

Review

Estrogen Signals through ER β in Breast Cancer; What We Have Learned since the Discovery of the Receptor

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Abstract: Estrogen receptor (ER) β (ER β) is the second ER subtype that mediates the effects of estrogen in target tissues along with ER α that represents a validated biomarker and target for endocrine therapy in breast cancer. ER α was the only known ER subtype until 1996 when the discovery of ER β opened a new chapter in endocrinology and prompted a thorough reevaluation of the estrogen signaling paradigm. Unlike the oncogenic ER α , ER β has been proposed to function as a tumor suppressor in breast cancer, and extensive research is underway to uncover the full spectrum of ER β activities and elucidate its mechanism of action. Recent studies have relied on new transgenic models to capture effects in normal and malignant breast that were not previously detected. They have also benefited from the development of highly specific synthetic ligands that are used to demonstrate distinct mechanisms of gene regulation in cancer. As a result, significant new information about the biology and clinical importance of ER β is now available, which is the focus of discussion in the present article.

Keywords: ER β ; breast cancer; ER β ligands; tumor progression; tumor microenvironment



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1. Introduction

Estrogen receptor α (ER α) was first identified by Elwood Jensen in 1958 [1] and cloned from MCF-7 cells in 1986. ER α had long been considered as the sole mediator of estrogen signaling until the discovery of ER β by the group of Jan-Åke Gustafsson in 1996 [2]. ER α and ER β are encoded from different genes that reside on separate chromosomes. The ER α gene (ESR1) is located on chromosome 6q25.1 and encodes for a protein that is 595 amino acids long, whereas the ER β gene (ESR2) locus is on chromosome 14q23.2 and gives rise to a 530 amino acid product [3]. As members of the nuclear receptor superfamily, both ER subtypes have three main functional domains: the N-terminal domain with activation function 1 (AF-1) that regulates target gene transcription independent of ligand activation, the DNA-binding domain (DBD) that facilitates binding to specific estrogen response elements (EREs) in regulatory regions of target genes, and the receptor dimerization and C-terminal ligand-binding domain (LBD) with AF-2 function. Despite the presence of common structural and functional domains, there is 95% and 59% homology in DBD and LBD between ER α and ER β , respectively, indicating the diverse nature of ligands that bind to ER subtypes and the similarity in the mechanism of DNA interaction during the regulation of target gene expression [4]. Minor differences in LBD and low homology in AF-1 that determines the interactions with co-regulators point to a distinct mechanism of action by the ER subtypes in target tissues.

Because of the different function of ER subtypes, the nature of estrogen response in target tissues primarily depends on the availability of the receptor subtype and the presence of co-regulators and partner transcription factors that control the transcriptional activity. ER α is mainly expressed in the mammary gland, uterus, thecal cells in the ovary, bone, male reproductive organs (testes and epididymis), prostate stroma, liver, and adipose tissue.

ER β , on the hand, is predominant in granulosa cells of the ovary, the prostatic epithelium, bladder, colon, bone, lung, breast and adipose tissue as well as in the central nervous, cardiovascular and immune system [5–8].

Our knowledge about the biology of ERs has primarily been derived from their study in the reproductive system, mammary gland, and in breast cancer [9]. It is now well accepted that increased signaling through ER α is essential for the growth of the mammary gland during development and pregnancy but has oncogenic properties in breast cancer. About 70% of breast cancers overexpress ER α , indicating its importance as a molecular target as well as in prognosis of the disease. The induction of ER α signaling in luminal tumors has long been the focus of endocrine therapy, including the ER α antagonist tamoxifen that has represented the gold standard for treatment of ER α -positive breast cancer for over five decades. On the other hand, owing to challenges in detection due to decreased protein expression, the use of non-specific antibodies, and the contribution of alternatively spliced and differentially expressed isoforms to the immunohistochemical signal, initial reports of ER β action and clinical importance in breast cancer were conflicting. Over two decades of extensive research using preclinical models and human specimens has reinvigorated interest in ER β by uncovering effects that link the expression and function of the receptor to the biology of the disease. While ER β is expressed in epithelial, myoepithelial and stromal cells of breast tissue during development and in adulthood, its levels have been reported to decline in breast cancer [10–14] due to oncogenic signaling that primarily represses the activity of the promoter of the ER β gene [14,15]. The decreased expression in malignant breast together with the anti-proliferative and anti-invasive effects of the receptor upon upregulation in cancer cells represents the strongest indication of a tumor suppressor function.

In this review we first discuss what we have learned about the biology of ER β from the complete and tissue-specific knockout mouse models. We also present information from studies looking at the role of the receptor in cell proliferation, invasion and metastasis. Since breast cancer is one of the most well-studied disease models for ER β function, we additionally focus on the involvement of the receptor in the prevention and treatment of refractory disease. Considering the potential of targeting ER β in breast cancer, we also discuss recent advancements in some of the most commonly used ER β synthetic agonists. Finally, given the well-documented implication of tumor immunity on cancer progression, we explore the theme of estrogen receptor action in the tumor microenvironment.

2. The Phenotypes of ER β Knockout Mouse Models

Several mouse models with constitutive deletion of ER β in all tissues have been generated to date [16–19]. Earlier models were developed involving either deletion of exon 3 by the Cre-LoxP system [17,19] or disruption of the gene with the insertion of a neo cassette into exon 3 of the receptor in embryonic stem cells by homologous recombination [16]. The key finding that was consistent throughout the analysis of the three original knockout models was the severe disruption of ovulation in the absence of ER β . The phenotyping of the latest mouse model with the crispr-cas9-mediated knockout of ER β reports a tumor suppressor function in the ventral prostate and mammary epithelium, where the loss of the receptor leads to increased activity of the androgen receptor (AR) and ER α , respectively [18]. Although the expression of genes that are involved in the promotion of prostate cancer increased upon ER β deletion, loss of the receptor alone did not lead to the development of prostate cancer [16]. Instead, ventral prostate epithelial hyperplasia and intraductal cancer-like lesions were observed in the absence of ER β , which is consistent with the significantly higher number of ki67- and p63-positive cells in the same tissue of six-month-old ER β knockout mice [18]. Of note, epithelial hyperplasia declined in the prostate of the same mice by the time they reached 18 months of age, linking this process to the physiology of the gland and the activity of the receptor in earlier stages of adult life [18].

Deletion of ER β in mice was also shown to reduce the differentiation of mammary gland epithelium and decrease the levels of the adhesion molecules E-cadherin, connexin 32,

occludin and integrin α [16]. The mammary gland of ER β knockout mice had increased expression of ER α , PR and ki67 as well as more invasive epithelium with a higher expression of matrix metalloproteinases (MMPs) [18]. These results point to a key role for ER β in controlling growth and promote the differentiation of prostate and mammary gland tissues in mice.

3. Effects of Tissue-Specific Deletion of ER β

Several tissue-specific ER β knockout mouse models have been developed and analyzed to characterize functions of the receptor on certain mouse organs. Muscle-specific deletion of ER β led to a decrease in muscle mass and strength in female mice only [20]. Although ER β is expressed in both male and female mice, genetic ablation of ER β in muscle stem cells (satellite cells) caused impaired muscle regeneration following injury specifically in young female mice, pointing to the importance of ER β in post-natal muscle growth [20]. On the other hand, deletion of ER β in intestinal epithelial cells revealed a protective role of the receptor in colitis-induced adenomas by countering TNF α - and NF κ B-mediated inflammation [21]. Knockout of ER β induced more colorectal tumors in male mice, while female mice developed significantly larger tumors in the absence of the receptor [21]. Intestine-specific depletion of ER β was also found to limit diversity in the gut microbiome during chemically induced colitis leading to colorectal cancer (CRC) [22]. In another study that was designed to test the selective activation of ER α or ER β with specific ligands on the oncogenic activity of high fat diet (HFD), activation of ER β elicited anti-inflammatory effects in the colon, as manifested by significantly reduced macrophage infiltration in both male and female mice [23], and protected against HFD-induced proliferation of colonic epithelial cells [23].

In addition to the intestine, effects of ER β were investigated in mouse mammary gland using tissue-specific knockout models. Effective synergism between ER β and the p53 tumor suppressor function was noted in breast cancer upon conditional deletion of both genes in mammary epithelial cells [24]. While knockout of ER β alone did not give rise to mammary tumors, loss of the receptor in p53-defective tissues significantly shortened tumor latency compared with the conditional deletion of p53 alone [24]. Because ER β has been reported to interact with both wild-type and mutant p53 enhancing the tumor suppressor function of the protein, the observed synergistic anti-tumor activity was linked to the function of the receptor in the developing mammary gland. During this period of extensive growth with an anticipated reduced capacity for genome surveillance due to p53 inactivation, loss of ER β signaling can lead to the induction of aberrant cell proliferation and impaired differentiation and DNA repair to malignant transformation [25].

4. Post-Translational Modifications of ER β

Serine residue (S) at positions 75, 87 and 105 in human ER β were found to be targets of ERK1/2 and p38 kinases. S105 of endogenous ER β was shown to undergo phosphorylation in MDA-MB-231 and BT-474 cells, enhancing the ability of the receptor to inhibit cell migration and invasion in vitro without affecting cell growth and cell cycle progression [26]. Consistent with the in vitro anti-tumor activity, phosphorylation of ER β at S105 has been associated with a favorable prognosis in breast cancer [27]. On the other hand, the highly conserved MAPK target site S87 of ER β was found to undergo phosphorylation by the stromal cell-derived growth factor 1 (SDF-1 or CXCL12) that enhances the occupancy of ER β at EREs and AP1 sites, even in presence of tamoxifen [28].

Phosphorylation of ER β at serine residues at positions 106 and 124 by MAP kinase has been shown to increase the interaction of ER β with the co-activator SRC-1. When MAP kinase is activated by Ras, EGF or IGF-1, it stimulates the phosphorylation of serines in AF-1 of ER β leading to an increased interaction with SRC-1 and ligand-independent activation of the receptor [29]. In addition to the human receptor, S16 in mouse ER β was shown to be either phosphorylated or modified through O-glycosylation. Phosphorylation

of S16 accelerated the degradation of mouse ER β , whereas O-glycosylation of the same residue increased the stability of the receptor and its transcriptional activity [30,31].

Interestingly, the phosphorylation status of tyrosine 36 (Y36) of ER β was found to be under the diametrically opposite control of c-ABL tyrosine kinase and EYA2 phosphatase. Phosphorylation of Y36 that is increased by the agonists 17 β -estradiol, diarylpropionitrile (DPN) and S-equol is required for recruitment of ER β co-activators to the promoters of target genes and causes inhibition of ER α -induced cancer cell growth in vitro and in xenografts [32,33]. In addition to regulating interaction with co-activators, the same phosphorylation has been reported to increase the turnover of the receptor by decreasing protein stability since mutant ER β (Y36F), where tyrosine was replaced with phenylalanine, was more resistant to ubiquitin-mediated protein degradation [32]. Lastly, another residue that was also found to undergo phosphorylation is S6. This phosphorylation is necessary for sumoylation of ER β at Lysine 4 that can be enhanced by constitutively active MAP/ERK kinases [34]. Sumoylation-deficient ER β mutants displayed greater transcriptional activity in response to estrogen treatment, indicating the negative effect of sumoylation on the activity of the receptor, considering the function of Lysine-4 of ER β as a suitable site for ubiquitination [34].

5. ER β and Breast Cancer Cell Proliferation

Early studies using Taqman-based qPCR analysis showed that in contrast to ER α , which is upregulated in luminal tumors, ER β mRNA levels were lower in breast tumor samples compared to normal breast tissue [11]. Consistent with the mRNA levels, quantitative immunohistochemistry clearly demonstrated higher expression of ER β in benign breast with a sharp decline in breast carcinoma in situ (CIS) [35]. In line with the higher levels of ER β in differentiated epithelium, the expression of the receptor exhibited a strong inverse correlation with that of the proliferation marker ki67 in ductal carcinoma in situ [35].

Despite the decrease of ER β expression in malignant breast, a significant number of specimens from patients with hormone receptor-positive breast cancer were reported positive for ER β . Expression of the receptor was strongly associated with pre-menopausal status and markers of less aggressive phenotypes, including axillary lymph node negativity and lower S phase fractions [36]. At the preclinical level, introducing ER β in ER α -positive MCF-7 cells inhibited their proliferation in vitro and in xenografts in vivo. Expression of ER β also caused G2 cell cycle arrest by repressing the transcription of cyclin D1, cyclin A and c-myc [37], and similar effects were observed in another luminal breast cancer cell line, the T47D cells [38]. ER β was also found to counter Akt signaling by downregulating the upstream HER2/HER3 receptor dimer and upregulating the tumor suppressor PTEN that is known to inhibit Akt signaling in breast cancer cells [39].

ER β can regulate the expression of oncogenes and tumor suppressors in luminal breast cancer cells in an ER α -dependent or independent fashion. ER α and ER β can form heterodimers that control the recruitment of the co-activator SRC-1 [40–42]. ER α /ER β heterodimers are less efficient than ER α homodimers in transactivating target genes, implying an inhibitory effect of ER β in the transcriptional activity of ER α . In support of this mechanism of action, a microarray-based transcriptomic analysis in T47D cells revealed inhibition of ER α target gene expression upon induction of ER β expression. Genes that were induced by ER α and repressed by ER β were involved in cell proliferation, a finding that was also supported by in vitro cell proliferation assays [43]. Consistent with these studies and other previous findings [37], over-expression of ER β in MCF-7 cells led to a marked decrease of estrogen-induced cell proliferation, and the analysis of binding sites in ER β -transfected cells identified gene expression signatures that correlate with the inhibition of cell proliferation [44]. In agreement with the in vitro studies, less orthotopic ER β -expressing T47D tumors were developed in SCID mice compared to xenografts that did not express the receptor [45]. Tumors expressing ER β also had fewer blood vessels and reduced expression of proangiogenic factors [45]. The anti-proliferative effects of ER β were primarily observed in ER α -positive breast cancer cells, suggesting that this specific

function may result from interference with the pro-proliferative activity of ER α . This interaction in cells with variable expression of artificially introduced ER β may also explain the publication of inconsistent data on cell proliferation in ER α -positive cells [46].

6. ER β in Cancer Cell Invasion and Metastasis

In one of the earliest studies, the tumor repressive role of ER β in prostate cancer was demonstrated through its adenovirus-driven expression in ER α -negative DU145 prostate cancer cells that caused a significant reduction of invasion in a matrigel-coated transwell assay [47]. Similar to DU145 cells, treatment of ER β -transfected PC3 prostate cancer cells with agonists decreased cell migration and induced the expression of INPP4B, a repressor of Akt signaling [48]. In addition, overexpression of ER β also reduced cell viability, migration, and inflammation and enhanced apoptosis in PC-3 and DU145 cells by suppressing lipopolysaccharide (LPS)-induced activation of NF κ B that represents another driver of prostate cancer progression and mediator of inflammation [49].

Unlike the documented tumor suppressor role of ER β in breast, prostate, ovarian, renal and thyroid cancer [50], reports of ER β action in lung cancer have been controversial [51], with several groups supporting an oncogenic function of the receptor [52–58]. A recent study in non-small-cell lung cancer (NSCLC) cells showed that ER β promotes invasion by directly binding to and inducing the expression of TMX4 circular RNA, which, through the inhibition of miR-622, leads to upregulation of the G protein-coupled receptor (GPCR) CXCR4 that promotes metastasis in several types of cancer [53]. Consistent with this function, knockdown of ER β in lung cancer cells led to reduced vasculogenic mimicry and invasion, whereas overexpression of ER β had the opposite effect [59]. ER β also promoted cell invasion by directly binding to the regulatory region of lnc-RNA MALAT1 and increasing its expression, which, in turn, downregulates miR-145-5p and upregulates the oncogenic factor NEDD9 [59]. In line with the preclinical data, female patients with ER β -positive NSCLC tumors had worse 5-year survival compared to those without ER β expression [59]. Although an oncogenic function of ER β in the lung should not be excluded and may be associated with the biology of the tissue, the pro-invasive effects in cancer cells following upregulation of the receptor need to be considered with extra caution and further validated to exclude non-physiological activation by the artificial expression.

In contrast to lung cancer, there is consensus regarding the anti-invasive and anti-metastatic role of ER β in breast cancer. Claudin-6 (CLDN6), a tight junction protein and tumor suppressor, was found to be a direct target of ER β in breast cancer cells [60]. Treatment of MDA-MB-231 and ER β -overexpressing SK-BR-3 breast cancer cells with the ER β -specific agonist DPN caused autophagy through CLDN6-mediated upregulation of the key mediator of autophagy beclin-1 (Figure 1). Overexpression of CLDN6 in MDA-MB-231 cells also led to reduced lung and liver metastasis in mice [60]. Upregulation of ER β in TNBC (triple negative breast cancer) MDA-MB-231 and Hs578T cell lines induced the expression of the epithelial marker E-cadherin and suppressed cell migration and invasion in vitro as well as in zebrafish embryos [61]. ER β was found to promote ubiquitination and degradation of EGFR along with induction of members of the miR200 family leading to subsequent inhibition of the transcriptional repressors of E-cadherin SIP1 and ZEB1 (Figure 1). Consistent with the association in cancer cells, ER β protein levels positively correlated with those of E-cadherin in clinical patient samples [61]. ER β was also found to transcriptionally repress EGFR, thereby indirectly downregulating IMP3 to counter invasion and migration in TNBC [62]. G protein-coupled estrogen receptor 1 (GPER) is a membrane-bound estrogen receptor that has been shown, like other GPCRs, to transactivate EGFR [63]. GPER expression positively correlates with disease progression in breast cancer patients [64]. Since ER β has already been shown to transcriptionally downregulate another G protein-coupled receptor—GPR141—to inhibit actin-based migration in inflammatory breast cancer [65], there is a possibility of functional crosstalk between ER β and GPER or other GPCRs, where ER β might oppose EGFR signaling by inhibiting the expression and activity of GPER. In addition to inhibiting EGFR, ER β was shown to reduce invasion in

TNBC cells by directly interacting with and blocking transcription by the oncogenic mutant p53 that exists in about 80% of TNBCs (Figure 1) [25]. Similarly, ligand-mediated activation of ER β in TNBC cells resulted in their decreased invasion and in vivo lung colonization through upregulation of several members of the family of cystatins via direct binding of the receptor to their regulatory elements (Figure 1) [66]. The importance of cystatins for TNBC metastasis was verified by their ability to decrease the invasiveness of TNBC cells by repressing TGF β signaling and their association with longer recurrence-free survival (RFS) in patients (Figure 1) [66]. In addition to TNBC, ER β was found to decrease the invasiveness of inflammatory breast cancer (IBC) cells by downregulating GPR141 and the guanine nucleotide exchange factor (GEF)-interacting protein ELMO1 that activate the mediator of IBC metastasis RhoC [65]. In contrast to ER β , a few studies provided conflicting evidence about the role of ER α in the invasiveness and metastatic potential of breast cancer cells. Initially, silencing of ER α has been shown to cause epithelial to mesenchymal transition (EMT) in ER α -positive breast cancer cells [67]. Subsequently, ER α has been reported to promote breast cancer cell migration and invasion by actin cytoskeletal remodeling through focal adhesion kinase (FAK) and N-WASP [68] and through Rho-associated kinase 2 (ROCK-2) [69].

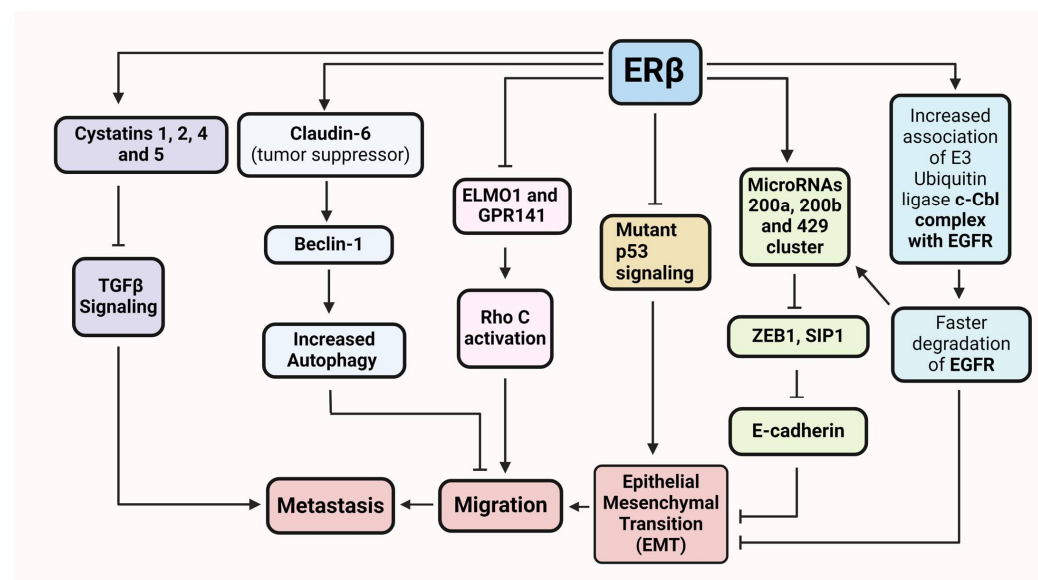


Figure 1. Flow chart depicting effects of ER β on EMT, cell migration and metastasis in breast cancer. Cystatins 1, 2, 4 and 5 are direct targets of ER β in triple negative breast cancer (TNBC) cells. Expression of ER β in TNBC cells followed by agonist activation inhibits metastasis in vivo by inducing the expression of cystatins that downregulate TGF β signaling. Beclin-1, a key regulator of autophagy is upregulated by Claudin-6, a direct target of ER β in breast cancer. Claudin-6 inhibits breast cancer cell migration and invasion. ER β represses transcription of the activators of the cytoskeleton remodeler RhoC, ELMO1 and GRP141, by directly binding to their regulatory regions, thereby preventing RhoC activation and actin-based cell migration. Approximately 80% of TNBCs harbor oncogenic mutations of p53. ER β directly interacts with mutant p53 and inhibits its pro-metastatic signaling. ER β also inhibits epithelial–mesenchymal transition (EMT) by inducing EGFR degradation that results in upregulation of the epithelial markers miR-200a-b-429 and E-cadherin.

7. ER β in TNBC

TNBC is marked by the absence of the receptors ER α , PR and HER2 that have been validated as oncogenic drivers in other subtypes of breast cancer. Although TNBC accounts for approximately 15% of total breast cancers, it is responsible for the majority of breast cancer-associated deaths [70]. This is, in part, due to the high propensity of TNBC tumors to develop metastasis, the high frequency of resistance to standard chemotherapy and the lack of effective targeted therapy.

Different isoforms of ER β have been associated with clinical outcomes in TNBC. These include the full length ER β (also known as ER β 1) that is composed of 530 amino acids and is the only isoform that forms homodimers and heterodimers and bind ligands [71]. On the other hand, the variants ER β 2, 3, 4 and 5 that result from alternative splicing of the last coding exon form heterodimers with ER β 1 and have an impaired ability to bind ligands (Figure 2) [71]. The expression of ER β 1 has been reported in about 18–27% of TNBC cases, and earlier studies indicated the importance of the receptor as an independent predictor of a favorable prognosis [72–76]. As per a recent report from Katzenellenbogen lab, ER β 2 and ER β 5 are the most abundant isoforms in TNBC cell lines and tumors, whereas ER β 1 is barely detectable. In contrast to ER β 1, the variants 2 and 5 were found to elicit an oncogenic function since knockdown in TNBC cells decreased proliferation, migration and invasion, whereas their overexpression had the opposite effect on these specific cellular phenotypes [71,77]. At the mechanistic level, overexpression of ER β 1 and treatment with specific ligands increased the protein levels of the epithelial and anti-invasive marker E-cadherin [61,62]. Upregulation of ER β 1 also reduced the expression of the oncogenic survivin (BIRC5), similar to the depletion of ER β 2 and ER β 5, suggesting opposing effects of ER β isoforms on gene regulation [77]. The oncogenic activity of the variants is in agreement with previous findings, suggesting an association of ER β 2 with poor prognosis in hormone receptor-negative breast cancer [73,77]. Similar to TNBC, in high-grade serous ovarian cancer, where more than 95% of the tumors harbor p53 mutations, ER β 2 was found to partner with mutant p53 to increase the transcription of FOXM1, leading to enhanced proliferation and therapy resistance [78]. The transcriptional activation of mutant p53 by ER β 2 represents another example of the opposite function of the variants in cancer considering the previously reported inhibitory interaction of ER β 1 with mutant p53 in TNBC [25].

In addition to mutant p53, the full length ER β has been shown to inhibit the function of other known drivers of TNBC. Expression of ER β 1 has been reported to induce apoptosis and reduce the proliferation and metastatic potential of androgen receptor (AR)-positive TNBC cells [76]. Similar, upregulation of ER β 1 increased the sensitivity of the same cells to the AR inhibitor enzalutamide [79]. ER β 1 decreased the activity of AR by forming heterodimers and inhibiting PI3K/Akt signaling. The same variant was also shown to inhibit proliferation and migration of TNBC cells by forming a co-repressor complex with PRC2/EZH2 to repress the transcription of p65/RelA and downregulate the NF κ B pathway [75,76]. Similar to upregulation, treatment with the ER β agonist liquiritigenin inhibited cell proliferation and increased the sensitivity of TNBC cells to doxorubicin [80]. As with the cell proliferation, treatment with ER β agonists greatly mitigated the invasion of TNBC cells when they were grown alone [81] and during their co-culture with MG63 osteoblasts [82]. Lastly, ER β 1 was also found in the same cells to downregulate the oncogenic pathway of cholesterol biosynthesis by binding to the promoter of SREBP1 [76]. The opposing effects of the variants together with their variable expression in tumors may have accounted for the initial controversy surrounding the role of ER β in TNBC [83,84]. Recent findings, however, have improved our understanding about the exact actions of the receptor and its clinical importance for the disease [85,86].

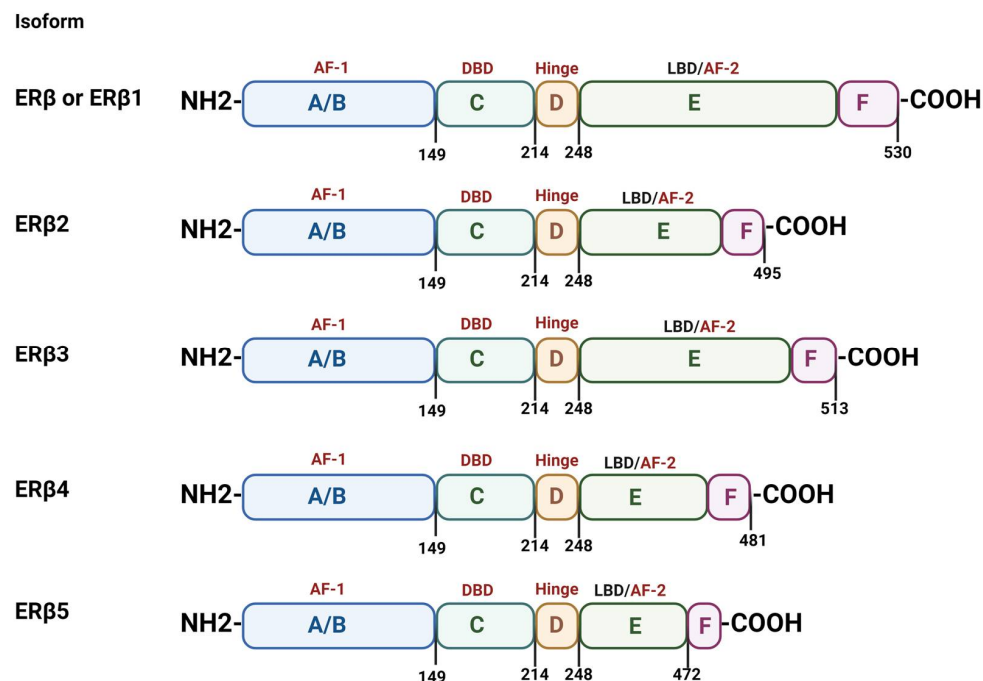


Figure 2. Structural and functional domains of estrogen receptor β . Domains are shown for both the full length ER β (also called ER β 1) and its isoforms ER β 2-5. Numbers indicate amino acid length of individual domains and the full length proteins. All ER β isoforms are identical until the hinge region, where they begin diverging from the C-terminal of the ligand-binding domain (LBD). The ligand-independent transactivation function (AF-1) resides in the N-terminus of the receptor and serves as an interaction site for regulatory factors. The DNA-binding domain (DBD) recognizes estrogen response elements (ERE) in regulatory regions of target genes, whereas the hinge region harbors a nuclear localization signal (NLS). The LBD consists of the ligand-binding transactivation function (AF-2) and provides an interface for receptor dimerization and co-activator binding.

8. Synthetic Ligands and ER β Activity

The nature of the estrogenic ligand effect largely depends on the conformation of the ligand-binding domain (LBD) of ER subtypes. ER α and ER β share a medium homology of 59% in the primary protein sequence of LBD [87]. The LBD in both isoforms has important features such as the ligand-dependent transcription activation function (AF2), a homo- or hetero-dimerization interface and an interaction surface for co-regulators [88]. The LBDs of ER α and ER β have a similar globular structure and consist of 11 α -helices organized as a three-layered sandwich structure with helices 4, 5, 6, 8 and 9 flanked on one side by helices 1 and 3, and by helices 7, 10 and 11 on the other [88,89]. 22 hydrophobic residues line the ligand-binding cavity in ERs and interact with the ligand [90]. The orientation of helix 12 with respect to the ligand-binding pocket determines whether a ligand serves as an agonist or antagonist. In an agonist-bound conformation, helix 12 is positioned at the entrance of a ligand-binding cavity and serves as an interaction surface for nuclear receptor co-activators [91]. Antagonists alter helix 12 positioning in a manner that blocks recruitment of co-activators [91]. The ligand-binding cavity of ER β is smaller in size and narrower compared to that of ER α . ER β also differs from ER α in two residues out of the 22 that form the ligand-binding cavity. Leu384 and Met421 in ER α are replaced by Met336 and Ile373, respectively, in ER β [87,89].

8.1. Raloxifene

Raloxifene binds both ER α and ER β with high affinity (Figure 3) [92]. It acts as an ER α antagonist in the mammary gland and uterus and as an agonist in bone and the liver [93]. In a clinical trial with more than 10,000 post-menopausal women, raloxifene significantly reduced the risk of invasive breast cancer, had no effects on outcomes that were

associated with coronary heart disease, but increased the risk of fatal stroke [94] and venous thromboembolism [94,95]. This ER ligand was also found to increase bone mineral density and lower the levels of total cholesterol and LDL in post-menopausal women [96] and, hence, it was approved for the treatment of osteoporosis in post-menopausal women in 2007. In addition to ER α , recent preclinical studies showed that the nano formulation of raloxifene inhibited TNBC tumor growth in vitro and in vivo, partially through regulating the activity of ER β [97]. Similar to TNBC, the drug was also shown to inhibit migration of hepatocellular cancer cells through ER β -mediated inhibition of the Akt signaling pathway [98]. Raloxifene was further found to inhibit the progression of pancreatic ductal adenocarcinoma (PDAC) in an orthotopic xenograft model through ER β [99]. Moreover, it mitigated metastasis and elicited tumor suppressive effects in AR-negative and castration-resistant prostate cancer (CRPC) [100]. These findings suggest a role for ER β in repurposing ER ligands for use in treatment of ER α -negative malignancies.

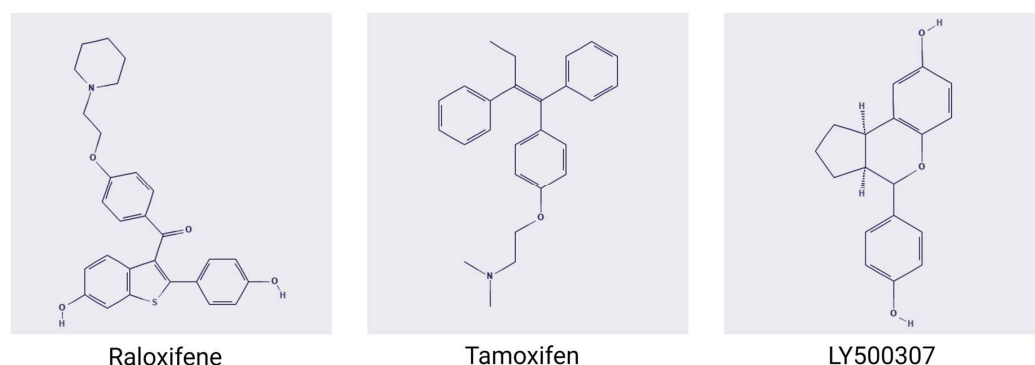


Figure 3. Chemical structure of synthetic ligands of estrogen receptors tamoxifen, raloxifene and LY500307.

8.2. Tamoxifen

Tamoxifen acts as an ER α antagonist in breast tissue and an agonist in the uterus, bone, and the liver (Figure 3) [101,102]. Tamoxifen was first approved by the FDA for the treatment of ER α -positive breast cancer in 1977 and later as an adjuvant treatment for primary breast cancer [103]. Application of tamoxifen therapy significantly benefited patients with the disease since it reduced breast cancer-associated mortality by a third [104]. Although ER α appears as the primary mediator of the clinical effects of the drug, new evidence suggests that tamoxifen can also affect breast cancer through ER β . Initially, low ER β protein levels in ER α -positive tumors were reported to predict resistance to tamoxifen [105,106], and treatment of breast cancer cells with ER β -selective agonists enhanced the growth-inhibitory effects of tamoxifen [107–109]. Similarly, ER β sensitized tamoxifen-resistant MCF-7 cells to endoplasmic reticulum (ER) stress apoptosis by downregulating the unfolded protein response (UPR) [109]. Tamoxifen was also shown to inhibit mutant p53-dependent oncogenic gene expression in ER β -expressing but not in control MDA-MB-231 TNBC cells [25]. On the other hand, the ligand was found to engage mitochondrial ER β , both as agonist and antagonist, thereby modulating the levels of manganese superoxide dismutase (MnSoD) and contributing to tamoxifen resistance [110]. In addition to breast cancer, targeting ER β with tamoxifen in diffuse large B-cell lymphoma (DLBCL) reduced cell viability in vitro, an effect that was significantly mitigated with knockdown of ER β [111] and corroborated in a xenograft lymphoma model [111].

8.3. LY500307

Several studies have reported the anti-tumor activity of the ER β -selective agonist LY500307 since its development by researchers at Eli Lilly in 2012 (Figure 3). Treatment with LY500307 caused suppression of TNBC and melanoma lung colonization by inducing recruitment of neutrophils to the metastatic site (Figure 4A) [112]. The recruitment of

neutrophils was associated with the expression and secretion of IL-1 β from cancer cells, and the involvement of this specific cytokine was verified by the absence of anti-metastatic effects of LY500307 in IL-1 β knockout mice [112]. Treatment with LY500307 also improved the efficacy of the PD-1 antibody in in vivo models of TNBC and colorectal cancer [113]. In addition to regulating neutrophils, LY500307 reduced the recruitment of CSF-1 receptor-positive myeloid-derived suppressor cells (MDSC) to the tumor microenvironment while also increasing CD8⁺ cytotoxic T cells by decreasing the production of CSF-1 by tumor cells (Figure 4D) [113]. Beyond TNBC, activating tumor-endogenous ER β by treating mice bearing orthotopically implanted inflammatory breast cancer tumors with LY500307 led to reduced lung metastasis (Figure 4B) [65]. In addition to IBC, ER β was also found to be enriched in ovarian cancer stem cells (OVSC), and treatment with LY500307 reduced their stemness and induced apoptosis [114]. Treatment with LY500307 also significantly impaired the tumor-initiating potential of OVSCs in orthotopically implanted xenograft models [114]. Similarly, glioblastomas (GBM) express ER β , and treatment of GBM cells with this selective agonist reduced proliferation and enhanced apoptosis in vitro (Figure 4C). More importantly, the ligand improved the survival of GBM tumor-bearing mice and inhibited in vivo tumor growth [115]. In an attempt to potentiate ER β signaling after treatment with LY500307 and driven by the observed increased acetylation of the ER β promoter, glioblastoma cells were incubated with HDAC inhibitors. HDAC inhibitors did indeed increase the expression of ER β and upregulated its target genes, and the combination of these inhibitors with LY500307 enhanced the survival of mice with orthotopic GBM tumors [116]. Similar results were observed in melanoma cell lines, where treatment with LY500307 reduced cell proliferation, increased apoptosis, decreased cell migration and partially reversed EMT [117]. Despite the enthusiasm generated as a result of the use of the compound in preclinical cancer models and its demonstrated in vivo anti-tumor activity, LY500307 failed to show clinical efficacy in clinical trials for other conditions, including men with benign prostatic hyperplasia (BPH) [118] and patients with cognitive impairment associated with schizophrenia [119].

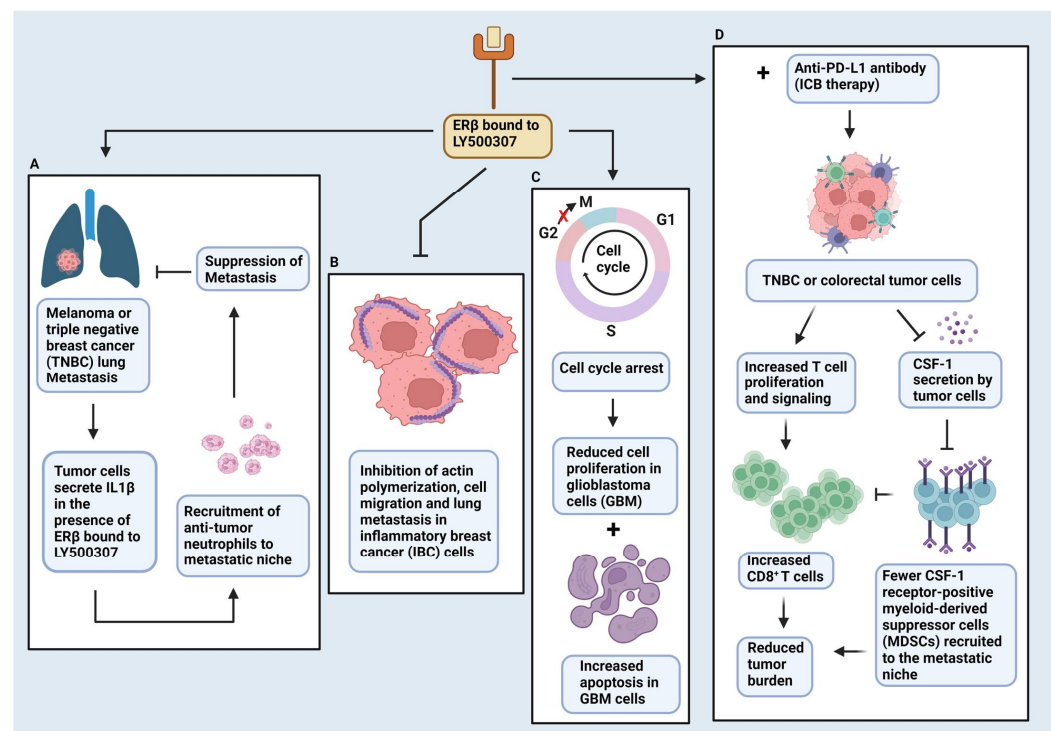


Figure 4. Anti-tumor activities of the selective ER β agonist LY500307. **(A)** Activating ER β with LY500307 enables triple negative breast cancer (TNBC) and melanoma cells to secrete interleukin-1 β (IL-1 β), which stimulates the recruitment of anti-tumor neutrophils to the metastatic niche suppressing lung metastasis. **(B)** Similar to TNBC, ER β and LY500307 prevent lung metastasis in inflammatory breast cancer (IBC) by inhibiting actin-based cell migration through the repression of the direct targets GPR141 and ELMO1 that activate the cytoskeleton remodeler RhoC. **(C)** Activation of ER β with LY500307 also inhibits tumor growth and increases the survival of mice with highly aggressive glioblastoma (GBM) by reducing cell proliferation and inducing cell cycle arrest and apoptosis. **(D)** LY500307 greatly improves the therapeutic efficacy of immune checkpoint blockade (ICB) therapy with anti-PDL1 antibodies in TNBC and colorectal cancer. Activating ER β in tumor cells with LY500307 prevents them from secreting CSF-1 in the tumor microenvironment, thus diminishing the recruitment of myeloid-derived suppressor cells (MDSCs), which, along with increased CD8 $^{+}$ T cells, leads to smaller tumors in mice. \rightarrow and \perp represent positive and negative regulation, respectively and Red X indicates blockade.

9. ER β and the Tumor Microenvironment

Despite the documented association of estrogen signaling with the function of the immune system in various pathophysiological conditions, the role of estrogen receptors in regulating tumor immunity still remains elusive, partially due to the lack of appropriate syngeneic and transgenic immunocompetent tumor models. Only a few studies have explored the function of ER β in the tumor microenvironment and how this impacts cancer progression and metastasis. One of these studies has focused on bladder cancer, where ER β has been detected as the predominant ER subtype in both cell lines and tumor samples [120]. Higher expression of ER β has been reported in metastatic tissue compared to benign urothelium and is strongly correlated with more aggressive phenotypes [121]. This correlation has been explained by the recruitment of ER β -expressing mast cells [122] and CD4 $^{+}$ T cells [123] in bladder cancer and by the association of mast and CD4 $^{+}$ T cells in the tumor microenvironment with enhanced invasiveness of bladder cancer cells. The effect of ER β was verified by treatment with the ER β antagonist PHTPP or ER β shRNA that abrogated the increased invasiveness of bladder cancer cells [122].

In addition to bladder cancer, estrogen signaling has been linked to the microenvironment of breast cancer. However, most of the evidence supporting this association has been

derived from the study of ER α . It is well accepted that immune cell infiltrates in the breast tumor microenvironment are altered based on ER α status [124]. For example, numerous studies have shown an association between high eosinophil count in peripheral blood and survival benefit in patients with ER α -negative breast cancer [125–127]. On the other hand, ER α is known to mediate the immune-suppressive effects of 17 β -estradiol that are, in part, dependent on FoxP3-positive regulatory T cells (Tregs) [128]. In addition to recruitment, treatment with 17 β -estradiol increased the activity of Tregs by inducing their intracellular expression of PD-1. The involvement of ER α was confirmed through the analysis of ER α knockout mice where the intracellular expression of PD-1 and Treg-mediated immune suppression were reduced [129].

The involvement of Tregs in estrogen-associated immunoregulation has also been observed in other conditions. These include the auto-immune disorders such as inflammatory bowel disease (IBD), where ER β as the predominant ER subtype in intestinal mucosa [130] was shown to elicit potent anti-inflammatory effects [130–134]. The population of ER β ⁺CD4⁺ T cells was significantly lower in experimentally induced IBD and was associated with increased disease severity [135,136]. Treatment with ER β -specific agonists countered inflammation in IBD by inducing differentiation of naïve T cells to Tregs and inhibiting pro-inflammatory T cell responses [135].

In addition to Tregs, ER β has been shown to affect breast cancer by regulating other components of the immune system. The selective ER β agonist LY500307 has been reported to suppress metastasis of TNBC by inducing tumor cell expression and secretion of IL-1 β , resulting in subsequent recruitment of neutrophils to the metastatic site [112]. The same ligand also increased the sensitivity of TNBC tumors to PD-1-based immunotherapy [113]. In addition to neutrophils, LY500307 inhibited the expression of CSF-1 in tumor cells, leading to reduced recruitment of myeloid-derived suppressor cells (MDSC) and an increase in CD8⁺ cytotoxic T cells in the tumor microenvironment [113]. The role of ER β signaling in the cells of the tumor microenvironment was evaluated by generating mice with a mutated mouse ER β , where the tyrosine 55 residue that is equivalent to Y36 in human ER β and essential for maintaining an active receptor through phosphorylation was replaced with a phenylalanine. Mice with whole body homozygous mutated ER β unable to undergo phosphorylation exhibited significantly faster growth of orthotopically implanted syngeneic mammary and melanoma tumors [137]. Replacing bone marrow of wild-type mice with bone marrow from ER β mutant mice led to fewer tumor-infiltrating CD8⁺ and CD4⁺ T cells compared to the control mice, indicating the impaired ability of host immune cells to control tumor growth in the absence of ER β signaling. Indeed, CD8⁺ T cells in mice with a mutated ER β phosphotyrosine switch (Y55) produced lower amounts of anti-tumor cytokines [137].

10. Concluding Remarks

Although initial reports of ER β action were conflicting, generating skepticism about the role and clinical importance of the receptor, extensive research in the last decade has increased our confidence for a tumor suppressor function in breast cancer. In addition to the direct effects of the receptor on tumor cells, recent studies have revealed entirely new courses of action through regulation of the tumor microenvironment. Activation of ER β in tumor cells is now known to alter tumor immunity through the secretion of immunomodulatory cytokines [112,113]. In addition to tumor epithelial cells, ER β is expressed in various cell types including endothelial cells, fibroblasts and tumor-infiltrating lymphocytes [138–141]. This underscores the importance of determining the ability of ER β to signal from the tumor microenvironment to regulate tumor development and metastasis and the capacity of ligands to enhance ER β signaling in these cells and achieve favorable clinical outcomes by potentiating the anti-tumor activity of the host immune system. As ER β has been reported to act as a tumor suppressor in various malignancies including breast, prostate, colorectal, ovarian and glioblastoma [50], further research to corroborate this function and define the mechanism of action will be essential in order to determine the

value of the receptor as biomarker and therapeutic target and its utility in new approaches to combat the resistant and metastatic states of these diseases.

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