Estrogen receptor alpha-36 (ER- α 36): a new player in human breast cancer

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Abstract

Prevailing wisdom is that estrogen receptor (ER)- α mediated genomic estrogen signaling is responsible for estrogen-stimulated cell proliferation and development of ER-positive breast cancer. However, accumulating evidence indicates that another estrogen signaling pathway, non-genomic or rapid estrogen signaling, also plays an important role in mitogenic estrogen signaling. Previously, our laboratory cloned a 36 kDa variant of ER- α , ER- α 36, and found that ER- α 36 is mainly expressed in the cytoplasm and at the plasma membrane. ER- α 36 mediates rapid estrogen signaling and inhibits genomic estrogen signaling. In this review, we review and update the biological function of ER- α 36 in ER-positive and -negative breast cancer, breast cancer stem/progenitor cells and tamoxifen resistance, potential interaction and cross-talk of ER- α 36 with other ERs and growth factor receptors, and intracellular pathways of ER- α 36-mediated rapid estrogen signaling. The potential function and underlying mechanism of ER- α in development of ER-positive breast cancer will also be discussed.

1. Introduction

Estrogen signaling is essential in the initiation and development of human breast cancer. In the past several decades, extensive efforts have been dedicated to understand the underlying mechanisms of this important signaling pathway in human breast cancer, 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) (Nilsson et al., 2001). Currently, there are two identified ERs, ER- α and ER- β , both of which are ligand-activated transcription factors that stimulate target gene transcription (Nilsson et al., 2001). Compelling evidence indicates that estrogens, especially 17 β -estradiol (E2 β), up-regulate the expression and function of c-Myc and cyclinD1, activate cyclin E-Cdk2 complexes and promotes cell cycle progression from G1 to S phase in mammary epithelial cells (Rochefort et al., 1995). 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, rapid or membrane-initiated signaling pathway) exists that involves cytoplasmic signaling proteins, growth factor receptors and other membrane-initiated signaling pathways (Segars et al., 2002; Driggers et al., 2002; Kelly and Levin, 2001). 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().

The identity of the membrane-based ER(s) that mediates rapid estrogen effects has been center for extensive research. ER- β and 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 (Razandi et al., 1999; Kelly and Levin, 2001; Li et al 2003). In addition, there is also evidence indicating that GPR30, an orphan G-protein-coupled-receptor, mediates the rapid, non-genomic responses to estrogens (Prossnitz and Barton 2011). In 2005, our laboratory identified and cloned a novel variant of ER- α that has a molecular weight of 36-kDa and thus we have termed it ER- α 36. This ER- α variant differs from the original 66 kDa ER- α (ER- α 66), lacking both transcriptional activation domains (AF-1 and AF-2) but retaining the DNA-binding domain and partial dimerization and ligand-binding domains (Wang et al., 2005; Wang et al., 2006). ER- α 36 is predominantly expressed outside of the cell nucleus and at the plasma membrane of both ER-positive and -negative breast cancer cells, mediates rapid estrogen and anti-estrogen signaling such as activation of the MAPK/ERK and PI3K/AKT signaling pathways and stimulation of cell proliferation (Wang et al., 2006; Lin et al., 2010). Thus, ER- α 36 is a novel membrane associated ER that mediates rapid estrogen and anti-estrogen signaling in both ER-positive and -negative breast cancer cells.

In this review, we highlight the historical understanding of the rapid estrogen signaling and its role in cell survival and proliferation, and specifically illustrate the biological function and the possible underlying mechanisms of ER- α 36 in rapid estrogen signaling of both ER-positive and -negative breast cancer cells.

2. Actions of estrogens

Estrogen signaling is pivotal in the establishment and maintenance of reproductive function in men and women. 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, extensive research has been conducted to understand the molecular mechanisms by which estrogen signaling functions. Currently two signaling pathways, genomic and non-genomic pathways, have been demonstrated.

2.1 Genomic estrogen signaling

It is well known that the physiological and pathological functions of estrogens are mediated by specific receptors designated as estrogen receptors (ERs). In 1986, the cloning of the 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).

ERs are members of the nuclear receptor superfamily that control various physiological processes. It was thought that this control occurs through the regulation of gene transcription (Reviewed by Katzenellenbogen & Katzenellenbogen, 2000; McDonnell & Norris, 2002). ER 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 co-regulators or mediator complexes (Reviewed by Klinge, 2001), (b) interaction of the ligand-occupied ER with other transcription factors such as AP-1 (Kushner et al., 2000), Sp1 (Safe, 2001) or NF- κ B (McKay & Cidlowski, 1999), (c) indirect modulation of gene transcription via sequestration of general/common transcriptional components (Harnish et al., 2000; Speir et al., 2000). (d) direct phosphorylation and activation of unliganded receptor by the MAPK/ERK pathway (Kato et al., 1995; Bunone et al., 1996). In addition, the ability of an ER 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 ER agonists or antagonists through these various mechanisms (Shang & Brown, 2002; Katzenellenbogen & Katzenellenbogen, 2002; Margeat et al., 2003).

2.2 Non-genomic estrogen signaling

It became 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 rapid signaling pathway that 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 almost 40 years ago (Pietras & Szego, 1975 & 1977), noting immediate calcium fluxes in endothelial cells induced by E2 β and specific binding sites at the cell surfaces of isolated endothelial cells. Rapid 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 one minute (Duffy 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., 1976 & 1977; Mermelstein et al., 1996), indicating that 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 (Migliaccio et al., 1996; Ahmad et al., 1999; Castoria et al., 1999 & 2001; Lobenhofer et al., 2000; Song et al., 2002). These findings strongly suggest that the rapid, non-genomic estrogen signaling is involved in estrogen-induced proliferation in breast cancer cells.

3. Receptors that mediate rapid estrogen signaling

While it is clear that membrane-initiated estrogen signaling exists, the identity of the ER that mediates these rapid estrogen effects, especially the effects in mammary epithelial cell proliferation and survival, has not been fully established.

3.1. ER- α and ER- β

Immunofluorescent staining of non-permeabilized pituitary tumor cells with anti-ER- α antibodies revealed a punctuated staining pattern on the cell surface although most ER- α were still localized in the 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). Laboratory evidence indicated that both ER- α and ER- β are involved in the rapid estrogen signaling; transient 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 . Thus, it is postulated that the membrane-associated ER- α is derived from the same transcript as the nuclear ER- α (Razandi et al., 1999). However, there is evidence indicating that the native, non-transfected 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 rapid estrogen signaling such as activation of the MAPK/ERK (Nethrapalli et al., 2005), suggesting these cells already possess some unknown proteins other than classical ERs that mediate the rapid estrogen signaling.

Experimental evidence also suggests that more than one rapid signaling pathway is associated with estrogen action. Data from several laboratories using the membrane-impermeable compound E2 β conjugated with bovine serum albumin (E2 β -BSA) indicates the existence of two functionally distinct 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 rapid estrogen signaling.

3.2. GPR30/GPER

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 Ca²⁺ currents and cAMP signaling in cells expressing GPR30 (Revankar et al., 2005) and activates the MAPK/ERK phosphorylation and the PI3K/AKT signaling 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 ER that mediates the rapid, non-genomic estrogen signaling (Prossnitz and Barton 2011; Barton 2012). Now, GPR30 is included in the official GPCR nomenclature and was named G protein-coupled estrogen receptor, GPER, by the International Union of Pharmacology in 2007.

However, there are reports that challenge the role of GPR30/GPER as a novel ER. A study showed that introduction of GPR30/GPER anti-sense oligonucleotides failed to block the MAPK/ERK activation and cell growth induced by E2 β in ER-positive breast cancer cells (Ahola et al., 2002). Pedram *et al.* (2006) failed to observe the cAMP increase or ERK activation in GPR30/GPER-positive and ER-negative breast cancer cells. Another study demonstrated that the GPR30/GPER selective agonist G1 failed to exert estrogenic effect in two classical estrogen target organs, the uterus and the mammary gland (Otto et al., 2008). Later, Otto *et al.* generated GPR30/GPER-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/GPER-deficient animals (Otto et al., 2009). Thus, the possibility that GPR30/GPER might act as a “collaborator” of rapid estrogen signaling has been proposed (Albanito et al., 2007; Gao et al., 2011).

3.3. ER-X

Another membrane-associated ER with an estimated molecular weight of 63-65 kDa, referred to as ER-X, has been reported (Toran-Allerand et al., 2002). 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. 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; Toran-Allerand 2005).

3.4. pER

A heterodimeric estrogen-binding protein, referred to as the putative ER (pER), was reported to bind to E2 β 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 the nucleus (Rao et al., 1998). Since the polyclonal anti-pER antibody failed to react with ERs and was unable to detect pER expression in reproductive organs (Rao et al., 1998), the role of this putative ER in rapid estrogen signaling of breast cancer cells remains elusive.

3.5. ER- α 46

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) that were differentially expressed in three types of breast 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- α 46 and the full-length ER- α 66. ER- α 46 forms homo-dimers and binds to an ERE, and it can also form heterodimers with ER- α 66 (Flouriot et. al., 2000). Furthermore, the ER- α 46/66 heterodimers form preferentially over the ER- α 66 homodimers and ER- α 46 acts competitively to inhibit transactivation mediated by the AF-1 domain of ER- α 66 but without effect on AF-2-dependent transactivation (Floutiot et. al., 2000). Therefore, ER- α 46 is a naturally occurring isoform of ER- α that regulates genomic estrogen signaling mediated by the AF-1 domain of ER- α 66.

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- α 46 in the rapid, non-genomic estrogen signaling. ER- α 46 was found at 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 indicating that the ER- α 46 isoform is involved in rapid estrogen signaling.

4. A novel ER- α variant, ER- α 36

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- α 66 and 46, and apply consistent nomenclature, this novel isoform of ER- α was named as ER- α 36. The cDNA sequence of the open-reading frame matches 100% to the DNA sequence of the exons 2 to 6 of the ER- α 66 gene. The 5'untranslated region (5'UTR) of the cDNA showed 100% homology to the DNA sequence of the first intron of the ER- α 66 gene. Thus, the transcript of this ER- α isoform is initiated from a previously unidentified promoter in the first intron of the ER- α 66 gene (Figure 1, Zou et al., 2009). A small, non-coding novel exon in the first intron of the ER- α 66 gene was designated as exon 1'. The exon 1' is then spliced directly into the exon 2 of the ER- α 66 gene and continues from exon 2 to exon 6 of the ER- α 66 gene. Exon 6 is then spliced to an exon located 64,141 bp downstream of the ER- α 66 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 exon of 4,374 bp located downstream of the ER- α 66 gene. This exon is thus designated as exon 9 to reflect the extra exon beyond the previous reported eight exons for ER- α 66 gene (Figure 1). All of these splicing events are supported by the identification of perfect splice donors and acceptors at the splice junctures. The protein ER- α 36 is produced from a perfect Kozak sequence located in the second exon, the same initiation codon used to produce ER- α 46 protein (Flouriot et. al., 2000). ER- α 36 differs from the ER- α 66 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- α 66 (Figure 2). Thus, ER- α 36 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).

5. Functional interactions of ER- α 36 and other ERs

Comparison of protein structures of ER- α 66 and ER- α 36 indicated that ER- α 36 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- α 36 has no intrinsic transcriptional activity in the presence and absence of E2 β (Wang et al., 2006). However, ER- α 36 strongly inhibited the transactivation activities mediated by the AF-1 and -2 domains of both ER- α 66 and ER- β (Wang et al., 2006). These findings indicate that ER- α 36 lacks intrinsic transcription activity. However, ER- α 36 acts as a naturally occurring dominant-negative regulator of the genomic estrogen signaling mediated by the AF1 and AF2 domains of ER- α 66 and ER- β .

Currently, the mechanisms by which ER- α 36 inhibits genomic estrogen signaling are not clear. ER- α 36 retains protein dimerization domains and is able to form heterodimers with ER- α 66 or ER- β , which may block transcription activities of both ER- α 66 and ER- β . Alternatively, ER- α 36 may bind to the same ERE DNA sequences as ER- α 66 and ER- β through its intact DNA binding domains and compete with ER- α 66 and ER- β for DNA binding. Previously, we found that breast cancer tissues expressing high levels of ER- α 36 exhibited cytoplasmic staining

of ER- α 66 (Lee et al., 1999). The same cytoplasmic expression pattern of ER- α 66 was also observed in ER-positive breast cancer MCF7 cells transfected with an ER- α 36 expression vector (Yin and Wang, unpublished observations). Thus, it is possible that ER- α 36 may retain ER- α 66 in the cell cytoplasm to block its genomic signaling in the cell nucleus.

Transcription of ER- α 36 is initiated from a previously unidentified promoter in the first intron of the ER- α 66 gene (Figure 1). The putative 5'-flanking region of the ER- α 36 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- κ B and AP-1 binding sites in the 5'-flanking region of ER- α 36. A perfect half ERE site was identified at the promoter region of ER- α 36 that is involved in suppression of ER- α 36 promoter activity by ER- α 66, indicating that ER- α 36 expression is subjected to negative regulation of ER- α 66 (Zou et al., 2009). ER- α 46 that lacks the AF-1 domain, however, had no effect on ER- α 36 promoter activity and ER- α 46 released the suppression activity of ER- α 66 when co-expressed with ER- α 66 (Zou et al., 2009). Thus, ER- α 66 suppresses the promoter activity of ER- α 36 presumably through the ligand-independent activity mediated by its AF1 domain, which can be blocked by ER- α 46. In addition, co-expression of ER- α 36 that lacks AF-1 domain also released the suppression activity ER- α 66 (Zou et al., 2009), suggesting that ER- α 36 expression may be regulated also by a positive-feedback mechanism.

Taken together, ER- α 36 blocks the genomic estrogen signaling of ER- α 66 and ER- β while ER- α 66 suppressed the promoter activity of ER- α 36, which can be released by ER- α 46 and ER- α 36 itself. In addition, ER- α 46 blocks the transcription activity of the AF-1 domain of ER- α 66. Thus, the relative expression levels of ER- α 66, 46 and 36 in a specific cell determine rations of genomic or non-genomic estrogen signaling.

6. Biology of ER- α 36

The fact that ER- α 36 posses no intrinsic transcription regulatory activity suggests that ER- α 36 may mediate rapid estrogen signaling. The sub-cellular fractionation assay in ER- α 36 transfected HEK293 cells revealed that a high percentage of ER- α 36 (~50%) is localized at 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 an ER- α 36 specific antibody that recognizes the last 20 amino acids that are unique to ER- α 36 exhibits a membrane expression pattern that is co-localized with caveolin-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- α 36 predominantly outside of the cell nucleus (Lee et al., 2008; Vranic et al., 2011). This anti-ER- α 36 specific antibody also blocked rapid 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- α 36 in intact cells and ER- α 36 is important in rapid estrogen signaling. These results thus demonstrated that ER- α 36 is expressed on or near the plasma membrane and suggested that ER- α 36 may be also shuttled to the cell cytoplasm and nucleus depending on cell context and extracellular signals.

Both E2 β and BSA-conjugated E2 β elicit the rapid estrogen signaling such as activation of the MAPK/ERK signaling pathway and stimulation of cell proliferation in ER- α 36 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), 17 α -estradiol (E2 α), estriol (E3) and estetrol (E4) all activate the ERK1/2 phosphorylation at a very similar level as E2 β , indicating that ER- α 36 possess a broader ligand-binding spectrum than ER- α 66 (Wang et al., 2006). Previously, it was reported that ER- α 36 even mediated testosterone-stimulated activation of the MAPK/ERK and PI3K/AKT signaling pathways in endometrial cancer Hec1A cells (Lin et al., 2009). ER- α 36 also mediates estrogen activation of the PKC δ /ERK signaling pathway (Tong et al., 2010). The finding that the rapid estrogen signaling mediated by ER- α 36 was insensitive to anti-estrogens suggests that ER- α 36 may be a receptor involved in the anti-estrogen-insensitive estrogen signaling described in different systems before. Taken together, ER- α 36 is a novel ER that mediates rapid, non-genomic estrogen signaling.

7. ER- α 36 in ER-positive breast cancer and anti-estrogen resistance

ER- α (ER- α 66) 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 66. At the onset, 46%-77% of breast cancers are ER-positive (Roberson et. al., 1996) and up to 70% of them respond to anti-estrogen therapy (Clark et. al., 2000). The ER-positive tumors are usually 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; Clark et. al., 2000).

Since mitogenic estrogen signaling plays a pivotal role in development and maintenance of ER-positive breast cancer, treatment with anti-estrogens such as tamoxifen has become a first-line therapy for advanced ER-positive breast cancer. However, laboratory and clinical evidence indicated that tamoxifen and its metabolites such as 4-hydroxy-tamoxifen 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).

ICI 182, 780 binds to ERs, impairs receptor dimerization and inhibits nuclear localization of receptor (Fawell, et. al., 1990; Dauvois et. al., 1992). In addition, ICI 182, 780 also accelerates degradation of the ER- α 66 protein without a reduction of ER- α 66 mRNA (Nicholson et. al., 1995). Thus, ICI 182, 780 binds ER- α 66 and accelerates degradation of ER- α 66 protein, resulting in a complete inhibition of estrogen signaling mediated by ER- α 66. However, ICI 182, 780 failed to induce degradation of ER- α 36 (Kang et al., 2010a) presumably because ER- α 36 has a truncated ligand-binding domain that lacks the last 4 helices (helix 9–12) of ER- α 66 (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- α 66 regulates functional differences between agonists and antagonists (Mahfoudi et al., 1995; Pearce et al., 2003). Indeed, ER- α 36 mediates agonist activities of tamoxifen and ICI 182, 780 in ER-negative breast cancer cells that express high levels of ER- α 36 (Zhang et al., 2012).

It is well-known that most breast tumors are eventually resistant to anti-estrogen therapy despite the significant anti-neoplastic activities of anti-estrogens. Essentially, two forms of anti-estrogen resistance occur: *de novo* and acquired resistance (Reviewed by Clarke et. al., 2001 & 2003; Ring & Dowsett, 2004). Although absence of ER- α 66 expression is the most common *de novo* resistance mechanism, about 40-50% ER-positive tumors are already resistant to tamoxifen

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 & Dowsett, 2004). In addition, most initially responsive breast tumors gradually acquire anti-estrogen resistance by loss of anti-estrogen responsiveness. Breast tumors with acquired tamoxifen resistance frequently but not always retain levels of ER- α 66 expression that would still define them as ER-positive tumors (Reviewed by Clarke et. al., 2001 & 2003). Therefore, a loss of ER- α 66 expression is not the major mechanism driving acquired anti-estrogen resistance. Up-regulation of growth factor receptor such as Erb-B2 (Her2/Neu) and constitutive activation of the MAPK signaling pathway, which usually bypass the requirement of estrogen signaling for cell proliferation was found to be involved in development of anti-estrogen resistance (Reviewed by Clarke et. al., 2001 & 2003; Ring & Dowsett, 2004).

Previously, a retrospective study of 896 cases of breast cancer patients revealed that about 40% cases of total human breast cancer patients were positive for ER- α 36; about 40% cases ER-positive breast cancer co-expressed ER- α 66 and ER- α 36 and about 40% cases of ER-negative breast cancer that lacked ER- α 66 expression were positive for ER- α 36 (Shi et al., 2009). The breast cancer patients with tumors expressing high levels of ER- α 36 less benefited from tamoxifen therapy than those with low levels of ER- α 36 expression did, and ER- α 36 expression is significantly associated with Her2/Neu expression (Shi et. al., 2009). These results suggest that enhanced levels of ER- α 36 expression in breast cancer cells is one of the underlying mechanisms of *de novo* tamoxifen resistance found in ER-positive breast cancer patients.

ER- α 36-mediated rapid estrogen signaling is insensitive to anti-estrogens such as tamoxifen and ICI 182, 780 (Wang et al., 2006). ER- α 36 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; Zhang et al., 2012 & 2013). In cells expressing ER- α 36, the effects of both 4-hydroxytamoxifen and ICI 182, 780 exhibited a non-monotonic, or biphasic dose response curve; anti-estrogens at low concentrations, elicited a mitogenic signaling pathway to stimulate cell proliferation while at high concentrations, anti-estrogens inhibited cell growth (Zhang et al., 2012). Thus, ER- α 36 is involved in anti-estrogen resistance by mediating agonist activities of anti-estrogens.

In ER-positive breast cancer cells, it has been well documented that the cross communication between EGFR and ER- α 66 leads to phosphorylation of ER- α 66 and ligand-independent activation of the ER- α 66-mediated transcription (Kato et al., 1995; Bunone et al., 1996). Conversely, EGF signaling is strongly enhanced by the ER- α 66 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: positive regulatory loops between ER- α 36 and EGFR/HER2 in ER-negative breast cancer cells (Zhang et al., 2011; Kang et al., 2013). In triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells, knockdown of ER- α 36 expression enhances EGFR protein degradation through the proteasome system while the EGFR signaling pathway up-regulates the promoter activity of ER- α 36 through an AP-1 binding site in the 5' flanking sequence of ER- α 36 gene (Zhang et al., 2011). In HER2 over-expressing breast cancer SKBR3 cells, ER- α 36-mediated signaling positively regulates HER2 transcription while HER2 signaling up-regulates the promoter activity of ER- α 36 (Kang et al., 2013). These results are consistent with the findings that ER- α 36 expression is significantly correlated with Her-2/Neu expression in breast cancer (Shi et al., 2009) and with EGFR expression in endometrial cancer (Tu et al., 2011). ER- α 36 was found to

be necessary for both EGFR membrane localization and E2 β -mediated stimulation of EGFR expression in TCam-2 cells (Wallacides et al., 2012). Thus, the interplay between growth factor receptors and ER- α 36 may play an important role in development and progression of subsets of breast cancer that highly express ER- α 36 and development of anti-estrogen resistance.

Many researches were conducted to understand the molecular pathways involved in acquired tamoxifen resistance and have revealed that multiple signaling molecules and pathways such as EGFR and HER2 are involved (Clarke et al., 2001 & 2003; Normanno et al., 2005). Gained signaling through these pathways often bypasses the requirement of estrogen signaling for growth of ER-positive breast cancer cells. Recently, we found that tamoxifen induced ER- α 36, EGFR and HER2 expression through the positive regulatory loops between ER- α 36 and EGFR/ HER2 in tamoxifen sensitive ER-positive breast cancer MCF7 cells (Yin et al., 2014). Thus, the ER- α 36-EGFR/HER2 positive regulatory loops are one of the underlying mechanisms of ER-positive breast cancer cells gained expression of the growth factor receptors during tamoxifen treatment.

8. ER- α 36 in ER-negative breast cancer and chemotherapy resistance

Because of the lack of ER- α 66 expression, it is prevailing 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 (Gupta et al., 2007). Surprisingly, however, the majority of breast cancers that developed following pregnancy are negative for either estrogen and progesterone receptors (Gupta et al., 2007). 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 reports suggest that subsets of ER-negative breast cancer cells retain rapid estrogen signaling which may contribute to development and progression of ER-negative breast cancers. Several studies demonstrated that ER- α 36 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). ER- α 36 is commonly over-expressed in apocrine and adenoid cystic carcinomas of the breast, most of which lack ER- α 66 and thus are ER-negative (Vranic et al., 2011). Pelekanou et al., (2011) also reported that triple-negative breast cancers that lack expression of ER- α 66, progesterone receptor and HER2 highly express ER- α 36. Thus, results from different laboratories indicated that subsets of ER-negative breast cancer still express the ER- α 36 variant although lack expression of ER- α 66. These ER-negative breast cancers may still response to mitogenic estrogen signaling.

Chaudhri et al., (2012) reported the presence of ER- α 36, in ER-positive MCF7 and ER-negative HCC38 breast cancer cells, that localized to the plasma membrane and rapidly activated PKC in response to E2 β , leading to deleterious effects such as enhancement of proliferation, protection against apoptosis, and enhancement of metastatic factors (Chaudhri et al., 2012). Our group also found that E2 β 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 knockdown of ER- α 36 expression using the ER- α 36 specific shRNA (Zhang et al., 2011). Knockdown of ER- α 36 expression also disrupted the ER- α 36-EGFR/HER2 positive regulatory loops in ER-negative breast cancer cells and inhibited growth of these cells (Zhang et al., 2011; Kang et al., 2012). Thus, ER- α 36-mediated rapid estrogen signaling and the ER- α 36-EGFR/HER2 positive regulatory loops are involved in malignant growth of ER-negative breast cancer.

Recently, Yu et al., (2014) reported that breast cancer patients with tumors highly expressing ER- α 36 less benefited from neoadjuvant chemotherapy and have worse prognosis. It was suggested that ER- α 36 may be used as a predictive biomarker for the efficacy of neoadjuvant chemotherapy, and the negative expression of ER- α 36 may be a favorable factor of prognosis in breast cancer patients treated with neoadjuvant chemotherapy.

9. ER- α 36 in breast cancer stem/progenitor cells

Accumulating experimental and clinical evidence supports that breast cancer may arise from mammary stem/progenitor cells which possess the ability to self-renew (Al-Hajj et al., 2003; Dontu et al., 2003; Charafe-Jauffret et al., 2009; Oliveira et al., 2010). Al-Hajj et al. enriched a CD44⁺/CD24^{-low} cell population from human breast cancer that displayed cancer stem/progenitor cell properties and was capable of forming tumors in immuno-compromised mice with higher efficiency than cells with alternative phenotypes. Later, aldehyde dehydrogenase (ALDH) 1 expression and/or its activity were identified to be a marker for breast cancer stem/progenitor cells; fewer ALDH1 positive tumor cells than CD44⁺/CD24^{-low} tumor cells are required to generate tumors *in vivo* (Ginestier et al., 2007). The breast cancers with ALDH1^{high} cancer stem-like cells are associated with more aggressive phenotypes such as ER negativity, high histological grade, HER2 positivity, as well as poor prognosis (Morimoto, et al., 2009).

Many signaling pathways involved in regulation of normal mammary stem cells including Hedgehog, Bmi-1, Wnt, NOTCH, HER-2, p53 and PTEN/Akt/ β -catenin pathways play roles in breast cancer stem/progenitor cells (Korkaya et al., 2008 & 2009; Han et al., 2009; Woodward et al., 2007). However, the involvement of estrogen signaling, a major signaling pathway profoundly influences mammary carcinogenesis, in regulation of breast cancer stem/progenitor cells has not been established for a long time, since expression of ER- α 66 in breast cancer stem/progenitor cells is controversial. It was reported that stem-like cells isolated from normal mammary gland and breast cancer tissues lack expression of the full-length ER- α 66 (Dontu et al., 2004; Sleeman et al., 2007). However, Clarke *et al.*, (2005) reported that ER- α 66 is expressed in putative normal breast stem/progenitor cells enriched by the “side population” method. Despite the fact that ER- α 66 expression in mammary stem cells is not clear, the significance of estrogen signaling for normal development and growth of the mammary gland are well established by studies in human and animal, which was explained as though an indirect paracrine pathway (Asselin-Labat et al., 2010; Fillmore et al., 2010; Mallepell et al., 2006). It was also proposed that the status of ER in breast cancer stem cells is dependent on the origin of breast cancer, ER-positive breast cancers originate from transformed ER-positive stem/progenitor cells while ER-negative breast cancers from ER-negative stem/progenitor cells (Dontu et al., 2004).

Recently, our group found that breast cancer stem/progenitor cells derived from ER-positive breast cancer cells express high levels of ER- α 36 (Deng et al., 2014a). Knockdown of ER- α 36 expression with the shRNA method in ER-positive breast cancer cells diminished rapid estrogen signaling in ER-positive breast cancer stem/progenitor cells and significantly reduced the percentage of breast cancer stem/progenitor cells (Deng et al., 2014a). ER- α 36 expression is well correlated with ALDH1 expression in tumor samples of breast cancer patients (Deng et al., 2014a). In addition, ER- α 36 knockdown also decreased ALDH1 positive cell population in ER-negative breast cancer SKBR-3 cells (Kang et al., 2013). Thus, ER- α 36 mediated rapid estrogen signaling plays an important role in maintenance of ER-positive and -negative breast cancer stem/progenitor cells.

The major features of cancer stem cells include the ability of self-renewal and generating tumors from very few cells, slow cell division, the ability to produce differentiated cells of different lineages, selective resistance to radio- and chemo-therapy (Dean et al., 2005; Diehn & Clarke, 2006). The possible involvement of breast cancer stem/progenitor cells in anti-estrogen resistance has been proposed (O'Brien et al., 2013) and demonstrated (Piva et al., 2013); the breast cancer stem/progenitor cells induced by Sox2 introduction are resistant to tamoxifen. Recently, our laboratory demonstrated that ER- α 36 mediated resistance of breast cancer stem/progenitor cells to anti-estrogens tamoxifen and ICI 182, 780 and anti-estrogen stimulation of luminal epithelial lineage specific ER-positive breast cancer progenitor cells (Deng et al., 2014b). Thus, it is possible that anti-estrogen treatment may enrich breast cancer stem/progenitor cells and even stimulate progenitor cell proliferation, which results in failure of anti-estrogen treatment.

10. ER- α 36-mediated rapid estrogen signaling

10.1 Early events of ER- α 36-mediated rapid estrogen signaling

Previously, Razandi et al., reported that HB-EGF released by matrix metalloproteinases (MMP)-2 and -9 that are activated by the G protein-coupled receptors via Src signaling is responsible for estrogen-induced activation of the EGFR and the MAPK/ERK in MCF7 cells (Razandi et al., 2003). Later, Song et al., reported that IGF-IR is also involved in the rapid estrogen signaling of MCF7 cells (Song et al., 2007). Previously, our group reported that ER- α 36 physically interacts with EGFR in ER-negative breast cancer cells (Zhang et al., 2011). After E2 β treatment, ER- α 36 was gradually dissociated from the EGFR and associated with Src and Shc, suggesting that ER- α 36 might dynamically change its partners with the EGFR/Src/Shc complex during rapid estrogen signaling (Zhang et al., 2011). Recently, our group further reported that E2 β induced several cycles of ERK activation in MCF7 cells expressing high levels of recombinant ER- α 36 (Zhang et al., 2014). Using neutralizing antibodies, we found that the estrogen-induced first and second peaks of the ERK activation was blocked by the anti-HB-EGF neutralizing antibody while the anti-amphyregulin antibody only blocked the second peak of ERK activation, suggesting HB-EGF is involved in the ERK activation induced by amphyregulin. With neutralizing antibodies, we also demonstrated that IGF-IR, EGFR and HER2 are all involved in the rapid estrogen signaling mediated by ER- α 36 in ER-positive breast cancer MCF7 cells (Zhang et al., 2014). Using different chemical inhibitors, we further revealed that Src and MMPs are also involved in the rapid estrogen signaling of ER-positive breast cancer MCF7 cells. Thus, initiation of the rapid estrogen signaling is a highly synchronized process that involves a number of growth factors and their receptors, consistent with the previous view that estrogen acts as growth factors (Migliaccio et al., 2002; Shupnik et al., 2004). Our results thus

provided a novel mechanism for the functional communication among these signaling pathways; ER- α 36 mediates rapid estrogen signaling that employs IGF-1R, EGFR and HER2 (Figure 3).

10.2. ER- α 36 downstream signaling pathways

Estrogen activation the MAPK/ERK pathways is a typical event of rapid estrogen signaling. Our laboratory reported an increase of ERK1/2 phosphorylation of HEK293 cells transfected with a ER- α 36 expression vector after the treatments with E2 β , and E2 β -BSA, a membrane-impermeable form of E2 β (Wang et al., 1996), indicating that ER- α 36 mediates rapid estrogen signaling. Such event was also noticed in ER-negative breast and endometrial cancer cells that express endogenous ER- α 36 (Kang et al., 2010; Zhang et al., 2011; Lin et al., 2009; Lin et al., 2010; Ohshiro et al., 2012).

Estrogen activates the ERK1/2 via the PKC (McMillan et al., 2006), rapidly enhancing phosphorylation of proteins that promote proliferation in plate chondrocytes. Tong et al. found that ER- α 36 mediated estrogen-induced activation of MAPK/ERK pathway via PKC δ in endometrial cancer cells (Tong et al., 2010). In breast cancer cells, ER- α 36 rapidly activated PKC in response to E2 β , leading to the increase of proliferation (Chaudhri et al., 2012).

The treatment of estrogen and anti-estrogens led to rapid activation of the MAPK/ERK1/2 and a substantial increase of cell migration and invasiveness in inflammatory microenvironment of breast cancer in the presence of ER- α 36 but not ER- α 66 (Chaudhri et al., 2012). Estrogen-induced migration and invasiveness were blocked by the MEK inhibitor U0126, indicating that estrogen activation of the ERK1/2 is involved in the regulation of the cell motility and invasiveness (Chaudhri et al., 2012). It was also found the up-regulation of p-ERK1/2 level in tumor samples from patients with inflammatory breast cancer (Ohshiro et al., 2012). Thus, ER- α 36-mediated estrogen activation of the MAPK/ERK signaling pathway is involved in proliferation and invasion of inflammatory breast cancer cells.

The c-Jun N-terminal Kinases (JNKs) are other principal members of the MAPK family that regulate cell proliferation, differentiation and migration (Wagner & Nebreda, 2009). In the absence of ER- α 36, the JNKs were less activated and cells were arrested at the G2/M phase after treatment with paclitaxel (Zhang et al., 2012). It seems that ER- α 36 attenuates the effect of paclitaxel via the activation of the JNKs pathway and ER- α 36-absent cancer cells are more sensitive to paclitaxel.

The serine/threonine kinase (AKT), or protein kinase B, plays an important role in cell proliferation and survival by inhibition of apoptosis in tamoxifen resistant cells (Jordan et al., 2004). Treatments with testosterone, E2 β , or tamoxifen induced rapid phosphorylation of AKT in Hec1A cells through ER- α 36 (Lin et al., 2009). Tamoxifen induced AKT phosphorylation in MCF7 cells transfected with ER- α 36 expression vector, which contributes to ER- α 36 mediated tamoxifen resistance (Lin et al., 2010; Zhang et al., 2013).

Taken together, these results suggested that most if not all of the signaling pathways downstream of tyrosine kinase receptors are involved in ER- α 36-mediated rapid estrogen signaling.

11. ERs in breast cancer: who has the last word?

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. Estrogens function as potent mitogens through promoting cell cycle progression from G1 to S phase (Prall et al., 1998; Altucci et al., 1996 & 1997). Thus, 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. This classic estrogen-signaling pathway is prevailingly thought to be responsible for estrogen-stimulated cell proliferation as well as initiation and progression of estrogen-related cancers such as breast and endometrial cancers. Consistent with its role in mammary carcinogenesis, ER- α 66 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 66 (Khan et. al., 1994).

ER- α 66 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 66 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). As ER- α 66 expression increases during breast cancer development, the inverse relationship between ER- α 66 expression and cell proliferation become dysregulated (Roger et al., 2000; Shoker et. al., 1999a & b; Jensen et al., 2001). Approximately 70% of breast carcinomas express the ER- α 66 and most of these tumors contain ER- α positive proliferating cells (Clarke et. al., 1997).

However, the explanation of the mitogenic estrogen signaling simply by the function of ER- α 66 is confounding. When ER- α 66 was expressed in ER-negative CHO cells (Kushner et al., 1990) and human cervical cancer HeLa cells (Touitou et al., 1990), estrogen did not stimulate cell growth. On the contrary, estrogen inhibited cell growth and even induced cell death. Likewise, the ER-null MCF10A breast epithelial cells (Pilat et al., 1996), and MDA-MB-231 breast cancer cells (Garcia et al., 1992; Barrón-González et al., 2004; Jiang & Jordan, 1992) were growth inhibited when they were stably transfected with ER- α 66 cDNA. These results strongly argue against a positive and direct role of the ER- α 66 in estrogen-stimulated cell proliferation.

ER- β also acts as a dominant-negative regulator of mitogenic estrogen signaling. ER- β expression is decreased in proliferative pre-invasive mammary tumors (Roger et al., 2001). In ER positive cells, ER- β attenuates estrogen activities such as transcriptional regulation and stimulation of cell proliferation (Reviewed by Deroo & Buensuceso, 2010; Harris, 2007; Ogawa et al., 1998; Lazennec et al., 2001; Galluzzo et al., 2007). ER- β inhibits the estrogen-induced proliferation in ER-positive breast cancer cells and blocks estrogen-stimulated tumor formation in nude mice (Paruthiyil et al, 2004; Hartman et al., 2006).

ER- α 46 is an isoform of ER- α 66 that has not been studied extensively. Forced expression of ER- α 46 inhibited proliferation of MCF-7 breast cancer cells and cyclin D1 promoter activity (Penot et al., 2005). Overexpression of both ER- α 46 and ER- α 66 in ER-negative MDA-MB-231 cells revealed that ER- α 46 inhibited basal transcription of the estrogen responsive gene pS2 while estrogen treatment released this inhibition (Metivier et al., 2004). Recently, the level of ER- α 46 expression was found to be down-regulated in tamoxifen-resistant breast cancer cells and re-introduction of ER- α 46 into these cells inhibited cell proliferation and ER- α 66-regulated gene transcription (Klinge et al., 2010). Thus, these results suggest that ER- α 46 may also function as a negative regulator of estrogen-stimulated cell proliferation.

Taken together, while it is well known that estrogen is a potent mitogen, the identity of the receptor that mediates estrogen-stimulated cell proliferation has not been well established.

Accumulating evidence indicated that the rapid estrogen signaling that involves the MAPK/ERK and the PI3K/AKT signaling pathways mediates proliferative and anti-apoptotic effects of estrogens in a variety of normal and cancer cells (Kelly & Levin, 2001; Levin 2009; Acconcial & Marino, 2011). The most intriguing data are those from Auricchio's group. An elegant and extensive series of experiments led to the conclusion that the mitogenic activity of estrogen is exclusively mediated by the rapid estrogen signaling involving Src and the PI3K/AKT pathway in ER-positive breast cancer MCF7 cells (Migliaccio et al., 1996; Castoria et al., 1999 & 2001).

Previous reports have indicated that ER- α 66 and ER- β mediate the rapid estrogen signaling such as the activation of the MAPK/ERK signaling based on experiments involving transfection of these ER cDNAs into reportedly ER-deficient cell lines such as CHO-K1 (Razandi et al., 1999), COS-7 (Migliaccio et al., 1996), Rat2 fibroblasts (Wade et al., 2001). Later, however, Nethrapalli et al., (2005) demonstrated that estrogen activated the MAPK/ERK signaling in native, non-transfected CHO-K1, COS-7 and RAT2 fibroblast cells. Previously, our laboratory also reported that non-transfected COS-7 cells expressed endogenous ER- α 36 and exhibited the rapid estrogen signaling (Kang et al., 2010b). Together, these results suggested that the cells null for ER- α 66 used as models to study the rapid estrogen action may already express endogenous ER- α 36 and are able to elicit the rapid estrogen signaling.

The membrane-bound ER GPR30/GPER belongs to the G-protein-coupled receptor superfamily was reported to mediate rapid estrogen signaling in different kinds of cells (Revankar et al., 2005; Filardo et al., 2000 & 2007; Prossnitz and Barton 2011). Our group reported that knockdown of GPR30/GPER expression in ER-negative breast cancer SK-BR-3 cells down-regulated ER- α 36 expression (Kang et al., 2010b). Introduction of a GPR30 expression vector into GPR30/GPER non-expressing cells induced endogenous ER- α 36 expression and GPR30/GPER activated the promoter activity of ER- α 36 via an AP-1 binding site located in the 5'-flanking region of ER- α 36 (Kang et al., 2010b). Recently, it has also been reported that E2 stimulates proliferation in the seminoma-like Tcam-2 cells through a GPR30/GPER-dependent induction of ER- α 36 (Wallacides et al 2012). In addition, we also observed that ER- α 36 physically interacted with GPR30/GPER (Kang and Wang, unpublished observations), suggesting a possible cross-talk between ER- α 36 and GPR30/GPER. Functional cross-talk between ER- α 66 and GPR30/GPER has been demonstrated in ovarian cancer cells (Albanito et al., 2007). Thus, ER- α 36 might be a downstream target gene of GPR30/GPER-mediated signaling and the previously reported activities of GPR30/GPER as a membrane-based ER may be partially mediated through its ability to induce ER- α 36 expression.

However, Lindsey et al. reported that under disease conditions, GPR30/GPER signaling exhibits renoprotective effects by reducing proteinuria in a model of salt-sensitive hypertension in kidney (Lindsey et al., 2011). These investigators failed to detect ER- α 36 expression in the kidney (Lindsey et al., 2011). Thus, it is possible that in the hypertensive renal disease model and the related proteinuria in experimental animals, ER- α 36 is not directly involved in the effects mediated by GPR30/GPER signaling. GPR30/GPER may signal dependently or independently on ER- α 36 in different systems. Currently, most studies focus on either ER- α 36 or GPR30/GPER, it would be more informative to examine cross-talk of both receptors in a suitable system.

Recently, we used the shRNA specifically targeting ER- α 66 or 36 to knock-down expression of ER- α 66 or 36 individually and found that the cells with knocked-down levels of ER- α 36 expression lacked the rapid estrogen signaling. On the contrary, the cells with ER- α 66 expression knocked-down still exhibited the rapid estrogen signaling (Zhang et al., 2014). Thus, our data, for the first time, demonstrated that the ER- α 36 is the ER responsible for the rapid estrogen signaling such as the ERK activation in ER-positive breast cancer cells.

Using the ER-positive breast cancer cells with ER- α 66 or 36 expression specifically and separately knocked-down, we also found that in ER-positive breast cancer cells, induction of the well-known estrogen responsive and growth-promoting genes such as cyclin-D1, c-Myc and PgR receptor, the ER- α 66 target genes without the ERE-like sequence in their promoter regions, were actually through the ER- α 36-mediated rapid estrogen signaling while the pS2 gene that contains a perfect ERE site was induced by ER- α 66 (Zhang & Wang, unpublished results). Recently, we reported that estrogen induced cyclin-D1 expression in ER-negative breast cancer cells that only express ER- α 36 through the Src/EGFR/STAT5 pathway (Zhang et al., 2013). It was also reported that STAT5, c-Src and EGFR play important roles in estrogen-stimulated proliferation of ER-positive breast cancer cells (Fox et al., 2008). Constitutive activation of STAT5 was detected in a panel of breast cancer cell lines such as MCF7 and T47D cell lines and introduction of a dominant-negative STAT5 into T47D cells inhibited estrogen-stimulated cell growth and induced apoptosis (Yamashita et al., 2003). Thus, estrogen responsive genes are regulated by both rapid and genomic estrogen signaling pathways, consistent with the hypothesis proposed before that estrogen signaling regulates gene transcription through multiple signaling pathways (Marino et al., 2006; Björnström & Sjöberg, 2005).

12. Conclusions

ER- α 36 is a novel isoform of ER- α 66 that mainly mediates rapid, non-genomic estrogen effects and inhibits the genomic activity of the nuclear ERs in breast cancer cells. ER- α 36 utilizes HB-EGF, SRC, EGFR, HER2 and IGF-IR to transduce rapid estrogen signaling by activating downstream signaling pathways. These signaling pathways eventually induce transcription of the growth-promoting genes such as c-Myc and Cyclin D1 and stimulate cell proliferation in breast cancer cells. The findings that ER- α 36 is highly expressed in both ER-positive and -negative breast cancer cells, contributes to anti-estrogen resistance and is involved in maintenance of breast cancer stem/progenitor cells, indicate that ER- α 36 is a new player that plays an important role in estrogen signaling during initiation and development of breast cancer. Thus, further investigation of the function and underlying mechanisms of ER- α 36 in initiation and development of both ER-positive and -negative breast cancer will greatly advance or even change our current knowledge on human breast cancer.

12.1 Perspectives.

Most previous studies of estrogen signaling in human breast cancer were focused on the ER- α 66, the only known ER for many years. The discovery of ER- α 36 in our laboratory, combined with the previous findings of membrane-based ER- α 46, raised the intriguing possibility that ER- α 36 and ER- α 46 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 ER- α isoforms.

A plethora of experimental evidence indicates that ER- α 66, ER- α 46, 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- α 36 is the only reported ER that mediates estrogen-stimulated cell proliferation in transfected cells and in ER-negative breast cancer cells that lack expression of ER- α 66, ER- α 46 and ER- β but express high level of endogenous ER- α 36. In addition, the finding that ER- α 36 functions as a potent dominant-negative regulator of the genomic estrogen signaling mediated by ER- α 66 and ER- β implicates that when ER- α 36 mediates mitogenic estrogen signaling, 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 indicates the importance of ER- α 36-mediated rapid estrogen signaling in malignant growth of endometrial cancer cells. ER- α 36 expression was detected at the plasma membrane and in the cytoplasm of cells in both endometrial cancer tissues and cell lines (Tu et al., 2011; Lin et al., 2009 & 2010). ER- α 36-mediated activation of the MAPK/ERK and PI3K/Akt pathways promoted the proliferation of endometrial cancer cells induced by different ligands, e.g. E2 β , tamoxifen, or testosterone (Lin et al., 2009 & 2010; Tong et al., 2010). In addition, ER- α 36 expression was positively associated with high stage and grade and poor disease-free survival rate of endometrial cancer cases (Sun et al., 2013). Thus, ER- α 36 may be an important biomarker for diagnosis and prognosis of endometrial carcinoma.

ER- α 36 expression at the plasma membrane and in the cytoplasm was also detected in different types of human cancer such as colorectal (Jiang et al., 2008), gastric (Deng et al., Wang et al., 2012) and hepatic cancers (Miceli et al., 2011), suggesting that ER- α 36 may be involved in initiation and development of human malignancy in non-classical estrogen targeting organs. Elucidating the functions of ER- α 36-mediated rapid 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 future investigation in different subtypes of human breast cancer and in the breast cancer stem/progenitor cells will lead to development of high efficacy, less toxic therapeutic agents for breast cancer patients through targeting breast cancer stem-like cells.

List of abbreviations:

ER: Estrogen receptor; E2 β : 17 β -Estradiol; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinases; JNK: c-Jun N-terminal Kinase; PI3K: Phosphatidylinositol 3-kinase; EGFR: Epidermal growth factor receptor; HER2: Human Epidermal Growth Factor Receptor 2; IGF-IR: Insulin-like growth factor receptor I; STAT: signal transducer and activator of transcription. GPCR: G protein-coupled receptor; GPER: G protein-coupled estrogen receptor.

Competing interests

All authors declare that they have no competing interests.

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Figure 1. Genomic structure of the ER- α 36 gene. The common exons between the ER- α 36 and ER- α 66 genes are shown as numbered boxes. The exon of ER- α 36 from the intron1 of ER- α 66 gene was indicated as 1' and the extra exon of the ER- α 36 gene that is beyond the 8 exons found in ER- α 66 gene is numbered as 9. ER- α 36 has a unique 27 amino acid domain at the C-terminus. Alternative splicing is indicated by a bended line.

Figure 2. Protein structures of ER isoforms. The A/B domain contains the transcriptional-activation function AF-1; the C domain represents the DNA-binding-domain (DBD); D contains the nuclear localization signal (NLS), E domain corresponds to the ligand-binding domain (LBD) and the transcriptional-activation function AF-2. ER-α36 lacks AF-1 and AF-2. The last 27 amino acids of ER-α36 are indicated. Representative Western blot result of MCF7 cells with the H226 antibody that recognizes the hinge region (D) is also shown.

Fig. 3. The signaling pathways of ER- α 36-mediated rapid estrogen signaling. ER- α 36 mediates E2-stimulated release of HB-EGF through the matrix metalloproteinases (MMP)-2 and -9 activated by the G proteins via Src signaling. HB-EGF then activates EGFR and HER2, which also involve IGF-IR. ER- α 36 then mediates rapid estrogen signaling via the signaling proteins such as c-Src and the adaptor proteins such as Shc leading to activation of the PI3K/AKT and MAPK/ERK signaling pathways as well as downstream transcription factors such as AP-1 and CREB. ER- α 36-mediated rapid estrogen signaling may also involve direct activation of the STAT3/5 through the EGFR/Src. The physical interactions between ER- α 36 and EGFR or HER2 are also indicated. The possible role of ER- α 36 in inhibition of genomic estrogen signaling mediated by ER- α 66 is also indicated.