# 16 Androgen Receptor and Estrogen Receptors

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*Contents*



## 1. GENERAL INTRODUCTION

The androgen receptor (AR) and the estrogen receptors (ER) are members of the nuclear receptor (NR) family. These NRs are distinguished from the other transcription factors by their ability to control gene expression upon ligand binding (steroids, retinoids, thyroid hormone, vitamin D, fatty acids, and other small hydrophobic molecules). Their combined effects are vast, influencing virtually every fundamental biological process, from development and homeostasis, to proliferation and differentiation.

All NRs display a modular structure, with five to six distinct regions, termed A–F *(1)*. (Fig. 1) The N-terminal A/B region contains the activation function (AF-1) that can activate transcription constitutively. Region C encompasses the DNA-binding domain (DBD). Region E consists of the ligand-binding domain (LBD), a dimerization surface, and the ligand-dependent transcriptional activation function 2 (AF-2).

The NR can be separated into two classes based on their association with other proteins in the absence of hormone *(2)*. Figure 2 shows the classic ligand-dependent activation of the steroid/thyroid hormone receptor family members that interact

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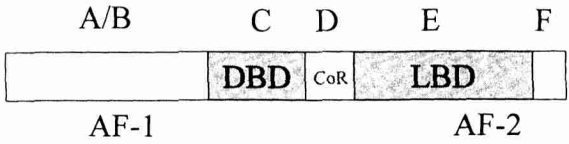


**Fig. 1.** Functional domains of nuclear receptors. A/B and F domains vary in size and primary sequence among the superfamily. CoR refers to corepressor binding site present in some nuclear receptors. AF-1 and AF-2 refer to two distinct activation functions.

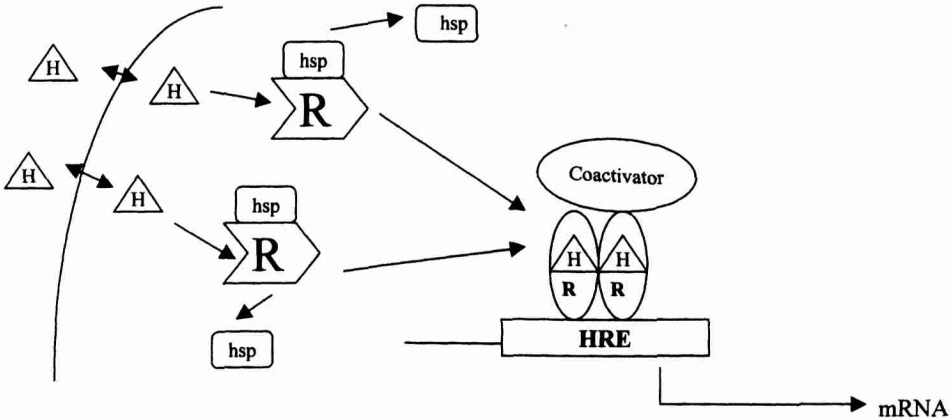


**Fig. 2.** Activation of steroid hormone receptors. In the absence of hormone, receptor monomers (R) are associated with heat shock proteins. Hormone (H) diffuses freely into the cell, binds to the receptor resulting in dissociation of the heatshock proteins, dimerization of the receptor and binding to target DNA sequences (HRE). Subsequently coactivators are recruited producing a transcriptionally active complex.

with heat shock proteins. Included in this group are the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor. In the absense of hormone, each receptor monomer is associated with a protein complex that contains heat shock protein 90 as well as a number of other proteins. This receptor complex is incapable of binding to DNA, and is either cytoplasmic or loosely bound in the nucleus. The steroid diffuses into the cell and binds to the ligand-binding domain of the receptor, inducing a conformational change that favors dissociation of the heat shock protein complex and tight binding to DNA. The receptors bind as homodimers or heterodimers to specific response elements that consist of inverted palindromes separated by three nucleotides *(3)*; they then interact with basal transcription factors, coactivators, and other transcription factors to induce and/or repress transcription of the target gene.

Receptors such as the thyroid hormone receptor, retinoic acid receptor, and vitamin D receptor do not interact with the heat shock proteins, and bind to DNA in the absence of ligand, repressing the transcription of the target gene. These receptors typically form heterodimers with retinoid X receptors. In the absence of ligand, these receptors interact with repressor proteins, blocking the activity of the dimer. Ligand binding induces dissociation of the corepressor, allowing interaction with other transcription factors and coactivators, which results in induction of target genes.

The activity of nuclear receptors is modulated by interactions with other proteins. Multiple coactivators have been identified that connect or bridge the DNA-bound NR to proteins in the preinitiation complex and thereby enhance transcription. Besides this bridging function, some coactivators can modify chromatin by histone acetylation and make promoters more accessible for the binding of other transcription factors. The opposites of coactivators are corepressors, which are recruited into the receptor-DNA-bound complex in the absence of ligand and actively inhibit transcription of the target gene through recruitment of histone deacetylases (HDACs) *(4)*.

It is becoming increasingly apparent that besides binding of their cognate ligands, non endocrine pathways, including those involving protein kinases and metabolic products, play a role in NR signaling. Whether a receptor can be activated in the absence of hormone appears to depend upon a number of factors, including the receptor type, the cell and promoter context, and the activation of the signalling-specific pathways.

## 2. THE ANDROGEN RECEPTOR

### *2.1. Introduction*

Prostate cancer is the second leading cause of death among men in Western countries *(5,6)*. The prostate is an androgen-regulated organ. Prostate development from the urogenital sinus, as well as its growth, differentiation, and maintenance of function in adult life, depend on androgen activity *(7,8)*. The androgen testosterone (T) is mainly produced and secreted by Leydig cells in the testis and is converted into dihydrotestosterone (DHT) by the 5α-reductase enzymes *(9)*. The 5α-reductase enzyme type II is expressed in the male urogenital tract and is responsible for the conversion of testosterone to dihydrotestosterone in the prostate. DHT is the more potent androgen, with a higher binding affinity to the AR *(10)*. Besides the testis, the adrenals secrete large amounts of the inactive precursor steroids dehydroepiandrosterone (DHEA), its sulfate (DHEAS), and androstenedione. DHEA and androstenedione can be converted to T in most peripheral tissues, including the prostate.

As is the case with normal prostate development, prostate cancer is, at least initially, largely dependent on androgens for growth and survival. Most patients

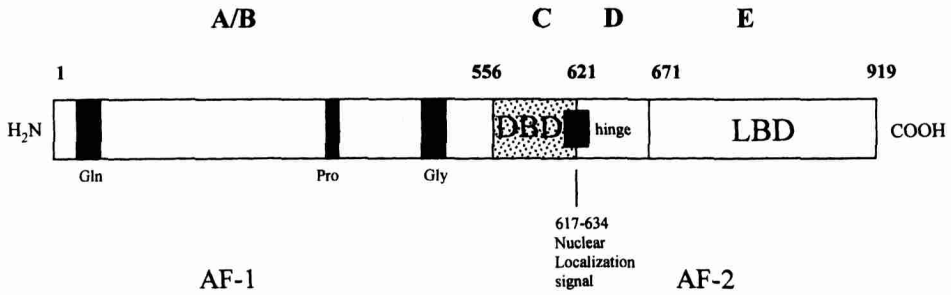


**Fig. 3.** Schematic illustration of the primary structure of the human androgen receptor. The letters A–E represent different functional domains. Within the N-terminal A/B region, variable glutamine(Gln) and glycine (Gly) are found.

respond favorably to androgen ablation therapy, which has become a standard treatment of metastatic disease. However, finally all patients with metastatic disease will relapse with clinically defined androgen-independent prostate cancer. In order to develop new therapies for this resistant disease, it is important to identify different molecular mechanisms that may be involved in prostate cancer development and progression. In this chapter, we will discuss the recent progress that have been made in the understanding of the mechanisms that play a role in androgen-(in)dependent prostate cancer.

## *2.2. Structure of the Androgen Receptor*

The AR was cloned in 1988 *(6,11)*. The AR gene is located on the short arm of the X chromosome *(12)*. The entire gene encodes eight exons *(13)*. The large first exon encodes the entire N-terminal domain. Three repeat sequences exist in this first exon: a CAG (glutamine) repeat, a GGN (glycine) repeat, and a GCA (proline) repeat (Fig. 3). The CAG repeats are polymorphic in length, varying from 11 to 31 repeats in normal individuals. It is suspected that abnormal lengths of this polymorphic region may be associated with prostate cancer *(14)*. Because of the above mentioned variable repeats, different lengths of the AR have been reported (910, 917, 918, and 919 residues) with molecular weights between 100 and 110 kDa *(15)*. A schematic representation of the primary structure of the AR is given in Fig. 3. The AR has two major transactivation domains *(6)*: AF-1 in the A/B region of the N-terminal domain *(16,17)* and AF-2 in the C-terminal ligand-binding domain (LBD) *(18)*. The DNA-binding domain referred to as the C region consists of 68 amino acids that fold into two zinc fingers involved in the recognition of androgen response elements (AREs) on androgen-regulated genes. At the distal end of the C region and within the hinge region (D region), there is a bipartite nuclear targeting sequence (amino acids 617–633) responsible for androgen-regulated nuclear import of the androgen receptor *(19)*.

## 2.3. Androgen Receptor Function

### 2.3.1. Activation by its Natural Ligand and Role of Steroid Receptor Coactivators

In its inactive state, the unliganded AR is associated with heat shock proteins and is unable to perform its transactivating function. This inactive complex can be located both in the cytoplasm and nucleus. Testosterone or DHT binding to the AR induces an allosteric conformational change of the protein that results in hormone-dependent, DNA-independent phosphorylation of the receptor at several sites and dissociation from heat shock proteins. This process allows the AR to dimerize, which is essential for binding to AREs in the promoter of target genes *(1,20)*.The DNA-bound AR next recruits coactivators resulting in stable assembly of the preinitiation complex and transcription initiation by RNA polymerase II *(4,21)*. Several AR coactivators, such as ARA70 *(22)*, ARA55 *(23)*, ARA54 *(24)*, ARA160 *(25)*, and Rb *(26)*, have been isolated and characterized. Besides, CBP [CREB (cAMP response element)-binding protein] was revealed as a coactivator for the AR. Upon ligand binding, the AR can recruit CBP to communicate with the transcription initiation complex and activate transcription. CBP also plays a role in the cross-talk between AR and AP-1, because its recruitment by AR titrates CBP away from AP-1 resulting in inhibition of AP-1 transactivation *(27–29)*.

### 2.3.2. Androgen-Regulated Genes

Various genes have been identified that are regulated by androgens. These include the prostate-specific antigen (*PSA*) gene, which encodes a prostate-specific protease, now widely used as a tumor marker for prostate cancer. *PSA* contains an upstream ARE; thus it is believed that androgens directly regulate its transcription rate. Human glandular kallikrein (hK2) is another androgen-regulated protein. It is primarily expressed in the prostate and also contains an ARE. Furthermore, the AR induces stimulation of epidermal growth factor receptor (EGFR), keratinocyte growth factor (KGF), CDK2, CDK4, p21, and vascular endothelial growth factor (VEGF), and it represses transforming growth factor β (TGF-β), p16/INK4A, and bcl-2 *(30)*.

### 2.3.3. Ligand Independent Activation of AR

Aberrant activation of the AR may be one of the mechanisms that contributes to progression of prostatic carcinoma to an androgen-independent stage. Similarly to other steroid receptors, the AR is involved in cross-talk with the signaling pathways mediated by protein kinases. In transient transfection assays, insulin-like growth factor-I (IGF-I), KGF, and EGF activate the AR to different extents in the absence of androgen *(31)*. This activitiy can be blocked by antiandrogens. Ligand-independent activation of the AR was also reported for substances that directly activate the protein kinase A and C signaling pathways *(32–36)*. All these

substances were able to potentiate the effects of low concentrations of androgen, thus reducing a concentration of steroid needed for maximal activation of the AR. This reduction may be particularly important in patients with advanced prostate cancer in which serum levels of androgen are continuously suppressed. However, all of the above mentioned studies are performed in cell cultures, and the outcome of this nonsteroidal activation of the AR depends on a cellular and promoter context. Mechanisms responsible for AR activation by protein kinase activators are only partly understood. Altered phosphorylation, enhanced nuclear localization, or increased DNA binding of the AR by protein kinase activators may all play a role.

Two groups demonstrated a role for HER-2 in the development of hormone-independent prostate cancer. Craft et al. *(5)* showed that androgen-independent sublines of human prostate cancer xenografts expressed higher levels of HER-2 receptor tyrosine kinase than did androgen-dependent sublines. Additionally, overexpression of HER-2 in a LNCaP prostate cancer cell line caused the cell line to become androgen independent. Furthermore, it was demonstrated that overexpression of HER-2 increased the expression of PSA, especially at low androgen levels, and activation of PSA transcription by HER-2 was shown to require functional AR. Chang's group *(37)* found that HER-2 activates AR through MAP kinase. The clinical implication of this HER-2 → MAP kinase → AR → PSA pathway remains to be demonstrated. There are conflicting data about the overexpression of HER-2 in different stages of prostate cancer *(38*, and references therein). Signoretti et al. *(39)* analyzed HER-2 at DNA, RNA, and protein levels in prostate tumors representing different clinical stages. They found increasing mRNA and protein HER-2 expression levels with progression to androgen independence. However, unlike what is seen in breast cancer, in prostate tumors no concordance between *HER-2* gene amplification and overexpression was seen. Trastuzumab (Herceptin), a monoclonal antibody against HER-2, has been shown to prolong survival in advanced, refractory, HER-2-positive breast cancer *(40)*. Whether there is a role for trastuzumab in treating androgen-independent HER-2-positive prostate cancer is under current study *(41)*. Other investigational therapies against the HER-2 signaling cascade are ansamycins, which produce a rapid reduction in the level of HER-2 expression in cell lines that overexpress HER-2 *(42)*, antisense approaches *(43)*, and novel antibodies directed towards the dimerization of HER-2 *(44)*.

Knudsen et al. *(45)* have shown that cyclin D1, which was found to induce ER activity, can also complex with AR, but instead inhibits its transcriptional activity.

An Italian group reported recently that prostate cancer cell proliferation can be triggered by steroid-induced formation of a ternary complex constituted of the AR, ERβ, and the tyrosine kinase Src, leading to activation of the Src/Raf-1/Erks signal-transducing pathway *(46)*.

### 2.3.4. Androgen Resistance

Most androgen-independent prostate tumors continue to express AR as well as the androgen-dependent gene *PSA*, which indicates that these cells maintain a functional AR signaling pathway despite very low levels of testosterone. In the literature, four possible mechanisms that can lead to the emergence of androgen-independent prostate cancer are suggested: (1) 30% of androgen-independent prostate carcinomas show amplification and overexpression of the wild-type AR gene *(47)*. (2) AR gene mutations can lead to altered hormone specificity of the AR. The first mutation reported to lead to androgen-independence was a missense mutation that caused a substitution of alanine for threonine at amino acid 877 (T877A) in the ligand-binding domain of the AR *(48)*. The T877A mutation expands the ligand specificity of the androgen receptor allowing it to bind estrogens, progestagens, and adrenal androgens, as well as many antiandrogens. This mutation is frequently found in cases of prostate cancer resistant to endocrine therapy*(49,50)*. Taplin at al. found that mutated AR (His874Tyr or Thr877Ser) from two patients with metastatic androgen-independent prostate cancer could be stimulated by estrogen and progesterone *(51)*.The total number of reported AR mutations is increasing *(52)*. Recently, Zhao et al. discovered that the L701H mutation in the ligand-binding domain of the AR in the prostate cancer cell line MDA PCa 2b (derived from a bone metastasis of a hormonal-therapy-resistant prostate cancer patient) makes the AR highly sensitive to cortisol and cortisone, but less sensitive to androgen stimulation *(53)*. Little is known about the frequency of this L701H mutation in prostate cancer. (3) One could speculate that altered function of AR coactivators and corepressors may play a role in the emergence of androgen-independent prostate cancer *(30,54)*. (4) In addition, as already mentioned in the previous chapter, mitogenic signaling pathways, such as those activated by HER-2 overexpression, might lead to androgen independence. Further understanding how AR is activated at low androgen levels will be important for the development of new therapies to treat this otherwise incurable disease.

## 3. THE ESTROGEN RECEPTORS

### *3.1. Introduction*

Breast cancer is the most common cancer in women in the Western world. Approximately 60–70% of all breast cancers are estrogen receptor (ER) positive *(55)*.Only about half of ER-positive patients will respond to the various hormonal therapies available. Of those who do initially respond, most will eventually develop hormonally unresponsive disease following a period of treatment, even though ER is often still present. Since estrogens and ER play a pivotal role in the development and progression of breast cancer as well as the treatment and outcome of

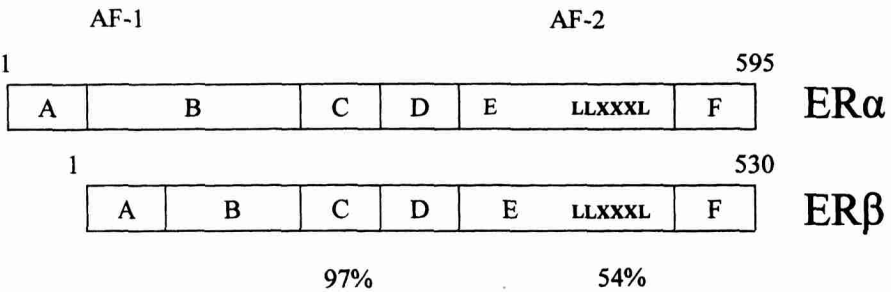


**Fig. 4.** Structure and functional domains of ERα and ERβ. **AB**: transcription activation; **C**: DNA binding and receptor dimerization; **D**: nuclear localization signal; **E**: ligand binding domain, coactivator binding, transcription activation, receptor dimerization; **F**: contributes to transactivation capacity, but function to a large extent unknown. The overall sequence identity between ERα and ERβ is 47%. There is little or no homology between their AF-1 domains. The DNA- and ligand-binding domains are well conserved.

breast cancer patients, mechanisms underlying regulation of ER gene expression and function are key areas of study.

### *3.2. Structure of the Estrogen Receptors*

The ER gene was initially cloned from the ER-positive human breast cancer cell line MCF-7 in 1986 *(56,57)*. It is located on chromosome 6q25.1 and consists of eight exons *(58)*. The structure of the ER, like the other nuclear receptors, can be divided into six conserved, functional domains, A–F *(59)* (Fig. 4). The A/B domain of the ER protein contains a constitutive, estrogen-independent transcriptional activation function, AF-1 *(60)*. Deletion mapping and mutagenesis of human ERα revealed that phosphorylation at $Ser^{118}$ is required for full AF-1 activity *(61)*. The C-domain possesses two zinc-finger DNA-binding motifs essential for binding to estrogen responsive elements (ERE) in the promoter regions of target genes *(59,62)*. The D or hinge region may be involved in estrogen-mediated transcriptional repression *(63)*, while the E domain contains the hormone binding site, the region required for stable dimerization of the receptor, and a second estrogen-inducible transcriptional activation function, AF-2 *(60)*. The function of the F-domain is presently unclear, but it may interact with cell-type-specific factors that regulate ER function *(64)*. AF-1 and AF-2 can act independently and synergistically to enhance transcription, and their activities have been shown to be influenced by promoter context and cell type *(60,65)*. Both AF-1 and AF-2 are presumed to interact with a complex array of coregulator proteins that mediate the interactions between receptors and the basal transcription machinery and remodeling of chromatin structure (*see* below).

It was previously assumed that ER was indispensable for maintenance of life, since no cases had been reported of humans or animals with an inactivated or

deleted receptor. However, in 1994 a case report was published describing a man with estrogen resistance *(66)*. This person lacks a functional ER due to a cytosine to thymine transition at codon 157 of both alleles, resulting in a premature stop codon. The patient was tall (204 cm [80.3 in.]) and had incomplete epiphyseal closure, with a history of continued linear growth into adulthood despite otherwise normal pubertal development. He also had severe osteoporosis and reduced fertility. This case demonstrated that deletion of ER is not lethal, and that a functional ER is necessary for bone maturation and mineralization in both women and men. Furthermore, in 1993, development of an ER knock-out mouse strain was reported *(67)*. In this mouse strain, specific estrogen binding could still be observed in some tissues, suggesting the existence of a second ER.

This second ER termed ERβ *(68,69)*, was discovered in 1996 with the previously defined ER now referred to as ERα. ERβ was initially cloned from rat prostate *(68)*, and the human clone was retrieved from testis *(69)*. ERβ is located on chromosome 14q22-24 *(70)*. ERβ is somewhat shorter than ERα, consisting of 530 amino acids *(71)* (*see* Fig. 4). Human ERα and ERβ share modest overall sequence identity (47%) *(69)*. The region of highest homology is in the DNA-binding domain (95%). The hormone-binding domain is also relatively well conserved (58% identity). The A/B domains are poorly conserved (only ~20%), suggesting that their AF-1 activities might be different, and possibly, that different coactivators interact with this region (*see* below).

## 3.3. Estrogen Receptor Function

### 3.3.1. Knock-out Studies and Tissue Distribution

Examination of the tissue distribution of ERα and ERβ and the results of knock-out studies suggest that the two receptors may have both distinct and redundant functions. Using commercial polyclonal antisera against peptides specific to human ERβ, Taylor et al. *(72)* have determined the sites of ERβ expression in archival and formalin-fixed human tissue and compared its expression to that of ERα. ERβ was localized to the cell nuclei of a wide range of normal adult human tissues including breast, ovary, Fallopian tube, uterus, lung, kidney, brain, heart, prostate, and testis. ERβ expression does not appear to be linked to ERα expression, raising the possibility that there are distinct ERα- and ERβ-dependent pathways. In the endometrium, both ERα and ERβ were observed in luminal epithelial cells and in the nuclei of stromal cells, but, significantly, ERβ was weak or absent from endometrial glandular epithelia. The prostate lacks ERα staining, but is immunopositive for ERβ. Increased ERβ immunoreactivity was noted in the glands of normal resting breast when compared with the glands of proliferating breast. In adult human bone, ERβ protein is expressed in cells of osteoblast lineage and in osteoclasts. In developing human bone ERα is predominantly expressed in cortical bone, whereas ERβ shows higher levels of expression in cancellous bone *(73)*.

Studies in mice lacking ERα, or ERβ, or both receptors reveal the distinct roles of each receptor in mammary gland development and reproduction. ERα knock-out mice (αERKO) show absence of breast development in females and infertility caused by reproductive tract and gonadal and behavioral abnormalities in both sexes *(67)*. In ERβ knock-out mice sexually mature females are fertile and exhibit normal sexual behavior, but have fewer and smaller litters than wild-type mice. Superovulation experiments indicate that this reduction in fertility is the result of reduced ovarian efficiency. The mutant females have normal breast development and lactate normally. Young, sexually mature male mice show no overt abnormalities and reproduce normally. Older mutant males display signs of prostate and bladder hyperplasia *(74)*. To further clarify the roles of each receptor in the physiology of estrogen target tissues, mice lacking both ERα and ERβ were generated *(75)*. Both sexes of αβ estrogen receptor knock-out (αβ ERKO) mutants exhibit normal reproductive tract development, but are infertile. Ovaries of adult αβ ERKO females exhibit follicle transdifferentiation to structures resembling seminiferous tubules of the testis, including Sertoli-like cells and expression of Müllerian-inhibiting substance, sulfated glycoprotein-2, and Sox9. Therefore, loss of both receptors leads to an ovarian phenotype that is distinct from that of the individual ERKO mutants, which indicates that both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary.

We will discuss the possible role of ERβ in the development of breast cancer and the resistance to hormone therapy later.

### 3.3.2. Activation by its Natural Ligand and Role of Steroid Receptor Coactivators

In the absence of ligand, ERα is predominantly located in the nucleus *(76–78)* as part of a complex with heat shock proteins. Ligand binding to ER displaces the heat shock proteins and allows phosphorylation of the receptor at serine residues within the AF-1 domain *(61,79)*. This ligand binding also causes a conformational change in the protein accommodating a productive interaction and transcriptional synergism between AF-1 and AF-2 *(80–82)*. These changes allow for receptor dimerization, and two ERα molecules complexed with hormone bind specifically to a consensus 13-bp palindromic estrogen responsive element (ERE) located upstream of target genes *(1,62,83)*. (Formation of ERα and ERβ heterodimers has been demonstrated both in vitro and in vivo *(71,84)*, but the physiological role of the heterodimer is unclear.) By binding to DNA, the transcription activation function AF-2 of ERα is activated, the basal transcription machinery is contacted directly, and other nucleoproteins are recruited. These nucleoproteins interact with the ERα protein dimer to modify the expression of the estrogen-responsive genes (reviewed in Horwitz et al. *(85)*, Glass et al. *(86)*, and McKenna et al. *(87–89)*. Some of these receptor-interacting proteins function as transcriptional coativators, such as the p160 coactivators SRC-1/N-CoA1 *(90,91)*, TIF-2/GRIP-1 *(92,93)*,

and AIB1/RAC3/ACTR/P/CIP *(94–96,97)*. A distinctive structural feature of these p160 coactivators is the presence of multiple LXXLL signature motifs, which comprise determinants for direct interactions with the nuclear-receptor ligand-binding domain. The p160 coactivators modify local chromatin structure through histone acetylation, thereby facilitating RNA pol II recruitment, and are thought to recruit additional transcriptional cofactors, P/CAF and p300/CBP *(91,98,99)*. P/CAF and p300/CBP make independent contacts with the nuclear receptor, as well as with one another *(97,100)*. In addition, P/CAF and p300/CBP have HAT activity *(101–103)*. A surprising deviation from the p160/CBP paradigm, and perhaps from our traditional view of transactivation in general, is the identification of an RNA that acts as a coactivator for steroid receptors. This SRA (steroid-receptor RNA activator) is present in a SRC-1 complex *(104)*. DiRenzo et al. *(105)* demonstrated that ATP-dependent chromatin remodeling plays a role in the regulation of ER signaling. It was shown that transcriptional activation by ERα requires functional BRG-1, and that the coactivation of estrogen signaling by either SRC-1 or CBP is BRG-1 dependent. In addition, a distinct multiprotein complex, called DRIP or TRAP, first found to be involved in thyroid hormone receptor *(106)* and vitamin D receptor signalling *(107)*, has also been implicated recently in ERα and ERβ action through a ligand dependent interaction with its PBP/TRAP220/DRIP205 subunit and the AF-2 domain *(108)*.

Other nucleoproteins can function as corepressors *(87,89)*. Recently, it was shown that tamoxifen can recruit the corepressors N-CoR and SMRT to the promoter of the ER target genes cathepsin D and pS2 *(109)*.

### 3.3.3. ERα and ERβ Mediated Transcription via AP-1

Besides this classical mechanism of direct DNA binding, the two ER subtypes can also regulate transcription via an activator protein (AP)1 response element *(110)*. AP-1 response elements are regulated indirectly through interactions between ER and the AP-1 transcription factors c-Fos and c-Jun. These transcription factors regulate genes involved in many cellular processes, including proliferation, differentiation, cell motility, and apoptosis. Thus, the ER–AP-1 interaction could be important clinically. When signaling is mediated via AP1, ERα and ERβ signal in opposite ways *(111)*. When bound to ERα, estradiol activates transcription, whereas with ERβ transcription is inhibited. However, antiestrogens bound to ERβ are potent transcriptional activators at an AP1 site, acting as estrogen agonists rather than antagonists. This finding could have important implications for the differential effects of selective estrogen receptor modulators (SERMS) and antiestrogen resistance.

### 3.3.4. ER Target Genes

Knowledge of which genes are actually regulated by ERα is of interest, since the expression of ERα in breast cancer is an important predictor of response to

hormone treatment. ERα regulated genes that have lost this regulation during malignant transformation could potentially cause resistance to hormone therapy. Thus far, reports have been published on seven genes of which expression has been found to be regulated by ERα. The genes are the progesterone receptor *(112)*, *cathepsin D (CATD) (113)*, *pS2 (114,115)*, *c-Myc (116)*, *TGFα (117)*, *c-fos (110, 118)*, and *VEGF (119)*.

### *3.4. Crystal Structure*

ER undergoes extensive conformational changes after ligand binding as revealed by recent crystal structures of ERα bound to various ligands *(120,121)*. The ER ligand binding EF domain (LBD) has been shown to be composed of 12 α-helices, forming a pocket to capture the ligand. Ligand binding only causes a shift in helix 12 at the C-terminal LBD without affecting the other regions. The conformation of the ERα LBD is determined by the nature of the particular ligand that is bound. In the estrogen or DES liganded complex, helix 12 containing the AF2 core is repositioned as a "lid" over the LBD cavity in a way that p160 coactivators can accommodate within a hydrophobic cleft of the LBD. This repositioning occurs through direct contacts with the LXXLL motif. Estrogen antagonists such as tamoxifen and raloxifene appear to alter the position of the AF2 core such that helix 12 itself occupies the hydrophobic cleft in the LBD, thereby precluding coactivator binding. In 1999, the crystal structure of the LBD of ERβ in the presence of the phytoestrogen genistein and the antagonist raloxifene was resolved showing similarity with the ERα-LBD three-dimensional structure *(122)*.

### 3.5. Ligand-Independent Activation of ER

Cross-talk with other growth-factor signaling pathways represents another way in which ER can affect important cellular processes. Phosphorylation at $Ser^{118}$ of ERα is required for full AF-1 activity *(61)*. This residue is a direct substrate for mitogen-activated protein kinase (MAPK), providing a link between ER action and the Ras–MAPK signaling cascade *(79)*. EGF *(123)*, insulin or coexpression of Ras can activate this MAPK pathway leading to phosphorylation at $Ser^{118}$. Tremblay et al. recently showed that phosphorylation of $Ser^{106}$ and $Ser^{124}$ in AF-1 of ERβ by MAPK resulted in increased interaction of ERβ with SRC-1 *(124)*. There is also considerable cross-talk between ERα and insulin-like growth factor (IGF) signal transduction pathways. ER functions to increase levels of several of the key IGF signaling molecules, and IGFs, in turn, may activate ER *(125,126)*. Additionally, ERα is a target for tyrosine phosphorylation. Activation of the HER-2 receptor in breast cancer cells by the peptide growth factor heregulin leads to direct and rapid phosphorylation of ER on tyrosine residues. This phosphorylation is followed by interaction between ER and estrogen-response elements in the nucleus, and production of an estrogen-induced protein, progesterone receptor *(127)*. A single tyrosine residue located immediately adjacent to the AF-2

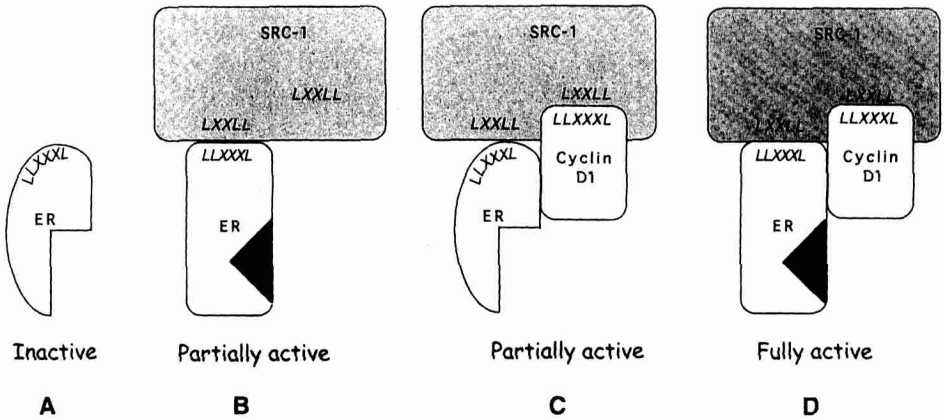


**Fig. 5.** Multiple ways to activate the estrogen receptor. In the absence of ligand, ER is unable to interact with steroid receptor coactivators (SRCs) directly as its leucine-rich coactivator interaction motif (AF-2), indicated as LLXXXL) is sterically unavailable for SRC interaction (**A**). Hormone binding by ER exposes AF-2 and allows recruitment of SRCs to ER, leading to activation of ER (**B**). Hormone-independent binding of cyclin D1 to ER provides a single leucine-rich interaction motif for SRCs on the cyclin D1/ER complex, which is present in the carboxyl terminus of cyclin D1. This results in partial activation of ER (**C**). Ligand binding of ER in the presence of cyclin D1 provides two independent interaction surfaces for SRCs: one is formed by the leucine-rich motif in AF-2 of ER and a second in the carboxyl terminus of cyclin D1 (**D**). The observed synergism between estrogen and cyclin D1 in ER activation may result from their cooperative recruitment of SRCs to the ER. The protein interaction motifs are shown in italics (L = leucine, X = any amino acid).

has been identified as a substrate for the src-family tyrosine kinases *(128)*. This cross-talk between signaling pathways could conceivably contribute to the development of estrogen independence and/or clinical resistance to hormone therapy.

Cyclin D1 can activate ERα in a ligand-independent and CDK-independent fashion *(129,130)*. By acting as a bridging factor between ERα and SRCs, cyclin D1 can recruit SRC-family coactivators to ERα in the absence of ligand, resulting in ligand-independent transcription *(131)* (Fig. 5). Cyclin D1 can also interact with P/CAF, facilitating the formation of a ternary complex in which P/CAF associates with ERα, leading to transcriptional activation *(132)*. Recently, it was reported that the functional interaction between cyclin D1 and ERα is regulated by a signal transduction pathway involving the second messenger, cyclic AMP *(133)*. The cyclin D1 gene is amplified in approx 20% of breast cancers, and the protein is overexpressed in 50% of cases *(134)*. Remarkably, cyclin D1 is overexpressed preferentially in ERα-positive breast cancers. Because ERα overexpressing breast cancers often occur in postmenopausal women–who have low levels of circulating estrogens–it is possible that the frequently overexpressed cyclin D1 in these tumors may, at least in part, be responsible for stimulating ER activity.

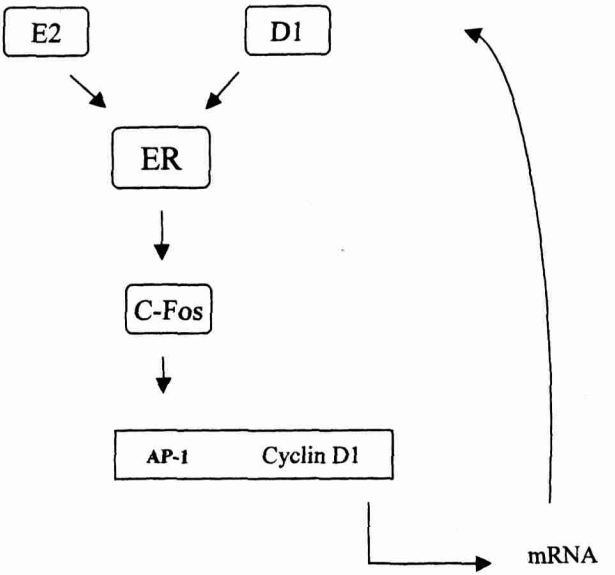


**Fig. 6.** Positive stimulatory loop of ER and cyclin D1. Estrogens bind to ER and stimulate the expression of cyclin D1 via AP-1. The increased cyclin D1 level leads to both CDK-dependent and CDK-independent cell cycle progression. The latter being the result of enhanced ER transcriptional activation mediated by cyclin D1.

The precise molecular mechanism by which estrogen and ER control cyclin D1 expression is at present poorly defined. Cyclin D1 does not represent a classical ER target gene, because the cyclin D1 promoter lacks an ERE. Altucci et al. *(135)* mapped the estrogen responsive region to a fragment between –944 and –136 of the cyclin D1 promoter. This region contains an AP-1 site, suggesting that estrogens can modulate cyclin D1 expression via AP-1. Combining the above-mentioned observations, we assume that ERα and cyclin D1 form a positive stimulatory loop in which estrogens stimulate the expression of cyclin D1 via AP-1, leading to both CDK-dependent and CDK-independent cell cycle progression. The latter being the result of enhanced ER transcriptional activation mediated by cyclin D1 (Fig. 6). Whether cyclin D1 has a role as a prognostic or predictive marker in breast cancer is still uncertain *(134)*.

In transient transfection assays, *BRCA1* was found to inhibit signaling by the ligand-activated ER-α through the estrogen-responsive enhancer element and to block the transcriptional activation function AF-2 of ERα. These results raise the possibility that wild-type *BRCA1* suppresses estrogen-dependent transcriptional pathways related to mammary epithelial cell proliferation, and that loss of this ability contributes to tumorigenesis *(136)*. However, the fact that $BRCA1^{-/-}$ tumors are mostly ERα negative argues against this model *(137–140)*.

### 3.3.5. Regulation by Antiestrogens

Inhibition of ER activity in cancer cells by antiestrogens such as 4-hydroxytamoxifen (OHT) has led to their use as therapeutic agents for the treatment of breast cancer. However, the mixed agonist/antagonist OHT inhibited only AF-2 function *(141)*. In fact, OHT functions as an agonist in uterine tissue and activates ERα in a cell-type and promoter-specific manner. The agonist activity of OHT at ERα requires an intact AF-1 domain *(142)*, and OHT cannot block the activation of ERα via the MAPK pathway *(79,123)*. The partial agonistic effect of OHT at ERα on a basal promoter linked to an ERE, was not seen in this setting with ERβ *(143,144)*.

Unfortunately, all patients eventually relapse on tamoxifen treatment. The mechanisms that lead to tamoxifen resistance are not completely understood, as a variety of mechanisms have been proposed but only limited evidence exists to substantiate them *(145)*. Mechanisms that can potentially give tamoxifen resistance in ERα positive tumors are altered metabolism of OHT; decreased intracellular drug concentrations; enhanced biological mechanisms for circumvention of tamoxifen cytotoxicity; stimulation of ERα positive cells by the agonistic component of OHT; altered levels of ERα in the tumor; variant or mutant ERs; and changes in expression of ER regulated genes.

In an effort to develop new and more effective antiestrogens, a new, pure antiestrogen was synthesized. ICI 182,780 (Faslodex) demonstrates a pure anti-estrogenic profile on all genes and in all tissues studied to date *(146)*. ICI 182,780 is also devoid of agonist activity in animal models and clinical trials, inhibiting growth of the breast and endometrium. In animal models, it does not cross the blood–brain barrier and appears to be neutral with respect to lipids and bone. ICI 182,780 down-regulates the estrogen receptor and is active in tamoxifen-resistant breast carcinoma. In a small, Phase II study, durable responses were seen. Phase III clinical trials are in progress comparing ICI 182,780 with anastrozole and tamoxifen in the treatment of patients with advanced breast carcinoma *(147)*.

### 3.3.6. The Role of ERα and ERβ in the Development of Breast Cancer

The number of normal breast epithelial cells expressing ERα is quite small, with estimates of between 7% and 17% ERα-positive ductal epithelial cells being reported in normal human breast tissue *(148,149)*. However, 60–80% of all human primary breast tumors express significant amounts of ERα *(150)*. This suggests that the expression of ERα signaling pathways is a selective advantage for breast cancer development.

The role of ERβ in carcinogenesis and progression of breast cancer is far from clear. Recent data considering the role of ERβ in the development of breast cancer show controversial results, probably due to small population samples, different techniques, and cut off points used. In Table 1, an overview of the results obtained in the different publications is given. Four studies *(151–154)* suggest that upon

Table 1

| *Author* | *No pts* | *Technique* | *Tissue* | *Outcome/comment* |
|---|---|---|---|---|
| Leygue et al. *(151)* | 18 | RT-PCR | normal breast vs primary breast tumor same patient | ERα/ERβ in tumor greater than in normal tissue $p < 0.02$ |
| Speirs et al. *(152)* | 83 | RT-PCR | normal breast tissue (n = 23) vs malignant breast tissue ($n = 60$) | In normal breast tissue ERβ predominates. Coexpressed ERα and ERβ associates with positive lymph nodes and higher tumor grade |
| Speirs et al. *(163)* | 17 | RT-PCR | primary breast tumor n = 8 tamoxifen S n = 9 tamoxifen R | ERβ ↑ in tamoxifen R. Comment: tam R group contains more pts with positive lymph nodes and high grade tumors |
| Jävinen et al. *(155)* | 92 | immunohistochemistry, confirmation by mRNA in situ hybridization. cut off ≥ 20% cells + | primary breast tumor | 55/92 (59.8%) ERβ+. ERβ+ was statistically significant associated with: PR +, ERα +, lymph node –, slow proliferation, premeno pauzal state |
| Iwao et al. *(164)* | 116 | real-time PCR | primary breast tumor | ERβ↑ in ERα – and PR – tumors |
| Iwao et al. *(153)* | 123 | real-time PCR | normal breast tissue (n = 11) vs malignant breast tissue (n = 112) | ERβ↓ and ERα↑ during carcinogenesis of breast cancer |
| Mann et al. *(157)* | 47 | immunohistochemistry Cut off ≥ 10% cells + | primary breast tumors | 33/47 (70%) ERβ+ 30/47 (64%) ERα+. How many for both receptors positive? |
| | 118 | same as above | primary breast tumors adjuvant treatment with tamoxifen | 78/118 (66%) ERβ+ ERα+ correlates with better survival $p = 0.0077$ |
| Roger et al. *(154)* | 130 | immunohistochemistry | 71 BBD 59 CIS vs 118 normal breast tissue | ERβ↑ normal breast nonproliferating BBD<br>ERβ↓ proliferating BBD and CIS |
| Omoto et al. *(156)* | 88 | immunohistochemistry | primary breast tumors | 52/88 (59.1%) ERβ+. No correlation with node status, grade. Significant correlation with ERα+ |

BBD, benign breast disease; CIS, carcinoma *in situ*.

transition from normal to cancerous tissue, the expression of ERβ decreases together with an increase of the ERα expression. This marked and early decreased level of ERβ protein expression suggests a protective effect of ERβ against the mitogenic activity of estrogens in mammary premalignant lesions. Two other groups, however, reported exactly the opposite, showing a correlation between ERα and ERβ expression in breast cancer specimens *(155,156)*. One group claims that it has found a correlation between ERβ positivity of a tumor and better survival after adjuvant tamoxifen treatment *(157)*. However, these authors did not report on how many of the ERβ positive cases were also positive for ERα. The possible survival benefit could therefore be the result of unreported ERα positivity.

Using the current assay for ER status, which uses immunohistochemistry with ERα specific antibodies, a significant number of women with invasive breast cancer whose tumors are ERβ positive, but ERα negative, will be determined to have a negative ER status and may not receive adjuvant hormonal therapy. Although there are no studies to date showing that ERα negative but ERβ positive tumors respond to treatment with antiestrogens, this could be a possibility, based on studies in cell lines.

In summary, we can conclude that although one has the expectation based on preclinical data that there is a different role for each estrogen receptor during breast development and breast carcinogenesis, clinical studies performed so far are unable to prove this.

## 4. CONCLUDING REMARKS

ERα and its regulated genes play an important role in the development and progression of breast cancer. Overexpression of ERα is of important predictive value for the response to tamoxifen treatment *(158)*. The role of ERβ in the development of breast cancer is not clear yet. Unfortunately, not all ERα positive tumors respond to hormone therapy, and eventually, all initial responders will relapse. In order to optimize the treatment for breast cancer patients, we need better tools to predict the outcome of therapy. One step forward is the FASAY in which functionality of the ER is tested *(159)*. The recently developed DNA microarray technique will further clarify the pathways involved in breast cancer develoment, and will help us to identify expression profiles, predicting response to (hormone) therapy *(160–162)*. These new expression profiles probably will completely replace our current classification system of breast tumors.

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