Published in final edited form as:
Steroids. 2012 June ; 77(7): 727–737. doi:10.1016/j.steroids.2012.03.008.

Estrogen receptor-beta and breast cancer: Translating biology into clinical practice

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Abstract

Estrogen receptor (ER) β was discovered over a decade ago. The design of most studies on this receptor was based on knowledge of its predecessor, ERα. Although breast cancer (BCa) has been a main focus of ERβ research, its precise roles in breast carcinogenesis remain elusive. Data from *in vitro* models have not always matched those from observational or clinical studies. Several inherent factors may contribute to these discrepancies: a) several ERβ spliced variants are expressed at the protein level, and isoform-specific antibodies are unavailable for some variants; b) post-translational modifications of the receptor regulate receptor functions; c) the role of the receptor differs significantly depending on the type of ligands, *cis*-elements, and co-regulators that interact with the receptor; and d) the diversity of distribution of the receptor among intracellular organelles of BCa cells. This review addresses the gaps in knowledge in ERβ research as it pertains to BCa regarding the following questions: 1) is ERβ a tumor suppressor in BCa?; 2) do ERβ isoforms play differential roles in breast carcinogenesis?; 3) do nuclear signaling and extranuclear ERβ signaling differ in BCa?; 4) what are the consequences of post-translational modifications of ERβ in BCa?; 5) how do co-regulators and interacting proteins increase functional diversity of ERβ?; and 6) how do the types of ligand and regulatory *cis*-elements affect the action of ERβ in BCa? Insights gained from these key questions in ERβ research should help in prevention, diagnosis/prognosis, and treatment of BCa.

Keywords

ERbeta isoforms; tumor suppressor; post- translational modification; extranuclear localization; co-regulators; phytoestrogen

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Introduction

Estrogen receptor beta (ERβ) is the second estrogen receptor (ER) identified in rat prostate and ovary in 1996 [1] and later in human testis in the same year [2], which is more than 30 years after discovery of the first ER (also referred to as ERα) [3]. Similar to the ERα, ERβ binds estradiol-17β (E2) with high affinity through its ligand-binding domain (LBD) but the two ERs share only moderate homology in their protein sequences (58% in human and 55% in rat) with the LBD of ERα [2]. Intriguingly, they have almost identical DNA-binding domains (DBDs, 96% homology in human and 95% in rat) capable of interacting with specific DNA elements (eg, estrogen-response element, ERE) and transactivating common and ER subtype-specific genes [4–7]. Significant information revolving around the involvement of ERβ in the development and progression of breast cancer (BCa) has emerged since its discovery [8], leading to new insights and directions in BCa research.

This review summarizes some of the major findings in this research area and highlights critical —missing pieces, with the hope that future investigations will fill these gaps. Throughout this review, we are aware that associations from clinical association data do not always agree with data obtained *in vitro*. No single cell model can truly duplicate the level of complexity found in tissues, which are subject to endocrine and paracrine influences from the surrounding macro- and micro-environment, respectively. We have chosen not to include tumor microenvironment in our review, as the topic is too broad. Instead, we have focused on six research areas we consider to be essential for improving our understanding of the function of ERβ in BCa: 1) ERβ as a possible tumor suppressor in BCa; 2) critical and distinct roles of ERβ isoforms in breast carcinogenesis; 3) differential roles of nuclear and extranuclear ERβ signaling in BCa; 4) the consequences of post-translational modifications of ERβ in BCa; 5) the increase in the diversity of the receptor's function by co-regulators and interacting proteins of ERβ; and 6) the differential behaviors of ERβ elicited by different ligands in BCa. These topics were selected because a) of the need to clarify existing controversies; b) the emergence of new research that has not yet been extensively reviewed; and c) that are, on the basis of our knowledge, at the leading edge of ERβ research in BCa.

ERβ as a possible tumor suppressor in BCa

Immunohistochemical analyses have identified ERβ as the major form of ER in the normal breast that is localized in luminal epithelium, myoepithelium, and in fibroblasts and lymphocytes in the stroma [9]. Murphy et al. [10] have recently reviewed the literature on the expression of ERβ in ERα-positive and -negative BCa Approximately 58% of BCa express both ERs, 18% express only ERβ, and 14% express only ERα [10;11]. High levels of ERβ expression, regardless of ERα status, were found to associate with a better response to tamoxifen and a longer survival time [12–20]. In contrast, several studies of ERα-negative BCa demonstrated a positive correlation between high ERβ expression and poor prognostic phenotypes, such as elevated proliferation [21;22] and basal phenotype [23]. However, several studies reported opposite results, in which stronger ERβ immunopositivity predicted longer disease-free survival [14;24]. For example, Honma et al. showed that positivity for ERβ was associated with better survival in patients with ERBB2-positive or ERα-, PR- and ERBB2- negative (triple-negative) BCa and a better response to tamoxifen monotherapy [14]. Whether ERβ has multiple distinct roles in ERα-negative BCa needs further clarification. These contradictory results may reflect different therapeutic regimens among the patients or heterogeneity of the patient populations (postmenopausal vs mixed populations).

Several lines of evidence suggest that ERβ functions as a tumor suppressor in *in vitro* models. Ectopic expression of ERβ in ERα-positive BCa cells slowed down the mitogenic

responses initiated by ERα [25–28], reduced cell motility and invasion [29;30], and inhibited tumor formation [26] and angiogenesis [31] in mouse xenografts. However, ERβ behaved differently in the ERα-negative MDA-MB-435 cells [32], whose identity as a true BCa cell line remains controversial [33]. The role of ERβ as a tumor suppressor is further supported by findings of several epidemiologic studies demonstrating a loss of ERβ in higher-grade vs lower-grade BCa tissue [27;34–38]. DNA methylation in the proximal promoter region of ERβ was identified as a potential cause of this gene silencing [39;40]. It has long been speculated that transcriptional silencing of ERβ is necessary for cancer progression, a phenomenon found not only in BCa but also in other hormone-sensitive cancers [41;42]. Overall, data from cancer-cell models and observational studies suggest that ERβ functions as a gatekeeper to inhibit tumor growth and progression.

Although ERβ seems to be a tumor suppressor in numerous cell models, its role in human breast carcinogenesis remains elusive. Presently, we know that E2 is a natural ligand for both ERα and ERβ. The proliferative effects caused by ERα are antagonized by the presence of ERβ signaling. ERβ, being the dominant ER should, in theory, be able to protect the normal mammary epithelial cells from any uncontrolled cell growth. But there is no consensus on how ERβ function should be studied in clinical BCa studies. Clearly, this remains as a gap in our understanding of the mechanisms of estrogen signaling and ERβ function in BCa. Association studies relying on immunodetection of nuclear ERβ in BCa specimens may be unable to define the native functions of ERβ in BCa, which may depend in large part on post-translational modifications of the receptor, the co-existence of ERβ with functionally unique ERβ isoforms, the involvement of extranuclear signaling, its differential modulations by interacting proteins and ligands, and hormone level (see below). Also, studies based on the measurement of ERβ transcripts with quantitative PCR in whole biopsy samples may not yield meaningful data because of the presence of a significant number of ERβ transcripts in the stroma, possibly masking expression in adjacent normal or malignant epithelial cells. Hence, future well-designed prospective population studies and/or large-scale clinical trials using specific ERβ agonists are needed to resolve these controversies.

Critical and distinct roles of ERβ isoforms in breast carcinogenesis

Immunohistochemical (IHC) analyses often are used to measure the expression of a protein in tissue sections. The success of this technique depends in large part on the specificity of an antibody against its target protein. Sometimes the alternative use of exons may result in the coexistence of multiple isoforms of the target protein in a tissue. Monoclonal antibodies that recognize only a common epitope or polyclonal antibodies that recognize multiple epitopes are necessary to differentiate the expression among various isoforms. ERβ is a prime example, whereby the use of a pan-ERβ antibody or isoform-specific antibodies for IHC studies may yield different results [43;44].

Early published data on human ERβ function/signaling were focused primarily on ERβ1, the originally cloned sequence [2]. Sequencing data suggested that multiple ERβ isoforms exist as a result of alternative splicing of the last coding exon (exon 8) (Figure 1) [45]. This is also supported by the availability of multiple ERβ isoform transcripts in the human genome project in the NCBI AceView database, as well as by our experimental data (Figure 1). With regard to nomenclature, the original ERβ is also called ERβ1. So far, four other ERβ isoforms (ERβ2, ERβ3, ERβ4, and ERβ5) have been identified. We and others have shown their existence as full-length transcripts, which have in common exon 1 through 7 plus one isoform-specific exon 8 (Figure 1) [46;47]. The molecular weights of ERβ1, 2, 4, and 5 have been determined as 59, 56, 54 and 53 kDa, respectively, according to protein sequence prediction programs, as well as ectopic protein-expression experiments [47]. Since all

isoforms share exons 1 through 7, they all have the same AF1 domain, DBD, hinge domain, and LBD, leaving the AF2 domain (C-terminus) specific to each of the isoforms.

An important question often asked in studying alternative-spliced variants is whether any of the endogenous proteins derived from the spliced variants are expressed in cells or tissues. Researchers tend to be skeptical about their existence because only transcripts, but not protein-products of the variants, are detectable in many cases. Studies carried out at the protein level depend on the availability of good antibodies, presenting a major obstacle in the study of protein isoforms such as ERβ because of the high sequence homology of the variants to their corresponding wild-type protein. Thus, the choice of antigenic region for raising antibody is limited. Fortunately, we [43] and others [12;44;48–53] have successfully raised antibodies to different ERβ isoforms. The specificity of a few isoform-specific antibodies has been validated [43;44], providing the needed tools for validating the clinical relevance of the expression of ERβ isoforms in BCa.

Shaaban and associates reviewed the role of ERβ isoforms in BCa in 2008 [54]. A few more studies have since been published. Up until 2011, at least 14 IHC studies on ERβ/ERβ1 in BCa have been published [55]. Seven studies found an association of ERβ1 expression with favorable outcomes [56–58] such as longer disease-free/overall survival [14;20], smaller tumor size, lymph-node negativity, lower histological grade [59], and responsiveness to tamoxifen [13]. Six studies did not find an association of ERβ1 expression with any clinical parameters [18;44;60–63]. Only one study showed an association of ERβ1 expression with increased cell proliferation in ERα-negative BCa [22]. Eleven of the fourteen studies also studied ERβ2. Five did not find a correlation of ERβ2 with any clinical outcome or survival [12;14;56;60;63]. Two investigations showed ERβ2 as a poor prognosticator [58;64], whereas two others found ERβ2 associated with better outcomes [59;61]. In one study, which also analyzed expression of progesterone receptor (PR), the presence of PR was found to associate with responsiveness to tamoxifen irrespective of ERβ2 level [65], but in a subset of PR-negative samples, ERβ2 expression was a predictor of resistance to tamoxifen [65]. In conclusion, with the use of ERβ-specific antibodies, it becomes clear that ERβ1 may be a predictor for good disease outcome; the role of other isoforms in BCa needs further studies using validated isoform-specific antibodies. Finally, only one study investigated ERβ1, 2, and 5 simultaneously and reported an association of expression of nuclear ERβ2 and cytosolic ERβ5 with BCa survival [44] (see Figure 2).

Our present understanding of the molecular function of ERβ and its isoforms is still quite limited. ERβ1 was shown to form functional homodimers and heterodimers with other ERβ isoforms, as well as with ERα, and negates ERα-signaling [47;66;67]. ERβ2 was found to heterodimerize with ERα and to inhibit ERα-mediated estrogen action [68;69]. The major difference between ERβ1 and ERβ2 is that ERβ1 can counteract ERα signaling in two ways: by neutralizing the action of ERα via heterodimer formation and by directly triggering the anti-proliferative signal to counteract the pro-proliferative function of ERα. In contrast, ERβ2 can inhibit ERα signaling only through heterodimer formation, as this ER isoform was nonfunctional by itself [47;69]. So far, nothing has been published regarding the action of ERβ4 or ERβ5 on ERα signaling. With regard to ERβ signaling, ERβ isoforms 2, 4, and 5 can heterodimerize with ERβ1 and enhance ERβ1-induced transactivation in a ligand-dependent manner. Only ligands like E2 and bisphenol A, but not phytoestrogen, can initiate the dimer formation between ERβ1 and the other ERβ isoforms [47]. However, the functional role of each ERβ isoforms in BCa remains to be characterized.

Differential roles of nuclear and extranuclear ERβ signaling in BCa

Since the ERα is the single most powerful predictor of BCa prognosis, all patients with BCa are routinely scored for the amount and presence of ERα, but not for ERβ, in the nuclei of

the normal or transformed epithelial cells. Clinical studies on the prognostic significance of ERβ in BCa have also focused on nuclear expression of ERβ [70;71]. Recent studies have noted, however, the existence of additional cellular ERβ pools in the cytoplasm, in the mitochondria, and at the plasma membrane of BCa cells (see Figure 2). One recent study found that nuclear ERβ1 and ERβ2 expression correlate with better overall survival and that nuclear ERβ2 correlates with better disease-free survival [44]. However, cytoplasmic ERβ2 expression alone, or in combination with nuclear ERβ2, predicted significantly worse overall survival. Patients with only cytoplasmic ERβ2 had a significantly poorer prognosis [44]. Cytoplasmic ERβ2 expression was also correlated with high-grade tumors, distant metastasis, recurrence, and death due to BCa. Nuclear ERβ2 also was strongly predictive of a twofold greater response to endocrine therapy. Thus, nuclear and cytoplasmic expressions of ERβ2 differentially affect outcome [58]. The distinct roles of ERβ isoforms at various cellular localizations have a clear prognostic significance. Here, we summarize what is known about ERβ in the nucleus, mitochondria, and plasma membrane in BCa cell models.

ERβ in the nucleus—Classical ERβ-mediated signaling involves binding of the ligand to the ERβ, resulting in translocation of ERβ to the nucleus, where it binds to DNA either directly to the classical ERE or indirectly to an NFκB-, AP1-, or Sp1-binding element via tethering with their respective transcription factors and recruits co-activators, thereby initiating downstream signaling cascades [72]. The ERβ1–5 isoforms retain the nuclear localization signal, and ERβ1, ERβ2, ERβ4, and ERβ5 are localized to the nucleus [43;70;71]. The question is whether the nuclear localization of the isoforms is really estrogen-dependent and whether ERβ1–5 isoforms are capable of activating transcription on their own when they are localized to the nucleus. In the yeast and HEK293 cell models, ERβ isoforms (ERβ2, 4, and 5) can form heterodimers with ERβ1, but not homodimers, and modulate gene expression in a ligand-dependent manner [47]. In ERα-expressing MCF7 cells, the constitutive expression of ERβ1 and ERβ2 diminished the ERE activity in these cells as compared with that of parental cells [65;73] as well as the expression of cathepsin D, a known target of ERα [74]. These findings indicate that both ERβ1 and ERβ2 inhibit ERα function, resulting in growth inhibition of ERα-positive BCa cells. These studies are especially relevant to the clinical situation, in which ERα is present initially and ERβ1 and its isoforms might repress ERα-targeted genes but have a different outcome in ERα-negative cancer.

ERβ in the mitochondria—Cytosolic ERβ was first demonstrated in MCF7 cells in experiments using pan-ERβ antiserum [75]. The observed cytoplasmic staining of ERβ was initially ignored as being either background staining or staining of inactive ERβ. At present, several lines of evidence support the presence of ERβ within the mitochondria and its association with mitochondrial proteins in BCa cells [76–79] (Figure 2). The mitochondrial ERβ in ERα-negative MCF-10F cells was involved in E2-induced expression of mitochondrial DNA (mtDNA)-encoded respiratory chain (MRC) proteins, cytochrome c oxidase subunits I and II, and NADPH dehydrogenase subunit 1. Using ERβ1 ectopic expression and tandem affinity purification followed by nano-LC-MS/MS, Nassa et al. identified the ERβ1 interactome in MCF7 cells, including the association of ERβ with several mitochondrial proteins [80]. Our laboratory performed yeast two hybrid–based interaction studies and also found the association of a number of mitochondrial proteins with the N-terminus of the ERβ (unpublished). The mitochondria are the energy powerhouse of a cell, and cellular processes such as cell proliferation, apoptosis, cell transformation, and tumorigenesis are closely related to MRC functions. Hence the physiological and pathological implications of ERβ-mediated mitochondrial effects in these cellular processes warrant further study. A putative mitochondrial targeting polypeptide signal (mtTP) has been identified in ERβ (between amino acids 220 and 270) and is present on all ERβ

isoforms [81]. However, to date, no investigation has examined whether the mitochondrial association is an ERβ isoform – specific event.

To explore the role of ERβ in mitochondria, the Russo laboratory [81] treated the benign MCF10F cells with E2 to induce transformation. They found that ERβ shifted from a predominantly mitochondrial localization in normal and early transformed cells to a nuclear localization in association with the expression of progressive stages of cell transformation. A separate study found that ERs, especially ERβ, in mitochondria strongly prevented radiation-induced cell death in a BCa cell model [82]. Furthermore, when BCa cells were exposed to ultraviolet light in the presence of E2, PPT (an ERα agonist) or DPN (an ERβ agonist) [83], DPN was more potent than PPT in inhibiting cytochrome C release. The upregulation of manganese superoxide dismutase activity to quench reactive oxygen species, thereby preventing cell death signaling pathways, was proposed as the mediator of the ERβ action.

ERβ on the plasma membrane—Rapid signaling through plasma membrane ERβ is now believed to be the major venue of non-genomic action of the receptor [84–90]. Endogenous ERβ has been identified in the caveolae and cell membranes of endothelial, non-small cell lung tumor, and BCa cells [91;92]. Our yeast two-hybrid screen for ERβ partners has also uncovered a number of novel membrane-associated proteins (unpublished), suggesting that cell membrane ERβ *per se* or through tethering of other protein partners participates in rapid signaling. In this regard, a preponderance of evidence has emerged indicating that the membrane-associated ERα and ERβ can activate mitogen-activated protein kinase (MAPK) (both the extracellular signal- regulated kinase (ERK) and the c-jun kinase) pathways, and the cytoplasmic free calcium ($[Ca^{2+}]_i$) flux [91;93–95], pathways known to be involved in BCa cell functions. Recent works have defined motifs in the LBD of steroid receptors that are critical to membrane localization and function [96–98]. Mutation of these motifs prevents both receptor dimerization and signaling through ERK, PI3K, and cAMP. Loss of the former signals prevents the cell survival activity of E2 in breast and lung cancer cells.

In short, it is crucial to identify the key factors contributing to differential distribution of ERβ among the various subcellular compartments during the progression of breast carcinogenesis to enhance our understanding of the differential roles of nuclear versus non-nuclear ERβ.

The consequences of post-translational modifications of ERβ in BCa

Post-translational modification (PTM) refers to the covalent addition of functional groups to proteins; it includes phosphorylation, ubiquitylation, nitrosylation, palmitoylation, acetylation, sumolyation, glycosylation, and methylation [97;99–101]. These modifications allow proteins to respond to extracellular signals, intracellular stress, pharmacological agents, and morphogens at different developmental stages. Studies on human ERβ PTM are sparse. Here, we review PTM data on mouse ERβ and relevant information on human ERα, aiming to provide insights into the role of PTM in human ERβ function.

Phosphorylation is the most extensively studied PTM, in part because of its relative frequency and stability [99]. It is a reversible process at serine, threonine, and tyrosine residues. Phosphorylation may modify the function of the ERβ, and different phosphorylation sites may indicate different normal and pathological states of the receptor. A recent clinical study demonstrated an association of phosphorylation of ERβ at S105 with better survival in BCa, even in tamoxifen-resistant patients [102]. Similar studies on ERα have shown that various serine phosphorylation sites are valuable for the classification and prognosis of BCa [103–105]. Reviews summarizing the prognostic value of ERα PTM sites

in BCa have been published [104;106], but since then the addition of new publications has been slow. In humans, stimulation of the p38 pathway enhanced the transcriptional function of ERβ (Figure 2) in endometrial adenocarcinoma Ishikawa cells [107] and BCa MCF-7 cells [108]. However, no single phosphorylation site on human ERβ has been identified *de novo* and functionally characterized. Using an unbiased mass spectrometry approach, our laboratory first identified three serines (S75, S87, and S105) as direct phosphorylation targets of ERK1/2 and p38 in the N-terminus of human ERβ. Functional analyses on the phosphorylation of ERβ at S105 demonstrated that this PTM inhibited migration and invasion in BCa cells [109].

Studies of the mouse ERβ have shed light on the functional role of phosphorylation in human ERβ [110;111]. For example, through prediction from mouse data, phosphorylation at S87 in the human ERβ was found to be a target of CXCL12/CXCR4 via activation of the ERK pathway in BCa cells [112]. Tremblay and co-workers extensively elucidated the function of mouse ERβ phosphorylation since they first cloned the gene in 1997 [111]. ERβ phosphorylation of S106 and S124 at the AF-1 domain of the mouse ERβ was ERK1/2-sensitive and associated with increased transactivation of the receptor [110]. Phosphorylation at these two sites was later shown to enhance recruitment of a steroid receptor co-activator 1 (SRC-1) and a co-activator CREB-binding protein (CBP) to the transcriptional complex [110;113] but ERβ phosphorylation at S255 was found to have an opposite effect [114]. Moreover, the same group reported that ERβ phosphorylation at S94 and S106 promoted degradation of the receptor through the ubiquitin-proteasome pathway [115]. A recent review provided a detailed summary of ERβ phosphorylation and its function in the mouse [116]. Although data on ERβ in mice has laid a foundation for human studies, not all information can be applied to human ERβ. The number of predicted kinase-specific motifs differs in humans and mice because of some major differences in the primary sequence, ie, some of the motifs are not preserved in humans, and the AF-1 domain of ERβ in humans is significantly shorter than that in mice. Emerging evidence indicates that phosphorylation of ERβ in mice may function differently from that in humans. For example, kinase p38 or ERβB2/ERβB3 activation repressed ERβ transactivation in mice [117], whereas p38 activation stimulated ERβ-mediated transcription in humans [107;108]. Therefore, further studies are necessary to understand the functions of each phosphorylation site and a combination of identified sites in human ERβ.

Ubiquitylation occurred at serine, threonine, and lysine residues of a protein [118]. Mono- and bi-ubiquitylation affects transcription, protein-protein interactions, and subcellular localization; and poly-ubiquitylation usually targets proteins for degradation through the 26S proteasome pathway. Human ERβ is degraded in an estrogen-dependent manner through ubiquitin/proteasome pathways in BCa cells *in vitro*, and the N-terminal 37-amino acid region is responsible in the recruitment of the ubiquitin ligase for ERβ degradation [119]. In addition, suppressor for Gal 1 (SUG1) interacts with and stimulates ubiquitin/proteasome-mediated degradation of human ERβ (and ERα), leading to reduced ER transactivation [120]. However, the exact sites of ubiquitylation have been difficult to identify owing to the instability of poly-ubiquitylated proteins. The exact ubiquitylation sites on ERβ remain to be revealed.

S-Nitrosylation is a liable and reversible reaction induced by nitric oxide on the cysteine residue of a protein. S-Nitrosylation of ERα at cysteine residues that coordinate Zn^{2+} within the two major DNA-binding Zn-finger domains inhibits the DNA binding of ERα at specific ERE [121]. Our laboratory, using mass spectrometry, recently identified three nitrosylation sites on human ERβ. In line with the findings for ERα, S-nitrosylation inhibited ERβ transactivation at ERE (unpublished).

A palmitoylation sequence was identified in the LBD of human ERβ based on sequence prediction, but the sites were not experimentally verified [98]. Palmitoylation has been shown to be essential for maintaining the juxtaposition of ERα with the plasma membrane, interacting with the membrane protein caveolin-1 (see Figure 2), and triggering non-genomic signaling pathways and cell proliferation [97]. Thus, it is reasonable to speculate that palmitoylation may function similarly to retain ERβ in the plasma membrane for rapid signaling, as the interaction between ERβ and caveolae has been shown to be crucial for the non-genomic action of ERβ [122].

Cell-surface and secreted proteins are usually modified by glycosylation. However, glycosylation with N-acetylglucosamine (O-GlcNAc) is more frequently detected in cytosolic and nuclear proteins on serine or threonine hydroxyl side chains [123]. S80 is a target for both phosphorylation and glycosylation in mouse ERβ, and these two modifications collaboratively modulate the degradation and activity of ERβ in the mouse [124]. Yet no glycosylation site has been identified on human ERβ. PTMs, including acetylation, sumolyation, and methylation, occur at lysine residues on steroid receptors [125;99;100]; however, no information on these modifications is currently available for ERβ.

Complex interplay among various types of PTMs on ERα exists: ERα S305 phosphorylation is reported to prevent K303 acetylation and to stimulate ERα activity [126]; and lysine sites (K266, K268, K299, K302, and K303) for acetylation are also common for sumoylation [127]. Until now, clinical studies on the *de novo* interaction between sumolyation and acetylation have been limited by the lack of antibodies specific to acetylated or sumoylated lysine; this area warrants further exploration. In conclusion, a greater effort should be made to investigate different ERβ PTMs and their inter-relationships because ERβ is expressed in 76% of BCa cases [11] and has been demonstrated to play important roles in cell functions in BCa and in its prognosis.

Increase in the diversity of the receptor's function by co-regulators and interacting proteins of ERβ

ERβ transactivation requires co-regulators and other transcriptional machinery. Ligands such as E2 enhance the formation of heterodimers between ERs and increase the binding of co-activators to the receptors. SERMS such as tamoxifen conversely facilitate the binding of the co-repressors (see Figure 2). Acquisition of tamoxifen resistance has been shown to be associated with changes in the expression of co-regulators in the cell culture [128]. Co-regulators may play a role in directing which ERβ-regulating gene or gene set can be activated or repressed, thus further contributing to the functional diversity of the receptor. In this section, we focus on the literature on human ERβ co-regulators and their protein-binding partners.

The co-expression of ERs and co-activators correlates with different prognoses of BCa; however, such information remains limited [13;19;129–131]. Young and co-workers found an inverse correlation between steroid receptor co-activator 1 (SRC-1) and ERβ in BCa [13;19]. Whereas the expression of ERβ was associated with better prognosis and responsiveness to tamoxifen, SRC-1 expression had the reverse association [13;19]. However, in another study with only 25 specimens, ERβ expression was associated with SRC-1, transcription intermediary factor 2 (TIF2), and nuclear receptor co-repressor (NCoR) in malignant specimens [130]. Moreover, the expression of ERβ, protein 300 kDa/CREB-binding protein (p300/CBP) and amplified in breast cancer 1 (AIB1) (SRC3) were higher in invasive ductal carcinomas than in normal mammary tissue [130]. The expression of SRC3 within epithelial cells of BCa was positively associated with ERα but inversely associated with ERβ [131]. Although various co-regulators were shown to co-localize with

ERβ in benign or malignant cells in BCa specimens, whether they are bona fide co-regulators of ERβ still needs to be determined experimentally. Cell and cell-free models have been used to analyze the ERβ co-regulatory activities of many co-regulators originally studied for their action in modifying ERα transcriptional activities. Both the type of ligand and *cis*-regulatory elements were important in determining the regulatory action of these co-regulators on ERβ; they often exert differential influences on the two ER subtypes [132].

Besides functioning as classical co-regulators that affect the transcriptional activities of ERβ, this nuclear receptor has also been shown to be influenced by its interaction with a variety of proteins with diverse functions. The better known ones now include inhibitor of differentiation protein (Id1) [133;134], insulin receptor substrate I (IRS-I)[135], and retinoblastoma protein 2 (pRb2/p130)[136]. With the advent of mass spectrometry, more than 300 proteins have been identified as putative ERβ1 binding partners [80]. Several previously proven ERβ1 co-regulators, such as SRC3 [132]; proline, glutamate, and leucine-rich protein 1 (PELP1) [137]; tripartite motif containing 24 (TRIM24) [137]; and mediator complex subunit 1 (MED1) [138] have been found. In addition, proteins involved in post-transcriptional modification of mRNA and actin filament-based processes were newly identified as ERβ protein partners [80]. ERβ was also shown to interact with proteins related to the regulation of apoptosis. They include a mitochondrial pro-apoptotic protein known as mitochondrial ribosomal protein S29 (MRPS29) [139], and Bcl-2-associated transcription factor 1 (BCLAF1), which is a transcriptional repressor localized to the nuclear enveloped and promotes apoptosis [140]. We recently identified BCL2-like 12 (proline rich) (BCL2L12) as a putative ERβ5 isoform-specific interacting protein that exerts anti- and pro-apoptotic functions in a cell-context manner (unpublished).

Looking ahead, the identification of ERβ-specific and ERβ isoform-specific co-regulators or protein-binding partners should help better define the functionality of ERβ in breast tissue, promising an improvement in BCa prognosis.

The differential behaviors of ERβ elicited by different ligands in BCa

E2 is the presumed ligand for ERβ. Will the diagnostic and prognostic value of ERβ vary with patients' estrogen status? ERβ—ERβ1 in particular—was significantly associated with diminished biological aggressiveness in premenopausal women [141] but correlated with worst outcome in postmenopausal women [22]. Another study reported opposite results for postmenopausal women [14]. Other reports demonstrated no effects of menopausal status on the prognostic value of ERβ (or ERβ1) in BCa, with results independent of ERα expression or tamoxifen treatment [12;14;18;20;21;24;142]. Unfortunately, many of the early studies on ERβ did not determine the menopausal status of the patients, making data interpretation difficult. Better designed studies are needed to clarify this important issue. Tamoxifen has been one of the most common endocrine therapies for ERα-positive BCa for 30 years [143]. However, a notable proportion (~ 30–40%) of patients with BCa relapse within 5 years of post-treatment [144]. Although tamoxifen is believed to target ERα in ERα-positive BCa, this mixed agonist- antagonist can also transactivate ERβ, thus raising the question of whether the latter has prognostic value for tamoxifen responsiveness/resistance. In this regard, in patients with ERα-positive BCa, ERβ was an independent predictor of tamoxifen responsiveness [12]; thus higher levels of ERβ expression correlated with longer disease-free and overall survival (DFS and OS) following tamoxifen therapy [15]. In ERα-negative patients, ERβ expression was also found to associate with a longer duration of distant disease-free survival (DDFS) [24]. This association is further supported by the significant association of promoter hypermethylation of the ERβ promoter, which leads to ERβ silencing, with tamoxifen resistance in BCa [145]. These findings, taken together, indicate that ERβ expression is a good prognostic marker for tamoxifen responsiveness in both ER-positive and ER–negative BCa, although some studies had contrary findings [22].

Fulvestrant is used when patients with BCa patients experience a relapse following tamoxifen therapy. Fulvestrant has also been approved for the treatment of ERα-positive metastatic BCa in postmenopausal women [146]. However, most studies have established correlations only between fulvestrant treatment and ERα but not with ERβ. In a BCa cell model, fulvestrant had little or no effect on the antiproliferative action of ERβ [28], although it stabilized the ERβ protein but promoted the degradation of ERα [147].

The traditional view that ER binds to estrogen and transactivates at the classical vitellogenin ERE was formulated largely from studies on ERα [148]. More recent publications [149;150] now reveal that ERβ can transactivate via different *cis*-regulatory elements in a ligand-specific manner and thereby influence different cellular functions (Figure 2). Endoxifen, the most important metabolite of tamoxifen, was shown to exert its antiproliferative action through stabilization of ERβ and enhancement of the number of ERα/β heterodimers [151]. In global transcriptome analyses, E2- or SERMs-activated ERα or ERβ action showed few overlaps in gene-expression profiles [152]. ChIP-cloning and -sequencing approaches revealed more commonalities among tamoxifen- and raloxifene-regulated gene sets than those activated by E2 [153]. Consistent with the clinical observations (see above), ectopic expression of ERβ increased the sensitivity of ERα-positive BCa cells to tamoxifen [154], whereas siRNA-mediated knockdown of the receptor reduced the responsiveness of MCF-7 to estrogen and tamoxifen, supporting a role for ERβ as a tumor suppressor [155]. Noteworthy is the finding that antiestrogens (e.g. fulvestrant) and SERMs (e.g. raloxifene) inhibit ERβ transactivation at ERE but enhance the transcriptional activity at other *cis*-elements, such as AP1, Sp2, NFκB [111;156]. Apropos to the concept of cross-talk with non-ERE *cis*-elements is the finding that a number of AP1-regulated genes are regulated by ERβ1 through tethering on cJun/cFos complex at the AP1 site [149;157;158]. Using ChIP-seq approach, other studies have identified non-ERE ERβ binding sites, including molecules such as AP2, E2F, and Sp1 [159–161].

Evidence supporting a protective role of phytoestrogens against BCa is conflicting [162;163]. Whereas the increase in soy intake correlated with lower cancer risk in studies of Asian populations, no strong correlations between these two factors were observed in Western populations [164–166]. These findings suggest that lifelong or early-life exposure to dietary soy diet among Asians may be critical to conferring the protective effects of soy on the breast [167]. Soy consumption also influences the type of BCa developed in various populations, in that a diet high in soy products was positively correlated with decreased risk of HER2-negative/ERα-positive/PR-positive BCa in Asian countries [168;169].

Because phytoestrogens can act like agonists in a low-estrogen environment but antagonists in a high-estrogen environment [170;171], the actions of phytoestrogens may vary depending on menopausal status [172]. Moreover, the stronger binding affinity of phytoestrogens to ERβ compared with ERα [173;174] and the significant estrogenic potency of phytoestrogens on ERβ may contribute to their antiproliferative and inhibitory effects on tumor growth [175–177]. Global gene expression profiling studies showed that phytoestrogens have biphasic activity in BCa cells, depending on the relative levels of expression of the two ERs [178–180]. In the absence of ERβ, phytoestrogens induced the same transcriptome changes as E2 in ERα-positive T47D cells [178;179]. The upregulated genes included those involved in cell cycle, DNA replication, chromosome segregation, and inhibition of apoptosis. Ectopic expression of ERβ in T47D resulted in opposite responses to phytoestrogen stimulation, causing inhibition of cell growth and the induction of cell- cycle arrest and apoptosis [179;180]. Of interest, apigenin, a flavone found principally in camomile, chives, garlic, and parsley, also inhibits cell growth in MDA-MB-231 cells via ERβ signaling [181]. Furthermore, the growth inhibitory action of phytoestrogens may also be mediated via their actions in regulating the expression of the various ERβ isoforms. For

example, genistein was found to induce the expression of ERβ1 and ERβ2 but not that of ERβ5 in T47D and BT20 BCa cells [182]. The ratio of the expression levels of these isoforms may be a key to determining whether an estrogen is pro- or antiproliferative. Finally, phytoestrogens can significantly affect the interaction between ERβ and co-regulators and thereby contribute to the tissue-dependent response [174]. Genistein preferentially promoted the binding of ERβ to SRC-1a (12,000-fold) and GRIP1 (33-fold) as compared with ERα [174].

Since breast carcinogenesis has been suggested to be related to estrogen-induced oxidative DNA damage [183;184], phytoestrogens may protect against tumorigenesis by reducing intracellular reactive oxygen species. Genistein, biochanin A, and resveratrol were shown to upregulate the expression of quinone reductase, a key enzyme in the maintenance of intracellular antioxidant capacity. The action of these phytochemicals was shown to be mediated preferentially through the transactivation of ERβ as opposed to that of ERα [185]. Biochanin A and resveratrol also significantly inhibited estrogen-induced oxidative DNA damage [186]. To conclude, the action of phytoestrogens in BCa may vary depending on the relative abundance of the two ER subtypes, the levels of E2, the utilization of *cis*-regulatory elements, and the expression levels of the various ERβ isoforms, hence making it difficult to predict their action in BCa prevention.

Finally, phytoestrogens may influence responses of patients with BCa to chemotherapy. Soy consumption was associated with a reduced risk of recurrence following tamoxifen treatment [187] or improved survival independent of tamoxifen use [188]. In BCa with both ERβ and HER2 expression, genistein was found to promote the growth inhibitory effect of trastuzumab, an anti-cancer drug targeting the HER2 receptor, in BT474 cells [189]. This finding suggests that ERβ-specific agonists potentiate the efficacy and enhance the potency of current BCa therapeutics.

Conclusion

ERβ is not simply a second ER. Its functions differ drastically from those of ERα and deviate more from those expected of a traditional nuclear receptor. It localizes in different cellular compartments and is susceptible to different PTMs. It is expressed in different variant forms, which interacts with multiple protein partners as well as ligands, utilizes canonical and non-canonical *cis*-elements, and heterodimerizes with ERα and its own isoforms, thereby creating a highly complex labyrinth of functions. In this review, we have summarized and discussed the existing literature in six key research areas of ERβ (see Figure 2). In our opinion, further investigations in these areas are essential to deepen our understanding of ERβ in BCa. Efforts should perhaps be focused on enhancing our understanding of the roles of ERβ isoforms and on devising ERβ-specific therapies that will help prevent or treat BCa.

We hope this review will stimulate additional studies in the following areas: 1) The function of ERβ isoforms in BCa. Do they respond to specific ligands? Do they regulate different gene sets and participate in physiological functions? Are SERMS or antiestrogens the ligands for the isoforms? 2) ERβ subcellular localization. How are ERβ isoforms targeted to different compartments and what are their physiological and pathophysiological roles? Does tamoxifen alter ERβ subcellular localization? 3) Determination of BCa relevant PTMs. What are the functional PTMs on human ERβ? Is there any cross-talk among PTMs? Is there a ligand-specific PTM signature? Is a PTM signature of a higher prognostic value than a single PTM? 4) Are there any ERβ interacting proteins that can dictate ERβ function in BCa? What is the mechanism? What are the relationships between these proteins with ERβ ligands? 5) Is E2 necessary to maintaining the basal function of ERβ in BCa? Is there any

ERβ-specific ligand that can be used as a BCa therapeutic? Why are phytoestrogens preferential ligands for ERβ? Can phytoestrogens be used as chemopreventive drugs for BCa on the basis of ERβ status?

Acknowledgments

We thank Nancy Voynow for her professional editing of this manuscript and thank Dr. Marian Miller for her valuable suggestions on the figures. This work was supported by grants from NIH (ES020988, ES019480, ES006096, CA15776, and CA112532 to SMH) and VA (BX000675 to SMH), and an internal funding source from the University of Cincinnati Medical Center.

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This review addresses the gaps in knowledge in ERβ research as it pertains to BCa regarding the following topics:

1. Tumor suppressive role of ERβ
2. ERβ isoforms, and its interacting proteins or co-regulators
3. Nuclear signaling and extranuclear ERβ signaling
4. Post-translational modifications of ERβ
5. Ligand and regulatory *cis*-elements of ERβ

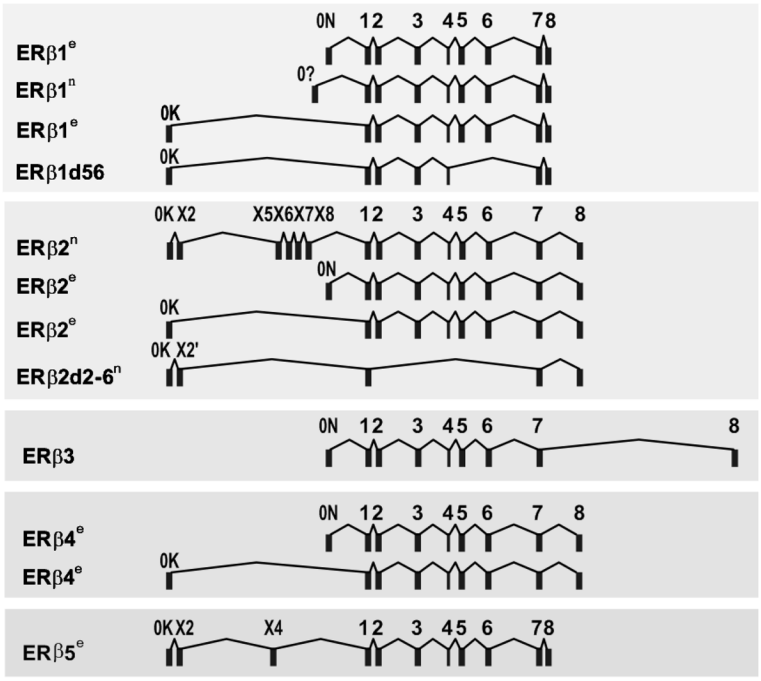

Figure 1.

Genomic arrangement of ERβ isoforms. Each ERβ transcript is composed of at least one 5′ non-coding exon (exon 0K, 0Xs, or 0N) and three to eight coding exons (exons 1–8). Light blue rectangular box represents an exon. Each full-length isoform (ERβ1, 2, 3, 4, 5) shares exons 1–7 plus an isoform-specific exon 8. Key: n=the transcripts published in NCBI database; e=transcripts discovered by sequencing experiments (unpublished).

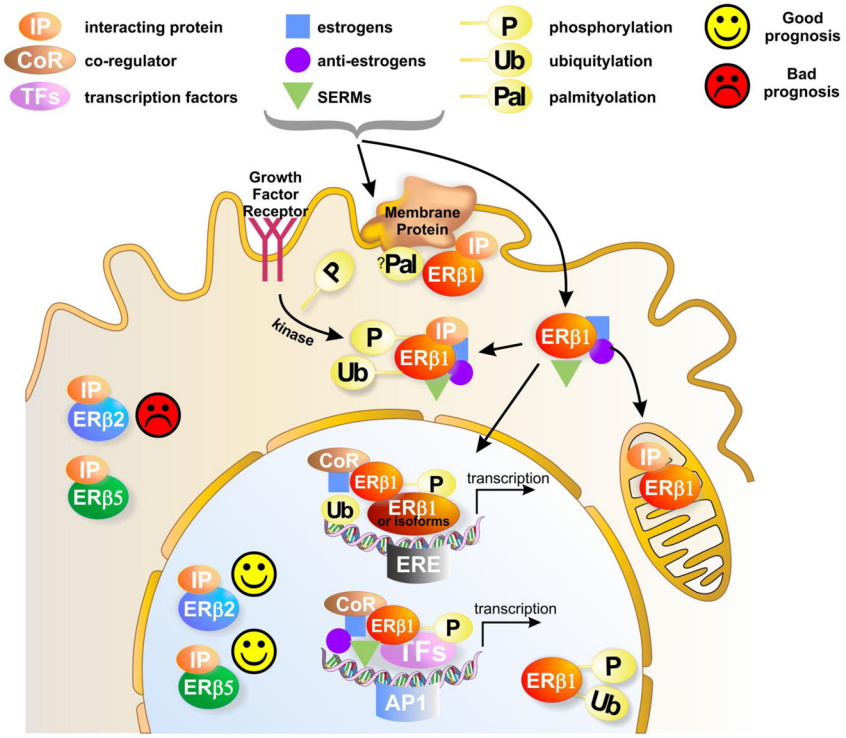

Figure 2.

Action of estrogen receptor β (ERβ) in breast cancer. Different ligands, such as estrogens, antiestrogens, and SERMs, activate non-genomic and genomic signaling of ERβ. In non-genomic signaling, ERβ interacts with membrane proteins (eg, G proteins, caveolin 1) and other interacting proteins (IPs) to activate kinase signaling pathways. In genomic signaling, liganded ERβ1 homodimerize or heterodimerize with ERβ isoforms or ERα and translocate from the cytoplasm to the nucleus. The homo- or heterodimer directly binds to estrogen response elements (EREs) or are tethered to other TFs (eg, AP1) in the promoter region or *cis*-regulatory sequences of target genes to facilitate gene transcription. Specific co-regulators (CoR) are believed to interact with ERβ to modulate gene transcription. ERβ may also translocate into mitochondria and interact with proteins involved in mitochrondrial ribosome synthesis and organization. ERβ has been found (or is expected to be) phosphorylated, ubiquitylated, or palmitoylated for gene transactivation, degradation, or membrane targeting, respectively. Expression of ERβ isoforms other than wild-type ERβ1 can be potential prognostic markers in breast cancer. For example, nuclear ERβ2 and ERβ5 were found to be associated with better patient survival; however, cytoplasmic ERβ2 was significantly correlated with worse outcome. Interactions of specific protein partners are believed to contribute to the functional roles of ERβ1 and its isoforms in breast cancer. Smiley and sad face represents good and bad prognosis in breast cancer, respectively.