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Loss of ERβ expression as a common step in estrogen-dependent tumor progression

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Abstract

The characterization of estrogen receptor beta (ERβ) brought new insight into the mechanisms underlying estrogen signaling. Estrogen induction of cell proliferation is a crucial step in carcinogenesis of gynecologic target tissues, and the mitogenic effects of estrogen in these tissues (such as breast, endometrium and ovary) are well documented both *in vitro* and *in vivo*. There is also an emerging body of evidence that colon and prostate cancer growth is influenced by estrogens. In all of these tissues, most studies have shown decreased ERβ expression in cancer as compared with benign tumors or normal tissues, whereas ERα expression persists. The loss of ERβ expression in cancer cells could reflect tumor cell dedifferentiation but may also represent a critical stage in estrogen-dependent tumor progression. Modulation of the expression of ERα target genes by ERβ or ERβ-specific gene induction could explain that ERβ has a differential effect on proliferation as compared with ERα. ERβ may exert a protective effect and thus constitute a new target for hormone therapy, such as ligand specific activation. The potential distinct roles of ERα and ERβ expression in carcinogenesis, as suggested by experimental and clinical data, are discussed in this review.

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Introduction

It is well documented that the mitogenic actions of estrogens are critical in the etiology and progression of human breast and gynecologic cancers (Henderson *et al.* 1988, Pike *et al.* 1993). The promoting effect of estrogens was recently highlighted by the results of large prospective studies, showing that estradiol intake during menopause increased the risk of breast cancer (BC) (Nelson *et al.* 2002, Rossouw *et al.* 2002, Beral *et al.* 2003, Chlebowski *et al.* 2003). In ovarian cancer, although the question is still debated (Coughlin *et al.* 2000), several recent prospective studies have indicated a risk of ovarian cancer for women undergoing long-term estrogen replacement therapy (Rodriguez *et al.* 2001, Lacey *et al.* 2002, Anderson *et al.* 2003, Folsom *et al.* 2004). In contrast, estrogens appear to exert a protective effect on the risk of colon cancer (Rossouw *et al.* 2002).

The effects of estrogens are mediated by estrogen receptor (ER)α and ERβ, which are members of the nuclear steroid receptor superfamily. ERα and ERβ classically mediate their action by ligand-dependent binding to the estrogen-response element (ERE) of target genes, leading to their transcription regulation (Green *et al.* 1986, Kuiper *et al.* 1996, Mosselman *et al.* 1996, Tremblay *et al.* 1997). Both of these proteins have a high degree of homology in the DNA-binding domain (Mosselman *et al.* 1996), but differ considerably in the N-terminal domain and to a lesser extent in the ligand-binding domain (E domain) (Kuiper *et al.* 1996, Mosselman *et al.* 1996). These differences suggest that the two receptors could have distinct functions in terms of gene regulation and biologic responses and may contribute to the selective actions of 17-β-estradiol (E2) in different target tissues (Gustafsson & Warner 2000).

Recently, various studies have shown decreased expression of ERβ mRNA and protein (or an increased ERα/ERβ mRNA ratio) in tumor versus normal tissues in many cancers, including breast, ovary, colon and prostate

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(Brandenberger *et al*. 1998, Pujol *et al*. 1998, Foley *et al*. 2000, Rutherford *et al*. 2000, Campbell-Thompson *et al*. 2001, Roger *et al*. 2001, Fixemer *et al*. 2003). The ERα/ERβ gene expression ratio thus appears to increase during carcinogenesis, suggesting that ERα- and ERβ-specific pathways may have distinct roles in this process (Leygue *et al*. 1998). The differential expression of ERα and ERβ in cancer cells and experimental data on their respective roles on proliferation are reviewed in this report.

Differential ERβ expression as a common feature of estrogen-dependent tumor progression in clinical studies

Analysis of ERα and ERβ expression in estrogen-sensitive cancers (Tables 1–4)

In breast tissues, several studies have indicated an increase in ERα/ERβ mRNA and protein ratios in cancer as compared with benign tumors and normal tissues. In immunochemical analyses, Roger *et al*. (2001) found a higher percentage of ERβ-positive cells in normal mammary glands than in nonproliferative benign breast disease (BBD) (85%), proliferative BBD without atypia (18.5%) and carcinoma *in situ* (33.8%). In contrast, an increase in ERα protein expression was noted during progression. Moreover, ERβ was inversely correlated with Ki67, a marker of cell proliferation. The authors thus suggest that ERβ protects against the mitogenic activity of estrogens in mammary premalignant lesions. This conclusion is also supported by the results of another study (Shaw *et al*. 2002), which revealed lower ERβ protein expression in carcinomas and demonstrated that ERα, but not ERβ, protein expression was correlated with tumor grade. Similar findings were obtained at the mRNA level by the RT-PCR method by Iwao *et al*. (2000), who also showed that ERα mRNA is increased and ERβ mRNA decreased during breast carcinogenesis. Recently, Park *et al*. (2003) compared ERβ mRNA levels in various breast tissues, using mRNA *in situ* hybridization. ERβ expression was decreased in BC and metastatic lymph node tissues as compared with normal mammary and benign breast tumor (BBT) tissues. The intensity and extent of ERβ expression were significantly higher in normal and BBT tissues than in BC or metastatic lymph node tissues (Park *et al*. 2003).

In invasive BC, other studies using immunohistochemistry (IHC) and *in situ* hybridization revealed that ERβ expression was associated with indicators of low biologic aggressiveness (low tumor grade, low S-fraction and negative lymph node status), suggesting that ERβ might be a good prognostic indicator (Jarvinen *et al*. 2000). Omoto *et al*. (2001) in a survival analysis showed that patients with ERβ-positive tumors had increased disease-free survival at 5 years as compared with those with ERβ -negative tumors. Fuqua *et al*. (2003) studied ERβ expression using IHC in a pilot series of 242 BC patients and showed that ERβ expression is not associated with clinical and biologic parameters, including progesterone receptor (PR) expression, tumor grade and S-phase fraction. ERβ was found to be correlated only with aneuploidy. The findings of this study suggested that ERβ could be a useful biomarker on its own in clinical breast tumors. To gain insight into the possible role of ERβ in breast carcinogenesis, Skliris *et al*. (2003) did an IHC analysis of ERβ in 512 breast specimens. Moreover, real-time PCR was used to investigate the ERβ gene methylation status in the ERβ-negative BC cell lines SK-BR-3 and MDA-MB-435. The results suggested that the loss of ERβ expression is one of the hallmarks of breast carcinogenesis, and that it may be a reversible process involving methylation. Zhao *et al*. (2003) also concluded that decreased ERβ mRNA expression may be associated with breast tumorigenesis and that DNA methylation is an important mechanism for ERβ gene silencing in BC (Table 1). Collectively, ERβ expression decreases in the process of BC development.

The ovary (Table 2) contains both ER isoforms, but ERβ seems to be the predominant species expressed in normal ovary in rats (Byers *et al*. 1997) and humans (Kuiper *et al*. 1996, Enmark *et al*. 1997). Our laboratory (Pujol *et al*. 1998) documented an increase in the ERα/ERβ mRNA ratio in ovarian carcinomas as compared with normal ovaries and cysts, and our findings suggested that overexpression of ERα relative to ERβ mRNA may be a marker of ovarian carcinogenesis. This conclusion was further supported by Brandenberger *et al*. (1998) and Rutherford *et al*. (2000). The latter revealed that the balance between ERα and ERβ receptors might be essential for maintaining normal cellular function, suggesting that, as ERβ decreases, uncontrolled cellular proliferation leads to a metastatic state. Lau *et al*. (1999) found no differences in ERβ mRNA expression between normal and cancer epithelial cells, but these authors analyzed only a few HOSE cell primary cultures ($n = 4$) and ovarian cancer cell lines ($n = 3$) by a nonquantitative PCR method. Decreasing levels of ERβ expression seem to be a common denominator between breast and ovarian carcinogenesis.

In prostate, it has been suggested that estrogens and their receptors may be involved in cancer development and progression (Santti *et al*. 1994, Farnsworth *et al*. 1999, Jarred *et al*. 2000). Estrogen exposure during prostate development may initiate cellular processes resulting in future neoplasia (Santti *et al*. 1994). In a study of Latil *et al*. (2001), ERα and ERβ mRNA expression was quantified by real-time RT-PCR in both benign and malignant prostate.

Table 1 Relative expression of ERα and ERβ in breast tumor progression

References	Tissues	Number	Methods	ERα		ERβ		Comments
				SQ Ov		SQ Ov		
Roger *et al.* (2001)	Normal	118	IHC	+	↑	+++	↓↓	ERβ positive
	NP-BBD	18		+		++		Cells decrease during
	P-BBD	37		++		++		preinvasive tumor progression
	P-BBDWA	13		++		+		
	CIS	25		++		+		
	High-grade CIS	35		++		−		
Iwao *et al.* (2000)	Normal	11	Real-time-PCR	++	↑	+++	↓	Changes in ERβ1 and ERβ2 mRNA
	Cancer	112		+++		++		levels in breast cancer
Park *et al.* (2003)	Normal	89	ISH			+++		ERβ mRNA level decreases during
	BBT	11				+++		tumor progression
	Breast cancer	85		/		+	↓	High ERβ level associated with
	Met. lymph node	10				+		poor differentiation
Skliris *et al.* (2003)	Normal	138	IHC			++++		Reduced expression of ERβ
	PDCIS	16				+++		in invasive breast cancer
	Invasive cancers	319				++		Loss of ERβ may be a reversible
	Met. lymph node	31		/		+	↓	process involving methylation
	Recurrences	8				+		
Speirs *et al.* (1999)	Normal	23	RT-PCR	+	↑	+++	↓↓	22% of normal breast expressing exclusively ERβ mRNA
	Cancer	60		+++		+		50% of breast tumors coexpressing ERα and ERβ
Leygue *et al.* (1998)	Normal (adjacent tissues)	18	Multiplex RT-PCR	+	↑	++	↓	Increase in ERα and decrease in ERβ during tumor progression
	Cancer	18		++/+++		+		
Gustafsson & Warner (2000)	Normal BBD Cancer	Total of 30 samples	RT-PCR Western blot, IHC		↑		↓	ERβ is the predominant form in normal mammary gland

The number of + indicates the ER's relative expression. The arrows indicate a decrease (↓), an increase (↑) or no variations in expression (⟷) between normal and cancer tissues. SQ = semiquantitative, Ov = overall trends, BBD = benign breast disease, NP-BBD = non proliferative BBD, P-BBD = proliferative BBD, P-BBDWA = proliferative BBD with atypia, BBT = benign breast tumors, CIS = carcinoma *in situ*, HGPIN = high-grade prostatic intraepithelial neoplasia, ISH = *in situ* hybridization, IHC = immunohistochemistry, Met = metastatic, RT-PCR = reverse transcription polymerase chain reaction.

ERβ mRNA level was decreased in most of the tumor samples as compared with normal prostate, suggesting that ERα and ERβ expression status could be used to identify advanced prostate tumor patients. This result is in agreement with those obtained at the protein level. Pasquali *et al.* (2001*a*) investigated ERβ expression in benign and malignant prostate tissue specimens, using a polyclonal antibody directed against the C-terminal domain of the ERβ protein. In contrast to normal tissues, ERβ nuclear immunostaining was undetectable in all cancer sections, showing that malignancy seems to be associated with the disappearance of ERβ expression in prostate tissue. Horvath *et al.* (2001), using IHC, also found that the ERβ protein was progressively lost in hyperplasia and neoplastic lesions. This is in agreement with the results of Fixemer *et al.* (2003) in a study in which a new monoclonal antibody revealed the differential expression of ERβ in tissue sections from 132 patients with prostate cancer. Moreover, these authors showed partial loss of ERβ in high-grade prostatic intraepithelial neoplasia (HGPIN) (Table 3). Once more, the change in ERα/ERβ ratio seems to be correlated with malignancy.

In colon cancer, the protective effect of estrogen replacement therapy is supported by a number of clinical observations (Calle *et al.* 1995, Newcomb *et al.* 1995, Persson*et al.* 1996, Kampman *et al.* 1997), including the results of recent randomized studies named 'WHI' (Nelson *et al.* 2002, Rossouw *et al.* 2002). These studies demonstrated that women with a history of current or past hormone replacement therapy had a significantly decreased risk of colon cancer. These findings have led many investigators to search for the biologic mechanisms

Table 2 Relative expression of ERα and ERβ in ovarian tumor progression

References	Tissues	Number	Methods	ERα		ERβ		Comments
				SQ Ov		SQ Ov		
Pujol *et al.* (1998)	Normal Cysts Borderline tumors Cancers	6 24 3 10	Competitive RT-PCR	+ + ++ ++	↑	+++ +++ ++ +	↓	ERα/ERβ mRNA ratio increases during tumor progression
Brandenberger *et al.* (1998)	Normal Cancer	10 10	Northern blot RT-PCR	++ +++	↑	++ +	↓	ERβ mRNA level decreases in cancer
Rutherford *et al.* (2000)	Normal Primary cancer Met. cancer	9 8 8	RT-PCR Western blot	++ ++ +++	↑	++ + –	↓↓	ERβ mRNA and protein levels decrease in ovarian cancer and metastases

by which estrogen may influence the pathogenesis of colorectal cancer. Since ERα is reported to be minimally expressed in normal colon mucosa and colon cancer cells (Waliszewski *et al*. 1997, Campbell-Thompson *et al*. 2001), the effects of estrogen on colon cancer susceptibility may be mediated by ERβ. Using semiquantitative RT-PCR, Campbell-Thompson *et al*. (2001) showed that ERβ is the predominant ER subtype in the human colon, and that decreased ERβ1 (ERβwt) and ERβ2 (ERβcx) mRNA levels are associated with colonic tumorigenesis in women. In a recent study using IHC analysis, Konstantinopoulos *et al*. (2003) showed that ERβ expression was significantly lower in colon cancer cells than in normal colonic epithelium, and that there was a progressive decline in ERβ expression, which paralleled the loss of malignant colon cell dedifferentiation. These findings are in accordance with a previous study of Foley *et al*. (2000), who also detected a selective loss of ERβ protein in

Table 3 Relative expression of ER and ERβ in prostate tumor progression

References	Tissues	Number	Methods	ERα		ERβ		Comments
				SQ Ov		SQ Ov		
Latil *et al.* (2001)	Normal Cancer	4 23	Real-time PCR	++ + to ++	↔	+++ +	↓	ERβ mRNA expression decreases in the hormone-resistant group
Pasquali *et al.* (2001*a*)	Normal Cancer	5 10	IHC	/ /		+++ +	↓	ERβ protein expression decreases in cancer
Pasquali *et al.* (2001*b*)	Normal Cancer	6 5	RT-PCR Western blot	++ ++	↔	++ +	↓	ERβ mRNA expression decreases in cancer
Horvath *et al.* (2001)	Normal Hyperplasia Cancer	5 157 159	IHC	/ / /		+++ – or + – or +	↓	Loss of ERβ protein expression during tumor progression
Leav *et al.* (2001)	Dysplasia - moderate grade - high grade Carcinoma - grade III - grade IV/V Metastasis	Total of 50 samples	IHC RT-PCR	– – –/+ –/+ – / /	↔	+ – + –/+ + / /	↔	ERβ protein and mRNA expression decrease in high-grade dysplasia and carcinoma
Fixemer *et al.* (2003)	HGPIN Adenocarcinoma Gleason grade: III IV V Metastatic	47 17 29 14 12	 IHC Monoclonal antibody	 / / / /		+++ + ++ + +	↓	ERβ protein expression decreases during tumor progression ERβ expression higher in Gleason grade IV than in grades III and V

Table 4 Relative expression of ERα and ERβ in colon tumor progression

References	Tissues	Number	Methods	ERα		ERβ		Comments
				SQ Ov		SQ Ov		
Campbell-Thompson *et al.* (2001)	Normal	26	RT-PCR	+	↔	+++	↓	ERβ1 and ERβ2 mRNA expressions decrease in cancer
	Cancer	26	Southern blot	+		+		
Foley *et al.* (2000)	Normal	11	RT-PCR	+	↔	+++	↓	Decrease ERβ protein but not mRNA expression in cancer Post-transcriptional mechanism?
	Cancer	11	Western blot	+		+		

malignant human colon by Western immunoblotting. Weyant *et al*. (2001) worked with a model of mice bearing germline mutations in murine Apc. These mice develop multiple intestinal tumors that show loss of wild-type Apc protein. In this model, E2-induced prevention of Apc-associated tumor formation was correlated with an increase in ERβ protein and a decrease in ERα in target tissues. Altogether, these results strongly suggest that ERβ protects against colon carcinogenesis (Table 4).

ERβ as a predictive factor for antiestrogen therapy?

Although many reports suggest the protective role of ERβ against tumor progression, controversies have arisen regarding the clinical value of ERβ expression in terms of predicting the adjuvant hormonal therapy response in breast cancer. Some studies suggest that the ERβ status in BC is a predictor of the response to tamoxifen (Leygue *et al*. 1998, Jarvinen *et al*. 2000, Mann *et al*. 2001) whereas others suggest that ERβ is significantly upregulated in tamoxifen-resistant breast cells and could be involved in tamoxifen resistance (Speirs *et al*. 1999).

The type of analysis, patient selection criteria, the type of splicing variants detected in RNA analyses or the small number of patients analyzed to date could ultimately explain these controversial results. The first findings were obtained in studies involving RT-PCR-based techniques, but the quantification of gene expression at the mRNA level may not be directly linked qualitatively or quantitatively to the protein expression. There have been very few studies in which ERs were measured by Western immunoblotting or IHC because of the lack of reliable antibodies. Finally, the choice of statistical analysis and different parameters selected for analysis could also influence the results.

ERβ as a potential tumor-suppressor gene?

The results of these different studies, showing a loss of ERβ expression in cancer as compared with normal cells, are in line with the hypothesis that the ERβ gene may act as a tumor suppressor (Iwao *et al*. 2000). This concept needs to be confirmed but could make sense in view of the location of ERβ on chromosome 14q (Enmark *et al*. 1997). A loss of 14q has been detected by comparative genomic hybridization in some breast cancers (Burki *et al*. 2000, Loveday *et al*. 2000). Interestingly, in ovarian cancer, two potential tumor-suppressor gene loci have been mapped to 14q (Bandera *et al*. 1997). 14q deletions are also observed in colon carcinoma (Young *et al*. 1993) and prostate cancer (Kasahara *et al*. 2002). These overall findings suggest a potential tumor-suppressive function for ERβ. However, further studies are required before definitive conclusions on the tumor-suppressive function of ERβ can be drawn.

What are the potential molecular mechanisms underlying ERα and ERβ differential actions?

Several *in vitro* studies have focused on the molecular mechanisms underlying the differential roles of ERα and ERβ. Differences in ligand affinity, transcriptional activation, interactions with cofactors or putative heterodimerisation have been proposed.

Structural properties of ERα and ERβ and effects on their transcriptional activities

ERα and β belong to the large nuclear steroid/thyroid hormone receptor family. Like most other members of the family, ERs have a modular architecture of four interacting domains: the N-terminal A/B domain, the C or DNA-binding domain (DBD), the D or hinge domain and the C-terminal E/F or ligand-binding domain (LBD) (Fig. 1). There is only 56% amino-acid identity between the two receptors in the LBD, whereas the homology in the DBD is 97%. This suggests that ERβ would recognize and bind to the same EREs as ERα, but that each receptor might have a distinct spectrum of ligands (Kuiper & Gustafsson 1997). A number of novel selective ER subtype ligands have now been developed. The propyl pyrazole triol (PPT) com-

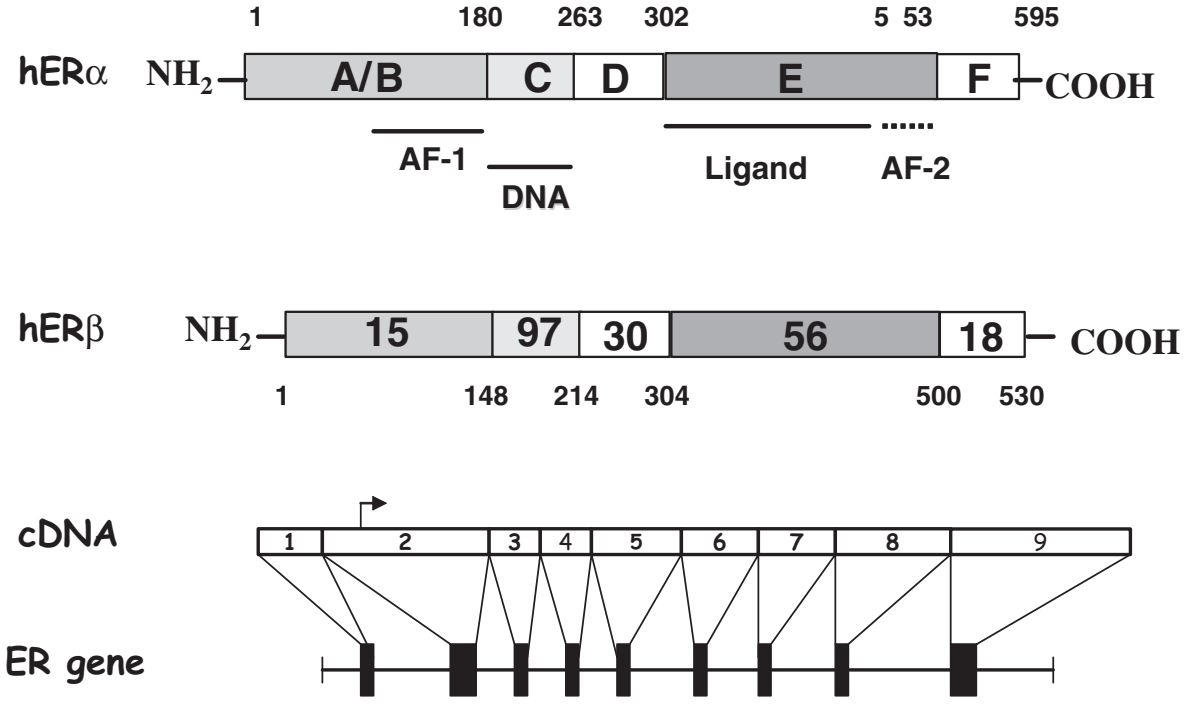

Figure 1 Schematic representation of the structure of human (h)ERα and ERβ nuclear receptors. The A/B domain at the NH-2 terminal contains the ligand-independent transcriptional-activation function AF-1, the C domain represents the DNA-binding-domain, the D domain corresponds to the hinge region, and the E domain contains the hormone-binding domain and the hormone-dependent transcriptional-activation function AF-2. Numbers outside each box refer to amino acid number, whereas the number inside each box of ERβ refers to the percentage of amino acid identity. The arrow indicates the translation starting site in ER cDNA.

pound was found to be an ERα-specific agonist, activating gene transcription only through ERα (Sun *et al*. 1999, Stauffer *et al*. 2000). A number of other known ligands are also somewhat ERβ selective. Some phytoestrogens, such as genistein and coumestrol, show a higher affinity toward ERβ than ERα (Kuiper & Gustafsson 1997). The diarylpropionitrile (DPN) compound is a potency-selective agonist for ERβ with a more than 70-fold higher binding affinity for ERβ than ERα (Meyers *et al*. 2001). Recently, Ghosh *et al*. (2003) have investigated a novel series of heterocycle ligands for the ERs based on a diazene core motif. In this process, they have found diazenes that have high binding affinity for the ERs, and some of these show preferential affinity for ERα or for ERβ.

The N-terminal domain of nuclear receptors encodes a ligand-independent activation function (AF-1) (Tora *et al*. 1989, Berry *et al*. 1990, McInerney & Katzenellenbogen 1996), a region of the receptor involved in protein–protein interactions (Onate *et al*. 1998), and transcriptional stimulation of target gene expression. The activation function-2 (AF-2) domain, located in the LBD (Tora *et al*. 1989), is responsible for hormone-dependent activation through recruitment of coactivator proteins (Tremblay *et al*. 1997, White *et al*. 1997). There is very little conservation in the N-terminal AF-1 domain, a fact which could explain why different sets of proteins in the transcription complexes may interact with ERα and ERβ and direct them to specific targets. Dissimilarity in the NH_2-terminal extremity of ERα and ERβ is one possible explanation for the difference in the response of the two receptors to various ligands. In fact, the two receptors are distinct in their responses to the synthetic antiestrogens tamoxifen, raloxifen and ICI-164,384. On an ERE-based reporter gene assay, tamoxifen, 4-OH-tamoxifen, raloxifen and ICI-164,384 have an ERα-selective partial agonist/antagonist function but pure ER antagonist effect through ERβ (McDonnell *et al*. 1995, Barkem *et al*. 1998, McInerney *et al*. 1998). Watanabe *et al*. (1997) showed that the agonistic effect of tamoxifen depends on the cell type, ERE-promoter context, and ER subtypes, and that this action is ERα specific. Tamoxifen is an ERα antagonist in breast (Jordan *et al*. 1992) but an agonist in bone (Love *et al*. 1992) and uterine tissues (Kedar *et al*. 1994). Raloxifene is also an ERα antagonist in breast tissue, but it exerts agonistic activity in bone, but not in uterine tissue (Black *et al*. 1994).

ERα and ERβ are capable of regulating gene transcription through a classical mechanism involving the consensus ERE, but ERβ seems to be a weaker transactivator (Cowley *et al*. 1999). Cowley and Parker (1999) have shown that the AF-1 activity of ERβ is weak compared with that of ERα on estrogen-responsive reporters, whereas their AF-2 activities are similar. In turn, when both AF-1 and AF-2 functions are active in a particular cell and/or on a particular promoter, the activity of ERα greatly exceeds that of ERβ, whereas ERα and ERβ activities are similar when only AF-2 is required (McInerney *et al*. 1998, Cowley *et al*. 1999). ERα and ERβ have similar but also different effects on gene transcription mediated via the ERE. To date, only a limited number of genes have been shown to be regulated by one of the two E2-liganded ER subtypes in this classical mode of action. In this way, the gene encoding the catalytic subunit of human telomerase hTERT is regulated by ERα, and not by ERβ in human ovary epithelium cells (Misiti *et al*. 2000) and in human prostate cancer (Nanni *et al*. 2002). In the same way, Lazennec *et al*. (2001) reported that, ERα, but not ERβ, was able to regulate c-myc proto-oncogene expression. The metallothionein gene is known to be specifically upregulated by E2 via ERβ in SAOS-2 cells (Harris *et al*. 2001). However, recently, Stossi *et al*. (2004) have compared the gene-regulatory activities of ERα and ERβ in bone and showed high similarity but also significant differences in gene targets for these two ERs. Thus, genes encoding for cystatin D, autotaxin or stromal antigen 2 appear to be E2-regulated specifically by ERβ in human osteosarcoma cells.

Estrogens (and antiestrogens) also transcriptionally regulate target genes via ERs though a non-ERE mode of action. These effects are mediated through promoter elements that bind various transcription factors, including AP-1-binding sites (Webb *et al*. 1995), Sp1 binding sites (Porter *et al*. 1997), SF1 response element (SFRE) (Vanacker *et al*. 1999), electrophophilic/antioxidant response element (EpRE/ARE) (Montano *et al*. 1997) and cyclic AMP response element (CRE) (Sabbah *et al*. 1999). At AP-1 sites, ERα and ERβ could have opposite transcriptional effects in some circumstances (Paech *et al*. 1997). In fact, ERβ is able to potentiate an AP-1-containing reporter in the presence of the antiestrogen tamoxifen, but not in the presence of estrogens in a tissue-specific manner. ERα stimulates AP-1 activity in the presence of antiestrogens in endometrial cells (Webb *et al*. 1995, Paech *et al*. 1997), but antiestrogens decrease or have no effect on AP-1 activity in BC cells (Philips *et al*. 1993, Webb *et al*. 1995). Of particular note, ERβ is more potent overall than ERα on AP-1 sites, whereas the contrary occurs on EREs (Paech *et al*. 1997, Cowley *et al*. 1999, Hall *et al*. 1999). Similar to AP-1, E2 binding to ERα induces transcriptional activation when associated with SP1 in GC-rich regions. However, E2 interaction with ERβ does not result in the formation of a transcriptionally active complex at a promoter containing Sp1 elements (Saville *et al*. 2000). Vanacker *et al*. (1999) found that the osteopontin gene promoter is stimulated through SFRE sequences by ERα, but not by ERβ.

Consequently, these differences in ligand interaction or transcriptional activity between the two ER subtypes may account for the major differences in their tissue-specific biologic actions. This complexity is further enhanced by ERβ isoforms, the ability of ERs to form homodimers and heterodimers, and their capacity to interact with various coregulators.

ER isoforms

Several groups have reported and cloned different ERβ isoforms with exon deletions (Lu *et al*. 1998), insertions (Hanstein *et al*. 1999), or C-terminal splice variants (Moore *et al*. 1998, Ogawa *et al*. 1998). These isoforms can also bind ligands, mediate estrogen signaling (Kuiper & Gustafsson 1997, Paech *et al*. 1997, Cowley *et al*. 1999, Bollig *et al*. 2000) and exhibit different properties, thus further enhancing the complexity in the spectrum of potential cellular responses to estrogen. The key element lies perhaps in the balance between the expression of these different variants and their relative quantities. It has been shown that ERβ splice variants have dramatically different localization patterns in living cells, and this localization can be altered by estrogen agonists and antagonists (Price *et al*. 2001). Interestingly, Poola *et al*. (2002) recently showed that ERβ splice variant mRNAs were differentially altered during breast carcinogenensis. ERβcx, which utilizes an alternative exon 8, is the most extensively studied splice variant. Ogawa *et al*. (1998) showed that this isoform may act as a potential inhibitor of ERα transactivation, possibly due to ERα/ERβcx heterodimer formation. Using IHC, it has been shown that differential expression of ERβwt and ERβcx may be used as a prognostic marker in human prostate (Fujimura *et al*. 2001). Peng *et al*. (2003) showed that all ERβ isoforms inhibited ERα transcriptional activity on an ERE, while only ERβwt had transcriptional activity of its own. It has been shown, using cDNA microarrays in MCF-7 cells stably transfected with ERβwt and ERβcx MCF-7, that these two isoforms inhibit ERα function differently (Omoto *et al*. 2003). Consequently, it can be hypothesized that the differential expression of ERβ isoforms may have a role in the modulation of estrogen action.

ER homo- and heterodimers

The functional formation of ERα and ERβ heterodimers has been demonstrated (Cowley *et al*. 1997, Pettersson *et al*. 1997). They are able to bind to DNA with an affinity similar to that of ERα and greater than that of ERβ homodimers, to interact with coactivators, and to stimulate the transcription of reporter gene in transfected cells (Cowley *et al*. 1997, Pettersson *et al*. 1997). The possible involvement of ERα and ERβ dimerization would increase the complexity of transcription activation in response to E2, suggesting the existence of two previously unrecognized estrogen-signaling pathways, that is, ERβ homodimers and ERα/ERβ heterodimers. Moreover, it has been reported that various ERα and ERβ ratios in different cells, resulting in different homodimer and heterodimer compositions, may constitute a key to gaining insight into the tissue-specific effects of estrogen and antiestrogens (Kuiper *et al*. 1997). Homodimers and heterodimers could bind to distinct response elements and consequently activate specific gene-expression patterns in given target tissues. For such interactions, ERα and ERβ must be coexpressed in cells, as noted in breast, ovarian and endometrium tissues. However, future studies will be required to determine the physiologic roles of ERα and ERβ homo- and heterodimers *in vivo*.

Interactions with coactivators and corepressors

There is one further confounding factor in the ER-mediated estrogen action equation. The ER-mediated transcriptional activity of estrogen is influenced by several regulatory factors, known as coactivators and corepressors, which activate or repress the transcription of ER-responsive genes (Klinge *et al*. 2000). The p160/SRC (steroid receptor coactivator) family is one of the most studied classes of coactivators, and it includes SRC1, SRC2 (GRIP1/TIF-2) (McKenna *et al*. 1999) and other more recently described coactivators such as ACTR (Chen *et al*. 1997), RAC3 (Li *et al*. 1997), AIB1 (Anzick *et al*. 1997) and TRAM-1 (Takeshita *et al*. 1997). Most of interactions of these coregulators with the ER are ligand-dependent, but some coactivators have also been shown to be recruited in a ligand-independent manner by the AF-1 domain of ERs (McInerney *et al*. 1996, Tremblay *et al*. 1999). SRC-1 activated ERβ AF-1 upon MAPK-induced phosphorylation of serine residues (Tremblay *et al*. 1999). Deblois *et al*. (2003) studied the steroid receptor RNA activator (SRA) and showed that SRA potentiated the estrogen-induced transcriptional activity of both ERα and ERβ. They demonstrated that the transcriptional activity of ERα can be enhanced by SRA in a ligand-independent manner through the AF-1 domain. However, this AF-1-dependent effect of SRA is not observed on ERβ. Very few receptor-specific ERβ cofactors have been identified so far. Warnmark *et al*. (2001) showed that TRAP220 displays a preference for ERβ and suggested that the coregulator selectivity of ER subtypes is an additional layer of specificity that influences the transcriptional response in estrogen target cells. Using multiplex RT-PCR, Kurebayashi *et al*. (2000) also showed that ERβ-expression levels were correlated with some activators such as AIB1, CBP, P/CAF, and a corepressor, N-CoR, but the significance of this correlation is unclear. Nuclear receptors usually bind the corepressors N-CoR and SMRT in the absence of ligand or in the presence of antagonists. Agonist binding leads to corepressor release and coactivator recruitment. Webb *et al*. (2003) recently demonstrated that, *in vitro* and *in vivo*, ERβ binds to N-CoR and SMRT in the presence of ER agonists, such as estradiol, and phytoestrogens, such as genistein, but not in the presence of antagonists. ERα and ERβ present completely distinct modes of action with coregulators, a fact which could be of major importance in terms of potential effects on physiologic behavior (Webb *et al*. 2003).

What do we know about the role of ERβ in cell proliferation and death?

ERβ and cell proliferation

Although the specific functions of ERβ in cancer are not known, there is some evidence that ERβ could have inhibitory effects on cellular proliferation. First, as indicated previously, the levels of ERβ are highest in normal tissue (breast, ovary and prostate) as well as in benign disease, and they decrease during carcinogenesis (Tables 1–3). Our laboratory obtained the first evidence that ERβ is an important modulator of proliferation and invasion of breast and ovarian cancer cells, thus supporting the hypothesis that the loss of ERβ expression could be one of the events leading to breast and ovarian cancer development (Lazennec *et al*. 2001, Bardin *et al*. 2004). Whereas ERα was able to regulate reporter genes and endogenous genes in a ligand-dependent manner, ERβ inhibited MDA-MB231 cell proliferation in a ligand-independent manner. This suggests that the two ERs inhibit cancer cell proliferation via different mechanisms (Lazennec *et al*. 2001).

Omoto *et al*. (2003) recently developed cell lines expressing ERβwt and ERβcx by stable transfection of each expression plasmid in MCF7 cells and demonstrated that this constitutive expression significantly reduced the percentage of cell population in S-phase and the number of colonies in an anchorage-independent assay. Recently,

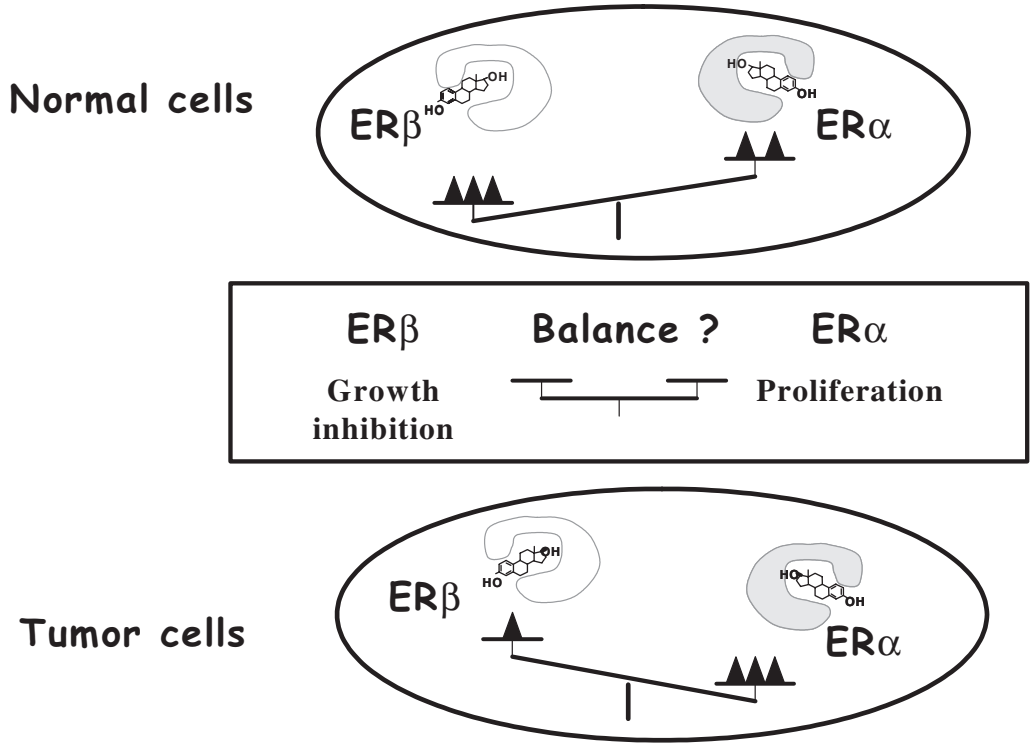

Figure 2 Schematic representation of ERα and ERβ imbalance in estrogen-dependent tumor progression.

two studies showed that the induced expression of ERβ in ERα-positive BC cells inhibits their growth (Paruthiyil *et al*. 2004, Strom *et al*. 2004). These reports also suggest that ERβ might reduce cell proliferation by inhibiting the cyclin D1 gene, a key factor controlling the G1-S transition of the cell cycle, and thus cell proliferation. Strom *et al*. (2004) also indicated that numerous other components of the cell cycle associated with proliferation, such as cyclin E or Cdc25A, were decreased. These results are in accordance with the study of Bièche *et al*. (2001), showing a negative correlation between ERβ and CCND1 (cyclin D1) expression. *In vitro* studies support the hypothesis of Liu *et al*. (2002), who showed that E2 activates cyclin D1 gene transcription through ERα, but inhibits cyclin D1 gene transcription through ERβ in HeLa cells.

The contrasting phenotypes observed in individual lines of $ER^{-/-}$ mice, that is, ERαKO and ERβKO, which exhibit phenotypes that generally mirror the respective ER-expression patterns, provides further evidence that the two ERs have distinct biologic functions. Weihua *et al*. (2000) observed that, in the immature uterus, ERα and ERβ are expressed at comparable levels in the epithelium and stroma, and E2 treatment decreases ERβ in the stroma. Increased cell proliferation and the exaggerated response to E2 in ERβKO mice suggests that ERβ plays a role in the modulation of the effects of ERα and also (or consequently) has an antiproliferative function in the immature uterus. A second study in $ERβ^{-/-}$ mice showed that ERβ is implicated in the regulation of epithelial growth, and its absence results in hyperplasia of the prostatic epithelium (Weihua *et al*. 2001). The inhibition of ERα transcriptional activity could be a molecular mechanism by which ERβ has antiproliferative effects. Previous *in vitro* data indicate that ERβ could act as a dominant negative regulator of ERα activity. Hall *et al*. (1999) have provided direct proof that ERβ modulates or represses ERα transcriptional activity in transient transfection cells. In bone, it has been shown that ERβ inactivation by gene targeting results in increased cortical bone formation. Windalh *et al*. (2001) showed that, when present, ERβ acts in a repressive manner on trabecular bone, possibly by inhibiting the stimulatory action of ERα. Finally, Lindberg *et al*. (2003) showed that in some mouse tissues, ERβ reduces ERα-regulated gene transcription, thus indicating that there is a balanced relationship between ERα and ERβ (Fig. 2).

ERβ and apoptotic pathways?

A decrease in the human cancer cell population *in vitro* or tumor regression *in vivo* reflects a change in the balance of cellular growth events and could involve arrested cell proliferation or an enhanced cell death, or both.

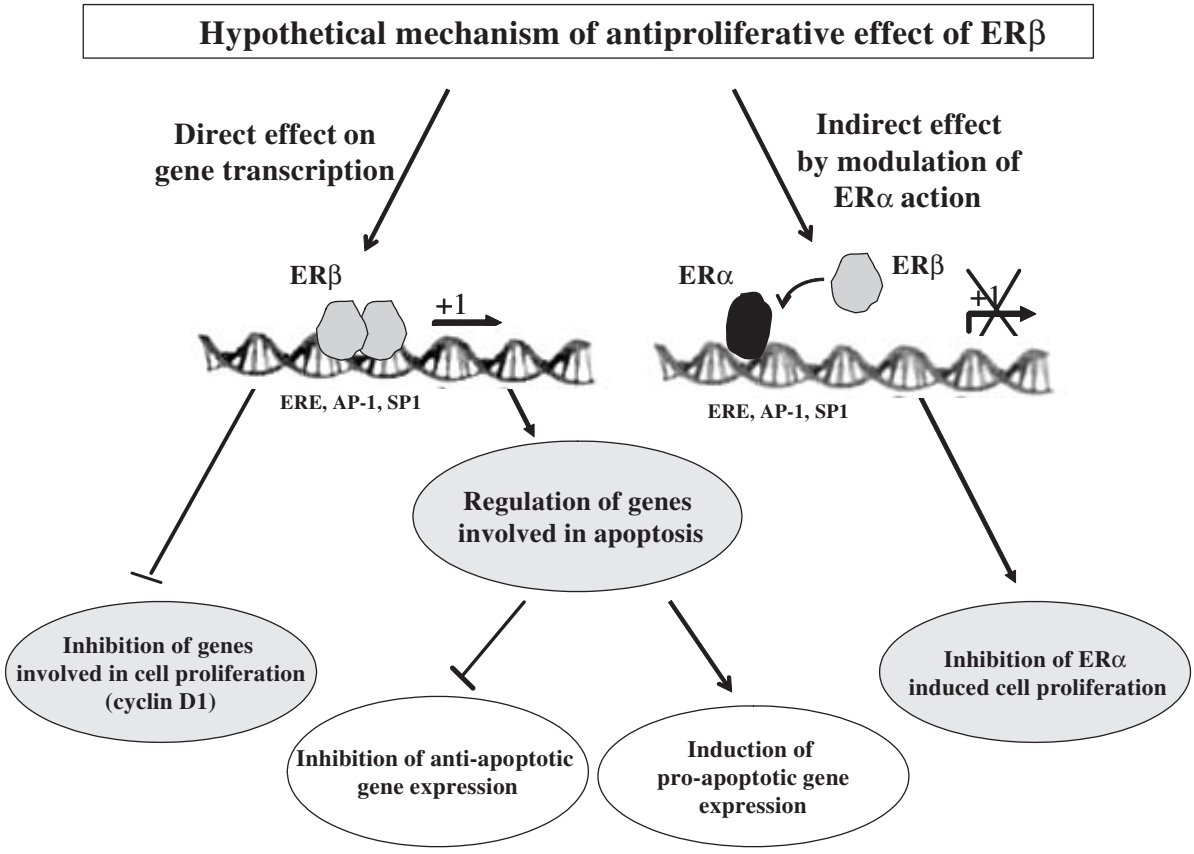

Figure 3 Hypothetical mode of ERβ action on cell proliferation pathways.

Several studies have suggested that estrogen may regulate apoptotic pathways in cancer (Kyprianou *et al*. 1991, Perillo *et al*. 2000, Choi *et al*. 2001). We could assume that E2 effects involve both proliferation induction and apoptosis inhibition. Choi *et al*. (2001) showed that E2 may be associated with upregulation of the antiapoptotic bcl-2 gene at the mRNA level. It has been suggested that ERα may play a role in ovarian tumorigenesis by preventing apoptosis, whereas the ERβ-induced inhibition of proliferation could be explained by the inhibition of the bcl-2 gene, as supported by a recent report of Nilsen *et al*. (2000). They showed that estradiol can function as a neuroprotective agent or an inducer of apoptosis, depending on the ER-subtype present in the cell. ERα is thus associated with a neuroprotective effect, while ERβ mediates the induction of apoptosis in neuronal cells. Similarly, Sapi *et al*. (2002) demonstrated estrogen-induced upregulation of FasL, an apoptotic protein ligand, in ovary. This may seem paradoxical since estrogen is known to be antiapoptotic in different cells. The authors proposed that in normal ovary the apoptotic protein ligand FasL is probably upregulated by ERβ, the predominant form of ER in this tissue (Fig. 3). Recently, we have demonstrated (Cheng *et al*. in press) that the expression of ERβ in prostate carcinoma cells triggers apoptosis, notably by increasing baxα levels, as well as cleavage of PARP and caspase-3 expression. We also observed pro-apoptotic effects of ERβ in ovarian cancer cells (Bardin *et al*. 2004).

Conclusions

Numerous clinical and *in vitro* studies suggest that imbalanced ERα/ERβ expression is a common feature and could be a critical step of estrogen-dependent tumor progression. ERβ seems to play a key role in the mitogenic action of estrogen by providing protection against ERα-induced hyperproliferation. A role in apoptosis might also be possible.

ERα and ERβ have some overlapping tissue distribution but also display high relative tissue-specific expression. Moreover, a number of molecular mechanisms may explain the differential roles of ERα and ERβ, including differences in ligand affinity and transactivation, distinct

cofactor interactions and putative heterodimerization. Splicing variant ERs isoforms may also be important in modulating the cellular response.

In conclusion, the imbalance in ERα/ERβ expression in estrogen-dependent cancer opens a new field in hormone therapy of cancer. Targeted ERβ therapies, including the development of ERβ specific ligands, may constitute a new therapeutic approach, particularly for pre-invasive or proliferative lesions. The clinical value of ERβ in cancer prognosis and its possible usefulness for prediction of the hormone response should be assessed in large-scale and prospective clinical studies.

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