Estrogen Receptors: How Do They Signal and What Are Their Targets

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I. Introduction

II. Estrogen Receptors
   A. Two ER subtypes and several isoforms
   B. Diverse structures of synthetic, dietary, and environmental estrogens
   C. Distinct molecular pathways involving ERs

III. Estrogen Signaling
   A. Estrogen regulation of growth factor pathways and branching morphogenesis in the mammary gland, prostate, and lung
   B. Imprinting by estrogen
   C. Estrogen as a trophic factor for neurons
   D. ERs and cell adhesion

IV. Anti-Estrogen Signaling
   A. Anti-estrogens: benefits and limitations
   B. Complete blocking of estrogen signaling
   C. Deleterious effects of anti-estrogens
   D. Structural basis for ER activity
   E. Role of helix 12 in the regulation of AF-2 activity
   F. Classical AF-2 antagonism
   G. Nonclassical AF-2 antagonism
   H. Coregulator recognition of antagonist-bound ER
   I. ER coregulators

V. Concluding Remarks

Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Ström A, Treuter E, Warner M, Gustafsson J-A. Estrogen Receptors: How Do They Signal and What Are Their Targets. Physiol Rev 87: 905–931, 2007; doi:10.1152/physrev.00026.2006.—During the past decade there has been a substantial advance in our understanding of estrogen signaling both from a clinical as well as a preclinical perspective. Estrogen signaling is a balance between two opposing forces in the form of two distinct receptors (ERα and ERβ) and their splice variants. The prospect that these two pathways can be selectively stimulated or inhibited with subtype-selective drugs constitutes new and promising therapeutic opportunities in clinical areas as diverse as hormone replacement, autoimmune diseases, prostate and breast cancer, and depression. Molecular biological, biochemical, and structural studies have generated information which is invaluable for the development of more selective and effective ER ligands. We have also become aware that ERs do not function by themselves but require a number of coregulatory proteins whose cell-specific expression explains some of the distinct cellular actions of estrogen. Estrogen is an important morphogen, and many of its proliferative effects on the epithelial compartment of glands are mediated by growth factors secreted from the stromal compartment. Thus understanding the cross-talk between growth factor and estrogen signaling is essential for understanding both normal and malignant growth. In this review we focus on several of the interesting recent discoveries concerning estrogen receptors, on estrogen as a morphogen, and on the molecular mechanisms of anti-estrogen signaling.
I. INTRODUCTION

Estrogens play key roles in development and maintenance of normal sexual and reproductive function. In addition, in both men and women they exert a vast range of biological effects in the cardiovascular, musculoskeletal, immune, and central nervous systems (73). The most potent estrogen produced in the body is 17β-estradiol (E2) (Fig. 1). Two metabolites of E2, estrone and estriol, although they are high-affinity ligands (121) are much weaker agonists on estrogen receptors (ERs). These metabolites were formally thought to be inactive, but recent evidence suggests that they may well have tissue-specific roles (71). The ground-breaking findings in the field were made in the late 1950s when Elwood Jensen (97) discovered and started the characterization of an estrogen binding protein, today recognized as ERα. In 1993, nearly three decades later, the first ERα knockout mouse was created (150), and the surprising discovery was made that life is possible without this receptor which, at the time, was thought to be the sole mediator of estrogen signaling. Soon after the characterization of the ERα knockout mouse, ERβ was discovered (122), and this discovery raised the question of whether survival of the ERα knockout mouse was due to ERβ substituting for the functions of ERα. ERβ knockout mice were made, and this was followed by double ERαβ knockouts (120). These different mouse models revealed that life is possible without either or both ERs but that reproductive functions are severely impaired (29). In addition, ERα and ERβ were found to have distinct, nonredundant roles in the immune, skeletal, cardiovascular, and central nervous systems (29, 73, 78). Some of the most remarkable phenotypes of the ERα and ERβ knockout mice are the effects on morphogenesis. Estrogen is well known to be a morphogen, and its role in morphogenesis is evident from the structure of the uterus (29), ovary (29), mammary gland (54), prostate (313), lung (181), and brain (301) of ERα and ERβ knockout mice. At the promoters of some genes, particularly those involved in proliferation, ERα and ERβ can have opposite actions (145), a finding which suggests that the overall proliferative response to E2 is the result of a balance between ERα and ERβ signaling. Anti-estrogens, designed to block ERα, are widely and effectively used clinically in the treatment of breast cancer (28, 43). However, although synthetic anti-estrogens are mainline ther-

FIG. 1. Molecular structures of the endogenous estrogen 17β-estradiol and of tamoxifen, 4-hydroxy-tamoxifen, raloxifene, ICI 182,780 (fulvestrant), toremifene, R,R-cis-diethyl-THC, and genistein.
apy for treating ERα-positive breast cancers, these drugs have unwanted side effects in nontarget tissues, and after prolonged treatment, cancers become resistant to anti-estrogen therapy (197, 256). The idea that ERβ agonists may be used to promote growth arrest offers new possibilities for pharmacological intervention in the treatment of cancers (179, 221). The mechanisms governing the action and development of resistance to anti-estrogens are poorly understood. The emerging picture is one in which ER is capable of adopting a multitude of response states depending on the nature of the bound ligand (Table 3 and references therein) and where each ligand induces a unique receptor conformation capable of recruiting a distinct pattern of coactivators and corepressors to the receptor-transcription complex.

In this review we focus on new insights into estrogen receptor modulation by synthetic anti-estrogens and coregulatory proteins and discuss the molecular concept of anti-estrogen signaling. We also discuss the roles of ERα and ERβ in mediating growth and morphogenesis.

II. ESTROGEN RECEPTORS

A. Two ER Subtypes and Several Isoforms

Cellular signaling of estrogens is mediated through two ERs, ERα (NR3A1) and ERβ (NR3A2), both belonging to the nuclear receptor (NR) family of transcription factors. Like many other members of the NR family, ERs contain evolutionarily conserved structurally and functionally distinct domains (Fig. 2). The central and most conserved domain, the DNA-binding domain (DBD), is involved in DNA recognition and binding, whereas ligand binding occurs in the COOH-terminal multifunctional ligand-binding domain (LBD). The NH2-terminal domain is not conserved and represents the most variable domain both in sequence and length. Transcriptional activation is facilitated by two distinct activation functions (AF), the constitutively active AF-1 located at the NH2 terminus of the receptor and the ligand-dependent AF-2 that resides in the COOH-terminal LBD (reviewed in Ref. 194). Both AF domains recruit a range of coregulatory protein complexes to the DNA-bound receptor. The two ERs share a high degree of sequence homology except in their NH2-terminal domains, and they have similar affinities for E2 and bind the same DNA response elements.

Ligand-dependent estrogen signaling begins with the binding of estrogen to ER. Thereafter, the cell-specific transcriptional response to estrogen depends on multiple factors, the most immediate being the composition of coregulatory proteins in a given cell and the characteristics of the promoters of estrogen responsive genes. Since hormones are modulators of transcription, the pattern of modulated genes also depends on what other signaling pathways are active in the cell at the time of hormone exposure (108, 110, 194).

The identification of the second estrogen receptor ERβ and several receptor isoforms has affirmed the complex nature of estrogen signaling and helped to explain estrogen action in tissues that do not express ERα (140). E Rβ and ERβ are products of separate genes located on different chromosomes (46, 172). Several splice variants have been described for both receptor subtypes (Fig. 2), but whether all the variants are expressed as functional
proteins with biological functions is not clear. Most ERα variants differ in their 5'-untranslated region (UTR), not in the coding sequence. In addition, shorter ERα isoforms lacking exon 1 and consequently the NH₂-terminal AF-1 (here termed hERα-46 and hERα-36) have been isolated and identified in different cell lines (52, 305). These receptor isoforms have not yet been identified or characterized in tissues, and their involvement in regulating estrogen effects in vivo remains to be determined. They are, however, interesting research tools since they have the ability to heterodimerize with the full-length ERα and thereby repress AF-1-mediated activity (52, 305). Possibly, they may also localize to the plasma membrane and may help to elucidate the mechanisms through which rapid, "nongenomic" estrogen signaling occurs (144, 305). Unlike ERα, several splice variants of ERβ are expressed in tissues. The 530-amino acid (aa)-long human ERβ isoform is currently regarded as the wild-type ERβ (rat and mouse, 549 aa) (140). Several alternative ERβ isoforms have been described (Fig. 2), and many of these are expressed as proteins in tissues (59, 252). Characterization of the functional isoform pattern in human samples is not complete, but several experiments indicate that ERβ isoforms can differentially modulate estrogen signaling and, as a consequence, impact target gene regulation (137, 167, 242). For example, the human ERβ isoform (also named ERβ cx) with 26 unique aa residues replacing the COOH-terminal part of the LBD is unable to bind ligand or coactivators and has no transcriptional activity in reporter assays. ERβ dimerizes with preferentially ERα, thereby silencing signaling via this ER isoform (204). Both species-specific and species-common ERβ isoforms have been identified (reviewed in Ref. 139) (Fig. 2).

B. Diverse Structures of Synthetic, Dietary, and Environmental Estrogens

In contrast to the situation for some NRs, such as the thyroid hormone receptor (TR) or the retinoic acid receptor (RAR), where the shape of the ligand-binding cavity is well adapted to fit the cognate hormone, ERs’ ligand cavity appears generous in size for E2 (22). This allows ER to bind a wide range of compounds with strikingly diverse structures. In addition to estrogens, ERs also show affinity for environmental contaminants such as polycyclic aromatic hydrocarbons, phthalates, and pesticides, a class of estrogens termed xenoestrogens (20). Certain plant constituents termed phytoestrogens (101, 209) also have estrogenic actions that are biologically relevant in humans as well as in farm animals.

Environmental contaminants with estrogenic activity are thought to interfere with endocrine signaling in estrogen-responsive tissues and are referred to as endocrine disruptors. Altered reproductive capacity in wildlife and cancers in the breast and endometrium are thought to be possible consequences of exposure to endocrine disruptors (133, 319). On the other hand, epidemiological studies suggest that diets rich in phytoestrogens, particularly soy and unrefined grain products, may be associated with decreased risk of some hormone-induced cancers (reviewed in Ref. 318). Numerous investigations have documented antiproliferative effects of genistein, the most abundant phytoestrogen in soy. Genistein (Fig. 1) has a ninefold higher affinity for ERβ than for ERα (5, 121). Because of its ERβ selectivity, genistein would be expected to have none of the unwanted side effects of estrogen replacement on the endometrium. Whether or not genistein by itself is adequate in replacing the effects of estrogen on bone, brain, and cardiovascular and immune systems is at present under intense investigation (reviewed in Refs. 212, 315, 324).

A variety of synthetic estrogen antagonists have been developed, and some of them are used clinically to reverse the growth-promoting effects of estrogens in breast cancers (Fig. 1) (reviewed in Refs. 247, 262). Additional compounds have been developed and characterized to obtain more favorable tissue and receptor subtype selective effects (reviewed in Refs. 109, 299). The term SERM (selective estrogen receptor modulator) describes synthetic ER ligands that display tissue-selective pharmacology. As anti-estrogens (or antagonists) they oppose the action of estrogens in certain tissues, while mimicking the action of endogenous estrogens (agonists) in others (108, 110). The mechanism of anti-estrogen signaling is discussed in detail below. Hereafter in the review, the term anti-estrogen will be used rather than SERM, since the latter term encompasses a wider concept.

C. Distinct Molecular Pathways Involving ERs

Emerging evidence suggests that there are several distinct pathways by which estrogens and ERs may regulate biological processes (74) (Fig. 3). Ligand-bound ERs can bind directly to estrogen response elements (ERE), in the promoters of target genes or can interact with other transcription factor complexes like Fos/Jun (AP-1-responsive elements) (127) or SP-1 (GC-rich SP-1 motifs) (255) and influence transcription of genes whose promoters do not harbor EREs. Ligand-dependent activation triggers recruitment of a variety of coregulators to the receptor in a complex that alters chromatin structure and facilitates recruitment of the RNA polymerase II (Pol II) transcriptional machinery. The details of ER-transcription factor interactions (i.e., ligand specificity, ER subtype specificity, interaction domains and motifs) remains to be characterized. Interestingly, in certain cell-type and pro-

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moter contexts, 4-hydroxytamoxifen and raloxifene, which function as antagonists at EREs, can behave as agonists through these indirect pathways. ER\textsubscript{H9252}, but not ER\textsubscript{H9251}, in the presence of E2 can oppose the actions of 4-hydroxytamoxifen and raloxifene on an AP-1 reporter gene (127, 216, 309, 312). Similar observations have been made for the SP-1 pathway (255, 331).

In addition to the well-studied transcriptional effects of E2, there are rapid effects, i.e., occurring within seconds or minutes after addition of E2 (283, 284, 307; reviewed in Refs. 274, 320). These rapid effects include activation of kinases and phosphatases and increases in ion fluxes across membranes. Although these rapid effects have been extensively studied, there is still no consensus as to whether or not the classical ERs are involved (33, 138, 243) or whether there is a distinct membrane-associated receptor (38). Tools such as pathway-selective ligands (77) or cell lines designed to selectively express ER\textalpha in the nucleus, cytoplasm, or membrane may prove useful in studying these extranuclear ER actions (253). Interestingly, evolutionary evidence suggests that early on, estrogen influenced reproduction through ER-independent pathways (111) and that the receptor was unresponsive to estrogens and acted as a constitutive transcriptional activator (111, 291).

When the two ER subtypes are coexpressed in cells, ER\textbeta can antagonize ER\textalpha-dependent transcription (167, 168). The molecular mechanisms of ER\textbeta-mediated inhibition of ER\textalpha signaling are currently under investigation. For example, it was shown that for ER\textbeta-mediated regulation of AP-1-dependent transcription, ER\textbeta expression alters the recruitment patterns of c-Fos to AP-1-regulated promoters (168). Moreover, expression of ER\textbeta and the ER\textbeta variant ER\textbeta2 increases the proteolytic degradation of ER\textalpha (168). Collectively, these data suggest that the ER\textbeta-mediated inhibition of ER\textalpha activity involves a combination of altered recruitment of key transcription factors and increased ER\textalpha degradation.

In addition to ligand-induced transcriptional activities of ER, ligand-independent pathways to activate ERs have been described. Growth factor signaling leads to activation through other signaling pathways, like growth factor signaling. In this case, activated kinases phosphorylate ERs and thereby activate them to dimerize, bind DNA, and regulate genes.
III. ESTROGEN SIGNALING

A. Estrogen Regulation of Growth Factor Pathways and Branching Morphogenesis in the Mammary Gland, Prostate, and Lung

Estrogens and androgens are necessary for initiating programs of morphogenesis at specific times during development, but it is growth factors and their receptors that are needed for communication between epithelium and mesenchyme. Without this communication, morphogenesis cannot occur. The major players in the cross-talk between epithelium and mesenchyme are the epidermal growth factor pathway, which includes epidermal growth factor receptor (stromal), epidermal growth factors (epithelial), and the proteases which activate them (epithelial and stromal); FGF receptors (epithelial) and their ligands (stromal); the transforming growth factor beta family of peptides (BMPs) (stromal) and their receptors; and the Notch signaling pathway (epithelial receptors).

Epidermal growth factor receptor (EGFR-1; also called HER-1 and ErbB-1) is essential for epithelial development, tissue repair, tumor growth, tissue-modeling phenomena, angiogenesis, and fibrogenesis. EGFR is activated by six peptide growth factors: EGF, transforming growth factor (TGF)-α, betacellulin, heparin-binding EGF (HB-EGF), amphiregulin, and epiregulin. These are all expressed as transmembrane precursors that need to be released from the cell surface by proteolysis (79). The proteases which activate EGF receptor ligands are members of a disintegrin and metalloproteinase family (ADAM) of cell surface proteases. Thus tissue selectivity in regulation of EGF signaling depends on the type of EGF receptor, the type of protease, and the type of ligand expressed in the tissue.

1. Mammary gland morphogenesis

Ductal elongation in the mammary gland is absolutely dependent on ERα and does not occur in ERα−/− mice. When pituitary hormones are normalized and prolactin, progesterone, and estrogen are administered to ERα−/− mice, a truncated mammary structure results. There is abundant alveolar growth but no ductal elongation. Kenney et al. (113) transplanted ERα−/− virgin mammary glands into the cleared mammary fat pad of virgin wild-type (wt) or ERα−/− mice and found that wt mammary tissue failed to grow in ERα−/− mammary fat pads. On the other hand, in ERα−/− mammary glands, there was lobulo-alveolar growth in wt mouse fat pads. Treatment of virgin ERα−/− mammary gland with TGF-α or heregulin β1 stimulated growth and secretory activity (113). It is not only in tissue recombinants that wt epithelium fails to penetrate ERα−/− fat pad. When 3-wk-old ERα−/− fat pad is implanted into the mammary fat pad of a 3-wk-old wt mouse, ducts avoid the ERα−/− implant. This strongly indicates that the ERα−/− fat pad secretes growth inhibitory substances. At 3 wk of age, the stroma of the mammary gland expresses both ERα and ERβ (Fig. 4). We speculate that ERβ stimulates secretion of a growth repressor. One possible factor is TGF-β, which is an estrogen-regulated gene (94).

The use of knockout mice (119, 133, 152, 161, 225, 250, 266) and tissue recombination methods (286) have revealed that ADAM-17 is responsible for processing of TGF and HB-EGF and that mammary ductal growth is dependent on epithelial amphiregulin and stromal EGFR. ADAM-17 is essential for branching, but other metalloproteinases (MMP) are involved in remodeling of the mammary gland (272, 317). Degradation of ECM proteins by MMPs clears a path for migration of epithelial cells during ductal growth. In addition, cleavage of some proteins (elastin, laminin-5, or collagen types IV or VII) releases growth factors that facilitate growth and migration of epithelial cells (104, 119). Inhibitors of metalloproteinase activity, TIMPs, modulate MMP functions. EGF and MMP signaling are not independent processes but are essential parts of a well-regulated system. Exposure of breast epithelial cells in culture to the EGFR ligand amphiregulin induces expression of MMP2 and MMP9 (173).

Tissue recombinant experiments with fetal tissues have shown that stromal, not epithelial, ERα is needed during mammary gland morphogenesis (32) and that EGFR phosphorylation and ductal development only occur when ADAM-17 and amphiregulin are expressed on mammary epithelial cells, whereas EGFR is required in the stroma (286). These tissue recombinant experiments indicate that ERα is not required for expression of EGFR ligands or for expression of ADAM-17 in epithelial cells during morphogenesis. One working scenario for the role of ERα in mammary gland morphogenesis is that stromal ERα induces ADAM-17, which stimulates release of amphiregulin from epithelial cells in the terminal end buds. Amphiregulin activates EGFR in nearby stromal cells. In this way epithelium and stroma communicate with each other in coordinating ductal growth. This mechanism is valid during the limited time when ERα is expressed in the stroma. Our own data show that this is during weeks 2–4 of postnatal life (Fig. 5). ADAM-17−/− (55, 286), amphiregulin−/− (151, 286), and ERα−/− mice (113) all show impaired ductal elongation. EGF receptor−/− mice do not survive the perinatal period (175).

Mammary lobular/alveolar growth does not require ERα or EGF (314). The requirement for estrogen in alveolar growth in ERα−/− mice suggests that ERβ may be involved in alveologenesis and epithelial cell differentiation. Such a role for ERβ is consistent with the finding of incomplete differentiation of the mammary epithelium in ERβ−/− mice (54). There is a normal ductal tree in ERβ−/− mice, but as these mice age, the epithelium in
the mammary gland continues to express the proliferation marker Ki67 and to proliferate. The gland becomes filled with large cysts. Mammary epithelial proliferation in ERβ−/− mice between 1.5 and 2 yr of age appears to be independent of estrogen, since at this age the ovaries are depleted of follicles (Fig. 5). Our working hypothesis is that loss of ERβ leads to loss of TGF-β and loss of a major growth repressor in the mammary gland.

2. Prostate morphogenesis

Some of the most compelling evidence for a role of estrogen during embryogenesis is the outcome of in utero exposure to the synthetic estrogen diethylstilbestrol (DES). Both males and females, who were exposed in utero, develop in adulthood a wide range of abnormalities in their urogenital tracts. In the 1980s, the McLachlan laboratory pioneered the use of DES in mice for investigating the mechanism of the human DES syndrome. Permanent alterations occur in the male and female genital tracts when mice are exposed to DES at certain critical times during development. In male mice, prenatal exposure to DES is associated with poor semen quality, prostatic disease, cryptorchidism, testicular neoplasia, feminization of the seminal vesicles (induction of the estrogen responsiveness and of the estrogen-regulated gene lactotransferrin), and stromal inflammation (9, 190, 240). Imprinting by DES in the developing prostate is absolutely dependent on the presence of ERα, and ERα−/− mice are resistant to imprinting by DES (238).

It is, therefore, not surprising that the structure of the prostates of ERα−/− mice are abnormal (206). Surprisingly, unlike the case in the mammary gland, the ERα−/− mouse ventral prostate is characterized by extremely long distended ducts with little branching and very sparse periductal stroma. This phenotype is compatible with the suggestion that ERα is a positive regulator of secondary growth.
branching and of stromal growth. How might ER\textsubscript{\beta} be a positive regulator of secondary branching? The ER\textsubscript{\beta} prostate is not unlike that of the prostate of mice treated with EGF, i.e., increased prostatic size, with reduced ductal branching and swollen ducts.

Tissue recombinant experiments have shown that like the mammary gland, prostatic growth requires ER\textsubscript{\beta} signaling in the mesenchyme, not in the epithelium, and this is compatible with the idea that a mesenchymal growth factor is ER\textsubscript{\beta} regulated. Testosterone produced by the fetal testes drives prostate morphogenesis, but ductal elongation and branching involve a close communication between the urogenital mesenchyme and epithelium, and this communication involves positive regulation by ER\textsubscript{\alpha}, FGF10, Shh, and repressive activity of BMPs (15, 37). Although FGF10 is a prostatic mesenchymal growth factor, available data show that it is not regulated by androgens (290).

BMP7 secreted from the stroma is a repressor of Notch signaling and thus of branching morphogenesis (132). Follistatin, an estrogen-regulated gene produced in the epithelium, is an inhibitor of BMP signaling (105). The transient expression of ER\textsubscript{\alpha} observed in prostate epithelium in the first 2 wk of life (206) leaves open the possibility that ER\textsubscript{\alpha} may, at this time, regulate follistatin expression. ER\textsubscript{\alpha} would then induce a repressor of a repressor and would thus be a positive regulator of notch signaling and branching. In ER\textsubscript{\alpha} mice, secretion of follistatin from the epithelium would be repressed and the BMP would be free to inhibit branching. BMP7 is expressed in the perirethral urogenital mesenchyme prior to formation of the prostate buds. In BMP7 null mice, Notch1 signaling is derepressed in the urogenital epithelium (70, 302, 303). Both ER\textsubscript{\alpha} and EB\textsubscript{\beta} oppose BMP signaling (327).

The original description of the ventral prostate of adult, neonatally estrogenized rats [elevated expression of the androgen receptor, a continuous layer of basal cells, an increase in interacinar stromal tissue, disorganized acini with epithelial hyperplasia, luminal sloughing, lack of epithelial differentiation, and a remarkable reduction in expression of ER\textsubscript{\beta} (236, 237, 239, 321)] are characteristics of the phenotype of the ER\textsubscript{\beta} mouse ventral prostate (92, 313), and we speculate that these
characteristics of the adult prostates of neonatally estrogenized mice are a consequence of the reduction in ERβ expression in the prostates of these mice (236, 237, 239, 321).

Recently, the Prins laboratory demonstrated that one of the genes that is downregulated by neonatal estrogen exposure is FGF10. This then leads to downregulation of sonic hedgehog-patched Gli (Shh-ptc-gli) and BMP7. Thus ERα directly or indirectly is a repressor of BMP7, and the branching defects in the dorsolateral prostate of neonatally estrogenized mice can be explained by the loss of Shh-ptc-gli and BMP7 signaling (90).

Interestingly, according to Prins, FGF10, Shh-ptc-gli and BMP7 are not reduced in the ventral lobes of the prostate following neonatal estrogenization. This difference in lobe-specific expression of these factors explains why there are more serious branching deficits in the dorsal than the ventral lobe. It leaves unanswered the questions of why the structure and size of the ventral lobe are so profoundly affected by neonatal estrogenization and why FGF10 in the ventral prostate lobe is insensitive to neonatal estrogen. FGF signaling is androgen dependent: the urogenital sinus of FGF10−/− mice in culture can be stimulated to produce prostate buds by FGF10 plus testosterone but not by FGF10 alone. The requirement for both testosterone and FGF10 for the growth of prostatic buds suggests that testosterone, not estrogen, is essential for expression of FGF receptors in the epithelium. The observed high expression of androgen receptors in the ventral prostate of neonatally estrogenized mice might help explain the maintenance of FGF10 signaling in this tissue.

If loss of ERβ results in profound morphological changes in the ventral prostate, what are the ERβ regulated genes that lead to these abnormalities? To answer this question we have to examine the epithelium of the adult ventral prostate for growth factors that are estrogen repressed or growth repressors whose expression is in the epithelium of the adult ventral prostate for growth factors that are estrogenized mice are a consequence of the reduction in ERβ expression in the prostates of these mice (236, 237, 239, 321).

3. Lung morphogenesis

The lungs of adult ERβ−/− mice are characterized by decreased elastic tissue recoil (164, 165) and an increased expression of MMP1 and TIMP2, and cleaved (active) MMP2 (181). Alterations in the proteolytic/antiproteolytic balance of the lung have been associated with several respiratory diseases characterized by changes in the lung extracellular matrix (ECM). So it is not surprising that in the lungs of ERβ−/− mice there is accumulation of collagen and that suboptimal lung function leads to overall hypoxia in these mice (181). ERβ is abundantly expressed in epithelial cells in the adult lung, and the elevated levels of expression of MMP1 and TIMP2 in ERβ−/− mice indicates that ERβ might normally suppress this complex and restrict activation of MMP2.

Both EGF and FGF signaling are essential for development of lungs. EGFR is essential for epithelial-mesenchymal interactions, and the lungs of EGFR−/− mice are so impaired that mice do not survive the perinatal period (175). Embryonic lung synthesizes several EGFR ligands and responds to EGF, TGF-β, and amphiregulin by precocious branching (259, 306). FGF10 is expressed in lung mesenchyme during branching morphogenesis (E10.5–16.5) and is an essential nonredundant growth factor (107). Transgenic mice expressing a dominant negative FGF receptor specifically in lung tissue do not develop lungs (226). The severe phenotypes of both the FGF10−/− and the EGFR−/− mice are not seen in the ERβ−/− mice. Lungs of ERβ−/− mice are morphologically indistinguishable from those of their wt littersmates until mice are 5 mo of age. Expression of ERα and ERβ in the embryonic lung has not been reported, and we do not know whether ERβ plays a role in lung development or whether it only functions in remodeling of the adult lung. Ryu et al. (249) have shown that fetal human lung is characterized by high levels of MMP-2 and little TIMP-3 expression while the adult human lung exhibits a more antiproteolytic profile with decreased MMP-2 and increased TIMP-3 expression. The MMP profile in the ERβ−/− mouse lung resembles that of the fetal rather than the adult human lung and may reflect a block in maturation as is seen in epithelium of other organs in ERβ−/− mice.

B. Imprinting by Estrogen

Several distinct critical periods or “windows in time” when estrogen influences tissue morphogenesis have been observed for many organs. In 1975, the Naftolin laboratory made the astounding observation that in rodents, estrogen masculinizes hypothalamic neurons on embryonic day 18 (185). The estrogen for this masculinization is synthesized in the embryonic brain from testosterone produced in the testes, and this transient exposure...
to estrogen initiates a critical period of sexual differentiation of the brain (reviewed in Ref. 184). We now know that there are even earlier time points in development when estrogen can influence brain function. In 1992 Beyer et al. (17) showed that sexual differentiation in prolactin expression of certain diencephalic neurons occurs before the testosterone surge, and in 2001 studies on the brains of ERβ−/− mice revealed that ERβ influences neuronal survival as early as embryonic day 15 (301). For the uterus, vagina, prostate, and lung, one critical period when estrogen influences morphogenesis is between embryonic days 9 and 16. There is a second critical period between postnatal days 1 and 6 when the prostate and the skeleton are imprinted (176). In the developing mammary gland on the other hand, estrogen influences morphogenesis at puberty.

C. Estrogen as a Trophic Factor for Neurons

Some 30 years ago, it was found that estrogen enhances the growth and arborization of axons and dendrites in hypothalamic neurons grown in organotypic cultures (294). Neonatal estrogen treatment of rats has been shown to increase synapse formation in the arcuate nucleus during postnatal development (3) and to restore deafferentation-induced loss of axodendritic synapses in adults (166). Synapse remodeling in adults does not occur only in response to axonal injury but is a continuously ongoing process involved in memory and learning. Synapses in the arcuate nucleus and in the CA1 region of the hippocampus exhibit a phasic remodeling, coordinated with fluctuation in hormone levels during the estrous cycle (205). Growth-promoting effects of estrogen have also been described in the midbrain (244), cortex (62), hippocampus (68), spinal cord (298), and pituitary (26).

It is generally accepted that in the early postnatal period as well as in adults, changes in the morphology of synapses and increased number of dendritic spines can account for many of the estrogen-induced changes in brain morphology, sexual behavior, and memory and learning.

Effects of estrogens on neuronal growth are mediated via regulation of endogenous neurotrophic substances. Estrogen regulates nerve growth factor (NGF) and its receptors in cholinergic neurons (293), TGF-β in the hypothalamus (155), brain-derived neurotrophic factor in the cortex (281), NGF receptors in sensory neurons (280), and the insulin-like growth factor and its receptor in the hypothalamus (42, 233).

As in peripheral tissues, the effects of estrogen on growth factor signaling are reciprocated, and growth factors can influence estrogen signaling. Thus NGF has been shown to regulate ER action in the forebrain, possibly via phosphorylation events (177). However, estrogen can also affect neurite elongation and differentiation directly through its regulation of cytoskeletal genes that are required for neurite growth (49, 154, 260).

Both ERα and ERβ are expressed in the adult rat brain with ERβ mRNA-containing cells more widely dispersed throughout the brain than those expressing ERα mRNA (12, 36, 129, 213, 270). Comparison of ER mRNA expression patterns in rat, mouse (125, 270), monkey (72), and human (214, 215) indicates that, although the general distribution is similar, data cannot be extrapolated from one species to another. The paraventricular nucleus of rats has been reported to express only ERβ, while in the mouse it contains cells that also express ERα. The human paraventricular nucleus has been reported to express mRNA for both ERα and ERβ, with ERα predominating.

In addition to the well-characterized role of estrogen on feedback regulation of hypothalamic and pituitary hormone secretion (102) and facilitation of female reproductive behavior (157), estrogen also has effects on the modulation of motor behavior (8) and on mood and mental state (50, 76). More recently, estrogen has been recognized as a neuroprotective agent. The use of estrogen to protect against Alzheimer’s disease, Parkinson’s disease, schizophrenia, and ischemic stroke (16, 153, 258, 275, 276) is being widely debated at present. Multiple epidemiological reports have suggested that estrogen replacement may delay the onset of and slow the cognitive decline associated with Alzheimer’s disease (reviewed in Ref. 264). In some studies estrogen showed no beneficial effects on cognition (182, 271). Suggestions for possible mechanisms through which estrogen might exert positive effects in the brain include prevention of amyloid β-plaques (10, 67, 325) and regulation of apoptosis. Several studies have shown that estrogen can modulate expression of the antiapoptotic proteins Bcl-2 and/or Bcl-xL (the long isoform of Bcl-α) in hippocampal, cortical, and dorsal root ganglion neurons (41, 193, 222). In addition, estrogen can downregulate expression of the proapoptotic Bad or Nip-2, which is a negative regulator of Bcl-2 (21, 66).

Perhaps the optimal strategy for estrogen replacement still has to be worked out. It seems that there is a critical period around the menopause, and if estrogen replacement therapy is used early after menopause it delays the onset of and slows the cognitive decline. However, estrogen replacement therapy at a later stage does not seem to improve the cognitive function. It also still remains to be determined whether selective ERα and ERβ agonists might be more effective than the conjugated estrogens currently used.

D. ERs and Cell Adhesion

Adhesion between neighboring cells and cell adhesion to the ECM influence the structure of normal epithe-
From studies on the ER/H9252 epithelial cells, ER and histone deacetylation complex (58). In mammary epithelial cell space, characterized by decreased levels of the adhesion molecules, E-cadherin, connexin 32, occludin, and integrin α2.

During tumor progression, epithelial-to-mesenchymal transition is associated with the loss of adherens junctions, profound morphological changes, and enhanced migratory and invasive capabilities (reviewed in Ref. 31). Expression of the cadherin and catenin families, CD44, integrins and metalloproteases are all changed in cancer. E-cadherin is highly expressed in ERα-positive, low-grade tumors (220, 282), while P-cadherin is present in ERα-negative, high-grade tumors (128, 218). From studies on the ERβ−/− mouse mammary gland (54), it appears that ERβ positively regulates the terminal differentiation of the mammary gland epithelium. The loss of ERβ results in a reduction of the ECM and lamina basalis and, frequently, in an increase of the interepithelial cell space, characterized by decreased levels of the adhesion molecules, E-cadherin, connexin 32, occludin, and integrin α2.

During tumor progression, epithelial-to-mesenchymal transition is associated with the loss of adherens junctions, profound morphological changes, and enhanced migratory and invasive capabilities (reviewed in Refs. 31, 103). Loss of E-cadherin is a key event in this progression. The E-box binding transcriptional repressors Snail, Slug, SIP1, and Twist are key repressors of E-cadherin transcription. Snail in turn is repressed by an E2 regulated gene, the metastatic tumor antigen 3 (MTA3), a component of the Mi-2/NuRD nucleosome-remodeling and histone deacetylation complex (58). In mammary epithelial cells, ERα is necessary for maintenance of mammary epithelial architecture by constraining Snail repression of E-cadherin. ERα with its corepressors NCoR and SAFB1 can bind to and repress transcription of the E-cadherin promoter (201). Thus E-cadherin expression is regulated by the cellular ER content, coactivators/corepressors, and active signaling from other pathways.

IV. ANTI-ESTROGEN SIGNALING

Many of the growth-stimulatory effects of estrogens in breast cancer have been linked to ERα (14, 248). Today, ERα expression is routinely checked in pathological diagnosis, and if ERα is expressed in the tumor, the patient receives the anti-estrogen tamoxifen. Seventy percent of women with ERα-positive breast cancer benefit from tamoxifen (43a, 100). The usefulness and importance of tamoxifen in breast cancer therapy is well established, and the disease-free survival rate has increased drastically due to the widespread use of tamoxifen (24).

The potential of ERβ as a predictive factor in breast cancer has been under intense scrutiny. Several publications have evaluated ERβ mRNA in breast cancer, but the usefulness of ERβ as a diagnostic marker is still not clear (39, 219, 251, 285). While ERβ is the predominant receptor in the normal breast, its levels are reduced in breast tumors, and some laboratories have reported on a correlation between the presence of ERβ and lower tumor stage and grade (96, 183, 207, 208). Others have found no correlation of ERβ protein with clinical parameters (60).

The unwanted agonistic effects of anti-estrogens in the breast and uterus have traditionally been linked to a strong AF-1 activity of ERα, since an effective and specific structural inhibition of AF-2 activity is seen with these ligands (Fig. 6). Although the tissue-selective agonist activity of anti-estrogens may be a function of the cell type-specific set of coregulators, surprisingly few of the well-known NR and ER coactivators (reviewed in Refs. 6, 163, 279) have been reported to be directly involved in anti-estrogen signaling (Tables 1, and 2). The contribution of ERβ to the tissue-selective agonistic effects of tamoxifen is considered minimal, since the activity of the AF-1 domain in this receptor subtype is almost negligible in reporter-mediated gene expression (75).

Anti-estrogens were traditionally thought to occupy the ligand-binding pocket blocking access to E2 and freezing ER in “inactive” conformations that are unfavorable to coactivator interactions. Current evidence suggests that cell type-specific profiles of coregulators determine transcriptional activities of antagonist-bound ERs (108, 110, 263). Furthermore, different antagonists induce ligand-specific conformational changes in ERs, allowing exposure of unique surfaces for coregulator interactions (82, 199, 217). These findings, together with recent gene expression profiling data (56, 57, 84), extend the traditional view of ER antagonist function to include “active anti-estrogen signaling.”

A. Anti-estrogens: Benefits and Limitations

When clinically employed as antiproliferative agents in the treatment of breast cancer, the agonist action of antiestrogens is desirable in certain tissues like bone and cardiovascular system, while highly inappropriate in tissues like uterus or breast. Optimization of therapeutic strategies for targeting hormone-dependent tumors is focused on agents with slightly modified pharmacological profiles. Raloxifene and toremifene are therapeutically established anti-estrogens, available for prevention of osteoporosis (18) and treatment of advanced hormone-sensitive breast cancer (80). Since the mechanism of action of tamoxifen, raloxifene and toremifene are closely related, similarities in their therapeutic profiles are expected. Although raloxifene and toremifene do exhibit the
same beneficial effects as tamoxifen on bone and on serum lipoprotein profile, unlike tamoxifen, raloxifene and toremifene do not induce uterine growth. Unfortunately, raloxifene causes an increase in hot flushes and also increases the incidence of thromboembolic diseases. Toremifene has not been in clinical use long enough to yield sufficient follow-up data. However, the few safety data that are accumulated suggest a favorable safety profile for toremifene (80).

B. Complete Blocking of Estrogen Signaling

There are two therapeutic strategies for completely blocking estrogen signaling; one is the use of a pure ER antagonist, having no agonistic properties, and the other is blocking of estrogen synthesis with the use of aromatase inhibitors. Fulvestrant (ICI 182,780) binds, inhibits, and promotes degradation of ERs (47, 162). Preclinical studies have indicated that fulvestrant in combination with growth factor receptor inhibition is an effective treatment option for postmenopausal women with advanced breast cancer. Because estrogen is essential for maintenance of brain function, complete blocking of estrogen receptors may be associated with cognitive disturbances and may render neurons more sensitive to stress and excitatory neurotoxins.

Aromatase inhibitors are effective in advanced breast cancer, but clinical trials have indicated that resistance develops with this strategy (reviewed in Ref. 40). Despite intense research, optimal agents targeting ER signaling have not yet been identified. Further investigation is likely to increase our understanding of the complexities of ER signaling and increase the chances of rational design of more optimal ER ligands for targeting estrogen-dependent tumors.

C. Deleterious Effects of Anti-estrogens

The acquired tamoxifen resistance developed in tumors after some time of treatment is a major problem and cause of serious concern to clinicians. Recent data indicate that growth factor signaling is likely to be involved in the development of tamoxifen resistance. There is reciprocal cross-talk between estrogen and growth factor signaling: estrogens regulate growth factor signaling and growth factors can activate ER (191, 257). The HER2 downstream signaling molecules ERK1 and ERK2 can phosphorylate ER, leading to enhanced sensitivity of the

### TABLE 1. Coactivators reported to be involved in tamoxifen signaling

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Mechanism of Activation</th>
<th>Involved ER Domain</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIB1</td>
<td>Recruit HATs, NR boxes</td>
<td>ERα-AF-1 and LBD</td>
<td>2, 210, 311</td>
</tr>
<tr>
<td>SRC-1</td>
<td>Recruit HATs, NR boxes</td>
<td>ERα-AF-1 and LBD</td>
<td>263, 278</td>
</tr>
<tr>
<td>L7SPA</td>
<td>Not known</td>
<td>ERα hinge</td>
<td>95</td>
</tr>
<tr>
<td>p68</td>
<td>Enhance tamoxifen activity when S118 phosphorylated, can recruit HATs</td>
<td>ERα-AF-1</td>
<td>45</td>
</tr>
</tbody>
</table>

ER, estrogen receptor; LBD, ligand-binding domain; AF, activation function.
receptor to its cognate hormone and potentially to ligand-independent receptor activation (106). Interestingly, ERK1/2 expression and activity are increased in several estrogen nonresponsive human breast cancer cell lines (30, 269). Coregulators are also subject to posttranslational modifications by the signaling kinases and thereby these enzymes may indirectly affect ER activity (53, 85, 134, 322). Data from xenograft models and cell lines show that overexpression of the growth factor receptor HER2 may constitute a primary mechanism of tumor resistance to tamoxifen (13, 117, 170, 228). Recent clinical evidence shows that patients with tumors expressing high levels of the transcriptional coactivator AIB1 in combination with HER2 have a poor response to tamoxifen. Those patients with tumors expressing high HER2 or AIB1 separately had a good disease-free survival (210). These data suggest that increased growth factor signaling via HER2 activates kinases that lead to phosphorylation of ER and AIB1, circumstances under which the agonistic activity of tamoxifen might be enhanced (reviewed in Refs. 211, 256).

D. Structural Basis for ER Activity

Structural analysis of ER domains over the past decade has greatly enriched our understanding of receptor function (232). While elucidation of the intact receptor’s three-dimensional architecture continues to pose formidable challenges to structure determination techniques due to interdomain flexibility and the poorly structured nature of the NH2-terminal region, analysis of the individual DNA- and ligand-binding domains has provided a wealth of information about sequence-specific DNA target recognition (27, 114) and the ligand-induced conformational changes that underpin receptor activation (188, 232).

The vast majority of structural studies of ER have focused on its COOH-terminal LBD due to the desire to develop subtype-specific ligands (115, 159). Consequently, numerous crystal structures have been determined for the LBDs of both ER subtypes bound to a range of different ligands and coactivator fragments (see Table 3). These studies have provided valuable information regarding the structural basis of receptor agonism/antagonism and the determinants that influence ERα’s and ERβ’s ligand-binding preferences (229, 232). The crystal structures demonstrate that ligand binding to a buried cavity in the interior of the ER-LBD stabilizes distinct receptor conformations that are subsequently interpreted by the transcriptional apparatus.

One of the main weaknesses of crystal structure analysis is that this technique typically provides a static picture/snapshot of the molecules being analyzed. However, the sheer variety of complexes that have been determined for ER-LBD has allowed us to build a comprehensive dynamic picture of the characteristic structural alterations that accompany binding of particular classes of receptor ligands.

E. Role of Helix 12 in the Regulation of AF-2 Activity

ER’s ligand-dependent transcriptional activation function (AF-2) is localized in a conformationally dynamic region of the LBD. The key element of the AF-2 conformational switch is a short helical region (helix 12; H12) located at the carboxyl terminus of the LBD. Different classes of receptor ligands influence H12’s orientation with respect to the rest of the LBD (Fig. 6). Agonist ligands stabilize a receptor conformation that is optimal for efficient interaction with coactivators and thereby facilitates transcriptional activation. In such a “transcriptionally active” conformation, the helical elements that comprise the AF-2 region (helices H3–5 and H12) assemble to form a shallow hydrophobic binding site for the leucine-rich LxxLL motifs of NR coactivators (Fig. 6, A and B). In this conformation, H12 is positioned across the entrance to the ligand-binding pocket and constitutes a key part of the coactivator docking surface (22, 195, 267). In contrast, receptor antagonists interfere with positioning of H12 through a variety of mechanisms resulting in ER conformations in which the coactivator recruitment site is incomplete (Fig. 6, C–E).

In cases where H12 is not stabilized in the “agonist conformation,” the helix is reoriented to bind along and occlude the AF-2 groove (Fig. 6, C and D), thereby physically blocking the recruitment site (22, 267). The commonly held view is that H12 is able to bind along the coactivator groove by mimicking the consensus LxxLL motif with its own intrinsic related sequence (267). Our recent analysis, however, suggests an alternate and more compelling explanation: ER’s H12 region contains an extended corepressor (CoRNR) box sequence that occludes the AF-2 site and prevents unwanted interaction.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ligand Class</th>
<th>Coregulator</th>
<th>PDB Code*</th>
<th>Comments</th>
<th>Reference Nos.</th>
</tr>
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<tr>
<td>ERα E2</td>
<td>Agonist</td>
<td>IERE</td>
<td>IA52</td>
<td>First structure of steroid receptor with agonist</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IQKT</td>
<td>Intermolecular disulfide crosslink generates nonphysiological</td>
<td>44, 61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IQKU</td>
<td>conformation of H12</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>IG50</td>
<td>Agonist conformation</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SRC2-3</td>
<td>IGRW</td>
<td>Agonist conformation with CoA peptide</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclic LxxLL</td>
<td>IPCG</td>
<td>Complex with cyclic antagonistic LxxLL peptide</td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td>Agonist</td>
<td>mSRC2-2</td>
<td>JERD</td>
<td>First illustration of CoA recruitment with LxxLL peptide bound along AF2</td>
<td>207</td>
</tr>
<tr>
<td>RAL core</td>
<td>Agonist</td>
<td>SRC2-2</td>
<td>IGWQ</td>
<td>Agonist conformation</td>
<td>308</td>
</tr>
<tr>
<td>GEN</td>
<td>Agonist</td>
<td>SRC2-2</td>
<td>IX7R</td>
<td>Agonist conformation. Provides clues to origins of ERα/β selectivity of GEN</td>
<td></td>
</tr>
<tr>
<td>THC</td>
<td>Agonist</td>
<td>mSRC2-2</td>
<td>IL21</td>
<td>Agonist conformation</td>
<td>268</td>
</tr>
<tr>
<td>WAY-244</td>
<td>Agonist</td>
<td>SRC2-2</td>
<td>IX7E</td>
<td>Agonist conformation with ERβ selective agonist</td>
<td>159</td>
</tr>
<tr>
<td>Benzopyran</td>
<td>Agonist</td>
<td></td>
<td>210J</td>
<td>Antagonist H12 conformation with ERβ selective agonist</td>
<td>106</td>
</tr>
<tr>
<td>Oxabicyclophe</td>
<td>Agonist</td>
<td>mSRC2-2</td>
<td>1ZKY</td>
<td>Agonist conformation with ERβ selective agonist based on oxabicyclic scaffold</td>
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<td>nols</td>
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<td>2BIV/2FAI</td>
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<td>Dihydroequilenin</td>
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<td>2BIZ</td>
<td>Agonist conformation</td>
<td>unpub</td>
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<td></td>
<td>Antagonist</td>
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<td>1ERR</td>
<td>Complex with SERM highlighting archetypal “agonist conformation” of H12 with AF2 groove occluded</td>
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</tr>
<tr>
<td>DES</td>
<td>Agonist</td>
<td>1Z7X</td>
<td>LxxLL</td>
<td>LxxLL-stabilized agonist conformation</td>
<td>This study</td>
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<tr>
<td>4-OHT</td>
<td>Antagonist</td>
<td>Affinity selected peptide</td>
<td>JERT</td>
<td>Antagonist conformation</td>
<td>118</td>
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<td></td>
<td></td>
<td>2B4J</td>
<td>Complex with ERα-specific phage-selected peptide antagonist highlighting novel putative coregulator docking site</td>
<td>207</td>
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<tr>
<td>Tetrahydro,</td>
<td>Antagonist</td>
<td>IUOM</td>
<td>ERα selective SERMs (SERAMs):</td>
<td>245, 246</td>
<td></td>
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<tr>
<td>isoquinolines</td>
<td></td>
<td>IXQC</td>
<td>antagonist conformation</td>
<td></td>
<td></td>
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<tr>
<td>GW56d8</td>
<td>Antagonist</td>
<td>IR5K</td>
<td>Antagonist conformation with orientation of H12 that differs slightly from the archetypal RAL/OHT structures</td>
<td></td>
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<tr>
<td>Dihydrobenzoxanthines</td>
<td>Antagonist</td>
<td>ISJ0/IXP1/IXP6/</td>
<td>Series of ERα-selective SERMs; antagonist conformation</td>
<td>19, 115</td>
<td></td>
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<td></td>
<td></td>
<td>IXP9/IXPC</td>
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<tr>
<td>Chromanes</td>
<td>Antagonist</td>
<td>IYIM</td>
<td>Series of ERα-selective SERMs</td>
<td>288</td>
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<tr>
<td>Aryl-naphthalene</td>
<td>Antagonist</td>
<td>IYIN</td>
<td>Complex with SERM effective against uterine leiomyoma</td>
<td>91</td>
<td></td>
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<tr>
<td>ERβ E2</td>
<td>Agonist</td>
<td>NcoA5 peptide</td>
<td>2J7X</td>
<td>LxxLL-stabilized agonist conformation</td>
<td>This study</td>
</tr>
<tr>
<td>17-Epiestril</td>
<td>Agonist</td>
<td>NcoA5 peptide</td>
<td>2J7Y</td>
<td>Ligand orientation dictated by hydroxyl positioning on D ring</td>
<td>This study</td>
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<tr>
<td>ERB041</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>IX7B</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>159</td>
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<tr>
<td>WAY-J97</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>IU9E</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>159</td>
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<tr>
<td>WAY-697</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>IX76</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>159</td>
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<tr>
<td>WAY-244</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>IX78</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>159</td>
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<tr>
<td>CL-272</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>103Q</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>158</td>
</tr>
<tr>
<td>WAY-338</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>IU3R</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>158</td>
</tr>
<tr>
<td>WAY-297</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>IU3S</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>158</td>
</tr>
<tr>
<td>2-aryldene-1-one</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>1ZAF</td>
<td>Series investigating ERα/β-selective agonist binding</td>
<td>171</td>
</tr>
<tr>
<td>2-Phenyl-naphthalene</td>
<td>Agonist</td>
<td>SRC1-1</td>
<td>1YY4/1YYE</td>
<td>Series investigating ERβ-selective agonist binding</td>
<td>174</td>
</tr>
<tr>
<td>Tetrahydrofuroene</td>
<td>Agonist</td>
<td>2GIU</td>
<td>ERβ-selective agonist; H12 bound along AF2 in suboptimal orientation</td>
<td>316</td>
<td></td>
</tr>
<tr>
<td>Benzopyran</td>
<td>Agonist</td>
<td>210G</td>
<td></td>
<td>H12 bound along AF2 in suboptimal orientation with ERβ selective agonist.</td>
<td>106</td>
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</table>

Physiol Rev • VOL 87 • JULY 2007 • www.prv.org
TABLE 3.—Continued

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<th>Coregulator</th>
<th>PDB Code</th>
<th>Comments</th>
<th>Reference Nos.</th>
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<td>GEN</td>
<td>Partial agonist</td>
<td>SRC-1</td>
<td>1QKM</td>
<td>H12 bound along AF2 in suboptimal orientation</td>
<td>160, 230</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>1X7J</td>
<td>Presence of LxxLL peptide forces H12 to adopt classic agonist orientation</td>
<td></td>
</tr>
<tr>
<td>RAL</td>
<td>Antagonist</td>
<td></td>
<td>1QKN</td>
<td>Antagonist conformation</td>
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<td>OHT</td>
<td>Antagonist</td>
<td></td>
<td>2PSZ</td>
<td>Nonnative H12 orientation stabilized by crystal contacts; additional OHT molecule bound to AF2 cleft</td>
<td>304</td>
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<tr>
<td>Triazine</td>
<td>Antagonist</td>
<td></td>
<td>INDE</td>
<td>ERb-selective SERM; antagonist conformation</td>
<td>83</td>
</tr>
<tr>
<td>THC</td>
<td>Antagonist</td>
<td></td>
<td>1L2J</td>
<td>Antagonist conformation related to that seen in 1QKM. First demonstration of &quot;passive&quot;/indirect antagonism</td>
<td>268</td>
</tr>
<tr>
<td>ICI 164,384</td>
<td>Full Antagonist</td>
<td></td>
<td>1HJI</td>
<td>Only pure antagonist structure; ICI side chain sterically blocks both agonist and antagonist docking of H12. AF2 accessible</td>
<td>231</td>
</tr>
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</table>

Coregulator peptide nomenclature is given as p160 subtype followed by NR box number. E2, 17β-estradiol; DES, diethylstilbestrol; RAL, raloxifene; OHT, 4-hydroxytamoxifen; THC, 5αR-11β-diethyl-5,6,11,12-tetrahydrochrysene-5-ol; GEN, genistein; ERβ, ERβ; ERβ041, 2-(3-fluoro-4-hydroxyphenyl)-7-vinyl-1, 3-benzoxazol-5-ol; ICI 164,384, (7α,17β)-n-butyl-3,17-dihydroxy-N-methyl-estra-1,3,5(10)trien-7-undecanamide. * Released PDB entries (www.resb.org) as of November 2006.

with traditional NR corepressors (Heldring, unpublished data). This would explain why removal of H12 greatly enhances ER’s ability to interact with traditional NR corepressors such as NCoR and SMRT (89, 310).

F. Classical AF-2 Antagonism

The AF-2 antagonists tamoxifen/raloxifene (Fig. 6C) and pure antagonist ICI 164,384 (Fig. 6E) all harbor a bulky side chain of varying length that cannot be contained within the ligand-binding pocket. As a consequence, H12 is sterically hindered from aligning in the proper agonist conformation. While tamoxifen and raloxifene are observed to induce similar receptor conformations, the rERβ-ICI structure (231) is slightly different since ICI’s long alkylamido side chain itself binds along the AF-2 recruitment site and completely abolishes the association between H12 and the rest of the LBD (Fig. 6E). Such differences in H12 positioning and concomitant AF-2 groove accessibility may underlie the pure antagonist effects seen with ICI (189, 231). Subtle differences in the positioning and stabilization of H12 along AF-2 may underlie the difference in tissue-specific actions of certain anti-estrogens. For example, a recent structure of hERα in complex with GW5638, an anti-estrogen that is mechanistically distinct from raloxifene and 4-hydroxytamoxifen, exhibits a novel orientation for H12 along the AF-2 binding groove (323).

It is worth noting that the structural snapshots provided by the various ligand complexes create a rather false impression that the orientation of H12 is limited to a few positions. This situation is exaggerated by the fact that all structural studies have been carried out with the isolated LBD, and we have little idea how other regions of the receptor influence H12 dynamics. Instead, the “agonist” and “antagonist” states of H12 observed in the LBD structures most likely represent stable end points in a continuum of possible conformations that reflect the diverse agonist/antagonist character of ER ligands.

G. Nonclassical AF-2 Antagonism

AF-2 antagonism can also be achieved without a bulky substituent (268). Such indirect antagonism occurs when a ligand fails to make the appropriate contacts within the ligand-binding cavity so that the antagonist rather than the agonist orientation of H12 is preferred. The crystal structure of hERα bound to genistein reveals that even though genistein binds within the ligand pocket in a similar manner to estrogen, H12 adopts an antagonist position rather than the typical agonist position (230) (Fig. 6D). The origin of this destabilizing effect on H12 is unclear even though it is known that the agonist positioning of H12 is more unstable in ERβ than in ERα possibly due to altered residues at positions involved in anchoring the NH2-terminal end of H12. The degree of destabilization appears to be marginal and the agonist orientation can be promoted by crystallizing ERβ-GEN in the presence of an LxxLL peptide (160). In contrast, THC, which acts as a full agonist on ERα while being a potent antagonist on ERβ, strongly favors the antagonist H12 conformation when bound to ERβ. THC appears to antagonize ERβ by affecting key residues in the ligand-binding pocket that leads to the stabilization of tran-
scriptionally nonproductive conformations (268). These findings provide key information for the rational design of novel ER ligands with enhanced subtype selectivity and diverse physiological effects. Yet another example of nonclassical AF-2 antagonism may emerge from a recent structure observation that 4-hydroxytamoxifen occupies a second binding site on the ERβ AF-2 surface (304). If this weak-affinity nonvalidated binding site is pharmacologically significant, antagonists might directly interfere with receptor-coactivator interactions.

H. Coregulator Recognition of Antagonist-Bound ER

As described above, coactivators (and in some cases corepressors) are able to bind to the LBD conformation stabilized by agonists via LxxLL motifs. However, receptor antagonists induce a conformation in which AF-2 exhibits little, if any, affinity for such motifs. It is well established, at least for certain members of the NR superfamily, that the AF-2 region also serves as the docking site for NR corepressors (87, 187, 224). In the case of antagonist-bound PPAR, the leucine-rich CoRNR box motif of SMRT binds as an extended helix along the AF-2 binding site (326). In the case of ER, the receptor conformation induced by anti-estrogens such as raloxifene and 4-hydroxytamoxifen, in which AF-2 is completely occluded, is difficult to reconcile with the known binding mode of the CoRNR motif. Recently, we have determined the crystal structures of two affinity-selected peptides, specific for the 4-hydroxytamoxifen-bound receptor conformation, in complex with the antagonist-bound ER LBD (Heldring, unpublished data). The two structures demonstrate that the AF-2 region of ER is, in principle, capable of directly interacting with corepressors. Both peptides bind along the AF-2 region in a manner similar to the PPAR-SMRT complex confirming that the general structural principles of corepressor motif binding to AF-2 are conserved within the NR superfamily. However, an internal CoRNR-box sequence motif within H12 serves as an effective “corepressor surrogate” and provides a considerable barrier to binding.

Alternatively, the AF-2 region may not serve as the dominant protein-protein interaction site in the anti-estrogen-bound state, and putative ER cofactors may target other alternative binding surfaces that are revealed in a ligand-specific manner. Recently, we characterized one such novel recruitment site for another phage-derived, ERα-specific interaction motif that lies on the opposite face of the LBD (118). Whilst the question remains as to whether this motif acts as a fortuitous conformational probe or is an actual mimic of an ER interaction partner, the docking site on the surface of ER appears to be a bona fide control surface involved in regulating receptor activity.

I. ER Coregulators

Studies in the past decade have provided much novel information on the general and cell-type specific actions of distinct coregulators involved in chromatin remodeling, histone modifications, transcription initiation and elongation, splicing, and coordinated degradation. Most of these proteins are bona fide coregulators for many members of the NR family and have been studied extensively using ERs (reviewed in Refs. 146, 223, 279; see also http://www.nursa.org). Coregulators are recognized to be critical for proper function of ER signaling in development, reproduction, and physiology, and alterations in

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Mechanism of Interaction</th>
<th>Involved ER Domain</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCoR</td>
<td>Class II</td>
<td>ERα-LBD</td>
<td>48</td>
</tr>
<tr>
<td>SHP</td>
<td>Class II</td>
<td>ERα-LBD, ERβ-LBD</td>
<td>99</td>
</tr>
<tr>
<td>RIP 140</td>
<td>Class II</td>
<td>ERα-LBD</td>
<td>23</td>
</tr>
<tr>
<td>DAX-1</td>
<td>Class II</td>
<td>ERα-LBD</td>
<td>131, 329</td>
</tr>
<tr>
<td>MTAl</td>
<td>Possibly class II</td>
<td>ERα-AF1, AF2, and DBD</td>
<td>124</td>
</tr>
<tr>
<td>DP97</td>
<td>Class III</td>
<td>ERα-LBD</td>
<td>241</td>
</tr>
<tr>
<td>REA</td>
<td>Class III</td>
<td>ERα-LBD</td>
<td>34, 273</td>
</tr>
<tr>
<td>MTA1</td>
<td>Class III</td>
<td>ERα-LBD</td>
<td>123, 169, 192</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Class III</td>
<td>ERα-LBD</td>
<td>156, 330</td>
</tr>
<tr>
<td>LIM domain only 4</td>
<td>Class III</td>
<td>ERα-DBD and LBD</td>
<td>277</td>
</tr>
<tr>
<td>Smad4</td>
<td>Class III</td>
<td>ERα-AF1</td>
<td>142</td>
</tr>
<tr>
<td>MRF-1</td>
<td>Class III</td>
<td>ERα-AF1 and LBD</td>
<td>64</td>
</tr>
<tr>
<td>pp32</td>
<td>Class III</td>
<td>ERα-DBD</td>
<td>147</td>
</tr>
<tr>
<td>CIP</td>
<td>Class IV</td>
<td>Indirect</td>
<td>7, 300</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>Class IV</td>
<td>Indirect</td>
<td>126, 210</td>
</tr>
<tr>
<td>SHARP</td>
<td>Class IV</td>
<td>Indirect</td>
<td>265</td>
</tr>
</tbody>
</table>

Class II, LxxLL recruitment motif; class III, lack an identified interaction motif; class IV, indirect recruitment.
coregulator function and expression are associated with cancer and other diseases.

The best-defined structure-function coregulator interaction is with the steroid receptor coactivator SRC (p160) family of coactivators. The important role of the p160 family members in ER signaling has been demonstrated with loss of function studies where ER activation is heavily affected, and severe phenotypes have been observed in estrogen target tissues when p160s are absent (63, 325). Interestingly, increased expression of AIB1 (SRC-3) has been observed in breast tumors, suggesting a possible involvement in carcinogenesis (2). However, many other proteins are also involved in ER signaling. Coactivators and corepressors have been shown to exist in multifunctional protein complexes (81, 88, 261, 292, 328). Even though the components of several complexes are identified, less is known about the processes controlling the recruitment by transcription factors. One model suggests that distinct coactivator and corepressor complexes are present in a preformed state and are recruited to the transcription site depending on the state of the transcription factor/NR/ER (65, 81). However, some coactivators and corepressors are, despite their opposite function, found in the same protein complexes (4, 143, 178, 287, 297).

With regard to corepressors, a number of recent studies have demonstrated that both agonist- and antagonist-bound ERs are able to recruit a variety of proteins that apparently repress activity (Tables 2 and 4). Simplified, these putative ER corepressors can be grouped into four major classes based on the reported interaction mechanisms (Fig. 7): those that contain a classical corepressor (CoRNR-box; class I) interaction motif (263), those that contain an LxxLL motif and are recruited in an estrogen-dependent manner acting as anti-coactivators (class II) (23, 48, 116, 329), those with less defined interaction mechanisms but most likely different from NR/CoRNR box type of interactions and possibly including an interaction-domain outside LBD (class III) (34, 47, 64, 124, 142, 169, 198, 203, 241, 330), and those that have indirect effects and possibly are recruited via complexes (class IV) (130, 210, 265, 300). However, the possibility also exists that some corepressors are recruited through multiple independent mechanisms and therefore would qualify to belong to several classes. For example, small heterodimer partner (SHP)

![FIG. 7. Schematic representation of ER corepressors based on the reported interaction mechanism. Class I: corepressors interacting through classical CoRNR-box motifs. Class II: corepressors interacting through NR boxes, thereby blocking coactivator binding. Class III: corepressors that most likely display interactions different from NR and CoRNR-box type. Class IV: corepressors with indirect effects possibly recruited via complexes.](http://physrev.physiology.org/)

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has been shown, in addition to acting as an anti-coactivator (class II) in an estrogen-dependent manner, also to repress the agonist activity of tamoxifen (116), and this function is most likely achieved through a different mechanism of recruitment. Several of the corepressors shown to act estrogen signaling may have the potential to be involved in antagonist signaling as well, provided that their mechanisms of repression are not due to anti-coactivator function by occupying the hydrophobic NR box site.

V. CONCLUDING REMARKS

The writing of this review coincides with a heated debate about the clinical use of estrogens and anti-estrogens provoked by the results of two large epidemiological studies, the Women’s Health Initiative (WHI) investigating the pros and cons of hormone replacement therapy (HRT) and the Early Breast Cancer Trialist’s Collaborative Group (EBCTG). It is clear that an understanding of the multifaceted estrogen receptor signaling is important from both a preclinical and a clinical perspective. A combination of interdisciplinary efforts including cell and molecular biology and genetically modified animals has generated a solid platform of knowledge but has also provided the basis for future research directions in the field. Much research effort has been focused on how ERs, in the absence of estrogens, can be activated by growth factor pathways and what the relevance of this phenomenon is to development of anti-estrogen resistance in cancer. The fact is that ERα and ERβ are modulators of growth factor pathways, and if this modulation is lost, growth becomes dependent solely on growth factors and their receptors. In this review we have discussed how estrogen influences growth factor pathways. It is a complex interaction between epithelium and stroma, depending on the changing presence of ERα and ERβ in these two tissue compartments. Current evidence suggests a cell-type specific involvement of coregulators in determining transcriptional activities of antagonist-bound ERs. However, molecular details of how antagonists trigger recruitment of these suggested coregulators remain unknown. One of the important issues for the future is to identify and characterize the proteins that bind or communicate with ERs in the presence of antagonists. It is likely that the currently known coregulators only represent a fraction of those mediating receptor activities. Considering that the two ERs appear to have overlapping but also unique biological functions, it is most likely that receptor subtype-specific coregulators exist. Identification of new coregulators may provide novel targets for development of new classes of therapeutic drugs with potential use in the treatment of diseases involving ER signaling. In this review we have discussed the advances in understanding the molecular basis of anti-estrogen signaling which is invaluable for the development of more selective and effective ER ligands and for future improvements of clinical applications.

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