A bi-faceted role of estrogen receptor β in breast cancer

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Abstract

Despite over 15 years of research, the exact role, if any, played by estrogen receptor β (ERβ) in human breast cancer remains elusive. A large body of data both *in vitro* and *in vivo* supports its role as an antiproliferative, pro-apoptotic factor especially when co-expressed with ERα. However, there is a smaller body of data associating ERβ with growth and survival in breast cancer. In clinical studies and most often in cell culture studies, the pro-growth and pro-survival activity of ERβ occurs in ERα-negative breast cancer tissue and cells. This bi-faceted role of ERβ is discussed in this review.

Key Words
- estrogen therapy
- estrogen receptor
- breast
- endocrine therapy resistance

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Introduction

The critical role of estrogen in human breast cancer is undisputed. The practical consequences of the concept of inhibiting the mitogenic action of estrogen on breast cancer cells have been the successful establishment of the endocrine therapies for treating breast cancer (Trialists' 1992, 1998) as well as providing options for preventing breast cancer (Fisher *et al*. 2005). While the concept itself is relatively simple, our understanding of the exact molecular mechanisms by which estrogen is involved in these processes continues to evolve and is more complex and multifaceted than originally thought (Zwart *et al*. 2011). In particular, one critical discovery has been the identification of a second estrogen receptor (ER), called ERβ (Kuiper *et al*. 1996), in contrast to the classical ERα, which can also mediate estrogen action in target cells. The discovery of ERβ has led to a full reevaluation of estrogen action in all target tissues, including human breast cancer (Fox *et al*. 2008). However, despite over 15 years of research, the exact role, if any, played by ERβ in human breast cancer remains elusive (Fox *et al*. 2008, Thomas & Gustafsson 2011, Murphy & Leygue 2012). Several reviews have recently covered the general topic of ERβ and tumorigenesis (Fox *et al*. 2008, Leygue & Murphy 2011, Thomas & Gustafsson 2011, Leung *et al*. 2012, Murphy & Leygue 2012). However, emerging data suggest that ERβ may have a bi-faceted role in breast cancer. We herein discuss the most recent data which suggest that ERβ plays a bi-faceted role in breast cancer. Interestingly, a bi-faceted role of ERβ in gynecological cancer (ovarian vs endometrial) has also been suggested (Haring *et al*. 2012).

ERβ has several variant isoforms, and generally, it is the ligand-binding form, ERβ1, that is being referred to. The variant ERβ protein isoforms derive from alternatively spliced transcripts that result in C-terminally truncated proteins that cannot bind ligand. Often the antibodies used for immunohistochemistry recognize epitopes that are common to all variant proteins and cannot distinguish among them. In this review, when this is the case, the terminology used is total ERβ or ERβ-like proteins. When an isoform-specific antibody is used, then the actual isoform name, e.g. ERβ1, is used.

What is meant by a bi-faceted role for ERβ?

The majority of published data have concluded that ERβ1 has both antiproliferative and pro-apoptotic activities,

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while a smaller number of studies suggest a proliferative and survival role for ERβ1. Therefore, the possibility of a bi-faceted role for ERβ1 in breast cancer development and progression can be suggested. The following will review both clinical and experimental data that support a bi-faceted role for ERβ1 in breast cancer.

Clinical correlation studies supporting the bi-faceted role of ERβ in breast cancer

There are several studies supporting a bi-faceted role of ERβ, in particular ERβ1, obtained using retrospective correlative biomarker analyses in cohorts of breast cancer cases linked to clinical outcome information. We have only considered studies in which ERβ-like proteins have been measured. Furthermore, these studies (except in one case where western blotting was used) also used immunohistochemistry, such that ERβ expression only in tumor cells was measured. These studies have been recently reviewed by us and others (Fox *et al*. 2008, Leygue & Murphy 2011, Leung *et al*. 2012, Murphy & Leygue 2012) in detail. A consistent finding is that, in contrast to ERα, total ERβ levels decline during breast tumorigenesis (Leygue *et al*. 1998, Roger *et al*. 2001), a phenomenon also observed in other cancers such as prostate (Prins & Korach 2008), colon, ovary, and lung (Bardin *et al*. 2004) but not endometrial cancer (Haring *et al*. 2012). This supports a potential tumor-suppressor role. Generally, higher levels of ERβ-like expression were found associated with the expression of good prognostic markers or better clinical outcome, usually in patients who have subsequently been treated with tamoxifen (Esslimani-Sahla *et al*. 2004, Fleming *et al*. 2004, Gruvberger-Saal *et al*. 2007). Other studies have, however, found that high vs low expression of ERβ-like proteins have either no (Esslimani-Sahla *et al*. 2004, Miller *et al*. 2006, Skliris *et al*. 2006, Honma *et al*. 2008, Shaaban *et al*. 2008) or poor (Saji *et al*. 2002*a*,*b*, O'Neill *et al*. 2004, Novelli *et al*. 2008, Shaaban *et al*. 2008) prognostic value in breast cancer.

Differences observed are potentially related to whether or not ERβ is expressed alone or co-expressed with ERα. It should be remembered that ER status (positive or negative) in human breast cancer is only defined by the measurement of ERα (Hammond *et al*. 2010). Approximately, 59% of primary breast cancers show ERβ co-expressed with ERα (ERβ+/ERα+) (Murphy *et al*. 2003) and ~17% only express ERβ (ERβ+/ERα−) (Murphy *et al*. 2003). Usually, only ER+ patients are treated with endocrine therapy and ER+ status is itself determined only by ERα. Therefore, most tumors being assessed in the majority of previous studies would be those co-expressing ERβ1 or total ERβ proteins with ERα. Furthermore, in most but not in all these studies, higher levels of ERβ1 or total ERβ proteins together with ERα are a better predictor of endocrine responsiveness than ERα alone. This supports the idea that nuclear ERβ-like proteins are having a restraining action on ERα-mediated growth and survival activities. However, in three studies where the cohorts studied were ERα negative and the patients had been subsequently treated with tamoxifen, high ERβ1 levels were predictive of a good response to tamoxifen therapy (Gruvberger-Saal *et al*. 2007, Honma *et al*. 2008, Yan *et al*. 2013). One of these studies (Yan *et al*. 2013) was a randomized placebo-controlled clinical trial, in which benefit was only found in the tamoxifen-treated but not in the placebo arm; therefore providing evidence that ERβ expression was predictive for response to tamoxifen inhibition of tumor growth and survival. These correlative data, together with the previous observations of a positive correlation of ERβ1 expression with Ki67 (a marker of proliferation), support the idea that ERβ1 is driving proliferation and/or survival in a subgroup of patients whose tumors were ERα negative. This subgroup seemed to be defined also by a high expression of a potential modulator of ERβ activity, called steroid receptor RNA activator protein (Yan *et al*., 2013). This ER co-regulator is encoded by a gene that in its own right is also bi-faceted, as alternative splicing of its transcripts results in a functional non-coding RNA and/or a protein able to modulate transcription (Cooper *et al*. 2011).

Another important finding, in one (Honma *et al*. 2008) of the three studies referred to the above, is that high expression of ERβ1 in triple-negative breast cancer cases was also significantly associated with good clinical outcome in patients treated with tamoxifen. While this may explain the historical observations that a small subset of patients with apparently ERα-negative breast cancers respond to tamoxifen treatment (von Maillot *et al*. 1980, Stewart *et al*. 1982), an implication of these findings is that ERβ1 may be a viable treatment target in some triple-negative breast cancers. Therefore, a group of patients previously considered only for aggressive chemotherapies would now be candidates for better tolerated hormonal-like therapies.

Experimental studies supporting a bi-faceted role of ERβ

In normal mammary tissue, ERβ is the most widely expressed ER and is expressed in both luminal and myoepithelial cells as well as in some cells in the

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surrounding stroma. ERα, in contrast, is less frequently expressed and generally its expression remains confined to the luminal epithelial compartment (Speirs *et al*. 2002). ERα, however, appears to play a more important role in the normal mammary gland. Indeed, knockout *Erα* mice do not develop a functional mammary gland (Bocchinfuso & Korach 1997, Feng *et al*. 2007), whereas knockout *Erβ* animals undergo an overall normal mammary gland development. Subtle effects associated with decreased differentiation and increased proliferation in the alveoli of lactating mammary glands are sometimes observed in these mice; these changes appear to be age related and are only observed in some (Forster *et al*. 2002, Palmieri *et al*. 2002), but not all, *Erβ* knockout mouse models (Couse & Korach 1999, Antal *et al*. 2008). Furthermore, it has been suggested that the effect on the development of the mammary gland might be indirect due to a deficiency in ovarian hormone synthesis rather than a direct result of lack of ERβ expression in breast epithelial cells (Antal *et al*. 2008). Data generated *in vitro*, on rodent or human mammary epithelial cells (nontumorigenic as well as neoplastic) in culture, showed that shutting down ERβ expression leads to an increased ligand-dependent and -independent growth (Helguero *et al*. 2005, Treeck *et al*. 2010). These results are consistent with the observed increased proliferation of cells in *in vivo* models following knockdown of *ERβ* expression (Weihua *et al*. 2000, Forster *et al*. 2002, Paruthiyil *et al*. 2004).

In apparent contrast to the common conclusion that ERβ1 is an inhibitor of proliferation, treatment of ovariectomized mice for 48 h with a selective agonist of ERβ1 called BAG has led, in the mammary epithelial cells of treated mice, to increased bromodeoxyuridine labeling (Cheng *et al*. 2004*a*). Interestingly, this incorporation, a marker of renewed DNA synthesis, was similar to that observed when mice were treated with 17β-estradiol (E_2) and tamoxifen but was not observed in uterine cells (Cheng *et al*. 2004*a*). As such, ERβ appeared to mediate cell proliferation in a tissue-specific way. Colocalization of Ki67 and ERβ in ~47% of mammary epithelial cells in primates has also been reported (Cheng *et al*. 2005). Such colocalization (Saji *et al*. 2000, Cheng *et al*. 2005) suggested that ERβ1 has a role, although not essential, in proliferation of some normal mammary epithelial cells. Or at least under specific circumstances, ERβ does not inhibit proliferation.

In most but not all studies where ERβ1 has been overexpressed in cell lines, antiproliferative and pro-apoptotic (Lazennec *et al*. 2001, Cheng *et al*. 2004*b*) activity was observed (Paruthiyil *et al*. 2004; Table 1). And intestinal tumorigenesis is enhanced in mice resulting from crosses between Apc (min) mice and ERβ-deficient mice (Giroux *et al*. 2008, Cleveland *et al*. 2009). ERβ1 can inhibit epithelial to mesenchymal transition in cancer cells (Mak *et al*. 2010, Thomas *et al*. 2012), consistent with a role in epithelial differentiation. More insight into the molecular mechanisms by which this occurs has recently been published using immortalized prostate epithelial and prostate cancer cell line models (Mak *et al*. 2013). Furthermore, it was also shown that these effects were mediated by a selective androgen-derived ligand for ERβ 5α-androstane, 3β, 17β-diol, and not estrogen (Mak *et al*. 2013). Altogether, these data support a role for ERβ as an anti-growth, pro-apoptotic, and pro-differentiation factor.

Interestingly, the development of a few breast cancer cell line models, where increased ERβ1 expression was associated with increased proliferation and survival, supported the idea that under some circumstances ERβ1 can associate with proliferation instead of apoptosis. These findings, however, may be due to alternative posttranslational modifications, i.e. short vs long (N-terminal) forms of ERβ1, a distinct cellular circuitry background associated with ERα negativity, clonal selection artifacts, and/or an insertional mutagenesis phenomenon of the transfected construct. The p53 status of cells may also affect ERβ1 activity (Choi & Pinto 2005, Lewandowski *et al*. 2005, Skliris *et al*. 2007) as might the microenvironment, and whether or not the cells are grown in a 2D vs 3D structure. Such differences alone or in combination could contribute to the bi-faceted nature of ERβ. Cotrim *et al*. 2012) recently published results, where they found that ERβ agonists as well as ERα selective ligands induced mammary gland hyperplasia and increase tumor growth of mice in which MC4-L2 mammary tumor cells had been implanted. This was in stark contrast to the MC4-L2 mouse mammary tumor cell model when grown under 2D cell culture conditions. MC4-L2 cells endogenously express both ERα and ERβ1. Under 2D culture conditions, selective ligands for ERα stimulate proliferation whereas selective ligands for ERβ have little effect on proliferation but instead increase apoptosis, increase p53 expression, and decrease cell numbers. However, when the cells are grown in Matrigel 3D culture, the ERβ agonists exert a slight but significant increase in cell numbers, which was inhibited by co-incubation with the MEK inhibitor U0126. It was hypothesized that activation of erk1/2 MAPK signaling by Matrigel, a surrogate for basement membrane, fully blocked the growth-inhibitory effects resulting from ERβ1 activation by agonist ligands (Cotrim *et al*.

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Table 1 Cell line models of human ERβ overexpression.

Cell line	ERβ isoform	Constitutive vs inducible	ERα expression	Phenotype	Reference
Growth inhibition					
MCF7	ERβ1	Constitutive (adenoviral transient)	Yes	Reduced E_2 growth in cells in culture and xenograft	Paruthiyil *et al.* (2004)
Tet-off-T47D	ERβ1	Inducible	Yes	Reduced E_2-induced growth	Strom *et al.* (2004)
MCF7	ERβ1	Constitutive (adenoviral transient)	Yes	Ligand-independent cell cycle arrest	Paruthiyil *et al.* (2011)
Tet-on MCF7	ERβ1	Inducible	Yes	Reduced E_2 growth Increase sensitivity to tamoxifen	Murphy *et al.* (2005)
Tet-off MCF7	ERβ2/cx	Inducible	Yes	Reduced E_2 transcription, reduced PR, and growth ND	Saji *et al.* (2002*b*) and Zhao *et al.* (2007)
Tet-off MCF7	ERβ1	Inducible	Yes	Reduced E_2 growth Increase sensitivity to antiestrogens	Hodges-Gallagher *et al.* (2008)
Tet-on HEK293	ERβ2/cx	Inducible	No	Reduced E_2 transcription, growth ND	Zhao *et al.* (2007)
Tet-off MCF7	ERβ1	Inducible	Yes	Reduced basal and E_2-induced growth	Liu *et al.* (2008)
MDA-MB-231	ERβ1	Constitutive	No	Growth inhibition – ligand independent	Lazennec *et al.* (2001)
Hs578T	ERβ1	Tet-on inducible	No	Reduced E_2-induced growth	Secreto *et al.* (2007)
Hs578T	ERβ2/cx	Tet-on inducible	No	No effect on growth	Secreto *et al.* (2007)
Hs578T	ERβ1	Tet-on inducible	No	Reduced E_2-induced growth	Shanle *et al.* (2011)
No effect on growth					
MDA-MB-231	ERβ1	Constitutive	No	No effect alone, but sensitized to RA inhibition	Rousseau *et al.* (2004)
Tet-on-MDAMB231	ERβ1	Inducible	No	No effect on proliferation	Murphy[b]
Tet-on-MDA-MB-231	ERβ2/cx	Inducible	No	No effect on proliferation	Murphy[b]
MCF-7	ERβ1	Constitutive	Yes	No effect on E_2-induced growth. Increases sensitivity to endoxifen	Wu *et al.* (2011)
Growth stimulation					
MDA-MB-231	Short ERβ1	Constitutive	No	Proliferation	Tonetti *et al.* (2003)
MDA-MB-435[a]	ERβ1	Constitutive	No	Increased proliferation and invasion	Hou *et al.* (2004)

RA, retinoic acid; ND, not determined.
[a]This is now known to be a melanoma-derived cell line (Rae *et al.* 2007).
[b]Murphy LC, Ung K & Peng B 2005, unpublished observations.

(2012). The authors also went on to show that when mammary epithelial cells, either normal-like or neoplastic, were grown in 2D culture in the presence of EGF (which activated erk1/2), ERβ agonists increased cell numbers. In a background of activated erk1/2, further experiments also implicated a role for activated PI3K/Akt signaling in this ERβ-driven proliferation. As ERβ phosphorylation was enhanced under conditions of growth stimulation, it was speculated that this posttranslational alteration may also play a role in ERβ-induced proliferation.

The results described earlier are the basis for suggesting a bi-faceted role of ERβ in breast cancer growth and survival. Furthermore, they also provide some insight into the potential mechanisms underlying a bi-faceted role.

What are possible mechanisms of the bi-faceted activity of ERβ?

To assess the potential processes underlying this bi-faceted aspect of ERβ's personality, it is helpful to outline briefly what is known about the structure and mechanism of action of this ligand-regulated transcription factor. ERα and ERβ belong to the thyroid/steroid receptor superfamily. As shown in Fig. 1, these receptors share the same structural and functional composition: an N-terminal functional domain (AF1), able to activate transcription

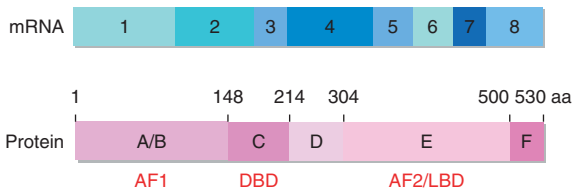

Figure 1
RNA and protein structure of ERβ. Exonic structure of ERβ messenger is indicated in blue boxes. The protein shares the classical structure of other steroid receptors with structural domains A–F shaded in various shades of pink. The positions of the functional domains, activation function 1 (AF-1), DNA-binding domain (DBD), activation function 2 (AF2), and ligand-binding domain (LBD) are indicated in red.

in the absence of ligand, a DNA-binding domain (DBD), consisting of a classical zinc-fingers motif, and a C-terminal functional domain (AF2), activated by the binding of estrogen on the ligand-binding domain (LBD). Several excellent reviews have described how these receptors can act (McKenna *et al.* 1999, Lonard *et al.* 2007, Kumar & McEwan 2012). Briefly, several mechanisms of action have been described. The first one, referred to as 'classical', is simplistically described in Fig. 2. In this model, the ligand enters passively into the target cells, binds to the receptor, and initiates a cascade of well-characterized events. The receptor is first released from a cytoplasmic chaperone complex containing several proteins including heat-shock proteins 70 and 90. The freed receptor, subjected to subsequent posttranslational events including multiple phosphorylations (Le Romancer *et al.* 2011), enters the nucleus, dimerizes, and binds to defined genomic enhancer regions, containing specific motifs known as estrogen-responsive elements (EREs). This binding is followed by the recruitment of cofactors, positive (coactivators) or negative (corepressors), the balance of which leads to either the activation or the repression of the expression of involved genes.

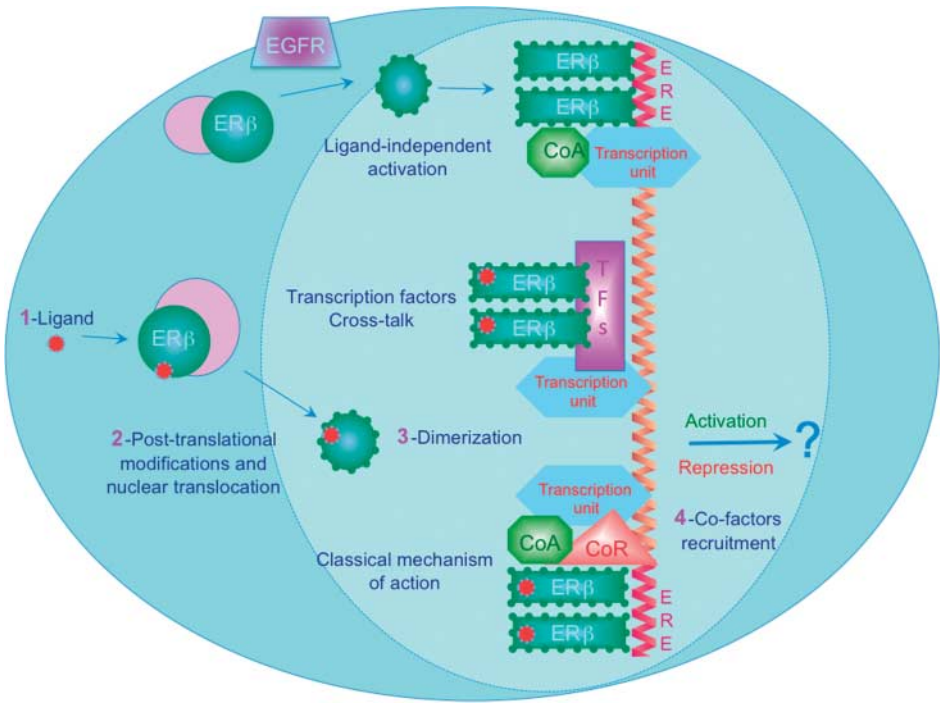

Figure 2
Simplified representation of three potential ERβ mechanisms of action. 'Classical' mechanism (lower part of the diagram): activation of ERβ1 occurs in four steps. First, the ligand penetrates passively in the target cells through the plasma membrane and binds to the receptor, inducing the release from a cytoplasmic chaperone complex (pink). This release is followed by a cascade of posttranslational modifications (indicated by dots on the surface of the receptor) and the targeting of the receptor to the nucleus. The receptor is then able to form dimers (homodimers or heterodimers with ERα), which binds specific enhancer regions (estrogen-responsive element, ERE) upstream or downstream of target genes. Depending on the ligand involved and the overall conformation of the receptors recruited, dimers will then interact with positive (coactivator, green) or negative (corepressors, red) regulators, leading to the activation or the repression of specific target genes. Tethering mechanism (middle part of the diagram): activated receptor can bind to transcription factors (such as AP-1) and modulate, positively or negatively, the activity of these factors. Unliganded activation (top part of the diagram): the receptor can be activated by posttranslational modification; phosphorylations resulting from EGFR signaling cascade, for example. Activated receptor can then act through ERE or tethering mechanism.

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This estrogenic action has been found to be cell-, gene-, and context specific. As outlined in previous reviews (McKenna *et al*. 1999, Lonard *et al*. 2007, Zwart *et al*. 2011, Kumar & McEwan 2012), the resulting effect on gene transcription is a dynamic process, involving multiple protein complexes, which contain chromatin-modifying molecules such as histone deacetylases, protein degradation units involving proteasome and ubiquitin ligase, as well as splicing regulatory units. Nonclassical mechanisms of action have also been described for steroid receptors: these include activation by EGF signaling through ligand-independent phosphorylations of the receptor; tethering of the receptor with other transcription factors, such as Sp1 and AP1 (Hall *et al*. 2001; Fig. 2); and non-genomic action involving receptors located on the cell membrane (Hammes & Levin 2011). Overall, the action of the ERβ will mechanistically depend on many parameters including but not limited to cyclical interactions between regulatory molecules (ligand, cofactors, ubiquitin, or histone deacetylases), cell context, specific protein degradation (proteasome involvement), and the exact gene considered (McKenna *et al*. 1999, Lonard *et al*. 2007, Kumar & McEwan 2012). Most of all, as with all other biological processes, the specific observation of a particular ERβ effect will depend on what endpoint is looked at and most importantly how it is observed. With that in mind, the bi-faceted aspect of ERβ action detailed earlier could result from differential modification and/or regulation of any of the steps involved in the mechanisms outlined earlier.

Ligand-dependent and -independent activity

First, the endogenous ligands able to bind and potentially regulate ERβ1 action are multiple (Kuiper *et al*. 1998, Guerini *et al*. 2005, Michael Miller *et al*. 2012) and their respective effects, in a tissue-specific context, remain to be fully characterized (Thomas & Gustafsson 2011). Even if ERβ1 is 'officially' defined as being an estrogen-binding protein, reports also indicate that compounds such as phytoestrogens (Shanle & Xu 2010, Shanle *et al*. 2011) DHEA (Michael Miller *et al*. 2012) and oxysterols (DuSell & McDonnell 2008) can also modulate the activity of this receptor. Importantly, some of these different ligands preferentially activate ERβ compared to ERα (Shanle & Xu 2010) and may alter the ER homo- and heterodimerization profiles (Powell *et al*. 2012). As such, one can easily see that such ligands can interfere with the 'normal or classical' pathway this receptor is otherwise directed toward.

ERβ variations

Alternatively spliced variants ERβ variant isoforms can be an important factor (Herynk & Fuqua 2004). Indeed, as previously emphasized, immunodetection implies the recognition of a specific epitope within a protein. Therefore, only a portion of the molecule is recognized, independently of the integrity of the whole protein. The characterization of multiple variants, mainly generated through alternative splicing (Figs 3 and 4), increases the complexity of interpreting the information gathered using one antibody for immunodetection of ERβ expression. Indeed, an antibody raised against the N-terminal extremity of the ERβ receptor will not differentiate between the full-length ligand-binding ERβ1 and a variant encoded by a well-characterized RNA, called ERβ2/cx. ERβ2/cx has an alternate exon 8 and encodes a protein missing the LBD. As such detecting the expression of this molecule, unable to bind ligand, but also able to heterodimerize with wild-type ERβ1 and ERα, could lead to erroneous interpretation (Murphy & Watson 2006).

Five major variants (ERβ1–5), resulting from alternative splicing events involving exons 7 and 8, have been identified (Fig. 3). ERβ1 (the first described), 2/cx, 3, and 4 variants contain exons 1–7 of the human *ERβ* gene followed by one of the several alternative exon 8. *ERβ5* variant contains an extended exon 7 and its exon 8 results from the splicing of an intron containing atypical CC and CA donor and acceptor sites.

The exact function of the alternatively spliced ERβ variants remains unclear and contradictory results concerning potential function have been published (Ogawa *et al*. 1998, Peng *et al*. 2003, Leung *et al*. 2006). For example, transient expression studies show that ERβ2/cx cannot bind ligand and when overexpressed can inhibit ERα transcriptional activity (Ogawa *et al*. 1998, Peng *et al*. 2003), with little effect on ERβ1 activity. However, subsequent studies have shown that ERβ2/cx as well as the other C-terminally truncated variants, ERβ3, 4, and 5, all of which cannot bind ligand and are missing the coactivator recruiting helix 12 (Fig. 4), can heterodimerize with ERβ1 and enhance its estrogen-mediated transcriptional activity (Leung *et al*. 2006). The differences in published results may be in part due to the different cell lines used to undertake these transient expression studies as well as different levels of expression and relative expression achieved. An overarching conclusion, however, is that the variant ERβ isoforms can modify both ERα and ERβ1 activity when co-expressed. Therefore, differential expression of the ERβ variants may play a role

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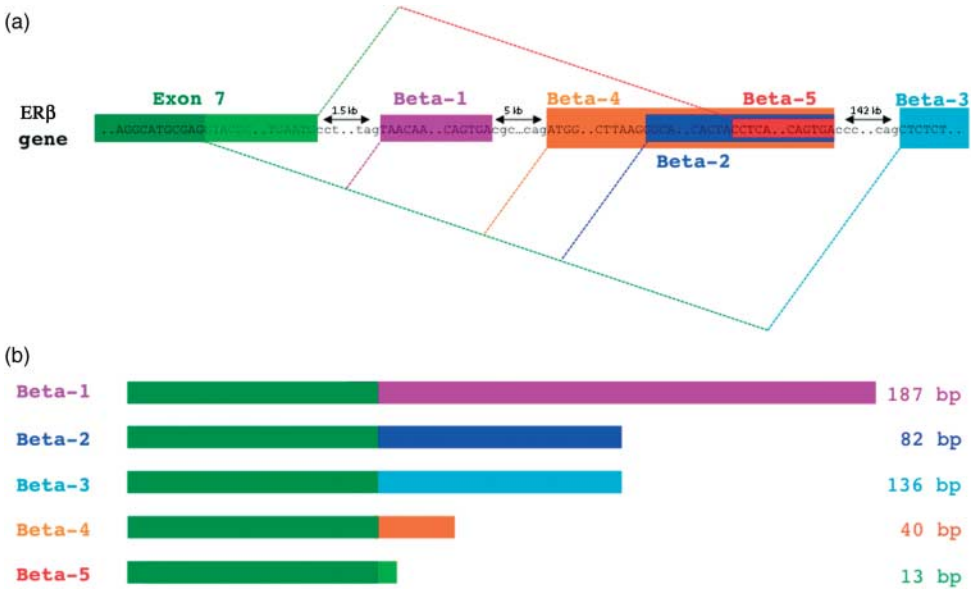

Figure 3

Schematic representation of alternative splicing events resulting in the production of multiple ERβ isoforms. (A) The use of alternative acceptor sites leads to the production of *ERβ1*, *-2/cx*, *-3*, and *β4* mRNAs. Indeed, these isoforms share a common exon 7 (exon 7 b1-4, dark green) but differ in their alternative exon 8 (purple, dark blue, light blue, and orange respectively). The combined use of alternative donor and acceptor sites produces *ERβ* mRNA, which contains an extended exon 7 (light green) and a shorter exon 8 (red). (B) Schematic representation of resulting common exon 7 (green) and additional coding sequences brought by the respective exon 8: purple, dark blue, light blue, and orange for exon 8 of ERβ1, -β2/cx, -β3, and -β4 respectively. The length of additional respective coding sequence is indicated on the right.

in altered and bi-faceted ERβ action and sensitivity to antiestrogens during breast tumorigenesis and breast cancer progression.

ERβ1, *-2/cx*, *-3*, and *-5* mRNAs have been detected in breast cancer tissues and cell lines. Using a specific assay allowing the co-amplification of ERβ1, 2/cx and 5, we found that not only breast cancer cell lines expressed different relative levels of these variants but also an increase in ERβ 2/cx and 5 RNA isoforms relative to the ERβ1 RNA isoform occurs during breast tumorigenesis (Leygue *et al.* 1999).

Co-expression with ERα The heterodimerization of ERβ1, as well as the proteins encoded by its known splicing variants, with ERα, further increases the complexity of the potential effect these ERβ-like proteins have on the estrogen-signaling pathway. Multiple articles have shown that homo- or heterodimers involving ERβ and ERα had significantly different gene targets (Monroe *et al.* 2005, Chang *et al.* 2006, Liu *et al.* 2008, Powell *et al.* 2012). The ability of ERβ variants to modify the activity of ERα is, *per se*, sufficient to drastically interfere with the expected mitogenic effect of estrogen on ER-positive cells as well as

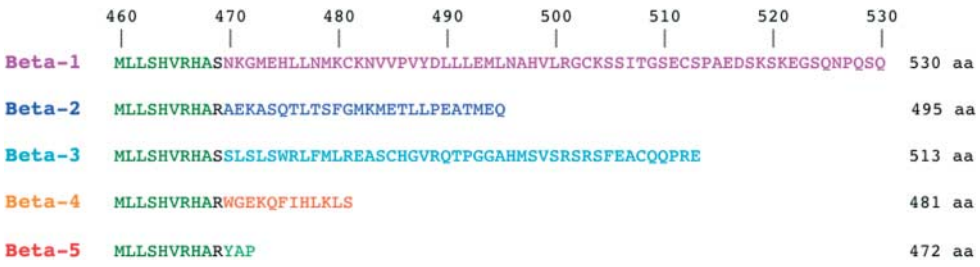

Figure 4

Alignment of the C-terminal extremities of ERβ-1, -2/cx, -3, -4, and β-5 proteins. These isoforms are identical in their first 468 amino acids (aa) but differ in the sequence corresponding to the end of the ligand binding of ERβ1. The total length of the resulting protein is shown on the right side. Underlined in ERβ1 sequence are the amino acid sequences involved in the ligand-binding domain of the receptor.

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to affect the sensitivity of the cells to antiestrogenic agents (Murphy *et al*. 2005, Wu *et al*. 2011).

Posttranslational modifications ERs are subject to multiple posttranslational modifications that may influence function (Le Romancer *et al*. 2011). It is well acknowledged that the presence of ERα is important in terms of diagnosis and prediction of response to endocrine therapy such as tamoxifen. More recently, it was shown that the specific phosphorylation profile of ERα, i.e. the specific detection of multiple phosphorylated residues (Skliris *et al*. 2010), might be a more accurate way to assess its prognostic and predictive value. It is easy to extrapolate that the same will happen regarding specific ERβ phosphorylation (Hamilton-Burke *et al*. 2010). Other posttranslational modifications of ERβ have recently been reviewed (Le Romancer *et al*. 2011) and another ERβ variant, an N-terminally truncated short form of ERβ1 generated posttranslationally by proteolysis (Savinov *et al*. 2006), has also been identified. The shorter ERβ1 protein may be more stable than the long form as it is potentially missing the binding site for the ubiquitin ligase, carboxyl terminus of HSC70-interacting protein (CHIP), required for inducing ERβ1 proteasomal degradation (Tateishi *et al*. 2006). Functional differences between the long and short forms of ERβ1 have been described, in particular associated with anti-inflammatory activities of ERβ1 (Bhat *et al*. 1998, Tateishi *et al*. 2006, Cvoro *et al*. 2008, Saijo *et al*. 2011). Furthermore, it has been shown that Pescadillo ribosomal biogenesis factor 1 (PES1) differentially affects ERβ1 and ERα at a posttranslational level and may, in part, be responsible for the altered ratios of ERα/ERβ seen consistently during breast tumorigenesis (Cheng *et al*. 2012, Thomas & Gustafsson 2012). The short form of ERβ1 may not be regulated by PES1 in the same way as the long form.

Therefore, differential posttranslational modifications may affect ERβ1 function including specific degradation pathways (Sanchez *et al*. 2010, 2012, Cheng *et al*. 2012, Picard *et al*. 2012) and kinetics of turnover, involving particular heterodimers for example, and contribute to a bi-faceted mechanism of action.

Nuclear vs non-nuclear activity The similarities and differences of ERβ1 and ERα with respect to structure of the full-length ligand-binding forms and their respective variant isoforms have recently been reviewed (Thomas & Gustafsson 2011, Murphy & Leygue 2012). The similarity of ERβ1 to ERα has led to a focus on its mechanism of action as a transcription factor and therefore on its localization to the nucleus. However, an extranuclear localization of ERβ has been reported in some cells and tissues including breast cancer (Hamilton-Burke *et al*. 2010, Leung *et al*. 2012, Razandi *et al*. 2012). The functions and potential mechanisms of action at the extranuclear sites are being explored. They are, however, less well described than the function and mechanisms of action of nuclear ERβ.

Similar to ERα, and other steroid hormone receptors, ERβ1 can homodimerize and directly bind to DNA sequences known as EREs, both distal and proximal, in target genes and regulate transcription. Four publications to date document genome binding (cistrome) studies of overexpressed ERβ1 in MCF7 breast cancer cells in culture (Liu *et al*. 2008, Charn *et al*. 2010, Zhao *et al*. 2010, Grober *et al*. 2011). These studies differ somewhat in their conclusions, although a common finding is that a reasonable degree of overlap exists between the cistrome of ERβ1 and ERα at least in MCF7 cells. Some differences were, however, noted, depending on the treatment conditions used. However, the transcriptional outcome of ERβ1 promoter binding compared to ERα often differs significantly as shown by transcriptome analyses (Chang *et al*. 2006, Vivar *et al*. 2010). Analysis of the ERβ1 target sequences within the genome identified ERE or half ERE binding sites as generally enriched, but each of the studies identifies distinct enrichment of other motifs not ERE related. The reasons for the differences, in these as well as those studies looking only at gene expression changes, may be due to the different experimental design: for example, in some cases, stable inducible overexpression of ERβ1 in MCF7 (Liu *et al*. 2008, Zhao *et al*. 2010) or T47D (Williams *et al*. 2008) cells was used, another used stable overexpression of ERβ1 in MCF7 cells (Grober *et al*. 2011) and others used transient adenoviral mediated ERβ1 overexpression (Paruthiyil *et al*. 2004, Chang *et al*. 2006, Charn *et al*. 2010). Furthermore, the resulting levels of ERβ1 overexpression may differ significantly among the studies. One study in particular found that when using a ChIP-on-chip approach to map ERβ1 genome-wide binding in MCF7 cells overexpressing ERβ1, around 60% of the identified genomic binding sites contained AP-1-like binding regions associated with ERE-like sites (Zhao *et al*. 2010). Differential signaling through AP-1 by ERβ and ERα has been reported (Paech *et al*. 1997). It is known that alterations in signaling pathways that impact directly on the ER and/or alternative transcription factor binding partners can also significantly alter the genome-wide binding of ER and estrogen signaling (Bhat-Nakshatri *et al*. 2008). As well other nuclear receptors, such as

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androgen, ER-related, and progesterone receptors, can be expressed variably in the different cell line models used (Muscat *et al*. 2013). Recently, it has been suggested that there may be overlapping transcriptomes and possibly cistromes for some of these receptors and ERα (Ni *et al*. 2011, Hickey *et al*. 2012, Deblois & Giguere 2013). Therefore, altered genome-wide binding resulting in altered transcriptomes due to altered signaling cascades or differential backgrounds of other nuclear receptors may also underlie a bi-faceted activity of ERβ in specific cells.

More recently, accumulating data have brought into focus the possible role(s) of ER proteins outside of the nucleus in breast cancer (Levin 2012, Welsh *et al*. 2012). Rapid, non-genomic activities of estrogen are thought to be mediated by ERs localized to the plasma membrane on some target cells (Levin & Pietras 2008). With respect to differential subcellular localization of ERβ-like proteins, extranuclear vs nuclear localization has been reported to provide differential prognostic information at least in breast cancer *in vivo* (Shaaban *et al*. 2008, Yan *et al*. 2011). It is likely that ERβ located in mitochondria and identified to interact with several mitochondrial proteins (Nassa *et al*. 2011) may have a dual role in mediating tamoxifen-induced apoptosis through increased ROS (Razandi *et al*. 2012). This effect, seen in tamoxifen-sensitive breast cancer cell lines, did not occur in tamoxifen-resistant cells. In contrast, other studies found an association between mitochondrial ERβ expression and protection against radiation and UV-induced cell death (Harrington *et al*. 2003, Pedram *et al*. 2006). Such data also support a potential bi-faceted role of mitochondrial ERβ-like proteins in apoptosis. In the first case, a pro-apoptotic role is likely, whereas in the latter cases, a protective role against cell death could be hypothesized. Differential localization of ERβ within the target cells may therefore also underlie altered function of ERβ as well as its variants.

Summary/conclusions

The importance of ER signaling pathways in breast cancer has been well established, with over 30 years of both basic and clinical research. Excitement surrounded the discovery of a second ER, ERβ, in 1996, mainly due to a rising hope that elucidating its function and mechanism would shed light and bring answers to some of the major discrepancies seen between clinical observations and the established molecular understanding of estrogen signaling based upon the existence of only one receptor, ERα. This excitement has now faded and stalled to some extent. There is no doubt significantly related to the dearth of cell model systems that naturally express detectable ERβ1 and/or its isoforms, as well as the use of less than well-characterized antibodies to detect a protein that is significantly downregulated in most types of immortalized or neoplastic cells.

However, the variability of results can also be explained in part, by the high degree of complexity that is emerging associated with the existence of ERβ-like proteins. The discussion above also highlights some of the issues raised clinically by what we have called the bi-faceted role played by ERβ in breast cancer. Importantly, there are some mechanistic data currently emerging that shed light on the mechanisms involved and to support how this may occur.

The potentially profound impact of ERβ1 being a target for therapy in some ER-negative breast cancers where only few options apart from aggressive chemotherapies are available, as well as emerging new concepts for selectively delivering ligands to specific tissues (Finan *et al*. 2012), supports a continued focus on understanding the molecular mechanisms for the bi-faceted role of ERβ.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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