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ER-Alpha36 Mediates Non-Genomic Estrogen and Anti-Estrogen Signaling in Breast Cancer Cells

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1. Introduction

Estrogen signaling is essential in the initiation and development of neoplasia in mammary gland. In the past several decades, extensive efforts have been dedicated to understand the underlying mechanisms of this important signaling pathway in mammary carcinogenesis, which have facilitated the development of anti-estrogen therapy, the first targeted therapy for human cancer.

It has been well documented that the diverse activities of estrogens and anti-estrogens are mediated by specific receptors designated as estrogen receptors (ERs). Currently, there are two identified ERs, ER-α and ER-β, both of which are ligand-activated transcription factors that stimulate target gene transcription. Compelling evidence indicates that estrogens, especially 17β-estradiol (E2β), up-regulate the expression and function of c-Myc and cyclin D1, activate cyclin E-Cdk2 complexes and promotes cell cycle progression from G1 to S phase in mammary epithelial cells. Thus, stimulation of target gene expression by ERs in response to estrogens is prevailingly thought to be responsible for estrogen-stimulated mammary carcinoma initiation and progression.

Despite the clarity with which ERs have been shown to act as ligand-dependent transcription factors, it became apparent now that not all of the activities mediated by ERs are accomplished through a direct effect on gene transcription. Another signaling pathway (also known as a non-classic, non-genomic, extra-nuclear or membrane-initiated signaling pathway) exists that involves cytoplasmic signaling proteins, growth factor receptors and other membrane-initiated signaling pathways. Several intracellular signaling pathways have been shown cross-talking with the membrane-initiated estrogen signaling: the adenylate cyclase pathway, the phospholipase C pathway, the G-protein-coupled receptor-activated pathways, the PI3K/AKT and the MAPK pathways.

Currently, the identity of the membrane-based estrogen receptor that mediates non-genomic estrogen effects has not been fully established. ER-β, two forms of ER-α, full-length (66-kDa) and short form (46-kDa) have been found on or near the plasma membrane and mediates membrane-initiated estrogen signaling. In addition, there is also evidence indicating that GPR30, an orphan G-protein-coupled-receptor, mediates the rapid, non-genomic responses to estrogens. In 2005, our laboratory identified and cloned a novel variant of ER-α, which

has a molecular weight of 36-kDa and thus we have termed it ER-α36. This ER-α variant differs from the original 66 kDa ER-α (ER-α66) by lacking both transcriptional activation domains (AF-1 and AF-2) but retaining the DNA-binding domain and partial dimerization and ligand-binding domains. ER-α36 is predominantly expressed on the plasma membrane of both ER-positive and –negative breast cancer cells, mediates membrane-initiated estrogen and anti-estrogen signaling such as activation of the MAPK/ERK and PI3K/AKT signaling pathways and stimulation of cell proliferation. Thus, ER-α36 is a novel membrane-associated estrogen receptor that mediates membrane-initiated estrogen and anti-estrogen signaling in both ER-positive and -negative breast cancer cells.

In this chapter, we highlight the historical understanding of non-genomic estrogen signaling and its role in cell survival and proliferation, and specifically illustrate the biological function and the possible underlying mechanisms of ER-α36 in non-genomic estrogen and anti-estrogen signaling in both ER-positive and –negative breast cancer cells. We also discuss the integral roles of EGFR, Src and STAT5 in the non-genomic estrogen signaling mediated by ER-α36 and the complex regulatory network among ER-α66, 46 and 36. In addition, the novel biology of non-genomic estrogen signaling mediated by ER-α36 also has significance for understanding the physiology of bone remodeling. Thus, ER-α36-mediated signaling has a broad appeal in physiology of non-classical estrogen targeting tissues and general cancer research.

2. Estrogen receptors and genomic estrogen signaling

Estrogen signaling is pivotal in the establishment and maintenance of reproductive function in men and women. It is also involved in normal development and physiology of bone, liver, cardiovascular and neuronal systems. Inappropriate estrogen signaling is involved in osteoporosis, atherosclerosis and Alzheimer's disease and is essential in the initiation and development of neoplasia in breast and endometrial cancers (Nilsson et al., 2001). Hence, it is important to understand the molecular mechanisms by which this important signaling pathway functions, so its participation in a wide variety of different biological processes can be clarified, including how its dysregulation contributes to different diseases and even neoplastic transformation.

The diverse physiological and pathological functions of estrogens are mediated by specific receptors designated as estrogen receptors (ERs). In 1986, the cloning of the estrogen receptor (ER) was first reported (Green et al., 1986; Greene et al., 1986). Until 1996, it was assumed that there was only one ER responsible for all of the physiological and pharmacological effects of natural and synthetic estrogens and anti-estrogens. However, in 1996, a second ER was cloned (Kuiper et al., 1996). Currently, the first ER discovered is referred to as ER-α, while the second is called ER-β.

ER-α and ER-β share a common structural architecture (Reviewed by Kong et al., 2003; Zheng et al., 2003). Both are composed of three independent but interacting functional domains: the N-terminal A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain. The N-terminal domain of ERs encodes a ligand-independent activation function (AF-1), a region involved in interaction with co-activators, and transcriptional activation of target genes. The DNA-binding domain or C domain contains a two zinc-finger structure, which is involved in receptor dimerization and binding to specific DNA sequences. The C-terminal E/F domain is a ligand-binding domain that mediates ligand binding, receptor dimerization, nuclear translocation, and a ligand-dependent

transactivation function (AF-2). The relative contributions that both AF-1 and AF-2 exert on transcriptional control vary in a cell-specific and DNA promoter-specific manner (Berry et al., 1990; Tzukerman et al., 1994).

It is well established that estrogen receptors are members of the nuclear receptor superfamily of ligand-activated transcription factors that control various physiological processes. It is prevailingly thought that this control often occurs through the regulation of gene transcription (Reviewed by Katzenellenbogen & Katzenellenbogen, 2000; McDonnell & Norris, 2002). The estrogen receptor utilizes multiple mechanisms to either activate or repress transcription of its target genes. These mechanisms include: (a) direct interaction of the ligand-occupied receptor with DNA at a consensus estrogen response elements (ERE, GGTCAnnnTGACC) followed by recruitment of transcriptional coregulator or mediator complexes (Reviewed by Klinge, 2001), (b) interaction of the ligand-occupied ER with other transcription factors such as AP-1 (Kushner at al., 2000), Sp1 (Safe, 2001) or NF-κB (McKay & Cidlowski, 1999), or (c) indirect modulation of gene transcription via sequestration of general/common transcriptional components (Harnish et al., 2000; Speir et al., 2000). In addition, the ability of an estrogen receptor to regulate transcription through these mechanisms appears to be cell-type specific, perhaps due to differences in the complement of transcriptional co-regulatory factors available in each cell type (Cerillo et al., 1998; Evans et al., 2001; Maret et al., 1999). Also, transcriptional regulation is dependent upon the nature of the ligand, with various natural and synthetic selective estrogen receptor modulators (SERM) acting as either estrogen receptor agonists or antagonists through these various mechanisms (Shang & Brown, 2002; Katzenellenbogen & Katzenellenbogen, 2002; Margeat et al., 2003).

3. Estrogen signaling and breast cancer

Experimental and clinical evidence for the role of endogenous estrogens in normal development of mammary glands and breast cancer etiology has been well documented (Reviewed by Feigelson & Henderson, 1996). Estrogen signaling is involved in mammary epithelial cell proliferation and differentiation. Dysregulated estrogen signaling increases the rate of cell proliferation and thus the risk for development of breast cancer.

ER-α is expressed in approximately 15-30% of luminal epithelial cells and not at all in any other cell types in the normal human breast (Clarke *et. al.*, 1997). However, dual label immunofluorescent technique revealed that ER-α expressing cells are separate from those labeled with proliferation markers such as Ki67 and cyclin A in both normal human and rodent mammary glands (Clarke et al., 1997; Zeps et al., 1999). This is in direct contrast to estrogen action in breast cancer cells, where estrogens, especially 17β-estradiol (E2β), functions as potent mitogens through promoting cell cycle progression from G1 to S phase (Prall et al., 1998; Altucci et al., 1996 & 1997). Thus, it is thought that estrogens directly interact with ERs in ER-positive human breast cancer cells, where they induce transcriptional activation of "immediate early" and cyclin genes, and promote cell cycle progression. Consistent with its role in mammary carcinogenesis, ER-α expression is increased at the earliest stages of ductal hyperplasia and increases even more with increasing atypia, such that most cells in atypical ductal hyperplasias and in ductal cancer in situ of low and intermediate grade contain ER-α (Khan et. al., 1994). As ER-α expression increases during breast cancer development, the inverse relationship between ER-α expression and cell proliferation become dysregulated (Shoker *et. al.*, 1999 a and b).

Approximately 70% of invasive breast carcinomas express the ER-α and most of these tumors contain ER-α positive proliferating cells (Clarke *et. al.,* 1997).

Currently, ER-α is the most widely used marker in diagnosis of human breast cancer. Breast cancers are diagnosed either as ER-positive or -negative depending on the existence or absence of ER-α. At the onset, 46%-77% of breast cancers are ER-positive (Robertson *et. al.,* 1996). The ER-positive tumors are histologically well differentiated and diploid, and patients with ER-positive tumors generally have a better prognosis since they respond well to anti-estrogen treatment (Clark *et. al.*, 1984; Osborne *et. al.,* 1980). ER-negative breast cancers that account for about one third of breast cancers diagnosed, however, are more malignant and aggressive through a still unknown mechanism (Sheikh *et. al.,* 1994), and they generally respond poorly to anti-estrogen treatment.

ER-negative breast cancers are often considered to be the result of tumor progression from ER-positive premalignant lesions or ER-positive breast cancers by epigenetic alterations such as promoter methylation (Ferguson et. al., 1995) or ER-α protein degradation by the proteasome system after hypoxia in non-vascularized tumors (Stoner et. al., 2002). However, immunohistochemical studies in human breast cancers showed that some proliferative ductal lesions and many high-grade ductal carcinomas in situ (DCIS) are ER-α-negative (Roger et. al., 2000), suggesting some ER-negative breast cancers may be progressed directly from ER-negative premalignant lesions.

4. Non-genomic estrogen signaling

It become clear now that estrogens elicit two signaling pathways; the first requires hours to days to result in transcriptional changes of target genes and is known as genomic, classic or nuclear signaling pathway while the second occurs in seconds to minutes after estrogen treatment and usually initiates at the plasma membrane. Thus, the second signaling pathway is also known as a non-classic, non-genomic, extra-nuclear or membrane-initiated signaling pathway which is mediated by estrogen binding proteins associated with the plasma membrane and employs various cytoplasmic signaling proteins, growth factor receptors and G-protein-coupled-receptor signaling pathways (Segars & Driggers, 2002; Driggers & Segars, 2002; Kelly & Levin, 2001; Levin, 2002; Hammes & Levin, 2007).

Pietras and Szego first described the rapid estrogen signaling more than 30 years ago (Pietras & Szego, 1975 &1977), noting immediate calcium fluxes in endometrial cells induced by estrogen and specific binding sites at the outer surfaces of isolated endometrial cells. Quick estrogen responses were also recorded in neuroendocrine tissue such as a rapid rise of intracellular calcium in pituitary cells, which leads to cell depolarization within 1 min (Dufy et al., 1979). Rapid estrogen signaling also induces prolactin secretion from pituitary tumor cells (Watson et al., 1999). Such signaling of estrogens has also been described in uterine (Aronica et al., 1994) and ovarian cells (Morley et al., 1992; Tesarik & Mendoza, 1997) as well as bone (Endoh et al., 1997; Longo et al., 2004; Sylvia et al., 2001), vascular endothelial (Russell et al., 2000; Lu et al., 2004) and neuronal cells (Kelly et al., 1977; Mermelstein et al., 1996), indicating that the rapid, non-genomic estrogen signaling is involved in various physiological and pathological estrogen activities.

Non-genomic estrogen signaling has been also documented in breast cancer cells for cell proliferation and survival (Song et al., 2002; Migliaccio et al., 1996; Ahmad et al., 1999; Lobenhofer et al., 2000; Castoria et al., 1999 & 2001). These findings strongly suggest that the

rapid, non-genomic estrogen signaling is involved in estrogen-induced proliferation in breast cancer cells.

5. Nature of membrane-based estrogen receptor

While it is clear that membrane-initiated estrogen signaling exists, the identity of the membrane-based estrogen receptor that mediates these rapid estrogen effects, especially the effects in mammary epithelial cell proliferation and survival, has not been fully established. Laboratory evidence indicated that both ER-α and ER-β are involved in the rapid, non-genomic estrogen signaling (Razandi et al., 1999). However, evidence also suggests that more than one membrane-initiated signaling pathway is associated with estrogen action. Data from several laboratories using the membrane-impermeable compound 17β-estradiol-bovine serum albumin (E2β-BSA) indicates the existence of two functionally distinct membrane-associated pathways: one sensitive to anti-estrogens and one resistant (Chen et al., 1999; Russell et al., 2000; Watters et al., 1997). For example, ER-α/knockout mice retained rapid estrogen-stimulated membrane effects in neurons, which were not blocked by anti-estrogen ICI-182, 780 (Gu, et. al., 1999). These findings suggest that another membrane-based ER may exist since all known ERs are sensitive to anti-estrogen inhibition. As a consequence, an orphan G-protein coupled receptor, GPCR30, and some other unknown proteins have been reported to mediate this anti-estrogen resistant non-genomic estrogen signaling.

6. ER-α

A large body of experimental evidence indicated that ER-α is involved in both genomic and non-genomic estrogen signaling. Immunofluorescent staining of non-permeabilized pituitary tumor cells with anti-ER-α antibodies revealed a punctuated staining pattern on cell surface although most ER-α were localized in cell nuclei (Watson et al., 1999). ER-α associated with the plasma membrane is also detected by a panel of antibodies targeting different domains of ER-α in intact breast cancer cells and in breast cancer specimens (Pietras et al., 2005; Kim et al., 2006). The findings that transfection of the nuclear ER-α cDNA into ER-"null" Chinese hamster ovary (CHO) cells leads to expression of both membrane and nuclear ER-α further support the notion that membrane-associated ER-α is derived from the same transcript as nuclear ER-α (Razandi et al., 1999). However, recent evidence indicates that the CHO-K1, Rat2-fibroblasts and COS7 cells previously considered as ER "null' cells and extensively used to transfect ER cDNAs in order to demonstrate rapid estrogen signaling actually exhibit strong non-genomic estrogen signaling such as activation of the MAPK/ERK before transfection (Nethrapalli et al., 2005), suggesting these cells already posses some unknown proteins other than classical ERs that mediate the rapid estrogen signaling.

It is widely accepted that estrogen signaling promotes cell proliferation in target cells. However, the explanation of this effect of estrogen signaling simply by the function of ER-α is confounding. When ER-α was expressed in ER-"null" CHO cells (Kushner *et. al*., 1990), and human cervical cancer HeLa cells (Touitou *et. al*., 1990), E2β failed to stimulate cell growth. On the contrary, E2β inhibited cell proliferation and even induced cell apoptosis. Likewise, the ER-negative immortal MCF10A breast epithelial cells (Pilat *et. al.*, 1996), and MDA-MB-231 breast cancer cells (Jiang & Jordan, 1992) were both growth inhibited by

estrogen when stably transfected with ER-α cDNA. These experimental results argue against a positive function of the well-known ER-α alone in mitogenic estrogen signaling.

7. ER-β

ER-β acts as a classical ligand-induced transcription factor. Like ER-α, ligand-bound ER-β functions by regulating downstream target genes. ER-β also heterodimerizes with ER-α and modulates ER-α function (Reviewed by Harris, 2007; Deroo & Buensuceso, 2010). ER-β has been shown to be S-palmitoylated, which facilitates the localization of ER-β at the plasma membrane to mediate the rapid, non-genomic estrogen effects (Marino & Ascenzi, 2008). Indeed, the involvement of ER-β in rapid, non-genomic estrogen signaling has been documented when ER-β cDNA was introduced into CHO cells (Razandi et al., 1999). In colon cancer, pro-apoptotic activities of ER-β have been reported to be mediated by membrane-initiated signaling; induction of ER-β via the MAPK/p38 signaling pathway, which in turn leads to downstream apoptotic events (Caiazza et al., 2007), indicating a potential role of ER-β as a tumor suppressor. In addition, inhibition of ER-β palmitoylation in colon cancer cells abrogated the pro-apoptotic activity of ER-β (Galluzzo et al., 2007), suggesting that non-genomic effects mediated by membrane–associated ER-β is important for its pro-apoptotic function.

The biological significance of ER-β in breast cancer has not been well established. Approximately 70% of breast tumor express ER-β and most tumor co-express both ER-α and –β (Dotzlaw et al., 1997; Fuqua et al., 1999 & 2003). Studies indicated that ER-β expression in human breast cancer is associated with a poorer prognosis, compared with tumors that only express ER-α (Speirs et al., 1999). ER-β expression is associated with elevated levels of proliferation markers, Ki67 and cyclin A, in human breast cancer (Jensen et al., 2001) These studies suggested that ER-β may promote cell proliferation and breast cancer progression.

In contrast, accumulating evidence indicated that ER-β acts as a tumor suppressor in breast cancer. ER-β is expressed in both normal and malignant mammary glands (Warner et al., 2000; Speirs et al., 2002). In the rodent mammary gland, ER-β expressing cells can proliferate but the majority cells that express proliferation markers do not express either ER (Saji et al., 2001). The levels of ER-β expression are highest in normal mammary gland and are decreased as tumors progress from pre-invasive to invasive (Leygue et al., 1998; Iwao et al., 2000; Roger et al., 2001). The presence of ER-β in breast cancer confers a more favorable prognosis and is associated with node-negative, low-grade tumors (Jarvinen et al., 2000) as well as a greater disease-free survival rate (Omoto et al., 2001). Several laboratory studies demonstrated that ER-β inhibited angiogenesis and malignant growth of T47D breast tumor xenograft and malignant growth of MCF7 breast cancer cells *in vitro* and in nude mice (Hartman et al., 2006; Paruthiyil et al., 2004). Intriguingly, ER-β also inhibited proliferation of ER-negative breast cancer MDA-MB-231 cells in a ligand-independent manner whereas ER-α inhibition of MDA-MB-231 cell proliferation is estrogen-dependent (Lazennec et al., 2001). These results indicate that ER-β negatively regulates mitogenic estrogen signaling.

8. ER-α46

Previously, it was reported that a 46-kDa antigen is tightly associated with ER-α in human breast cancer samples (Diaz-Chico *et. al*., 1988). This 46-kDa antigen is enriched in the cell

cytosol and could be recognized by an isolated monoclonal antibody, E476, raised against the human ER-α (Diaz-Chico *et. al.,* 1988). Similarly, Jozan (1991) later reported that there are two species of ER with different molecular weight (65 and 47 kDa), and three species of tumors (36% containing the highest form of ER alone, 49% bearing the two forms in variable amounts, and 15% bearing only the minor form). These results strongly suggest that two different forms of ER-α exist at different ratios in human breast cancer.

In 2000, Flouriot *et. al.* cloned a 46-kDa isoform of ER-α and demonstrated that the 46-kDa isoform lacks the first 173 amino acids (A/B or AF-1 domain) and is derived from alternative splicing of the ER-α gene by skipping exon 1. This alternative splicing event generates an mRNA that has an AUG in a favorable Kozak sequence for translation initiation in frame with the remainder of the open reading frame of ER-α (Flouriot et. al., 2000). This new isoform of ER-α is named as ER-α46 and the original one as ER-α66. ER-α46 forms homo-dimers and binds to an ERE, and it can also form heterodimers with ER-α66 (Flouriot et. al., 2000). Furthermore, the ER-α46/66 heterodimers form preferentially over the ER-α66 homodimers and ER-α46 acts competitively to inhibit transactivation mediated by AF-1 domain of ER-α66 but without effect on AF-2-dependent transactivation (Floutiot et. al., 2000). Therefore, ER-α46 is a naturally occurring isoform of ER-α that regulates genomic estrogen signaling mediated by the AF-1 domain of ER-α66.

Previously, two forms of ER-α, full-length (66-kDa) and short form (46-kDa) were co-purified with 5′ nucleotidase, a plasma membrane-marker enzyme (Marquez *&* Pietras, 2001), suggesting a possible role of ER-α46 in the rapid, non-genomic estrogen signaling. Recently, ER-α46 was localized on the plasma membrane, in the cytosol, and nucleus of endothelial cells and mediated rapid estrogen signaling such as activation of the Src/PI3K/AKT signaling and stimulation of NO synthesis (Kim & Bender, 2005; Li *et. al.,* 2003; Reviewed by Moriarty et al., 2006), further confirming that the ER-α46 isoform functions as a membrane-associated estrogen receptor.

Although it is clear now that ER-α46 is involved in both genomic and non-genomic estrogen signaling, its function in breast cancer has been less investigated. Forced expression of ER-α46 inhibited proliferation of MCF-7 breast cancer cells and cyclin D1 promoter activity (Penot et al., 2005). Overexpression of both ER-α46 and ER-α66 in ER-negative MDA-MB-231 cells revealed that ER-α46 inhibited basal transcription of the estrogen responsive gene pS2 while estrogen treatment released this inhibition (Metivier et al., 2004). Recently, expression levels of ER-α46 was found to be down-regulated in tamoxifen-resistant breast cancer cells and re-introduction of ER-α46 into these cells inhibited cell proliferation and ER-α66-regulated gene transcription (Klinge et al., 2010). Thus, these results suggest that ER-α46 may function as a negative-regulator of mitogenic estrogen signaling in ER-positive breast cancer cells.

9. ER-α36

In 2005, our laboratory identified a 5.4 kb cDNA clone from a normal human edometrium cDNA library (RZPD clone number: DKFZp686N23123) and later cloned this cDNA from a human placenta cDNA library (Wang et al., 2005). This cDNA clone harbors a 310 amino acid open-reading frame that can produce a protein with a predicted molecular weight of 35.7 kDa. To differentiate it from ER-α66 and 46, and apply consistent nomenclature, this novel isoform of ER-α was named as ER-α36.

The cDNA sequence of the open-reading frame matches 100% to the DNA sequence of the exons 2 to 6 of the ER-α66 gene. The 5'untraslated region (5'UTR) of the cDNA showed 100% homology to the DNA sequence of the first intron of the ER-α66 gene. Thus, the transcript of this ER-α isoform is initiated from a previously unidentified promoter in the first intron of the ER-α66 gene (Figure 1, Zou et al., 2009). A small, non-coding novel exon in the first intron of the ER-α66 gene was designated as exon 1'. The exon 1' is then spliced directly into the exon 2 of the ER-α66 gene and continues from exon 2 to exon 6 of the ER-α66 gene. Exon 6 is then spliced to an exon located 64,141 bp downstream of the ER-α66 gene (Figure 1). The cDNA sequence encoding the last 27 amino acids and the 4,293 bp 3'untranslated region (3'UTR) was matched 100% to a continuous sequence from the genomic sequence of clone RP1-1304 on chromosome 6q24.2-25.3 (GeneBank accession number AL78582), indicating the remaining cDNA sequence of this novel ER-α isoform is transcribed from one big exon of 4,374 bp located downstream of the ER-α66 gene. This exon is thus designated as exon 9 to reflect the extra exon beyond the previous reported eight exons for ER-α66 gene (Figure 1). All of these splicing events are supported by the identification of perfect splice donors and acceptors at the splice juncture. The protein ER-α36 can be produced from a perfect Kozak sequence located in the second exon, the same initiation codon used to produce ER-α46 (Flouriot *et. al.,* 2000). ER-α36 differs from the ER-α66 by lacking both transcriptional activation domains (AF-1 and AF-2) but retaining the DNA-binding and dimerization domains, and partial ligand-binding domains. It also possesses an extra, unique 27 amino acid domain to replace the last 138 amino acids encoded by exon 7 and 8 of the ER-α66 (Figure 2). Thus, ER-α36 is another naturally occurring novel isoform of ER-α that may play an important role in both genomic and non-genomic estrogen signaling. Currently, there are three major ER-α isoforms observed in ER-positive breast cancer MCF7 cells that correspond to three mRNA variants generated from different promoter usage and alternative splicing (Figure 2).

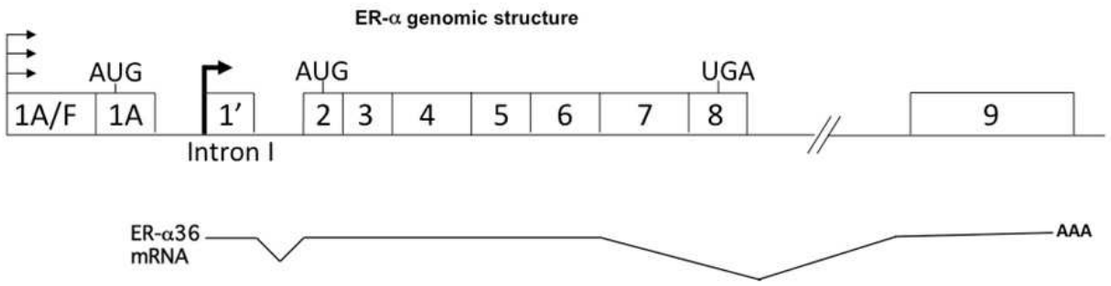

Fig. 1. Genomic organization of the human ER-α36 gene. The locations of multiple promoters of human ER-α66 gene are shown as arrows. The translation start and stop codons are indicated as AUG and UGA. The common exons are shown as numbered open boxes. The extra exon that is beyond the 8 exons of the human ER-α66 gene is numbered as 9 in the open box. The intron 1 is also shown with the exon 1' in the open box. The lower panel shows mRNA structure of human ER-α36 isoform.

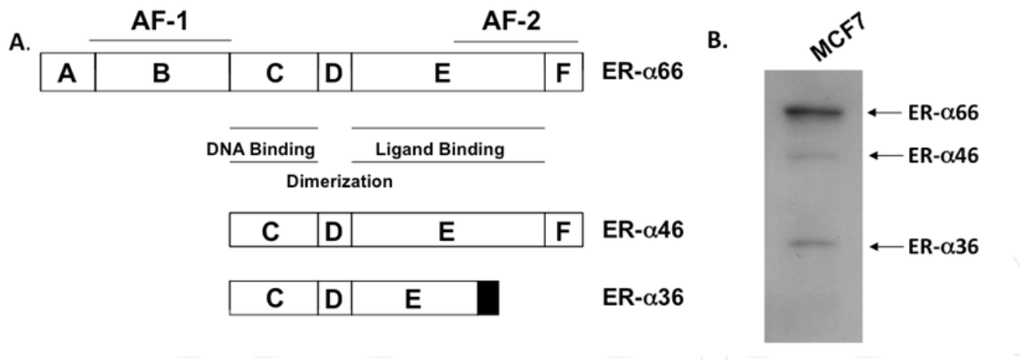

Fig. 2. Domain structure and expression of human estrogen receptor-α variants in ER-positive breast cancer MCF7 cells. (A). Domain structure representation of human ER-α isoforms. Domains (labeled A–F), and activation function domains (AF-1 and -2) are shown. The function of each domain is indicated. The last 27 amino acids of human ER-α36 are indicated as a filled box. (B). Western blot analysis of three human ER-α isoforms in MCF7 cells with an anti-ER-α66 antibody (H222).

10. GPR30

Previously, different laboratories demonstrated the existence of two distinct membrane-associated pathways: one sensitive to anti-estrogens and one resistant (Chen et al., 1999; Russell et al., 2000; Watters et al., 1997). These results suggest that another membrane-associated estrogen receptor may exist since both ER-α and -β are sensitive to anti-estrogen inhibition.

An orphan G protein-coupled receptor, GPR30 was reported to mediate the rapid, non-genomic estrogen signaling that was insensitive to ICI 182,780; estrogen stimulates changes of Ca^{2+} currents and cAMP signaling in cells expressing GPR30 (Revankar et al., 2005) and activates the MAPK/ERK phosphorylation and the PI3K/Akt signalling pathways via transactivation of the EGFR pathway in ER-negative but GPR30-positive breast cancer cells (Filardo et al., 2000 & 2007). Thus, GPR30 was considered as a novel type of membrane-associated estrogen receptor that mediates the rapid, non-genomic estrogen signaling.

There are also reports that challenge the role of GPR30 as a novel estrogen receptor. A study showed that introduction of GPR30 anti-sense oligonucleotides failed to block the MAPK/ERK activation and cell growth induced by estrogen in ER-positive breast cancer cells (Ahola et al., 2002). Pedram *et al.* (2006) failed to observe the cAMP or ERK activation in GPR30-positive, ER-negative breast cancer cells. Another study demonstrated that the GPR30 selective agonist G1 failed to exert estrogenic effect in two classical estrogen target organs, the uterus and the mammary gland (Otto et al., 2008). Recently, Otto *et al*. generated GPR30-deficient mice and demonstrated that the development of reproductive organs was unimpaired in these mice and the estrogenic responses in the uterus and the mammary gland were completely maintained in GPR30-deficient animals (Otto et al., 2009).

Recently, our group reported that knockdown of GPR30 expression in ER-negative breast cancer SK-BR-3 cells down-regulated the expression levels of ER-α36 (Kang et al., 2010b). Introduction of a GPR30 expression vector into GPR30 non-expressing cells induced endogenous ER-α36 expression and GPR30 activated the promoter activity of ER-α36 via an AP-1 binding site located in the 5'-flanking region of ER-α36 (Kang et al., 2010b). Thus, ER-

α36 is a downstream target gene of GPR30-mediated signaling and the previously reported activities of GPR30 as a membrane-based estrogen receptor are through its ability to induce ER-α36 expression.

11. Other putative estrogen receptors

Other unknown proteins that may be involved in the rapid, non-genomic estrogen signaling have been also reported. For an example, a report that E2β activated the MAPK/ERK signaling in un-trasnfected CHO-K1, COS7 and Rat2-fibroblasts (Nethrapalli et al., 2005) suggested the existence of an unidentified membrane-associated mER. Recently, we reported that un-transfected COS7 cells express high levels of endogenous ER-α36 (Kang et al., 2010b) and found that CHO-K1 cells also express ER-α36 (Kang et al., unpublished observations), suggesting that ER-α36 may mediates the rapid, non-genomic estrogen signaling observed in these cells.

Another unique membrane-associated ER with an estimated molecular weight of 63-65 kDa, referred to as ER-X, is developmentally regulated differently from both ER-α and –β. Its pharmacological profile was also different from ER-α and –β, even with some features opposite to those shown for these two receptors (Toran-Allerand et al., 2002). For examples, ER-X mediated rapid estrogen signaling was not sensitive to anti-estrogen, and the association of Hsp90 is required for the inactive state of ER-α while ER-X requires to be associated with hsp90 for its activity (Toran-Allerand et al., 2002). Whereas ER-X shares limited similarities with ER-α36, such as reaction with antibodies to the ligand-binding domain of ER-α66 and responding equally to 17α- and β-estradiol, the molecular similarity of these two receptors awaits for the cloning and sequencing of ER-X.

Additionally, a heterodimeric estrogen-binding protein, referred to as the putative ER (pER), was reported to bind to E2β at a sub-nanomolar affinity but was unable to bind other estrogens or anti-estrogens. Depending on cell types, pER is expressed on the plasma and/or nuclear membranes or in the cytoplasm and nucleus (Rao et al., 1998). Since the polyclonal anti-pER antibody failed to react with estrogen receptors and was unable to detect pER expression in reproductive organs (Rao et al., 1998), the role of this putative estrogen receptor in non-genomic estrogen signaling of breast cancer cells remains elusive.

12. Negative regulation of genomic estrogen signaling by ER-α36

Comparison of protein structures of ER-α66 and ER-α36 indicated that ER-α36 lacks both AF-1 and -2 transcription activation domains but retains the DNA-binding domain and dimerization domain. Transient co-transfection assays using a luciferase-expressing reporter construct that contains two Estrogen Response Element (ERE) placed upstream of the thymidine kinase promoter (2 X ERE-tk-Luc) revealed that ER-α36 has no intrinsic transcriptional activity in the presence and absence of E2β (Wang et al., 2006). However, ER-α36 strongly inhibited the transactivation activities mediated by the AF-1 and -2 domains of both ER-α66 and ER-β (Wang et al., 2006). These data indicate that ER-α36 itself is unable to mediate genomic estrogen signaling by regulate target gene expression. However, ER-α36 acts as a naturally occurring dominant-negative regulator of the genomic estrogen signaling mediated by the AF1 and AF2 domains of ER-α66 and ER-β.

13. Membrane-association and mitogenic signaling of ER-α36

The fact that ER-α36 posses no intrinsic transcription regulatory activity suggests that ER-α36 may be a membrane-associated estrogen receptor. The sub-cellular fractionation assay in ER-α36 transfected HEK293 cells revealed that a high percentage of ER-α36 (~50%) is localized on or near the plasma membrane and a low percentage of it in cytosol (~40%) and nucleus (~10%) (Wang *et al*., 2006). Immuno-fluorescence staining of intact breast cancer MCF7 cells and endometrial cancer Hec1A cells using the ER-α36 specific antibody recognizing the last 20 amino acids that are unique to ER-α36 exhibits a membrane expression pattern that is co-localized with caviolin-1, a typical cell-surface protein (Lin et al., 2010). Immunohistochemistry analysis of specimens from breast cancer patients also demonstrated an expression pattern of ER-α36 predominantly on the breast cancer cell surface (Lee et al., 2008; Vranic et al., 2011). This anti-ER-α36 specific antibody also blocked ER-α36-mediated non-genomic estrogen signaling such as activation of the MAPK/ERK signaling in breast cancer cells presumably through steric hindrance of estrogen accesses to its binding pocket (Kang *et. al*., 2010b), indicating that the antibody is accessible to the C-terminal region of the plasma membrane-associated ER-α36 in intact cells. These results thus demonstrated that ER-α36 is expressed on or near the plasma membrane and suggested that ER-α36 may be also shuttled to the cell cytoplasm and nucleus depending on cell context and extracellular signals.

Both 17β-estradiol (E2β) and BSA-conjugated E2β elicit the rapid, membrane-initiated estrogen signaling such as activation of the MAPK/ERK signaling pathway and stimulation of cell proliferation in ER-α36 transfected HEK293 cells, which is not blocked by anti-estrogens such as tamoxifen, 4-hydroxy-tamoxifen and ICI 182, 780 (Wang et al., 2006). In addition, other estrogens including estrone (E1), 17α-estradiol (E2α), estriol (E3) and estetrol (E4) all activate the ERK1/2 phosphorylation at a very similar level (Wang et al., 2006). Recently, it was reported that ER-α36 even mediated testosterone-stimulated activation of the MAPK/ERK and PI3K/Akt signaling pathways in endometrial cancer Hec1A cells (Lin et al., 2009). ER-α36 also mediates estrogen activation of the PKCδ/ERK signaling pathway (Tong et al., 2010). The finding that the non-genomic estrogen signaling mediated by ER-α36 was insensitive to anti-estrogens suggests that ER-α36 may be a receptor involved in the anti-estrogen-insensitive estrogen signaling described in different systems before. Taken together, ER-α36 is a membrane-associated estrogen receptor that mediates rapid and mitogenic estrogen signaling.

14. ER-α36 in anti-estrogen signaling and anti-estrogen resistance

Since mitogenic estrogen signaling plays a pivotal role in development and maintenance of ER-positive breast cancer, treatment with anti-estrogens such as tamoxifen (TAM) has become a first-line therapy for advanced ER-positive breast cancer. However, laboratory and clinical evidence indicated that TAM and its metabolites such as 4-hydroxytamoxifen (4-OHT) have mixed agonist/antagonist or estrogenic/anti-estrogenic actions depending on cell and tissue context, and the agonist activity of tamoxifen may contribute to tamoxifen resistance observed in almost all patients treated with tamoxifen. As a consequence, a more potent and "pure" anti-estrogen, ICI 182, 780 (Fulvestrant, Faslodex) has been developed (Reviewed by Howell et al., 2000).

TAM and 4-OHT are thought to function as antagonists by competing with E2β and other estrogens for binding to ERs. Further structural studies revealed that TAM induces an ER-α conformation that does not recruit coactivators to trans-activate target genes but recruits co-repressors (Shang *et. al.,* 2000), suggesting that TAM- and 4-OHT-bounded ER-α66 is unable to effectively activate genes involved in cell growth and breast cancer development. On the other hand, ICI 182, 780, a 'pure' antiestrogen without estrogenic activity, works in a different mechanism. ICI 182, 780 binds to ERs, impairs receptor dimerization and inhibits nuclear localization of receptor (Fawell, *et. al.,* 1990; Dauvois *et. al.,* 1992). Furthermore, ICI 182, 780 also accelerates degradation of the ER-α66 protein without a reduction of ER-α66 mRNA (Nicholson *et. al*., 1995). Thus, ICI 182, 780 binds ER-α66 and accelerates degradation of ER-α66 protein, resulting in a complete inhibition of estrogen signaling mediated by ER-α66.

Although ICI 182, 780 has been depicted as a non-agonist or 'full' or 'pure' anti-estrogen, different laboratories documented estrogenic agonist activities of ICI 182, 780 in different systems. Estrogenic agonist activity of ICI 182, 780 has been found in hippocampal neurons and in bone cells where ICI 182, 780 promoted bone growth (Zhao et al., 2006; Sibonga et al., 1998). Agonist-like activities of ICI 182, 780 have also been reported in tamoxifen-resistant KPL-1 breast cancer cells (Kurebayashi et al., 1998) and Yeast (Dudley et al., 2000). Both tamoxifen and ICI 182, 780 were also reported to induce phosphorylation of the adhesion molecules p130Cas/BCR1, FAK and Src in ER-positive breast cancer MCF7 cells (Cowell et al., 2006). The molecular mechanisms by which ICI 182, 780 acts as an estrogenic agonist have never been elucidated. Studies from a number of laboratories suggested that a membrane associated estrogen-binding receptor mediates the agonist actions of ICI 182, 780 in neurons (Reviewed by Brinton 2001; Zhao et al., 2005; McEwen, 2002).

As described above, ER-α36 mediated non-genomic estrogen signaling is insensitive to anti-estrogens such as TAM and ICI 182, 780 (Wang et al., 2006). ER-α36 also mediates agonist activities of tamoxifen and ICI 182, 780 such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways in breast and endometrial cancer cells (Wang *et. al.,* 2006; Lin *et. al*., 2010). ICI 182, 780 failed to induce degradation of ER-α36 (Kang et al., 2010a) presumably because ER-α36 has a truncated ligand-binding domain that lacks the last 4 helixes (helix 9–12) of ER-α66 (Wang et al., 2005). The helix-12 domain is critical in protein degradation induced by ICI 182, 780 and different positioning of the helix 12 and the F domain of ER-α66 regulates functional differences between agonists and antagonists (Mahfoudi et al., 1995; Pearce et al., 2003). Thus, it is possible that ER-α36 is a receptor that mediates agonist activities of TAM and ICI 182, 780 recorded in different systems.

Despite the significant anti-neoplastic activities of anti-estrogens, most breast tumors are eventually resistant to anti-estrogen therapy. Essentially, two forms of anti-estrogen resistance occur: *de novo* and acquired resistance (Reviewed by Clarke *et. al.,* 2001 & 2003; Ring & Dowestt, 2004). Although absence of ER-α66 expression is the most common *de novo* resistance mechanism, about 40-50% ER-positive tumors are already resistant to anti-estrogens by the time of diagnosis; this *de novo* resistance mechanism in these ER-positive tumors is largely unknown (Reviewed by Clarke *et. al*., 2001 & 2003; Ring & Dowestt, 2004). Furthermore, most initially responsive breast tumors gradually acquire anti-estrogen resistance by loss of anti-estrogen responsiveness. The underlying mechanism of breast tumors loss their anti-estrogen responsiveness remains unknown. Breast tumors with acquired TAM resistance frequently but not always retain levels of ER-α66 expression that

would still define them as ER-positive tumors (Reviewed by Clarke *et. al*., 2001 & 2003). Therefore, a loss of ER-α66 expression is not the major mechanism driving acquired anti-estrogen resistance. Up-regulation of Erb-B2 (Her2/Neu) and activation of the MAPK signaling pathways are usually associated with development of anti-estrogen resistance (Reviewed by Clarke *et. al.,* 2001 & 2003; Ring & Dowsett, 2004).

A recent retrospective study of 896 cases of breast cancer patients revealed that about 40% cases of human breast cancer patients were positive for ER-α36; about 40% cases ER-positive breast cancer co-expressed ER-α66 and ER-α36 and about 40% cases of ER-negative breast cancer that lacked ER-α66 expression were positive for ER-α36 (Shi et al., 2009). The breast cancer patients with tumors expressing high levels of ER-α36 were less benefited from tamoxifen therapy than those with low levels of ER-α36 expression did, and ER-α36 expression is significantly associated with Her2/Neu expression (Shi *et. al.,* 2009). These data suggest that high levels of ER-α36 expression in breast cancer cells is one of the underlying mechanisms of *de novo* tamoxifen resistance found in ER-positive breast cancer patients.

The aromatase inhibitors (AIs), on the other hand, inhibit the action of the enzyme aromatase, which converts testosterone to E2 (estradiol) and androstenedione to E1 (estrone). The third generation AIs exemplified by anastrazole and letrozole, provides a second line therapeutic strategy in advanced ER-positive patients (Reviewed by Santen, 2003). However, breast cancer cells that express high levels of ER-α36 are super sensitive to estrogen; activation of the MAPK/ERK in response to an extreme low concentration of estrogen, 1 X 10^{-16} M/L (Zhang et al., 2011). The anastrozole usually suppresses a plasma level of E2β to a mean of 2.6 pmol/L and letrozole to a mean of 2.1 pmol/L (Geisler et al., 2002). These data suggest that breast cancer patients with tumors expressing high levels of ER-α36 may also be refractory to the third generation of AIs.

15. ER-α36 in ER-negative breast cancer

Because of the lack of ER-α66 expression, it is prevailingly thought that estrogen signaling is not involved in development and progression of ER-negative breast cancer. However, early study showed that ovariectomy prevents formation of both ER-positive and –negative breast cancers (Nissen-Meyer, 1964). In addition, BRCA1 mutation related tumors, the vast majority of which are ER-negative, are also effectively prevented by prophylactic ovariectomy (Rebbeck et al., 1999). The increased risk developing breast cancer following pregnancy is assumed due to the ability of pregnancy-associated hormones such as estrogen to stimulate mammary epithelial cell proliferation. Surprisingly, however, the majority of breast cancers that develop following pregnancy are negative for either estrogen and progesterone receptors. To explain this contradictory findings, Gupta et al., (2007) proposed that estrogen may promote the growth of ER-negative breast cancer via a systemic increase in host angiogenesis.

Rapid activation of the PI3K/Akt pathway in ER-negative breast cancer MDA-MB-231 cells that could not be blocked by estrogen antagonists was reported (Tsai *et. al.,* 2001), which was explained as estrogen signaling through an ER-independent pathway. Taken together, these data suggest that subsets of ER-negative breast cancer cells may retain non-genomic estrogen signaling, which may contribute to development and progression of ER-negative breast cancers. Several studies demonstrated that ER-α36 variant is expressed in established

ER-negative breast cancer cell lines such as MDA-MB-231 and MDA-MB-436 and about 40% specimens from ER-negative breast cancer patients (Wang et al., 2006; Lee et al., 2008; Shi et al., 2009). Our group recently reported that E2β treatment activated the MAPK/ERK signaling pathway, induced expression of growth-promoting genes, c-Myc and cyclin D1, stimulated cell proliferation and accelerated tumor growth *in vivo* in ER-negative breast cancer MDA-MB-231 and MDA-MB-436 cells, all of which could be abrogated by knock-down of ER-α36 expression using ER-α36 specific shRNA (Zhang et al., 2011). Thus, ER-α36-mediated mitogenic estrogen signaling contributes malignant growth of ER-negative breast cancer cells.

16. Underlying mechanism of mitogenic estrogen signaling mediated by ER-α36

Compelling evidence demonstrated that estrogens up-regulate the expression and function of c-Myc and cyclin D1, and activate cyclin E-Cdk2 complexes, all of which are rate limiting factors for cell cycle progression from G1 to S phase (Prall *et. al*., 1998; Altucci *et. al.,* 1996 & 1997). Based on these findings, it is believed that estrogens directly interact with ERs in ER-positive human breast cancer cells, where they induce transcriptional activation of "immediate early" and cyclin genes and promote cell cycle progression. However, membrane-initiated estrogen signaling, including rapid changes of the signal transduction cascades, has been proposed to be also essential for the mitogenic action of estrogen signaling. Castoria *et. al.,* (1999) reported that NIH3T3 fibroblasts are made equally E2β-responsive in terms of DNA synthesis by transient transfection with either the wild-type or the transcriptionally inactive ER-α66 mutant. Castoria *et. al.,* (2001) later demonstrated that the PI3K/Akt signaling pathway and Src together mediates activation of cyclin D1 promoter activity and promotion of the S-phase entry in estrogen–stimulated ER-positive breast cancer MCF7 cells. These findings together with other reports (Song et al., 2002; Migliaccio et al., 1996; Ahmad et al., 1999; Lobenhofer et al., 2000) highlight the importance of the non-genomic action of estrogen signaling in estrogen-stimulated cell proliferation and mammary tumorigenesis.

The epidermal growth factor receptor (EGFR) is essential for ductal morphogenesis during normal mammary gland development and its overexpression either at the gene or protein levels is well documented in human breast cancer (Reviewed by Troyer & Lee, 2001). Overexpression of EGFR alone usually does not constitute efficient transformation and tumorigenesis in breast cancer models. However, co-expression with the non-receptor kinase c-Src dramatically increases tumorigenesis (Maa et al., 1995; Tice et al., 1999; Biscardi et al., 1998). Co-expression of EGFR and c-Src in breast cancer cell lines results in their association and c-Src-mediated phosphorylation of the EGFR at tyrosine 845 (Tyr845) within its catalytic domain, which contributes to enhanced proliferation *in vitro* and tumor formation *in vivo* (Biscardi et al., 1998; Maa et al., 1995; Tice et al., 1999; Biscardi et al., 2000). Accumulating evidence indicated that c-Src is involved in non-genomic estrogen signaling by interacting with ER-α66 in ER-positive breast cancer cells (Shupnik, 2004). In ER-negative breast cancer cells, E2β induced the MAPK/ERK activation through a mechanism that involves the interaction between ER-α36 and the EGFR/Src/Shc complex (Zhang et al, 2011). Intriguingly, ER-α36 interacts strongly with EGFR in the absence of estrogen and is progressively dissociated from EGFR after estrogen treatment (Zhang et al., 2011), which is

in contradictory to the finding that estrogen stimulates recruitment of ER-α66 to the EGFR complex (Reviewed by Levin, 2003). Like ER-α66 (Song et al., 2002), interaction between ER-α36 and the Src/Shc was estrogen-dependent (Zhang et al., 2011), which results in Src-Tyr-416 phosphorylation and phosphorylation of the EGFR-Tyr-845 but not the major auto-phosphorylation sites of EGFR such as Tyr-992, -1068 and -1073. Tyr-845 in the EGFR is not an auto-phosphorylation site and is not required for EGFR kinase activity but is phosphorylated by Src (Biscardi et al., 1998).

Signal transducer and activator of transcription 5b (STAT5b), c-Src and EGFR play important roles in estrogen-stimulated proliferation of ER-positive breast cancer cells (Fox et al., 2008). Estrogen-induced Src activation and Src-dependent phosphorylation of EGFR-Tyr-845 recruit STAT5b as a downstream effector of phosphorylated EGFR-Tyr-845 to induce c-Myc and cyclin D1 expression (Fox et al., 2008). Introduction of a dominant-negative STAT5a into ER-positive T47D breast cancer cells inhibits E2β-stimulated cell proliferation and induces apoptosis (Yamashita et al., 2003). The involvement of STAT5 in ER-α36-mediated estrogen activation of the cyclin D1 promoter activity has been established (Zhang et al., unpublished data). Thus, it is possible that ER-α36 mediates mitogenic estrogen signaling through the EGFR/Src/STAT5 pathway in breast cancer cells.

17. Transcriptional regulation of ER-α36

Transcription of ER-α36 is initiated from a previously unidentified promoter in the first intron of the ER-α66 gene (Figure 1). The putative 5′-flanking region of the ER-α36 has been cloned and sequenced (Zou et al., 2009). Computer analysis revealed a TATA binding protein (TBP) recognition sequence upstream of the cDNA start site and several Sp1, NF-κB and Ap1 binding sites in the 5′-flanking region of ER-α36. A perfect half ERE site was identified at the promoter region of ER-α36 that is involved in suppression of ER-α36 promoter activity by ER-α66, indicating that ER-α36 expression is subjected to negative regulation of ER-α66 (Zou et al., 2009). This is consistent with the finding that ER-positive breast cancer cells tend to express lower levels of ER-α36 compared to ER-negative breast cancer cells (Wang et al., 2006; Zou et al., 2009). ER-α46 that lacks the AF-1 domain, however, had no effect on ER-α36 promoter activity while ER-α46 released the suppression activity of ER-α66 when co-expressed with ER-α66 (Zou et al., 2009). Thus ER-α66 suppresses the promoter activity of ER-α36 presumably through ligand-independent activity mediated by its AF1 domain, which can be blocked by ER-α46. In addition, co-expression of ER-α36 also released the suppression activity mediated by ER-α66 (Zou et al., 2009), suggesting that ER-α36 may be regulated by a positive-feedback mechanism.

BRCA1 mutations and downregulation are found in familial and sporadic breast cancers and BRCA1-related tumors are more likely to be ER-negative than are non-BRCA1 related breast cancers. It is still unknown why dysfunctional BRCA1 only predispose to cancers of estrogen responsive tissues and why these BRCA1-related breast tumors are often ER-negative. BRCA1 mediates the ligand-independent transcriptional repression activity of the ER-α66 through its AF-1 domain (Zheng *et. al.,* 2003). Since ER-α66 represses the promoter activity of ER-α36 through its AF-1 domain, it is reasonable to postulate that loss of BRCA1 function either by mutations or downregulation may activate ER-α36 expression, which then activates ER-α36-mediated mitogenic estrogen signaling that eventually leads to development of breast cancers characterized as ER-negative since they lack ER-α66 expression.

The analysis of ER-α36 promoter revealed several important features of transcriptional regulation of this potentially important player in estrogen signaling. The finding of several NF-κB binding sites indicates that ER-α36 is subject to regulation by the NF-κB signaling pathway and by different cytokines that activate the NF-κB signaling pathway. The existence of Ap-1 binding sites in the ER-α36 promoter region raised the possibility that growth factor signaling may regulate ER-α36 expression, which was confirmed by our recent report that EGFR-mediated signaling induces ER-α36 expression via one of the Ap-1 binding sites (Zhang et al., 2011). Thus, further study of the transcription regulation of ER-α36 will provide more information about the mechanisms underlying regulation of ER-α36 mediated non-genomic estrogen signaling in cells and tissues other than mammary gland.

18. Cross-regulation of ER-α36 and members of the EGFR family

In ER-positive breast cancer cells, it has been well documented that the cross-communication between EGFR and ER-α66 leads to serine phosphorylation of ER-α66 and ligand-independent activation of the ER-α66-mediated transcription (Kato et al., 1995; Bunone et al., 1996). Conversely, EGF signaling is strongly enhanced by the ER-α66 in ER-positive MCF7 cells and anti-estrogen ICI 182, 780 was able to block the EGF signaling (Migliaccio et al., 2006). In addition, our group has reported another level of cross-talk mechanism by which EGFR and ER-α36 positively regulate each other's expression in triple-negative breast cancer cells (Zhang et al., 2011); EGFR signaling activates the promoter activity of ER-α36 and ER-α36 stabilizes the steady state levels of EGFR protein. This positive feedback loop provides a molecular explanation to the aggressiveness of triple-negative breast cancer. A similar cross-regulation between Her2/Neu and ER-α36 was also found (Kang et al., unpublished observations). However, unlike EGFR, ER-α36-mediated estrogen signaling up-regulates the promoter activity of Her2/Neu gene, suggesting that ER-α36 positively regulates Her2/Neu expression. This data is consistent with the finding that ER-α36 expression is significantly correlated with Her-2/Neu expression in specimens from breast cancer patients (Shi et al., 2009). Thus, the interplay between growth factor receptors and ER-α36 may play an important role in development and progression of subsets of breast cancer that highly express ER-α36.

19. ER-α36 in osteoporosis

Non-genomic estrogen signaling plays an important role in bone protection; estrogen is able to protect the adult skeleton against bone loss by maintaining a focal balance between bone formation and resorption, which mainly results from the opposite effects of osteoblasts (OBs) and osteoclasts (OCs). Estrogen has anti-apoptotic effects on OBs and pro-apoptotic effects on OCs through an extra-nuclear signaling that leads to activation of the MAPK/ERK signaling pathway and kinase-dependent changes in transcription activities (Reviewed by Manolagas, 2000; Manolagas et al., 2004). However, the underlying mechanisms of this opposite effects of estrogen signaling have not been established. Xie et al., (2011) recently reported that the postmenopausal level of E2β induces mitogenic, anti-apoptotic and anti-osteogenic effects in postmenopausal OBs and pro-apoptotic effects in postmenopausal OCs, respectively. ER-α36 mediates the effects of post-menopausal-level E2β on proliferation, apoptosis, and differentiation of OBs through transient activation of the MAPK/ERK

pathway, whereas ER-α36 mediates post-menopausal-level E2β induced apoptosis of OCs through prolonged or sustained activation of the MAPK/ERK pathway. The levels of ER-α36 expression in bone are positively associated with bone mineral density in post-menopausal women. Thus, the high levels of ER-α36 expression are required for preserving bone mass in post-menopausal and menopausal women.

20. Conclusions

Most previous studies of estrogen signaling in human breast cancer were focused on the ER-α66, the only known estrogen receptor for many years. The discovery of ER-α36 in our laboratory, combined with the previous reports of membrane-based ER-α46, raised the intriguing possibility that ER-α36 and ER-α46 are also involved in estrogen signaling. Net estrogen response in a specific target cell thus depends on absolute and relative levels of the regulated expression of full-length and alternatively processed estrogen receptor-α isoforms.

It is well established that estrogen stimulates mammary epithelial cell proliferation. However, the function of ER-α66, ER-β and ER-α46 in mitogenic estrogen signaling is confounding. A plethora of experimental evidence indicates that ER-α66, ER-α46, and ER-β negatively regulate mitogenic estrogen signaling in transfected cells, which argue against the positive role of these receptors in estrogen-stimulated cell proliferation. Thus far, ER-α36 is the only reported estrogen receptor that mediates mitogenic estrogen signaling and stimulates cell proliferation in transfected cells and in ER-negative breast cancer cells that lack expression of ER-α66, ER-α46 and ER-β but express high level of endogenous ER-α36. In addition, the finding that ER-α36 functions as a potent dominant-negative regulator of the genomic estrogen signaling mediated by ER-α66 and ER-β implicates that when ER-α36 mediates mitogenic estrogen signalling, it may require the silence of genomic estrogen signaling. This again challenges the well-known role of genomic estrogen signaling in estrogen-stimulated cell proliferation.

Accumulating evidence highlights the importance of ER-α36-mediated non-genomic estrogen signaling in malignant growth of breast cancer and endometrial cancer cells. The expression of ER-α36 has also been detected in other types of human cancer such as human colon cancer and liver cancer (Jiang et al., 2008; Miceli et al., 2011), suggesting that ER-α36 may also involved in initiation and development of human malignancy in non-classical estrogen targeting organs. Elucidating the functions of ER-α36-mediated non-genomic estrogen signaling in different types of human malignancy could provide more informed approaches to better understand the underlying mechanisms of mitogenic estrogen signaling in mammary carcinogenesis. We hope that further investigation of the function and underlying mechanisms of this ER variant in different subtypes of human breast cancer and even in the putative breast cancer stem/progenitor cells will lead to development of high efficacy, less toxic therapeutic agents for patients affected with mammary malignancies.

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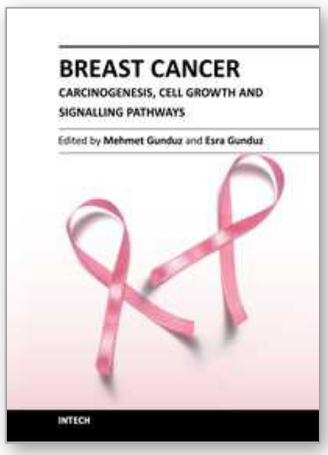

Breast Cancer - Carcinogenesis, Cell Growth and Signalling Pathways

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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