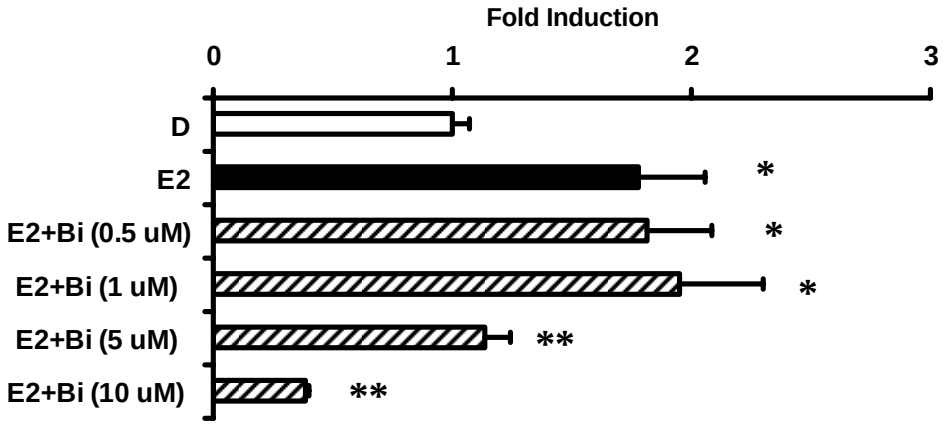

Fig. 35. Inhibition of E2-dependent activation of pLDH-7 by the PKC inhibitor bisindolylmaleimide I. MCF-7 cells were transfected with pLDH-7 and treated with DMSO (D), E2 (10 nM) alone, or E2 plus different concentration of bisindolylmaleimide I (Bi) for 24 hr. Luciferase activities were determined for three separate determinations as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk. Significant inhibition ($p<0.05$) of E2-induced luciferase activity is indicated by double asterisks.

The effects of kinase inhibitors on hormonal activation pLDH-7 construct are summarized in Table 2. The results indicate that the induction of LDHA by estrogen is mediated through activation of PKC pathway.

Table 2

Effects of kinase inhibitors on activation of pLDH-7 constructs by E2

Kinase pathway	**Inhibitors**	**Effects**
MAPK	PD98058	None
PI3K	wortmannin	None
	2’5’-dideoxyadenosine	
PKA	SQ22536	Minimal
	H89	
CaMKIV	W7	None
PKC	bisindolylmaleimide I	decreased

The role of PKC was further confirmed by inhibiting the E2-inudced activation of pLDH-7 using 5 uM Bi and by showing that activation of pLDH-7 by the PKC activator TPA was also inhibited after cotreatment with Bi (Fig. 36).

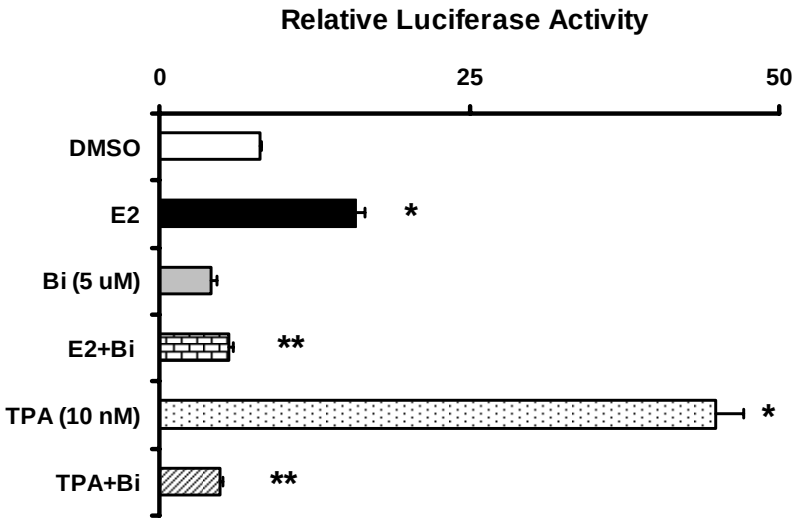

Fig. 36. E2-responsiveness of pLDH-7 is inhibited by the PKC inhibitor bisindolylmaleimide I. MCF-7 cells were transfected with pLDH-7 and treated with DMSO, E2 (10 nM) alone, E2 plus bisindolylmaleimide I (Bi), TPA alone or TPA plus Bi for 24 hr, Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 and TPA is indicated by an asterisk and significant inhibition ($p<0.05$) of E2 or TPA-induced luciferase activity is indicated by double asterisks ($p<0.05$).

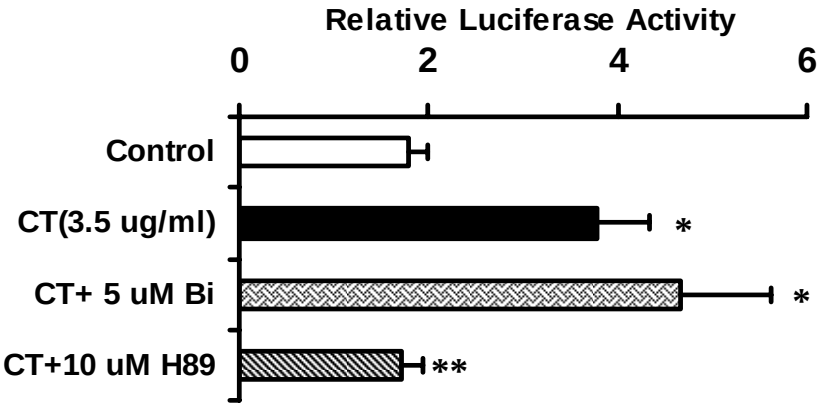

Fig. 37. Induction of pLDH-7 by cholera toxin . MCF-7 cells were transfected with pLDH-7 and treated with DMSO, cholera toxin (CT) alone, CT plus Bi, CT plus H89 for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by CT is indicated by an asterisk and significant inhibition ($p<0.05$) of CT-induced luciferase activity is indicated by double asterisks ($p<0.05$).

Activation of pLDH-7 by E2 is in direct contrast with the activation by cholera toxin (CT), a cAMP/PKA inducer. CT-induced luciferase activity was inhibited by the PKA inhibitor H89 but not the PKC inhibitor Bi (Fig. 37). This demonstrates that cholera toxin and E2 activate the LDHA construct through different mechanisms. Cholera toxin acts via PKA, while E2 acts mainly via PKC.

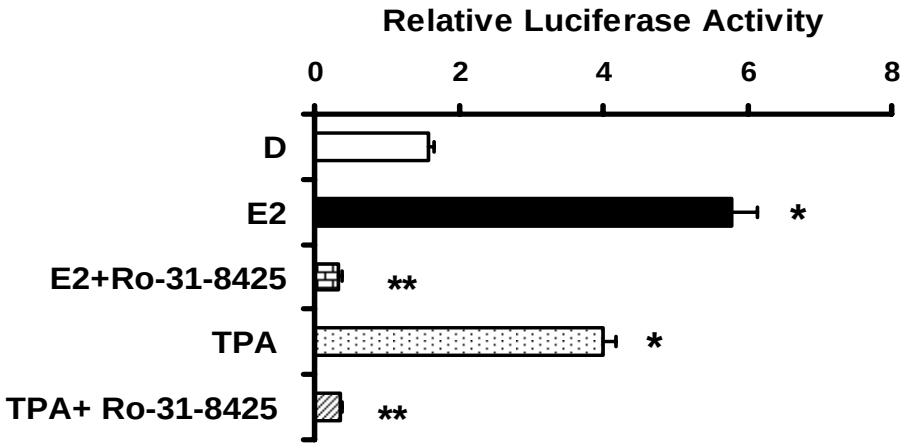

Fig. 38. E2-responsiveness of GAL4-CREB is dependent on PKC. MCF-7 cells were transfected with GAL4-Luc reporter gene and GAL4-CREB. Cells was treated with DMSO (D), E2 (10 nM) alone, or E2 plus 5 uM Ro-31-8425 (RO), TPA (10 nM) alone or TPA plus Ro-31-8425. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to control (DMSO) is indicated by an asterisk and significant inhibition of E2- and TPA-induced luciferase activities is indicated by double asterisks ($p<0.05$).

Since we have demonstrated that estrogen upregulated LDHA via PKC-dependent activation of CREB, we next investigated whether PKC activated CREB in MCF-7 breast cancer cells. In cells transfected with GAL4-CREB fusion protein, GAL4-Luc reporter gene and hER, E2 induced the luciferase activity, indicating that CREB is activated by E2 treatment. The activation was inhibited by the PKC inhibitor Ro, suggesting the event is PKC dependent. As a control, the PKC activator TPA activated CREB and the activation was also inhibited by Ro (Fig. 38).

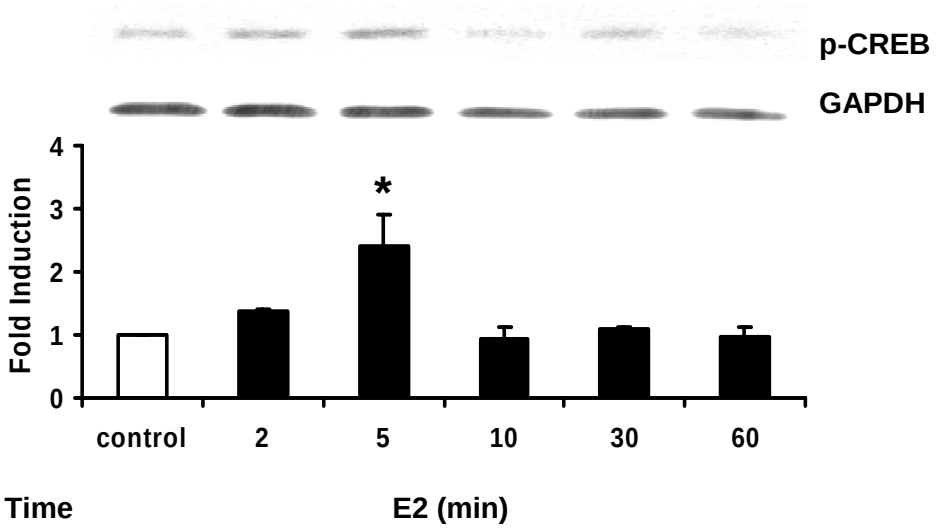

Fig. 39. E2-induced phosphorylation of CREB. MCF-7 cells were treated at various time points with E2. Whole lysates were obtained and levels for phospho-CREB were determined for three separate determinations as described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to control is indicated by an asterisk ($p<0.05$). GAPDH was used as a loading control.

Phosphorylation on Ser133 is essential for the transcriptional activity of CREB (Gonzalez and Montminy, 1989). Therefore, we use a phospho-Ser133 CREB antibody to investigate the phosphorylation of CREB upon E2 treatment. E2 rapidly induced phosphorylation of CREB at Ser133 within 5 min and the phosphorylation soon returned to basal levels (Fig. 39). Both E2 and the PKC activator TPA induced CREB phosphorylation and these responses were blocked by PKC inhibitor Bi (Fig. 40). These results confirmed that in MCF-7 cells, E2 induces LDHA by activating CREB family proteins through the nongenomic PKC pathway.

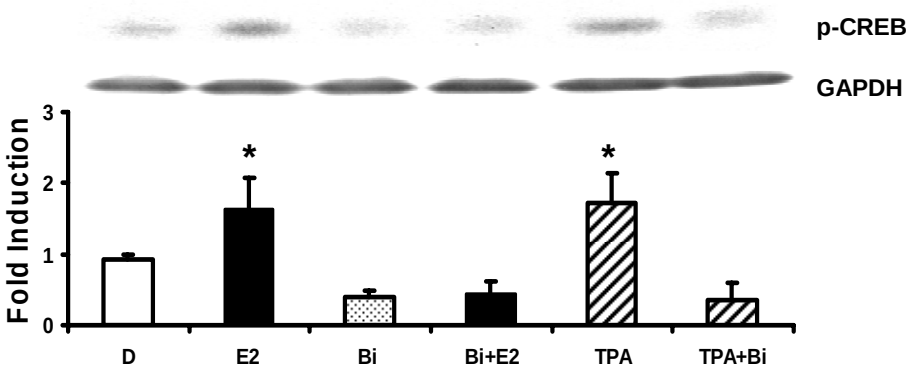

Fig. 40. Inhibition of CREB phosphorylation by the PKC inhibitor. MCF-7 cells were treated with E2 or TPA for 5 min with or without the cotreatment with Bi. Whole cell lysates were obtained and the levels for phospho-CREB were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to DMSO (control) is indicated by an asterisk ($p<0.05$) and significant inhibition of E2- and TPA-induced CREB phosphorylation is indicated by double asterisks. GAPDH was used as a loading control.

3.4 GAL4-protein chimeras can be used to assess estrogen-induced kinase cascades

ERα modulates an array of kinases, but detailed mechanisms of these responses are not well understood (Migliaccio et al., 1996; Duan et al., 2001) (Aronica et al., 1994; Farhat et al., 1996; Kelly et al., 1999; Simoncini et al., 2000; Hisamoto et al., 2001; Sylvia et al., 2001; Duan et al., 2002; Qin et al., 2002) . Previous studies have shown that E2-dependent activation of MAPK, PI3K, CaMKIV and PKA/PKC can be determined in MCF-7 cells transfected with GAL4-Elk-1, GAL4-SRF, GAL4-p65 and GAL4-CREB expression plasmids, respectively (Castro-Rivera et al., 2001; Duan et al., 2002; Qin et al., 2002; Chen et al., 2004a; Li et al., 2004). Moreover, since endogenous ERα is limiting in this system and estrogen-induced transactivation is only observed after cotransfection of ER, this system can be used to assess the role of variant ERα in mediating hormonal activation of kinase pathways.

In MCF-7 cells transfected with GAL4-Luc, GAL4-Elk-1, and ER/variants/empty vector, only wild type hER and ERα variant TAF1 can mediate the E2-induced MAPK activity. TAF1 is an ER variant with three amino acid mutations in helix 12 (D538N, E542Q and D545N) that facilitate nuclear ERα-coactivator interactions (Tzukerman et al., 1994), thus it is not surprising that mutations on these amino acids do not affect estrogen-dependent activation through non-genomic pathways (Fig. 41). In these experiments, ER variants HE11 (DBD deletion mutant), HE15 (E/F domain mutant) and HE19 (A/B domain mutant) were not active. Similar results were observed in E2-induced PI3K activity (Fig. 42) and PKA/PKC activity (Fig. 43) in cells transfected with GAL4-SRF and GAL4-CREB, respectively. However, an exception was observed for induction of CaMKIV activity by E2 (Fig. 44). ER variant HE19 mediated the activation of CaMKIV by estrogen whereas TAF1 exhibited minimal activities in modulating E2-induced CaMKIV.

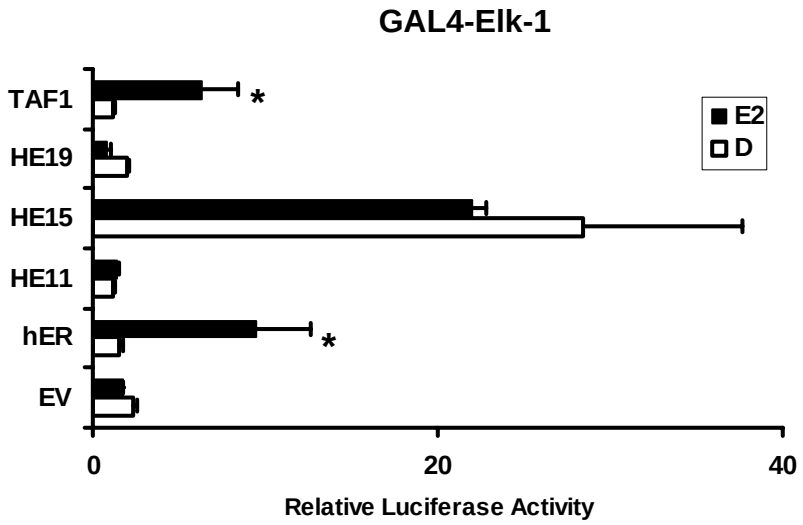

Fig. 41. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-Elk-1 in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector (EV), and 1 ug GAL4-Elk-1 expression plasmid. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

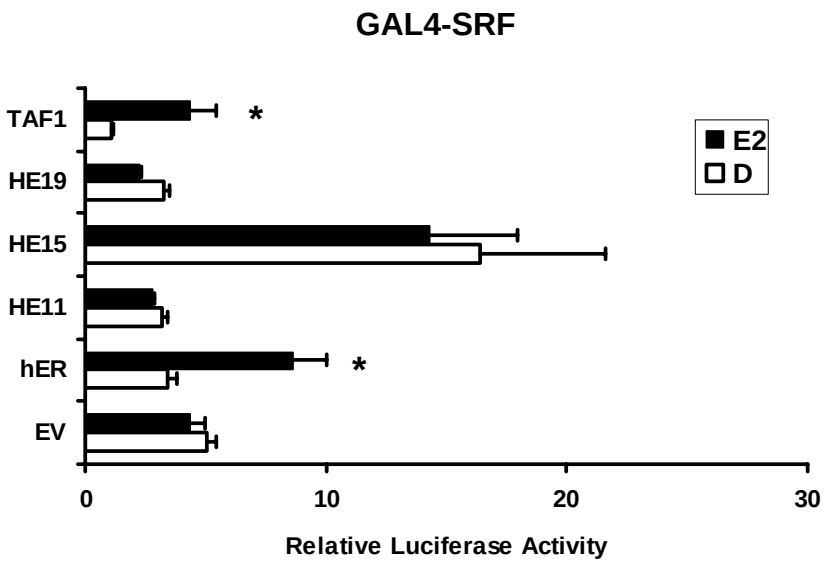

Fig. 42. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-SRF in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector, and 0.1 ug GAL4-SRF expression plasmid. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

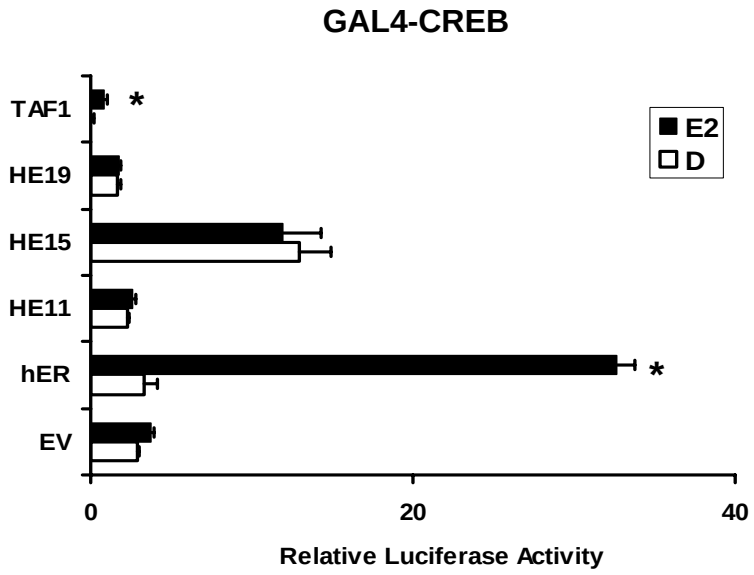

Fig. 43. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-CREB in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector, and 0.1 ug GAL4-CREB expression plasmid. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

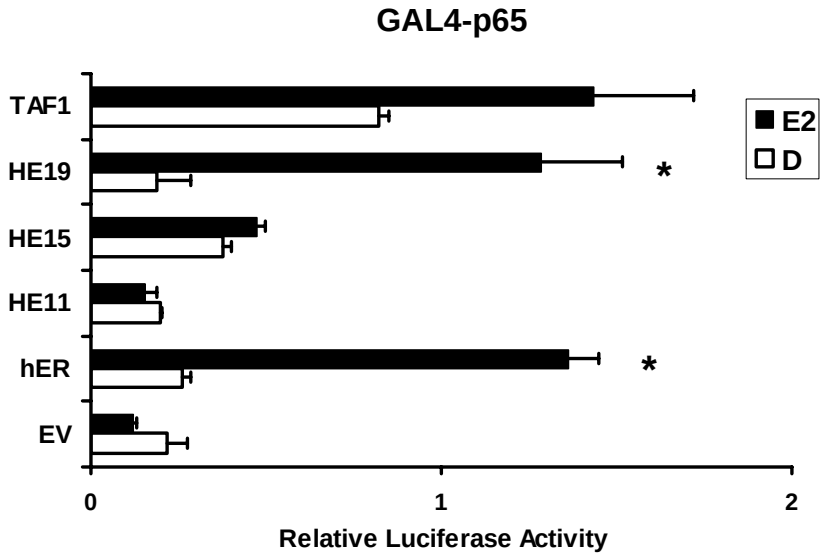

Fig. 44. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-p65 in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector, and 0.1 ug GAL4-p65 expression plasmid. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

To rule out the role of possible interference of endogenous ERα in these assays, we carried out a similar set of experiments within the MCF-7 C4 clone, an ERα-negative cell line derived from MCF-7 cells (Figs. 45-48) (Oesterreich et al., 2001). The patterns of kinase activation in C4 cells transfected with wildtype and variant ERα were comparable to that observed for MCF-7 cells (Figs. 41-44), indicating that the endogenous ER is not interfering with the assay system.

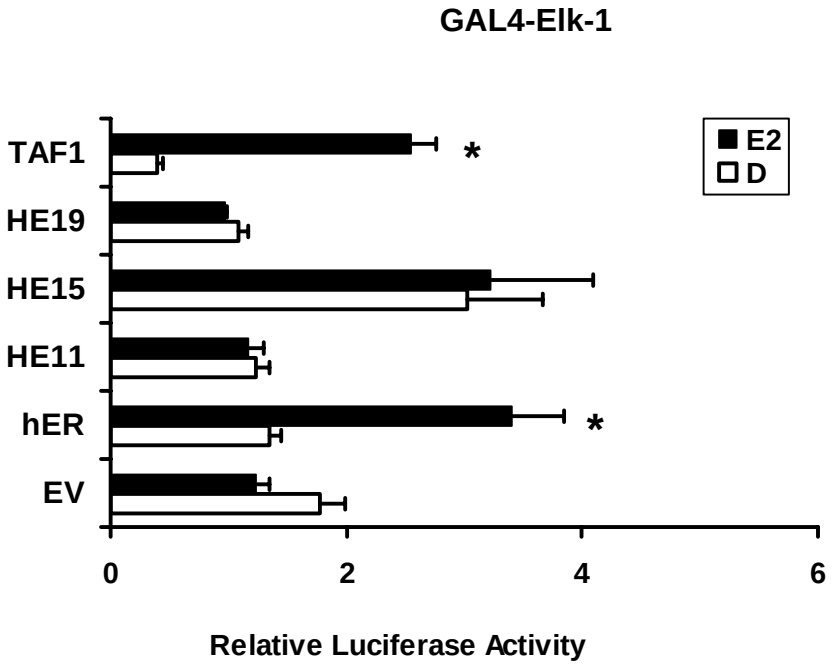

Fig. 45. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-Elk-1 in C4 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector, and 1 ug GAL4-Elk-1 expression plasmid. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

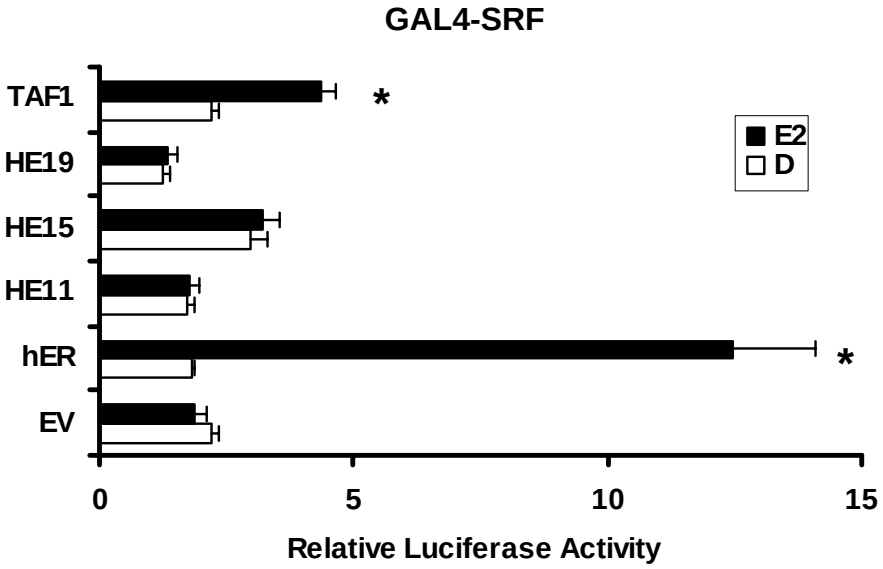

Fig. 46. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-SRF in C4 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector, and 0.1 ug GAL4-SRF expression plasmids. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

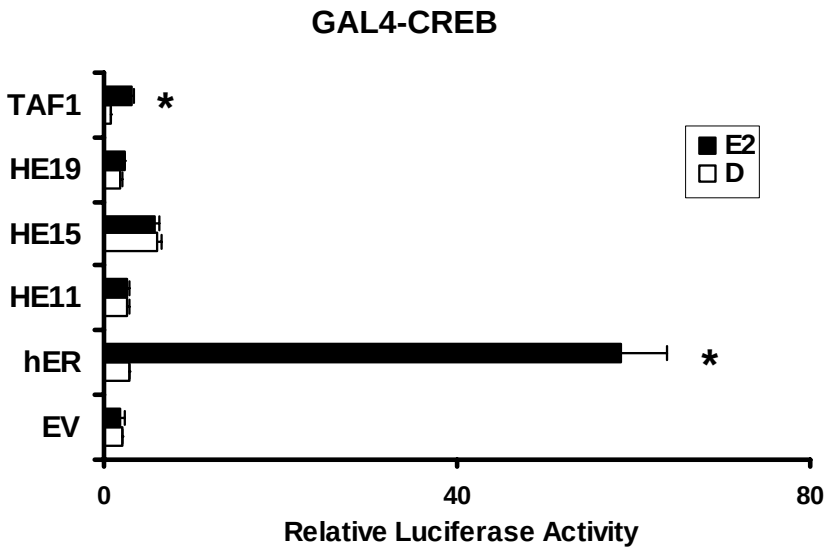

Fig. 47. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-CREB in C4 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector, and 0.1 ug GAL4-CREB expression plasmid. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

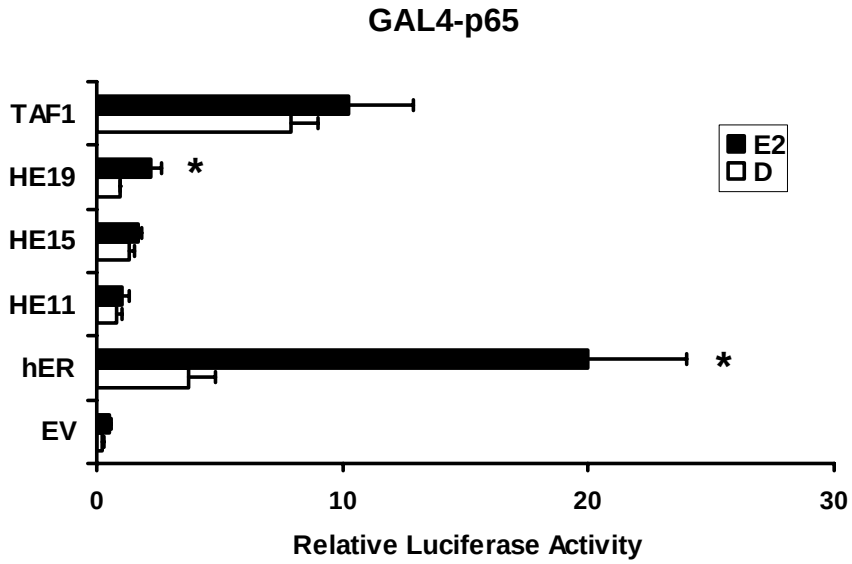

Fig. 48. Effects of wildtype/mutant ERα on E2-dependent activation of GAL4-p65 in C4 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ERα/mutants/empty vector, and 0.1 ug GAL4-p65 expression plasmid. Cells were treated with 10 nM E2 or D (DMSO) for 24 hr and luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk.

The effects of kinase-specific inhibitors on hormonal activation of GAL4-Elk-1, GAL4-SRF, GAL4-CREB and GAL4-p65 were also investigated. The results showed that activation of GAL4-Elk-1, GAL4-SRF and GAL4-p65 by E2 were inhibited MPAK inhibitor PD98059, PI3K inhibitor LY294002 and CaMKIV inhibitor KN93, respectively (Fig. 49-51). Activation of GAL4-CREB by E2 was primarily inhibited by the PKC inhibitor Ro-31-8425, whereas no inhibition by the PKA inhibitor H89 was observed (Fig. 52). These data support the validity of these assays, which were used in subsequent studies on the activation of non-genomic pathways by structurally-diverse estrogenic compounds.

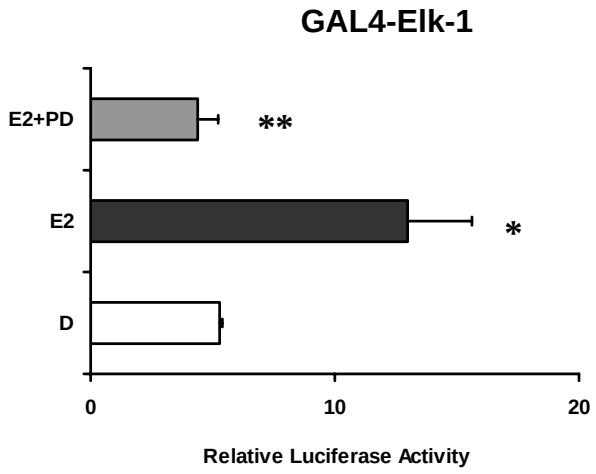

Fig. 49. Inhibition of E2-dependent activation of MAPK in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ER and 1 ug GAL4-Elk-1 expression plasmids. MCF-7 cells were treated with DMSO (D), E2 or E2 plus 30 uM PD98059 (PD) for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk. Significant inhibition ($p<0.05$) of E2-induced luciferase activity is indicated by double asterisks.

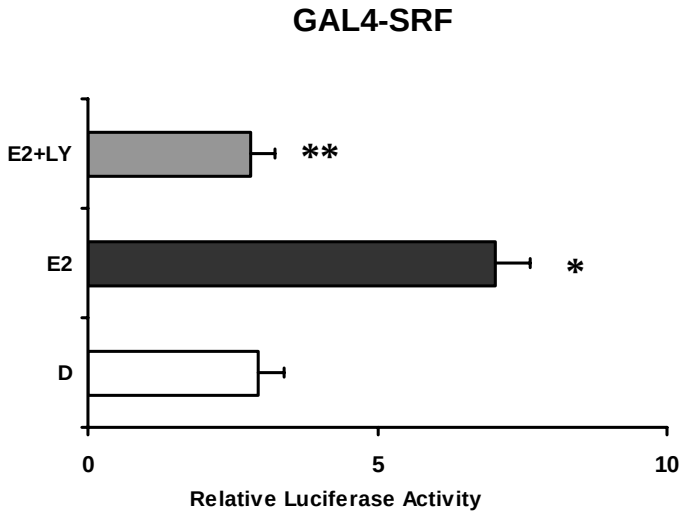

Fig. 50. Inhibition of E2-dependent activation of PI3K in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ER and 0.1 ug GAL4-SRF expression plasmids. MCF-7 cells were treated with DMSO (D), E2 or E2 plus 25 uM LY294002 (LY) for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk. Significant inhibition ($p<0.05$) of E2-induced luciferase activity is indicated by double asterisks.

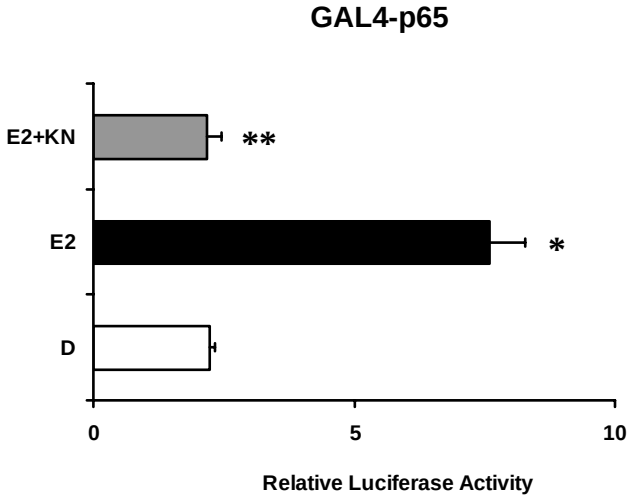

Fig. 51. Inhibition of E2-dependent activation of CaMKIV in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ER and 0.1 ug GAL4-p65 expression plasmids. MCF-7 cells were treated with DMSO (D), E2, E2 plus 20 uM KN93 (KN) for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk. Significant inhibition ($p<0.05$) of E2-induced luciferase activity is indicated by double asterisks.

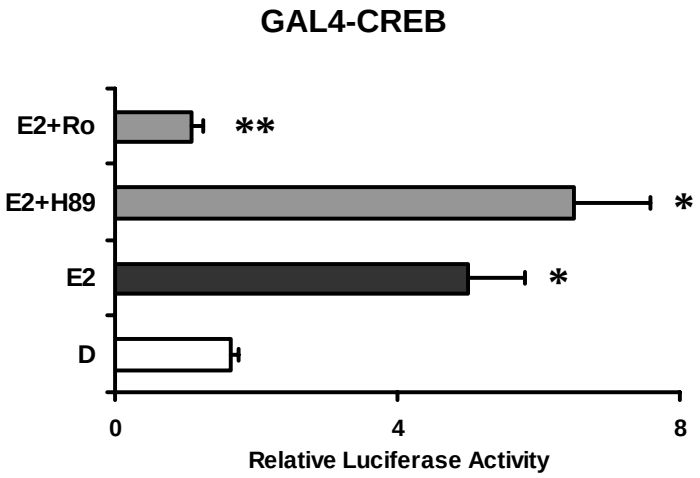

Fig. 52. Inhibition of E2-dependent activation of PKA/PKC in MCF-7 cells. Cells were transfected with 1 ug GAL4-Luc, 0.5 ug ER and 0.1 ug GAL4-CREB expression plasmids. MCF-7 cells were treated with DMSO (D), E2, E2 plus 10 uM H89 or E2 plus 5 uM Ro-31-8425 (Ro) for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) by E2 is indicated by an asterisk. Significant inhibition of E2-induced luciferase activity ($p<0.05$) is indicated by double asterisks.

3.5 The activation of various kinase pathways by structurally diverse estrogenic compounds

A number of structurally-diverse estrogenic compounds were used to test their abilities to activate kinases in MCF-7 cells (Fig. 53). Cells were transfected with GAL4-Elk-1/ERα, treated with 10 nM E2 and DES and various concentrations (20-75 uM) of other estrogenic compounds. All compounds, with the exception of resveratrol, induced luciferase activity, and thereby activated MAPK activity (Fig. 54)

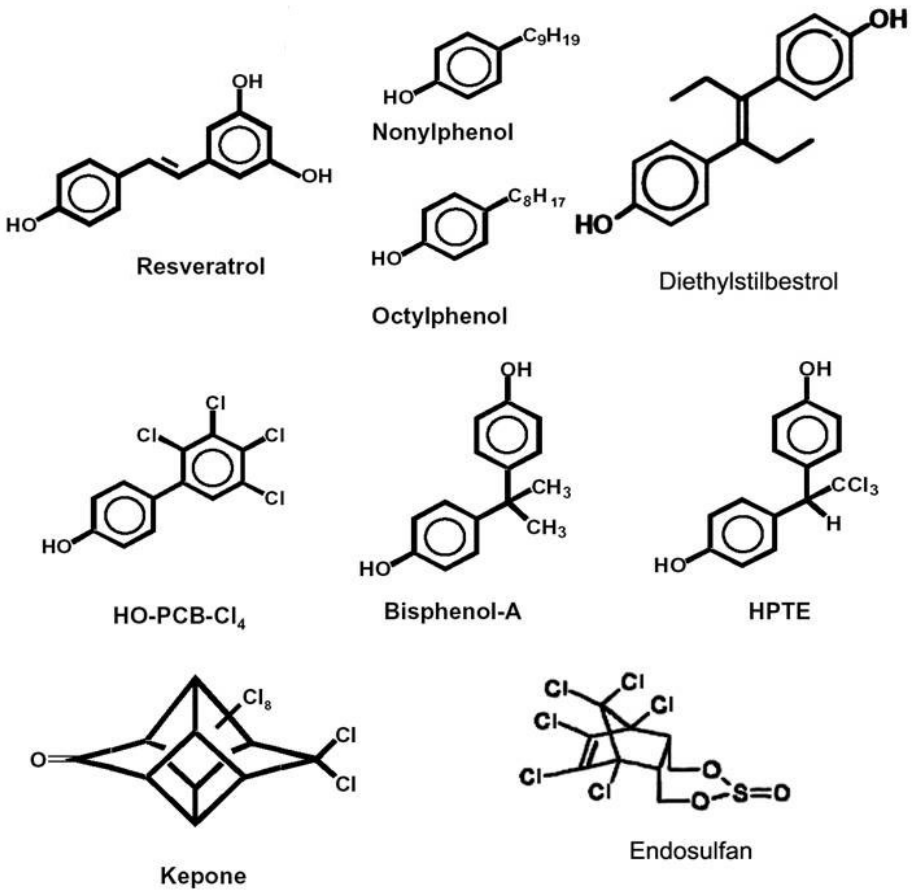

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Fig. 53. Estrogenic compounds tested for their activation of kinase pathways in MCF-7 cells.

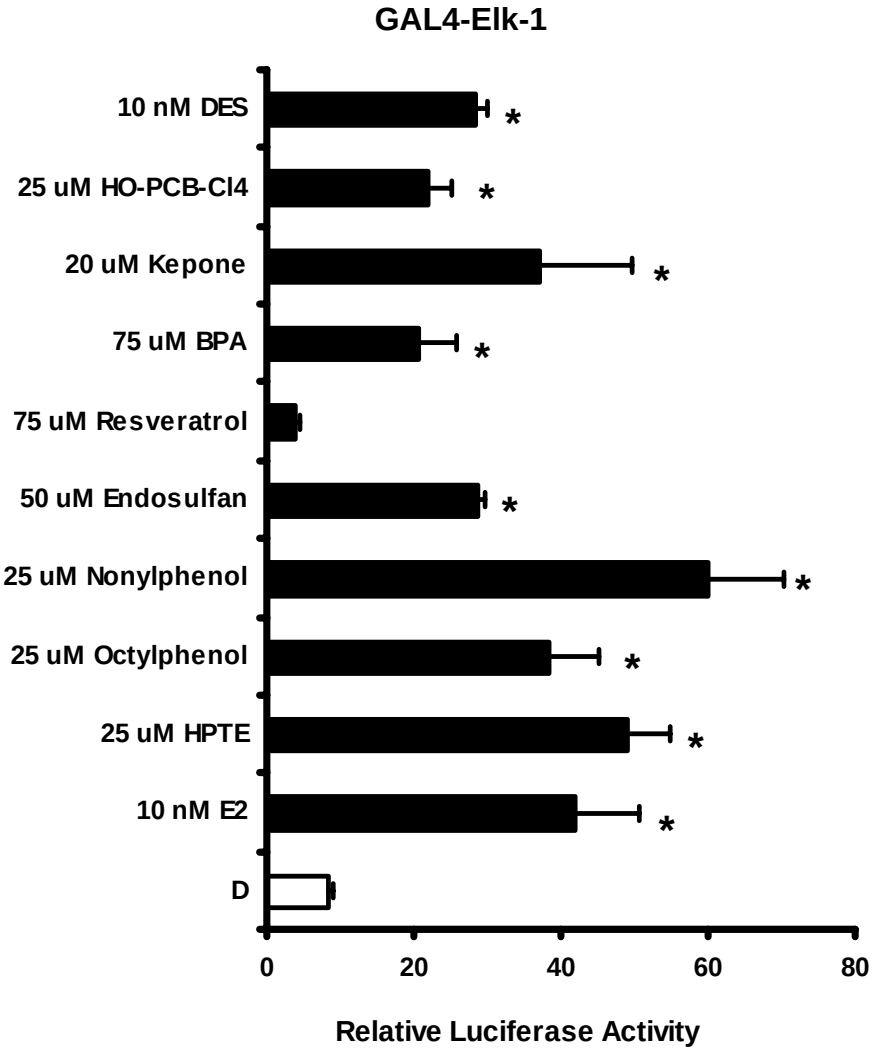

Fig. 54. Activation of GAL4-Elk-1 by estrogenic compounds. MCF-7 cells were transfected with 1ug GAL4-Luc, 0.5 ug ER, and 1 ug GAL4-Elk-l. Transfected cells were treated with D (DMSO), E2, or the estrogenic compounds for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to DMSO control is indicated by an asterisk.

Results from Western blot analysis for phospho-ERK confirmed results of transient transfection assays. With the exception of resveratrol, E2 and other estrogenic compounds induced phosphorylation of ERK within 10 min after treatment (Fig. 55). Inhibition studies with PD98059 indicated that E2 and two other xenoestrogenic compounds, HPTE and nonylphenol, also induced phosphorylation of ERK (Fig. 56).

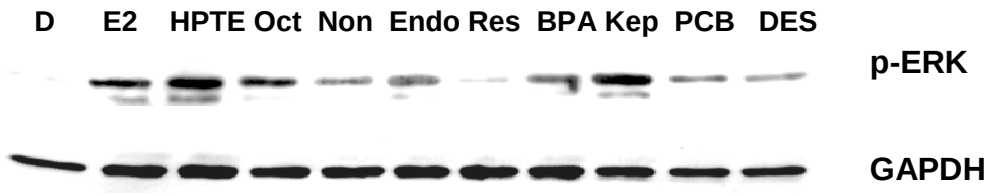

Fig. 55. Effects of estrogenic compounds on the phosphorylation of ERK. MCF-7 cells were treated for 10 min with D (DMSO), E2, HPTE, Oct (*p-t*-octylphenol), Non (nonylphenol), Endo (endosulfan), Res (resveratrol), BPA, Kep (Kepone), PCB (HO-PCB-Cl_4) and DES. Whole cell lysates were prepared and Western blots were performed as described in the *Materials and Methods.* GAPDH was used as a loading control.

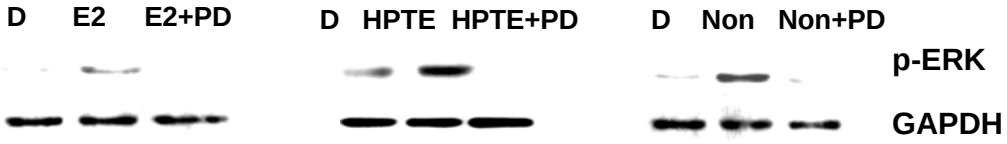

Fig. 56. Inhibition of ERK phosphorylation by PD98059 (PD). MCF-7 cells were pretreated with 30 uM PD98059 for 2 hr and then treated with D, E2, HPTE or Non in the presence or absence of PD. Whole cell lysates were prepared and Western blots were performed as described in the *Materials and Methods.* GAPDH was used as a loading control.

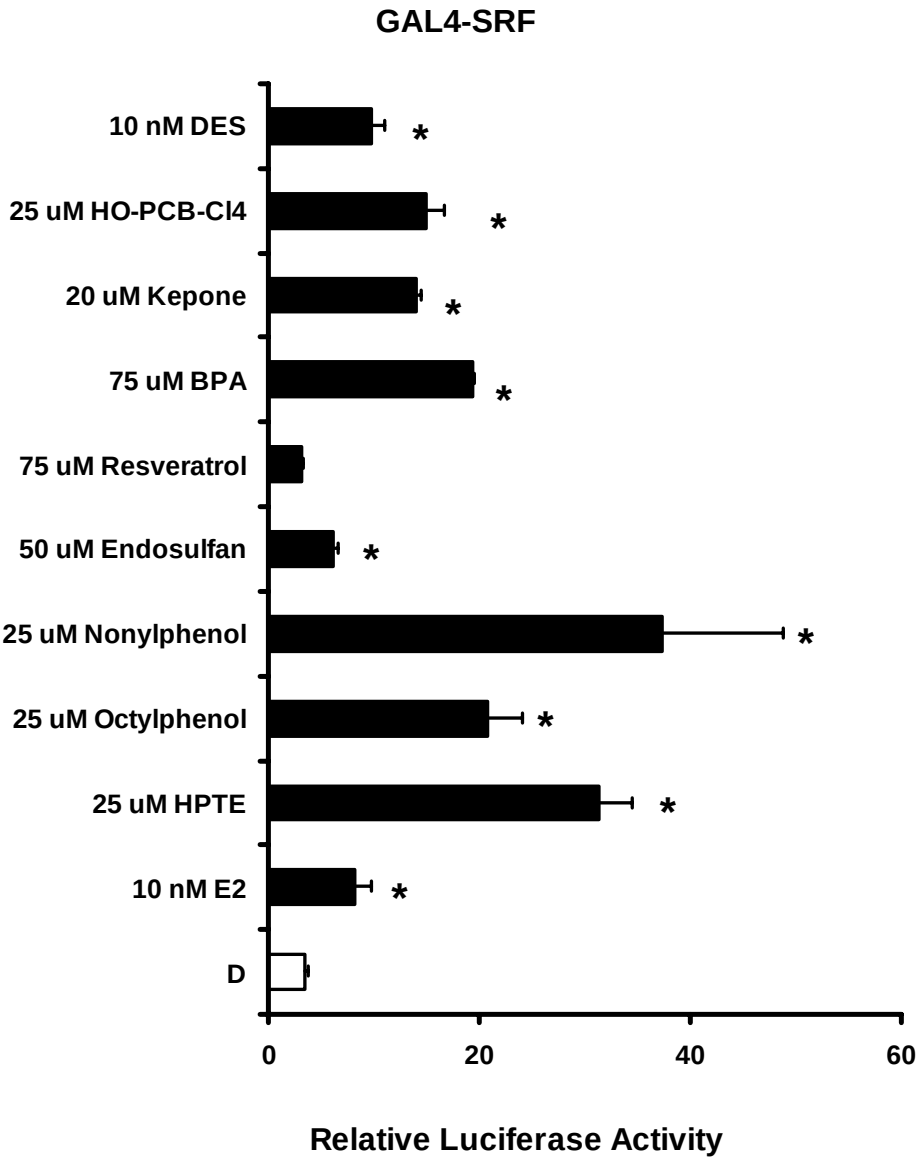

Fig. 57. Activation of GAL4-SRF by estrogenic compounds. MCF-7 cells were transfected with 1ug GAL4-Luc, 0.5 ug ER, and 0.1 ug GAL4-SRF. Transfected cells were treated with D (DMSO), E2, or the estrogenic compounds for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to DMSO control is indicated by an asterisk.

Estrogen-induced PI3K plays a major role in proliferation of MCF-7 cells (Castoria et al., 2001). Results in Figure 57 showed that E2, DES and seven xenoestrogens all induced PI3K activity whereas induction was not observable for resveratrol. Western analysis for phosphorylation of AKT, a downstream target of PI3K, confirmed this observation (Fig. 58). The relative potencies of these compounds varied among the transient transfection and Western blot assays, possibly due to the fact that transient transfection assay evaluates long term (24 hr) effects whereas short term (10 min) effects are observed in the immuno blots. The results in Figure 59 confirmed that the induction of AKT phosphorylation by E2, nonylphenol and HPTE is inhibited by the PI3K inhibitor LY294002.

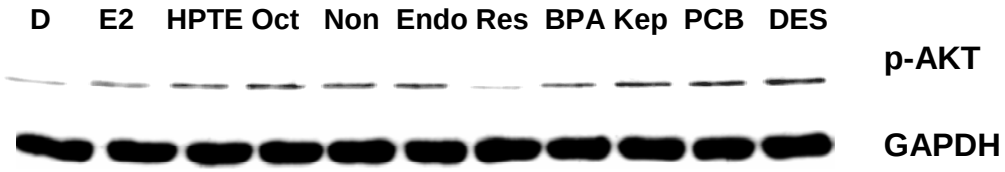

Fig. 58. Effects of estrogenic compounds on phosphorylation of AKT. MCF-7 cells were treated for 10 min with D (DMSO), E2, HPTE, Oct (*p-t*-octylphenol), Non (nonylphenol), Endo (endosulfan), Res (resveratrol), BPA, Kep (Kepone), PCB (HO-PCB-Cl_4) and DES. Whole cell lysates were prepared and Western blots were performed as described in the *Materials and Methods.* GAPDH was used as a loading control.

Fig. 59. Inhibition of AKT phosphorylation by LY294002 (LY). MCF-7 cells were pretreated with 25 uM LY for 2 hr and then treated with D, E2, HPTE or Non in the presence or absence of LY. Whole cell lysates were prepared and Western blots were performed as described in the *Materials and Methods.* GAPDH was used as a loading control.

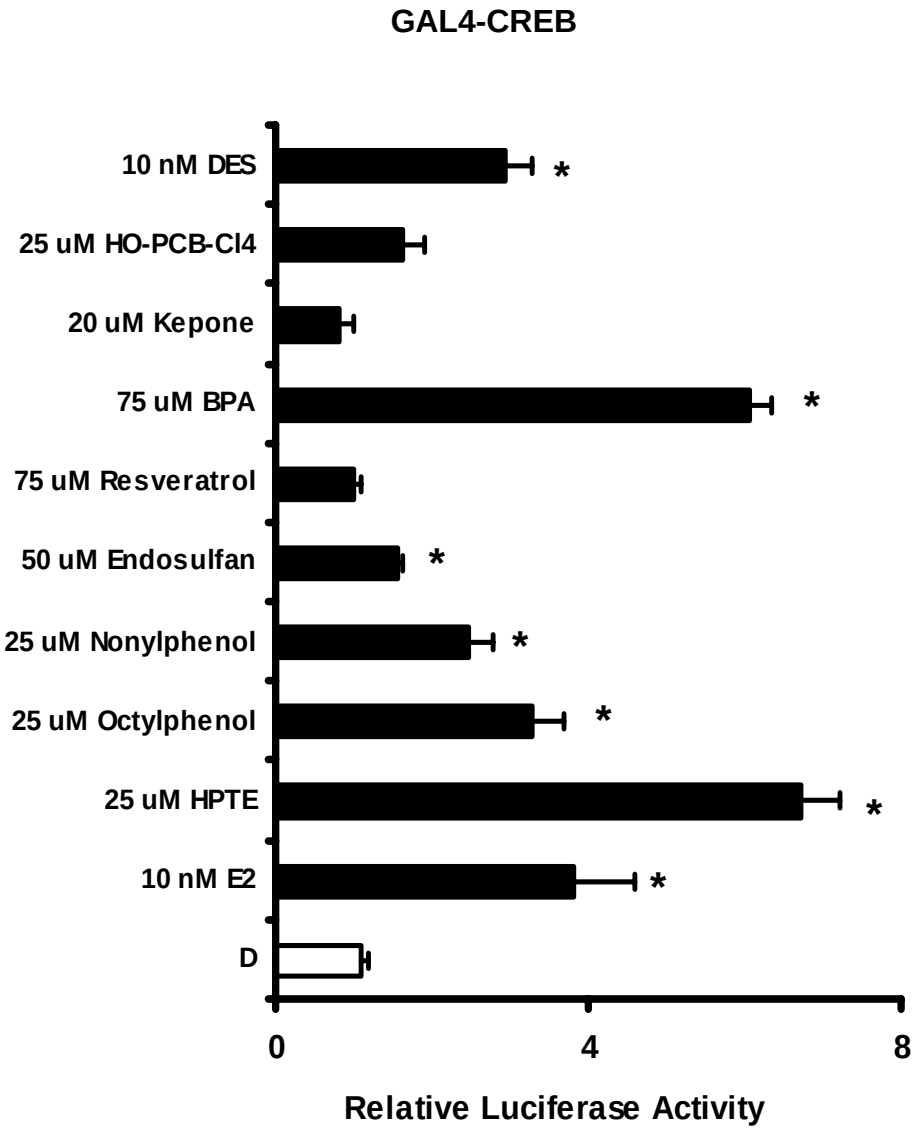

Fig. 60. Activation of GAL4-CREB by estrogenic compounds. MCF-7 cells were transfected with 1ug GAL4-Luc, 0.5 ug ER, and 0.1 ug GAL4-CREB. Transfected cells were treated with D (DMSO), E2, or the estrogenic compounds for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to DMSO control is indicated by an asterisk.

Activation of GAL4-CREB is observed in MCF-7 cells treated with E2, DES, HPTE, BPA, octylphenol, nonylphenol and endosulfan, but not kepone, resveratrol and HO-PCB-Cl_4. These results (Fig. 60) differentiated kepone and HO-PCB-Cl_4 from other xenoestrogens. Transactivation studies suggested that E2 primarily activates CREB through PKC pathways in MCF-7 cells (Fig. 51), and this was further investigated by analyzing induction of CREB phosphorylation by PKC in the presence or the absence of PKC inhibitor Ro-31-8425 and PKA inhibitor H89. Minor (E2/HPTE) or no detectable inhibition (nonylphenol) of CREB phosphorylation were observed in cells cotreated with H89. Significant inhibition was observed after treatment with E2, HPTE and nonylphenol in cells cotreated with Ro-31-8425 (Fig. 61). These results confirmed that E2 and some xenoestrogens activate PKC in MCF-7 cells.

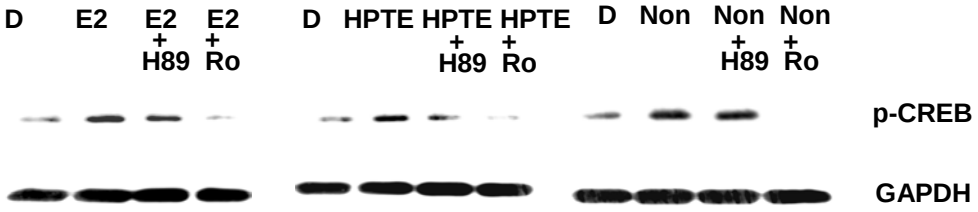

Fig. 61. Effects of estrogenic compounds on the phosphorylation of CREB. MCF-7 cells were pretreated with 10 uM H89 or 5 uM Ro-31-8425 (Ro) for 2 hr and then treated with D, E, HPTE or Non (nonylphenol) in the presence or absence of inhibitors for 10 min. Whole cell lysates were prepared and Western blots were performed as described in the *Materials and Methods.* GAPDH was used as a loading control.

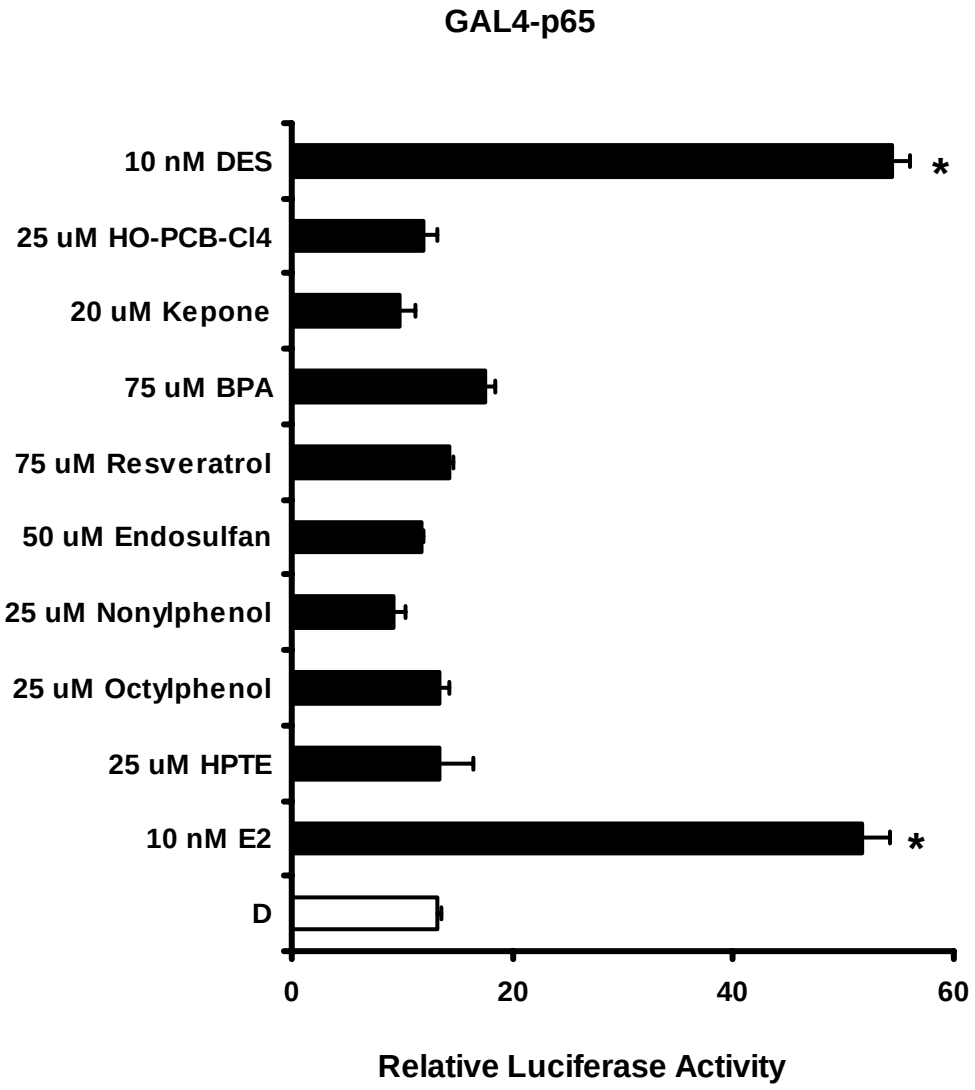

Fig. 62. Activation of GAL4-p65 by estrogenic compounds. MCF-7 cells were transfected with 1ug GAL4-Luc, 0.5 ug ER, and 0.1 ug GAL4-p65. Transfected cells were treated with D (DMSO), E2, or the estrogenic compounds for 24 hr. Luciferase activities were determined as described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to DMSO control is indicated by an asterisk.

Of all the compounds tested, only E2 and DES induced transactivation in MCF-7 cells transfected with GAL4-p65/ERα (Fig. 62). However, preliminary studies showed that in cells transfected with GAL4-p65, HO-PCB-Cl_4 inhibited the E2-induced transactivation, suggesting that this compound may be a CaMKIV inhibitor. This inhibitory effect was further investigated in MCF-7 cells transfected with GAL4-p65/ERα and treated with E2 alone or in combination with different concentrations of HO-PCB-Cl_4. The results showed that HO-PCB-Cl_4 significantly inhibited CaMKIV-dependent activation of GAL4-p65 by E2 in a dose-dependent manner (Fig. 63). Antibodies for detecting phospho-CaMKIV are not available. Therefore, the inhibitory effects of HO-PCB-Cl_4 were investigated using CaMKIV-dependent induction of p53 by E2 as a model (Qin et al., 2002). The results (Fig. 64) demonstrated that E2-dependent inductions of p53 protein levels in MCF-7 cells was inhibited by the antiestrogen ICI 182,780 and by both KN93 and HO-PCB-Cl_4, confirming that HO-PCB-Cl_4 is a CaMKIV inhibitor.

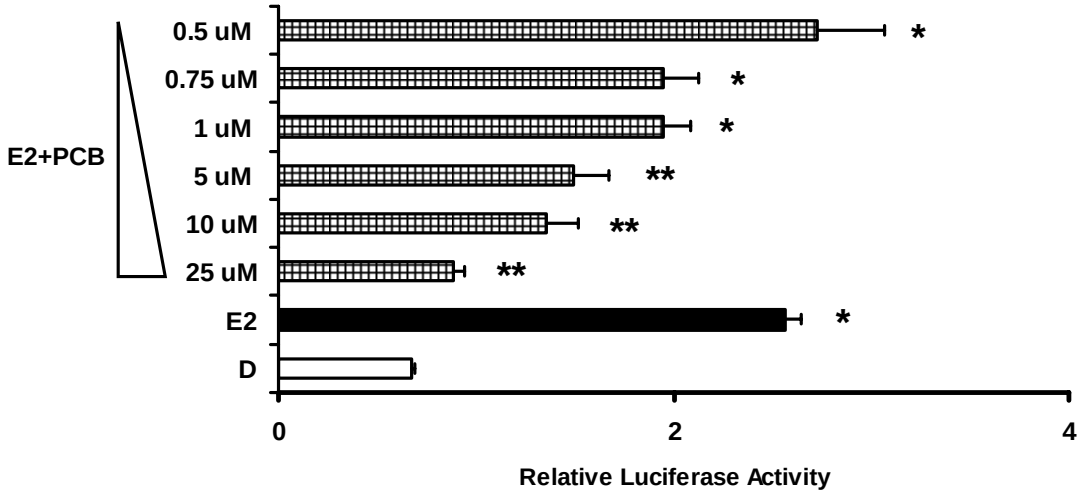

Fig. 63. Inhibition of transactivation of GAL4-p65 by HO-PCB-Cl_4. MCF-7 cells were transfected with 1ug GAL4-Luc, 0.5 ug ER and 0.1ug GAL4-p65. Cells were treated with D, E2 or E2 plus various concentrations of HO-PCB-Cl_4 for 24 hr. Luciferase activities were determined described in the *Materials and Methods.* Significant induction ($p<0.05$) compared to DMSO control is indicated by an asterisk.

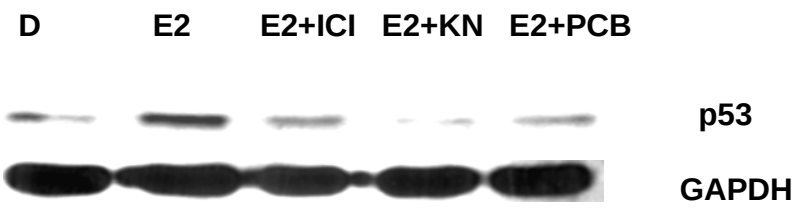

Fig. 64. Inhibition of hormone-induced p53 protein expression by ICI 182, 780, KN93 and HO-PCB-Cl_4. MCF-7 cells were treated with DMSO (D), E2, or E2 plus ICI 182,780 (ICI), KN93 (KN) and HO-PCB-Cl_4 (PCB) for 6 hr. Whole cell lysates were prepared and Western blots were performed as described in the *Materials and Methods.* GAPDH was used a loading control.

To evaluate the role of ER in the nongenomic actions of estrogenic compounds, we chose E2 and two compounds HPTE and nonylphenol to test whether their activation of MAPK and PI3K is sensitive to antiestrogen inhibition. The phosphorylation of AKT through E2, HPTE and nonylphenol were inhibited by ICI 182, 780, suggesting that activation of PI3K by these three chemicals is ER-dependent. E2-induced phosphorylation of ERK is also inhibited by ICI 182,782, however, HPTE- and nonylphenol-induced phosphorylation of ERK were partially (HPTE) or completely (nonylphenol) resistant to the inhibitory effects of ICI 182,780 (Fig. 65). These results indicate that some nongenomic actions of xenoestrogen may not be mediated by ER.

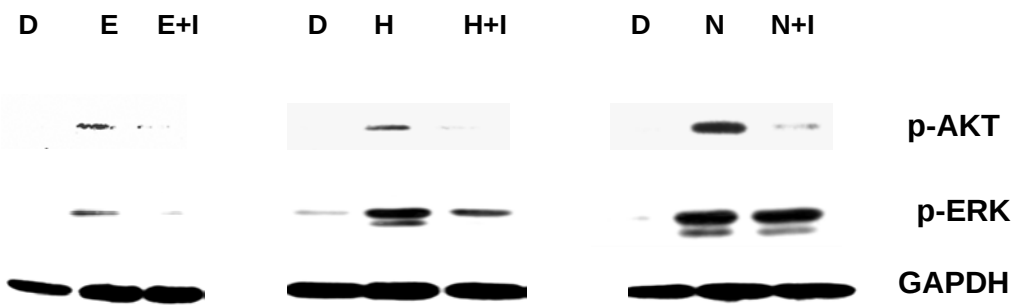

Fig. 65. Effects of the antiestrogen ICI 182,780 on MAPK and PI3K activation by estrogenic compounds. MCF-7 cells were pretreated with ICI 182,780 (I) for 6 hr and then treated with DMSO (D), E2 (E), HPTE (H) and nonylphenol (N) for 10 min. Whole cell lysates were prepared and Western blots were performed as described in the *Materials and Methods.* GAPDH was used as a loading control.

CHAPTER IV

DISCUSSION AND SUMMARY

4.1 The E2-responsive DNA motif in LDHA gene promoter is a CRE (-48/-41)

Estrogen-induced breast cancer growth requires upregulation of a plethora of cellular components. Traditionally, the molecular mechanisms of hormonal activation of genes were thought to be mediated by the classic ER-ERE pathway. However, during the past 10-15 years, multiple novel pathways of hormonal actions have been discovered. For example, c-fos, a proto-oncogene essential for cell proliferation, is induced by estrogen through activation of MAPK and PI3K pathways (Duan et al., 2001). Heat shock protein 27 (Hsp 27), an important chaperone protein, is regulated by estrogen through ER-Sp1 interactions with both GC-rich and ERE half sites or through GC-rich sites alone (Porter et al., 1996).

One goal of this study has been to elucidate the mechanism of induction of LDHA by estrogen. LDHA has been well characterized as an E2-inducible gene. For example, in MCF-7 breast cancer cells, LDHA were induced by estrogen but not by progesterone, hydrocortisone, prolactin, insulin, or triiodothyronine (Burke et al., 1978). Rodent mammary tumors and normal mammary glands had increased LDHA expression after administration of E2 (Richards and Hilf, 1972). LDHA levels increased significantly in the uterus of immature mice treated with DES and the LDHA promoter-reporter constructs are also responsive to E2 and DES in CHO cells (Li and Hou, 1989). Preliminarily results of Northern blot analysis (Fig. 20) indicate that upon treatment of MCF-7 cells with E2, LDHA mRNA levels are significantly increased within 1 hr and increased further 6 and 48 hr after treatment. The maximum induction (about two-fold) was achieved at 48 hr time point. It is possible that LDHA mRNA levels may be further induced if cells were treated with E2 for longer than 48hr. These results confirm that LDHA mRNA levels are induced by E2 at the transcriptional level.

Analysis on the LDHA prompter indicates that it contains multiple cis-elements (Fig. 21). Results of transient transfection assays (Fig. 23-25) show that the cis-element mediating estrogen-induction in MCF-7 cells is a CRE site located at -48/-41 in the

LDHA promoter. This CRE appears to be an important regulatory element and other studies showed that the element is activated by cAMP signaling (Short et al., 1994). The cAMP-responsiveness of the CRE (-48/-41) site was also confirmed in our study through the induction of LDHA promoter-reporter constructs by constitutively active PKA (Fig. 30) and cholera toxin, a cAMP inducer (Fig. 31). CRE binds CREB family proteins and results of a gel mobility shift (Fig. 27), and supershift assays (Fig. 28) and the inhibitory effects of Killer CREB on a LDHA promoter construct (Fig. 29) have verified the binding of CREB family proteins to the CRE (-48/41).

Active CRE sites are frequently observed in close proximity (200 bp) to transcription initiation sites in gene promoters (Fig. 66) and become less cAMP-responsive when moved further upstream (Tinti et al., 1997). In agreement with this observation, the E2-responsive-CRE in LDHA promoter is located near the initiation start site (-48/-41) and this motif is not only important for E2-responsivenes, but also for basal activity. Mutation of the CRE in promoter-reporter construct results in a 75% decrease in basal activity compared to the wildtype construct (Fig. 25).

Somatostatin	TTGGC**TGACGTCA**GAGAGAGAG	-32
PEPCK	GCCCC**TTACGTCA**GAGGCGAGC	-74
VIP	TACTG**TGACGTC**TTTCAGAGCA	-60
PTH	GGGAC**TGACGTCA**TCT	-65
Enkephalin	GGGCCTG**CGTCA**GC	-87
αCG	AAAAT**TGACGTCA**TGG	-113
fos	CCCAG**TGACGT**AGGA	-57
Secretogranin II	GCCGG**TGACGTCA**GCGT	-74
Chromogranin B	CTCCG**TGACGTCA**GCGT	-108
Synapsin I	CGCGC**TGACGTCA**CTCG	-166
LDHA	CACTC**TGACGTCA**GCGC	-48
Aromatase	TATG**CACGTCA**CCCA	-161
Fibronectin	CCC**TGACGTCA**CCC	-170
αA Crystallin	ACCAGACT**GTCA**TCCC	-148

Fig. 66. Locations of active CRE sites in gene promoters. CRE sites are underlined and indicated in bold and their starting positions are indicated as numbers in the right column. Adapted from Montminy (1997).

The consensus CRE sequence is TGACGTCA, however, a CGTCA half site is also functional, although less active (Fink et al., 1988; Craig et al., 2001). Many CRE-regulated genes are involved in metabolic pathways and this includes genes such as phosphoenol pyruvate carboxykinase (Liu et al., 1991), cytochrome C (Herzig et al., 2000) and the gene in this study, LDHA. Some important cell cycle and cell survival factors, such as Bcl-2 (Dong et al., 1999) and cyclin D1 (Castro-Rivera et al., 2001), are also regulated in breast cancer cells by these CRE motifs. Other examples of CRE-regulated genes are growth factors (insulin, fibroblast growth factor 6), transcription factors (glucocorticoid receptor, STAT3) and immune system regulators (Cox-2, interleukin-2) (Mayr and Montminy, 2001). Since CREs can be regulated by estrogen, these genes are potential targets for hormonal activation through their respective CREs. Many of them, such as Bcl-2 and cyclin D1, have already been characterized as E2-responsive in breast cancer cells (Dong et al., 1999; Castro-Rivera et al., 2001).

CRE-regulated genes are particularly important for mediating brain functions. For example, trkB, a receptor for brain-derived neurotrophic factor (BDNF)/neurotrophin (NT)-4/5, is regulated by a proximal CRE within its promoter. The product of trkB plays a crucial role in brain development and maintenance, and its adaptation to injury or pathological conditions (Deogracias et al., 2004). CART, a peptide involved in addiction and feeding behavior, is also responsive to cAMP, which subsequently activates a CREB-CRE complex (Lakatos et al., 2002). Given the important role of CREB in the nervous system, it is possible that some of these effects of estrogen on the nervous system are mediated through activation of CRE-regulated genes (McEwen, 2001).

LDHA promoter-reporter constructs are E2-inducible only when cotransfected with wildtype ER. The ER variants HE11 (deletion on DBD), HE15 (deletion on AF2) and HE19 (deletion on AF1) were not active (Fig. 22), suggesting that AF1, AF2 and DBD in ER are all necessary for induction of LDHA by E2. This observation is consistent with the study on ER domain requirement for E2-induced GAL4-CREB activation, which also showed that HE11, HE15 and HE19 did not mediate GAL4-CREB activation whereas wildtype ER was active (Fig. 43, 47). The E2-induced LDHA promoter activity

can be inhibited by the antiestrogens ICI 182,780 and tamoxifen (Fig. 26), further confirming the requirement of ER for hormone-responsiveness.

Gel mobility shift assays demonstrated that nuclear factors specifically bound to CRE (-48/-41) since coincubation with excess unlabeled probe (LDHA) or a consensus CRE competitively decreased the retarded band intensity whereas unlabeled probe with a CRE mutation had no effect on retarded band intensity (Fig. 27). Supershift assays showed that ATF1 antibody supershifted the nuclear-proteins-DNA complex, suggesting that CREB family proteins are components of this complex. The fact that other antibodies against CREB and CREM did not form supershifted complex does not exclude their potential roles in the complex, since supershift assays are conducted *in vitro,* and do not necessarily reflect the situation in living cells. Furthermore, some epitopes in the complex may be inaccessible to specific antibodies and thus cannot be detected and previous studies in this laboratory with other protein-CRE complexes were not supershifted by these same antibodies.

Cholera toxin, a cAMP inducer, and PKAc, a constitutively active form of PKA, both activated the LDHA promoter constructs (Fig. 30, 31). Responsiveness to cAMP/PKA stimuli is a common trait for CRE-regulated genes and this confirms that the CRE in the LDHA promoter is cAMP-responsive. The Killer CREB inhibition study suggests the transcriptions factors binding to CRE (-48/-41) include CREB proteins (Fig. 29). CREB family proteins have several closely related members, including CREB, ATF1 and CREM and for CREB and CREM, several alternatively spliced products have been identified (van Dam and Castellazzi, 2001; Lonze and Ginty, 2002).The splicing variants exhibit distinct activating/repressing ability (Table 3). For example, a truncated CREM gene product, ICER (inducible cAMP response element repressor), is a potent transcriptional repressor for CRE (Molina et al., 1993) and is involved in the stress response in pituitary gland (Mazzucchelli and Sassone-Corsi, 1999). A highly conserved basic region/Leucine Zipper (bZIP) in C-terminus of CREB family proteins is essential for their dimerization and DNA-binding (Yun et al., 1990). Dimerization can form homodimers, or heterodimers between different CREB family members (Hurst and Jones, 1987; Yamamoto et al., 1988; Laoide et al., 1993). Killer CREB has a DNA binding domain deletion and exerts its inhibitory effects by sequestering CREB family

protein through dimerization (Walton et al., 1992). Thus, the Killer CREB experiment confirmed the role of CREB family proteins in mediating LDHA induction by E2.

Table 3
Examples of splicing variants of CREB and CREM. Adapted from Mayr and Montminy (2001).

CREB family member	Splicing variants	activities
CREB	CREB 341/CREB-α	Activation
	CREB 327/CREB Δ	Activation
	CREB Δ-14	Inhibition
	CREB Δ-35	Inhibition
CREM	CREM-τ	Activation
	CREM-α	Conditional activation
	S-CREM	Inhibition
	ICER I	Inhibition

GAL4-CREB was also activated by E2 when cotransfected with ER and GAL4-Luc (Fig. 32). Furthermore, similar to observations with the LDHA promoter-reporter constructs, GAL4-CREB was non-responsive to E2 without cotransfection of ER. Thus, these results indicate that E2 activates CREB in MCF-7 cells in an ER-dependent manner.

In summary, this study identified a CRE (-48/-41) site that is responsible for the E2-inducibility of the LDHA promoter. The responsiveness of LHDA to E2 is dependent on ER and cannot be mediated by ER mutants with deletions of AF1, AF2 and DBD. This CRE is the specific binding site for CREB family proteins.

4.2 E2-induced activation of CREB is mediated predominantly through nongenomic activation of PKC by E2 in MCF-7 Cells

CREB requires phosphorylation of Ser133 to be transcriptionally active (Gonzalez and Montminy, 1989) and this phosphorylation can be achieved through multiple kinase cascades, including PKA (Gonzalez and Montminy, 1989), CaM Kinase (Matthews et al., 1994; Sun et al., 1994), PKC (Xie and Rothstein, 1995), PI3K/AKT (Du and Montminy, 1998) and MAPK/pp90RSK (Xing et al., 1998). The MAPK kinase inhibitor PD98059, the PI3K inhibitor wortmannin and CaM kinase inhibitor W7 did not inhibit the E2-inducibility of LDHA promoter-reporter constructs, suggesting that MAPK/pp90RSK, PI3K/AKT and CaM kinase are not involved into E2-mediated activation of CREB (Fig. 33).

PKA is a major pathway for phosphorylation of CREB Ser133 (Gonzalez and Montminy, 1989) and estrogen has been shown to elevate intracellular cAMP levels (Aronica et al., 1994). Thus it is biologically plausible that E2 activates LDHA by activating CREB family through the cAMP/PKA pathway. In fact, some CRE-regulated genes, such as Bcl-2 and cyclin D1, are induced by estrogen-activated PKA (Dong et al., 1999; Castro-Rivera et al., 2001). However, three cAMP/PKA inhibitors that we employed (H89, SQ22536 and 2’5’-dideoxyadenosine) had only minimal impact on the E2-responsiveness of LDHA promoter constructs (Fig 34). The cAMP/PKA pathway is functional and capable of inducing LDHA promoter-reporter constructs in MCF-7 cells, because cholera toxin and PKA can activate those constructs (Fig. 30, 31). However, induction of LDHA by estrogen is not predominantly mediated by activation of PKA pathway.

In contrast to the cAMP/PKA inhibitors, the PKC inhibitors Ro-31-8425 and bisindolylmaleimide I abolished E2-induced activation of LDHA promoter-reporter constructs (Fig. 35) and GAL4-CREB (Fig. 36), suggesting that the E2-induced activity is mainly through activation of PKC. This finding is further corroborated by Western blot analysis showing that E2-induced CREB phosphorylation was also inhibited by bisindolylmaleimide I (Fig. 40). The effects of estrogen are clearly distinguished from the traditional cAMP inducers such as cholera toxin in the activation of LDHA. Cholera-toxin-induced activation of LDHA was inhibited by the PKA inhibitor H89 but not the

PKC inhibitor bisindolylmaleimide I (Fig. 37), while activation of LDHA by E2 was inhibited by bisindolylmaleimide I but not H89 (Fig. 34, 35). Activation of CREB family protein by PKC is a relatively rare event compared to that of PKA, but has previously been reported (Xie and Rothstein, 1995; Xie et al., 1996; Johannessen et al., 2004). Therefore, PKC is an effector of nongenomic actions of E2 (Kelly et al., 1999; Sylvia et al., 2001) and this study shows that estrogen induces PKC, which in turn activates CREB family proteins and induces LDHA transcription.

Previous studies in this laboratory have identified two genes, Bcl-2 and cyclin D1, that are regulated by E2 through activation of CREB in a PKA-dependent manner (Dong et al., 1999; Castro-Rivera et al., 2001). In the case of cyclin D1, the induction is mediated through a nonconsensus proximal CRE (TAACGTCA) in ZR-75 cells. This nonconsensus CRE was not active in MCF-7 cells. In the case of Bcl-2, the nonconsensus CRE motif (TGACGTA) was activated by E2 in MCF-7 cells. In the LDHA promoter, the E2-responsive CRE is a consensus motif (TGACGTCA). These results suggest that the cell context, the nucleotide sequence of the CRE and its flanking regions have a role in determining whether the target gene will be preferentially activated by PKA or PKC. E2-induced activation of GAL4-CREB (Fig. 52) and phosphorylation of CREB protein (Fig. 61) were all predominantly inhibited by the PKC inhibitor Ro-31-8425 rather than by the PKA inhibitor H89, suggesting that there is a certain degree of preference over kinase activation pathways linked to phosphorylation of CREB. However, at present, little is known about the mechanism of activation of PKA or PKC by E2/ER and it is difficult to speculate about the underlying mechanisms for this preferential PKA/PKC activation.

Phosphorylation of CREB is significantly increased within 5 min after treatment of E2. This rapid action is within the typical time-frame of the nongenomic actions by E2 (Fig. 39). We also observed that the phosphorylation levels of CREB soon returned to basal levels. This transient phosphorylation is also a typical feature of CREB activation. The underlying mechanism may be the rapid dephosphorylation of CREB at Ser133 by protein phosphatases, such as protein phosphatase-1 (PP-1) and protein phosphatase PP2A (Hagiwara et al., 1992; Wadzinski et al., 1993). Possibly due to this reason,

many CREB-regulated genes are induced only transiently. For example, in PC12 cells, the transcription of somatostatin can be rapidly induced by cAMP inducers but soon decreases to basal levels within 4 hr (Hagiwara et al., 1992). However, the induction of LDHA transcription appears to be more long-term because Northern blots (Fig. 20) showed that the induction on LDHA mRNA levels persisted for up to 48 hr after treatment with E2. The gel mobility shift assay also demonstrated that two-hour E2 treatment before harvesting nuclear extracts enhanced nuclear protein binding to the CRE. Thus, after initial phosphorylation and following dephosphorylation, there may be a secondary sustainable CREB activation in cells through some unidentified mechanism. Another possibility is that some CREB isoforms, or other members of CREB family, may be less susceptible to dephosphorylation and can substitute for CREB in maintaining upregulated gene expression for longer periods.

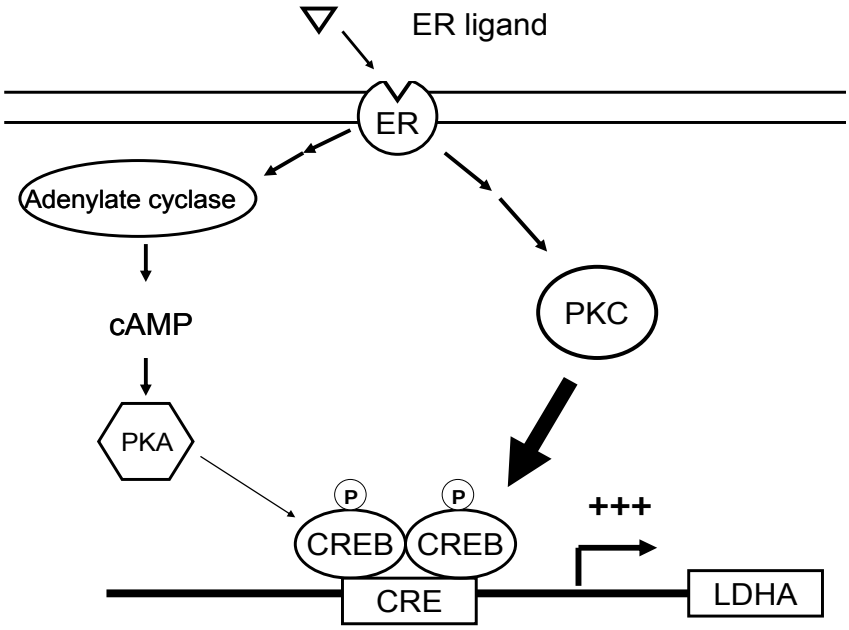

Fig. 67. Model of E2-induced LDHA transactivation in MCF-7 cells.

In summary, this study showed that E2-induced activation of CREB in MCF-7 cells is mediated predominantly through PKC. The PKA pathway in MCF-7 cells is intact and

fully functional after stimulation with a cAMP inducer, however, PKC but not PKA is the major pathway for hormone-dependent phosphorylation of CREB (Fig. 67).

4.3. Nongenomic actions of E2 require different ER domains

Estrogen is not unique in its activation of nongenomic actions in cells/tissues. Rapid, nongenomic effects have been extensively reported for other steroids, including progesterone (Morrill and Kostellow, 1999; Patrat et al., 2000), androgens (Benten et al., 1997; Machelon et al., 1998), glucocorticoids (Qiu et al., 1998; Venero and Borrell, 1999) and mineralocorticoids (Gekle et al., 1996; Christ et al., 1999). The large number of examples of steroid-mediated activation of kinase pathway suggests that steroid hormones are important signaling molecules in metazoans. Phylogenetic analyses indicate that the first steroid hormone receptor was an estrogen receptor and other steroid receptors evolved from the prototypical estrogen receptor through ligand exploitation and serial genome expansions (Thornton, 2001). Thus, some nongenomic activities by the diverse group of steroids may also have evolved from a common mechanism which originated with ancient ER.

Steroid-mediated nongenomic actions have also been characterized in plants. A transmembrane receptor kinase BRI1 can be activated by binding to brassinosteroid (BR), a plant steroid (Wang et al., 2001c). BR binding to BRI1 leads to its dimerization with BRI1-associated receptor kinase 1 (BAK1) and mutual transphosphorylation. The BRI1-BAK1 complex inhibits the activity of BIN2, a kinase which shares homology with mammalian glycogen synthase kinase 3 (GSK3). BIN2 is a negative regulator of BR signaling and its inhibition leads to the activation of two downstream nuclear proteins, BZR1 and BZR2/BES1 and target gene expression (Fig. 68) (Tichtinsky et al., 2003; Wang and He, 2004). The steroid binding domain of BRI1 has a unique structure from that of the metazoan steroid receptors, suggesting that they are not closely related (Kinoshita et al., 2005). The plant steroid signaling systems may share a distant ancestral prototype with metazoan systems, or it may have arisen independently. Whatever the case, steroid-induced nongenomic actions appear to be an ancient mechanism and have important physiological functions. For example, in breast cancer cells hormone-dependent growth is not only inhibited by antiestrogens but also by PI3K

inhibitors such as LY294002, suggesting that direct or indirect actions of this kinase by E2 is essential for cell growth (Zhang et al., unpublished data).

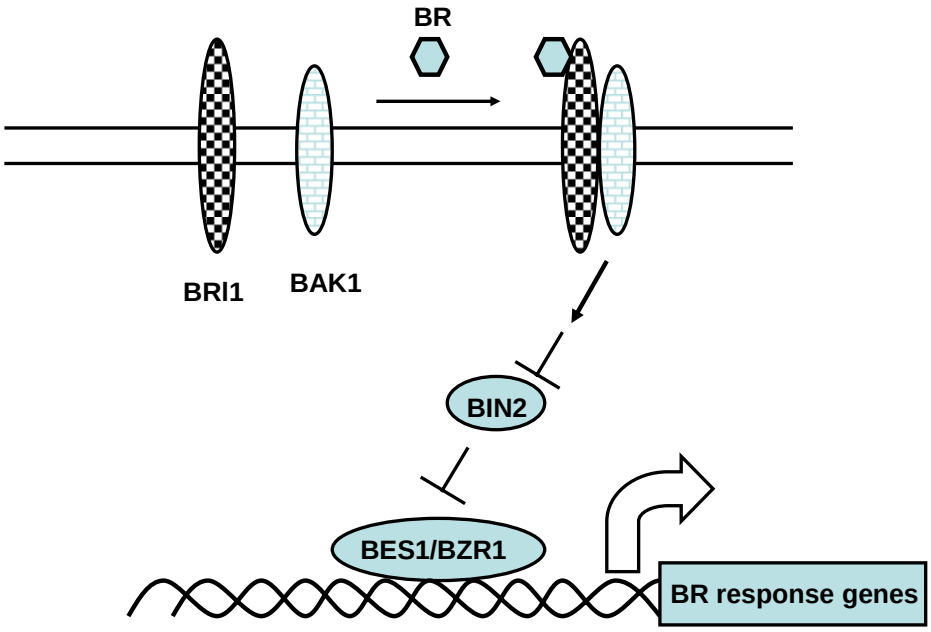

Fig. 68. Brassinosteroid (BR)-mediated kinase pathway in *Arabidopsis*. Adapted from Tichtinsky et al. (2003).

When transfected chimeric proteins are used to assess E2-induced kinase activation, cotransfection of ER is required for activity even though MCF-7 cells express endogenous ER (Fig. 41-44). As observed for LDHA promoter-reporter constructs and many other E2-responsive constructs, endogenous ER is not sufficient to observe E2-responsiveness due to overexpression of constructs in the transfected cells. Therefore we conducted parallel experiments in ER-negative C4 cells and observed results similar to those observed in transfected MCF-7 cells (Fig. 45-48). These results indicate that at least, a portion of kinase-inducing membrane associated ER and nuclear ER are encoded by the same gene (Razandi et al., 1999). This is an important issue since some lines of evidence suggest that the membrane-associated ER is distinct from the nuclear ER (Gu et al., 1999; Nadal et al., 2000; Singh et al., 2000; Tsai et al., 2001; Thomas et al., 2004). In our studies, transfected ER was capable of mediating the activation of MAPK, PI3K, PKA/PKC and CaMKIV, in ER-negative C4

cells and ER-positive MCF-7 cells, suggesting that the same ER can mediate both genomic and nongenomic actions of estrogen.

The validity of the chimeric protein assays were confirmed with the specific kinase inhibitors. E2 induced transactivation in MCF-7 cells transfected with GAL4-Elk-1, GAL4-SRF and GAL4-p65 and these responses were inhibited by the MAPK inhibitor PD98059, PI3K inhibitor LY294002 and CaMKIV inhibitor KN93, respectively (Fig. 49-52). E2-dependent activation of GAL4-CREB was inhibited by the PKC inhibitor Ro-31-8425 but not the PKA inhibitor H89, confirming that E2 preferential induced activation of CREB through PKC in MCF-7 cells. Thus GAL4-Elk-1, GAL4-SRF, GAL4-CREB and GAL4-p65 have been validated as assays to detect activation of MAPK, PI3K, PKA/PKC and CaMKIV pathways, respectively, in MCF-7 cells. All the assays were performed in MCF-7 cells and their use in other cell lines would require similar validation studies.

For MAPK, PI3K and PKA/PKC, their activation can be mediated by transfected wildtype ER or TAF1. ER with deletions of AF1 (HE19), AF2 (HE15) and DBD (HE11) (Fig. 69) are ineffective for activating MAPK, PI3K and PKA/PKC (Fig. 41-43). In contrast, for CaMKIV, activation can be mediated by wildtype ER and the AF1 deletion mutant (HE19), but not by HE11, HE19 or TAF1 (Fig. 44). The different patterns of ER domain requirements for hormonal activation of CaMKIV and MAPK/PI3K/PKA/PKC suggest that the association of ER with other membrane/cytosolic proteins may differ for specific kinases.

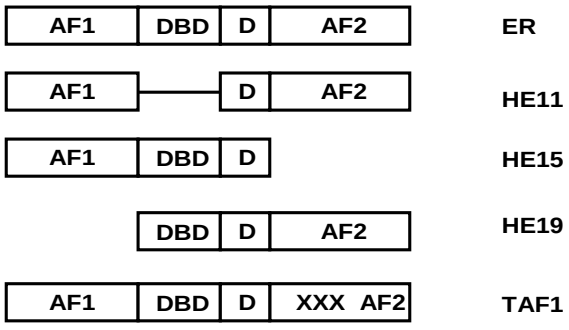

Fig. 69. Domains of wild-type, deletion and mutant ER constructs.

The AF2 domain of ERα is important for regulating E2-induced kinase cascades since this domain can associate with SH2 domain of c-Src, triggering downstream activation of MAPK in MCF-7 cells. This association is stimulated by estrogen and inhibited by antiestrogens, suggesting a ligand-dependent mechanism of kinase activation (Migliaccio et al., 1996; Migliaccio et al., 2002). In our study, the HE15 construct, in which the AF2 region is deleted, did not mediate E2-dependent transactivation in cells transfected with the chimeric proteins although basal activities were increased (Fig. 41-44). The estrogen-insensitivity is not surprising because HE15 lacks the ligand binding domain. However, the elevated basal activities suggest that AF1/DBD of ER may constitutively enhance activation of kinases.

The constitutive activity of the AF1 domain of ER has been well documented in its genomic actions (Tremblay et al., 1999); (Schlegel et al., 2001). Our study provides indirect evidence that the AF1/DBD domain contributes to the constitutive nongenomic activity. It is possible that the constitutive nongenomic activity of AF1/DBD is inhibited by AF2 domain and can be released only by adding ligand and by deleting the AF2 domain. The TAF1 ER mutant mediated E2-induced transactivation in cells transfected with kinase-dependent constructs. However, the basal activities and fold induction were variable compared to those observed in cells transfected with wildtype ER (Fig. 41-43). TAF1 is mutated on three amino acids that are important for the ER genomic actions (D538N, E542Q and D545N) and ER genomic pathways are decreased or not observed using this construct (Tzukerman et al., 1994). Since TAF1 activates MAPK, PI3K and PKA/PKC, this suggests that the genomic and nongenomic actions of ER are mediated through distinct mechanisms.

CaMKIV activation distinguished itself as a unique pathway in this study. Transfecting cells with HE19, an ER mutant with deletion on AF1, activated CaMKIV-dependent GAL4-p65 and this was also observed in cells transfected with wildtype ER (Fig. 44, 48). HE19 did not activate other kinase-dependent chimeric proteins, suggesting that AF1 domain is not essential for CaMKIV activation. In contrast, TAF1 mutant, although active in regulating the E2-inducibility of MAPK-, PI3K- and PKA/PKC-dependent constructs, did not mediate CaMKIV-dependent activation of GAL4-p65 by E2, however, basal activities were significantly elevated (Fig. 44, 48). These unique features suggest

that hormonal activation of CaMKIV differs from the other kinases and this may be due to association of ER with other cytosolic/membrane factors.

In summary, this study used chimeric proteins to evaluate the nongenomic actions of E2 mediated by wildtype and mutant ER in MCF-7 cells and C4 cells. The results demonstrate differential activation among kinases, with wildtype ER and TAF1 activating MAPK, PI3K and PKA/PKC, while wildtype ER and HE19 activating CaMKIV (Fig. 41-48). Phylogenetic studies suggest that the ancestral estrogen receptor was an orphan receptor and its ligand-binding capacity was acquired later during evolution (Escriva et al., 1997; Laudet, 1997). It is possible that the ancestral prototypes of AF1 and AF2 were sufficient for induction of nongenomic activities before acquiring the capability for activating genomic pathways. A sequential acquisition of nongenomic and genomic pathways of estrogen receptor may be an integral component of the evolution of ER structure and function.

4.4 Structurally diverse estrogenic compounds have different profiles for ER-dependent activation of kinase pathways

The estrogenic compounds examined in this study are structurally diverse. Some closely resemble the backbone structure of estradiol, such as DES. Others such as kepone bear no apparent semblance to estradiol. Kepone is a cubic molecule and does not contain a single aromatic ring (Fig. 53). These compounds all bind to the ER and exhibit estrogenic activities. With the exception of DES, which has comparable potency to E2, the compounds investigated in this study are weak ER agonist compare to E2. For example, kepone has only about 0.01-0.04% of the affinity for ER compared to estradiol (Hammond et al., 1979).

Estrogenicity is a loosely used term and literally means estrogen-like property. However, what constitutes an estrogen-like property has not been well-defined. Usually an ER-binding compound will be considered “estrogenic” if it can mimic some activities of estrogen, such as inducing vaginal cornification in immature mice, increasing the proliferation of uterine epithelium and uterine wet weight in ovariectomized mice, or upregulating estrogen-inducible genes and cell proliferation in estrogen responsive cell

cultures or tissues. The compounds examined in this study have all been intensively studied and are estrogenic based on at least one of the criteria indicated above (Hammond et al., 1979; Gould et al., 1998; Steinmetz et al., 1998; Hodges et al., 2000; Yoon et al., 2000; Yoshida et al., 2000; Yoon et al., 2001; Recchia et al., 2004).

In these studies, we used concentrations of estrogenic compounds that induced maximal activities but were not cytotoxic. With the exception of resveratrol, E2 and the other eight compounds activate MAPK and PI3K in MCF-7 cells in both transient transfection assays and by Western blot analysis for phospho-ERK or phospho-AKT (Fig. 54-55, 57-58). There were some differences on the relative potencies of these compounds between the two assays and these were not further investigated. Two representative chemicals, HPTE and nonylphenol were selected as prototypes for inhibition studies using the MAPK inhibitor PD98059 and the PI3K inhibitor LY294002. The results confirmed that the activities mediated by these estrogenic compounds are indeed modulated through activation of MAPK and PI3K pathways (Fig. 56, 59).

In MCF-7 cells, PI3K activation is crucial for E2-induced cell proliferation (Castoria et al., 2001). With the exception of resveratrol, the estrogenic compounds all induced PI3K activity, in agreement with reports showing that they stimulate MCF-7 cell proliferation (Soto et al., 1995). In our studies resveratrol was inactive and did not modulate MAPK-, PI3K-, PKA/PKC- and CaMKIV-dependent activities. There are reports demonstrating the activation of ER-ERE by resveratrol (Gehm et al., 1997). However, with regard to kinase activation, our observations are supported by studies showing that resveratrol inhibits some kinase activities and inhibits growth of human cancer cells (Lu and Serrero, 1999).

E2, DES, HPTE, BPA, octylphenol, nonylphenol and endosulfan activated PKA/PKC, however, kepone, resveratrol and HO-PCB-Cl_4 were inactive in this assay (Fig. 60). Using HPTE and nonylphenol as representative compounds, Western blots demonstrated that the phosphorylation of CREB by xenoestrogens is mediated mainly through PKC, not PKA (Fig. 61). Kepone and HO-PCB-Cl_4 activated MAPK and PI3K but not PKA/PKC, showing that activation of PKA/PKC by xenoestrogens was structure-

dependent and required ligand-dependent recruitment of different sets of cytosolic/membrane proteins associated with activation of kinases.

The uniqueness of CaMKIV was also observed by its selective activation by estrogenic compounds. Among the compounds that were used, only E2 and DES induced CaMKIV-dependent activation of NFkB p65 subunit (Fig. 62). DES is a synthetic estrogen that closely mimics estradiol and it is not surprising that DES and E2 consistently showed the same kinase activation patterns in this study (Fig. 54, 57, 60, 62). It was surprising that HO-PCB-Cl_4, which binds ERα and induces MCF-7 cell proliferation (Ramamoorthy et al., 1997), inhibited E2-induced GAL4-p65 activity. Significant inhibition of this CaMKIV dependent-activity was observed at concentrations as low as 5 uM, suggesting a potency similar to the widely used CaMKIV inhibitor KN93 (Fig. 63). Estrogen-induced p53 upregulation is activated by through CaMKIV-dependent action of NFkB subunit p65 (Qin et al., 2002) and both KN93 and HO-PCB-Cl_4 inhibited p53 protein expression by E2 in MCF-7 cells (Fig. 64). These results confirmed that HO-PCB-Cl_4 is a potent inhibitor of CaMKIV, quite the contrary to what might be expected of an estrogenic compound.

These results, coupled with previous findings on the structure-dependent differences in activation of genomic ER (Gould et al., 1998; Sonnenschein and Soto, 1998; Gaido et al., 2000), demonstrate profound structure-dependent differences among various estrogenic compounds. In this study, DES and had the same kinase activation profile. However, even DES can be distinguished from E2 in some respects. In MCF-7 cells, E2 but not DES activates GC-rich promoters through ER/Sp1 interactions (Wu et al, unpublished data). Thus, each compound exhibits unique estrogenic activities and also shares some of the same estrogenic activities with E2.

The activation of MAPK and PI3K by E2 can be inhibited by the antiestrogen ICI 182, 780. Similarly, ICI 182, 780 abolished the activation of PI3K by HPTE and nonylphenol (Fig. 65). These results are consistent with the hypothesis that estrogen-dependent nongenomic actions are mediated by the ER. However, MAPK induced by HPTE and nonylphenol could not be inhibited by ICI 182,780, suggesting that some nongenomic actions were independent of ER and mediated by membrane-associated estrogen-

binding factors not related to the traditional nuclear ER. Based on these results, the nongenomic actions of estrogenic compounds can be view as a combinational effect of ER and non-ER mediated pathways. The relative weight of each pathway maybe variable for each specific kinase and is determined by cell context and ligand structure. The effects of ER-dependent and –independent pathways can be distinguished through the ER-inhibitory effects of antiestrogen ICI 182,780 (Fig. 70). Future studies will investigate the mechanisms associated with the differential responses induced by xenoestrogens.

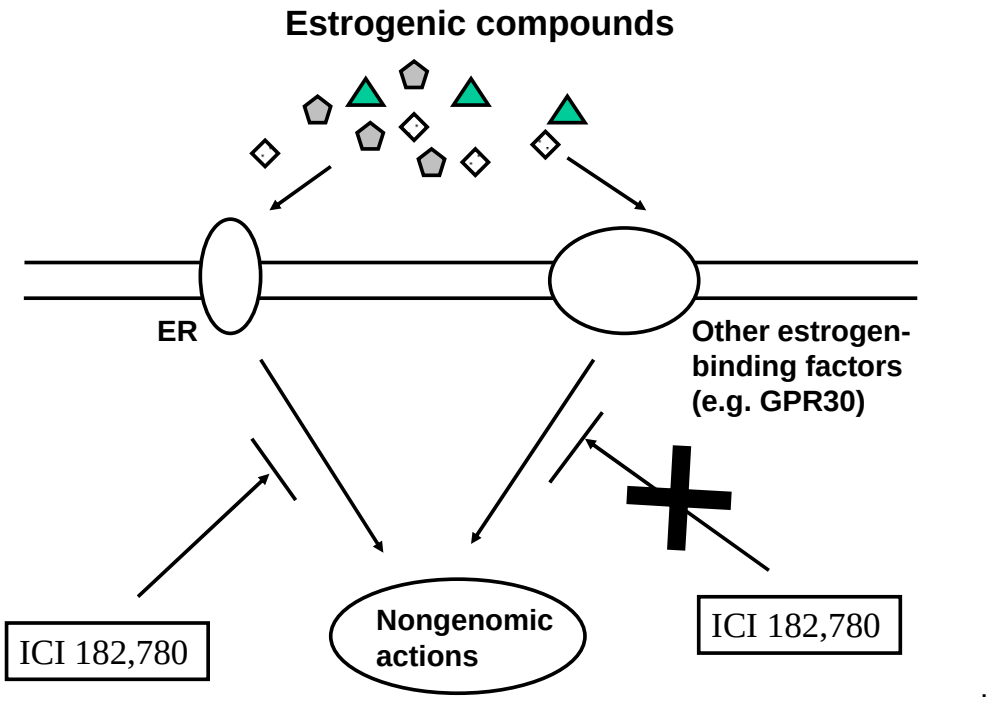

Fig. 70. The nongenomic actions of estrogenic compounds.

Parallel phenomena have been observed for the nongenomic actions of other steroid hormones. A distinct membrane progestin receptor (mPR) has been identified and this protein mediates the nongenomic actions of progesterone. This putative mPR has been cloned and resembles a G protein-coupled receptor (GPCR) and is unrelated to the traditional nuclear progesterone receptor (PR) (Zhu et al., 2003). The androgen receptor also appears to have a membrane-associated form unrelated to the nuclear

form. The membrane androgen receptor (mAR) mediates the nongenomic actions of androgens on the cell membrane of T cells. This mAR apparently is different from nuclear androgen receptor because AR antibodies do not recognize mAR and cyproterone, a blocker of the classical AR does not inhibit activation of mAR (Benten et al., 1999). In another report, a putative membrane androgen receptor (mAR) has been biochemically characterized in the ovary of Atlantic croaker. Again, the mAR in this case has different ligand-binding affinity from that of nuclear AR, suggesting that it is not nuclear AR, or at least is a modified form of nuclear AR (Braun and Thomas, 2004).

Several candidate proteins have been proposed as membrane-associated estrogen-binding factors (Luconi et al., 1999; Nadal et al., 2000; Thomas et al., 2004). One factor that has been relatively well-characterized is a transmembrane G protein-coupled receptor named GPR30. GPR30 possesses specific, high affinity binding capacity for E2, the antiestrogens tamoxifen and ICI 182,782, and the xenoestrogen o-p'-DDE (Thomas et al., 2004). Upon E2 treatment, GPR30 rapid induces adenylate cyclase and elevates cAMP levels (Filardo et al., 2002). E2 and phytoestrogens genistein and quercetin also activated MAPK cascades and upregulated c-fos gene through GPR30 (Maggiolini et al., 2004). Recently, GPR30 has been identified in the endoplasmic reticulum and rapidly mobilizes intracellular calcium and induces PIP_3 production in the nucleus in response to estrogen stimulation (Revankar et al., 2005). These findings suggest that GPR30 is an integral part of estrogen signaling and may have important physiological functions.

Some steroid hormone receptor agonists, such as estradiol, progesterone and tamoxifen, elicit nonspecific nongenomic actions by altering cell membrane fluidity and other properties (Clarke et al., 1990; Shivaji and Jagannadham, 1992). These actions usually require ligand concentrations in the micromolar range, which is significantly higher than most physiological concentrations of E2. However, these concentrations are similar to those of the estrogenic compounds we used in this study. Although it is unlikely, it is possible that some kinase activities activated by specific estrogenic compounds in this study may be caused by nonspecific effects which are receptor-independent.

Based on current reports, the nongenomic actions of estrogen and other estrogenic compounds are highly complex, ligand-structure-dependent and mediated by multiple mechanisms. Results obtained from routine receptor binding or transactivation assays are an oversimplification and cannot accurately reflect the estrogenic activity of a compound and novel approaches should be explored to address this inadequacy.

In summary, this study demonstrated that structurally diverse estrogenic compounds differentially activate multiple kinase pathways in MCF-7 cells. With the exception of resveratrol, all the compounds we tested activated MAPK and PI3K. In contrast, the activation of PKA/PKC and CaMKIV are by these compounds are structure-dependent. One xenoestrogen, HO-PCB-Cl_4, was identified as a potent CaMKIV inhibitor. Thus, the nongenomic actions of estrogenic compounds should not be evaluated based solely on data from their genomic action. Current studies in this laboratory are focused on both genomic and non-genomic ER-dependent and –independent effects of structurally diverse estrogenic compounds.

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