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REVIEW

Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer

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Abstract

Evidence for a role of ovarian factors in the growth of metastatic breast cancer was first recognized over 100 years ago. Today, anti-estrogens are central to the treatment of breast cancer of all stages. We now understand that the action of estrogen is mediated by the estrogen receptors (ER) which are members of the nuclear receptor family of ligand-regulated transcription factors. In this article we review the molecular mechanisms through which ER activates transcription of target genes and through which available anti-estrogens mediate their therapeutic effects. We discuss possible mechanisms of failure of treatment with current anti-estrogens and how newer anti-estrogens under development attempt to address these problems. In addition an expanded view of the molecular mechanisms of estrogen action is leading to the development of novel selective ER modulators or SERMs.

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Introduction

The estrogen receptor (ER) plays an important role in the clinical care of breast cancer patients both as a prognostic factor as well as a therapeutic target. By immunohistochemistry, ER expression is found in only 6–10% of normal breast epithelial cells, whereas 60% of primary breast cancers are ER-positive (1, 2). Of advanced ER-positive breast cancers, two-thirds will respond to therapy with anti-estrogens such as tamoxifen (Nolvadex) (3). In addition, the majority of ER-positive tumors, even if initially responsive to anti-estrogenic treatment will eventually develop resistance to this treatment, in general without altering their ER profile (4). For the most part, tamoxifen is an effective adjuvant therapy for early-stage breast cancers only in ER-positive tumors (5, 6). The extensive use of tamoxifen as successful breast cancer therapy and the reduced incidence of contralateral breast cancer during tamoxifen treatment have encouraged the testing of tamoxifen as a preventive agent (5). The breast cancer prevention trial found that a median of 55-months treatment with tamoxifen decreased the risk of invasive breast cancer by 49% and estrogen receptor-positive breast cancer by 69% (7). Importantly, tamoxifen had no effect on the development of ER-negative cancers. The BCPT thus provides strong evidence for the ability of tamoxifen to reduce the risk of ER-positive breast cancers (8).

The endocrine therapy of breast cancer was first reported in 1896 by Sir George Beatson, a British surgeon who described that oophorectomy could lead to tumor regression (9). A review of the response rate to oophorectomy showed it to be in the order of 30% in all breast cancer patients (10). This is approximately equivalent to endocrine therapy seen today in unselected patients. Although certain clinical features predicted a positive response to endocrine therapy the reason for the heterogeneous response of breast cancer remained unknown until the 1960s with the identification of the estrogen receptor by Toft and Gorski (11) and Jensen *et al.* (12). Measurement of the level of ER expression in the primary breast tumors has allowed an accurate prediction of response to endocrine therapy.

Interestingly, estrogens were among the first hormonal agents to be studied for the treatment of metastatic disease, and partial response rates of 20–40% in postmenopausal women treated with diethylstilbestrol (DES) could be achieved. Due to the greater side effects of high dose estrogens compared with other hormonal agents they are currently infrequently used. Nevertheless, it has been shown by many clinical trials that response rates to estrogens are similar to current therapy with anti-estrogens (13, 14). For the treatment of advanced breast cancer both nonsteroidal anti-estrogens such as tamoxifen, and progestins such as megestrol acetate, are widely used. In addition, anti-estrogens

with pure antagonistic effects like ICI 182,780 (Faslodex) and new targeted anti-estrogens are being tested. To date, there is no compelling insight into the apparent paradox that both estrogens and anti-estrogens exhibit similar efficiency in breast cancer treatment.

ER is a member of the nuclear receptor family

The molecular cloning of the first human estrogen receptor, ERα, revealed it to be a member of a family of nuclear receptors for small hydrophobic ligands that regulate growth, differentiation and homeostasis in eucaryotic cells (15). This family of receptors includes those for steroid hormones, thyroid hormone, vitamin D, retinoic acid (vitamin A) and eicosanoids (16–20). As a class, these receptors are transcription factors whose activity is regulated allosterically by ligand binding. The 66 kDa ER protein is encoded by 8 exons of a gene which encompasses ~140 kb in length. The ERα gene has been localized to human chromosome 6q24-27 (21).

The estrogen receptor and its ligand estradiol had been thought to be essential for survival, fertility, sexual differentiation and development (22). The disruption of the mouse ERα gene has generated new and unexpected insights. The lack of ERα does not lead to embryonic lethality nor does its absence affect the processes leading to sex determination. Mice lacking ERα survive until adulthood but reproductive functions are severely compromised. Both males and females are infertile (23, 24). Importantly, mammary glands of 4-month-old females showed only a very primitive ductal rudiment as compared with a fully developed ductal tree in wild-type animals. ER-deficient mice also lacked terminal end buds seen during normal mammary ductal morphogenesis (23). In 1994, the first null-mutation in the ERα gene in a man was described (25), leading to the conclusion that a disruptive mutation of the ERα in humans is not lethal.

The dissection of the molecular mechanism by which estrogen activates its receptor and thereby modulates target gene transcription is central to the understanding of the complex biology governed by estrogen. After diffusion into the cell, estradiol binds the estrogen receptor. The binding of estradiol to ER induces an allosteric change which subsequently leads to dissociation of heat shock proteins (Hsp) from the ER followed by homodimerization or heterodimerization of the receptors which allows the receptor–hormone complex to bind to its specific DNA target, the estrogen responsive element (ERE) (Fig. 1) (26). These DNA sequences are found in the promoter region near the start site of transcription of many but not all genes directly regulated by estrogens. Upon ERE binding the liganded receptor activates gene transcription, which ultimately leads to increased expression of the proteins encoded by these

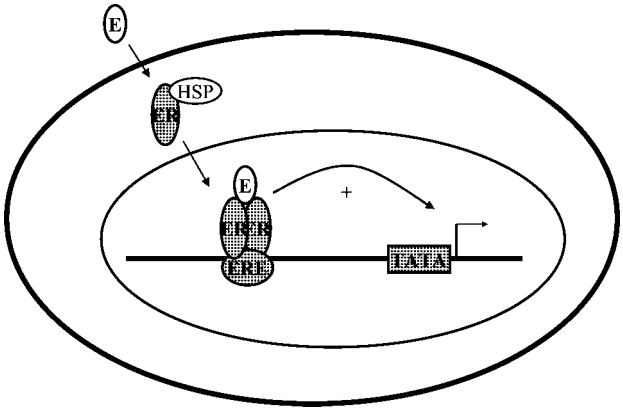

Figure 1 Model for ER-mediated signal transduction pathway. Estradiol (E) diffuses through the cellular membranes into the nucleus where it binds to the estrogen receptor (ER). Binding of estradiol to the estrogen receptor leads to dissociation of heat shock proteins (HSP) from ER followed by homodimerization and specific DNA binding to the estrogen response element (ERE) in target genes.

genes (19, 27). A surprisingly small number of genes has so far been shown to be direct targets of ER action. In terms of breast cancer these include the progesterone receptor (PR) (28) and pS2, a gene of unknown function (29). In addition, evidence exists that both growth factors such as transforming growth factor (TGF)-α and insulin-like growth factor (IGF)-I and growth factor receptors such as the epidermal growth factor (EGF)-receptor and erbB2 can be upregulated in breast cancer cells following treatment with estradiol (30). Furthermore, estrogen increases expression of cathepsin D, Hsp27, c-Myc (31), c-fos, c-jun (32) and retinoic acid receptor alpha (RARα) (33). Also expression of cyclin A, B1, D1 and E was found to be induced by estradiol in human breast cancer (34). Studies indicate that increased cyclin D1 levels after estradiol (E2) treatment recruit the p21 cdk inhibitor from its association with cyclin E–cdk2 complexes, thereby offering a mechanism for estradiol-regulated G1 cyclin dependent kinases and cell growth (35, 36). Interestingly, recent studies suggest that the cell cycle regulatory protein, cyclin D, may also function as a potentiator of ER signaling. This potentiation has been demonstrated to be independent of cyclin dependent kinase (CDK) (37, 38) activity and may explain, in part, the selective advantage for cyclin D amplification and overexpression seen in some breast cancers. Moreover, estrogen has been demonstrated to regulate expression of prothymosin alpha in ER-positive breast cancer cells (39). Interestingly, prothymosin alpha interacts with a repressor of ERα-mediated transcription, thereby providing a positive autoregulatory loop for ER-activated transcriptional regulation (40). More recently, the availabiltiy of microarray technologies has further extended the list of estrogen-target genes. Besides other newly identified

ER-target genes, Hewitt *et al.* could demonstrate that estrogen stimulation *in vivo* specifically regulates members of the histon H1 family and histon deacetylase (HDAC) 5, thereby altering chromatin structure and general transcription (41).

The mechanism of ER-mediated transactivation is thought to involve the ligand-dependent association with a large complex of proteins that integrate and transmit transactivating signals to the basal transcription machinery. These proteins are thought to act, at least in part, as bridging factors between the receptor and the transcription initiation complex. Moreover, there is evidence to suggest that this complex can integrate signals initiated by serum growth factor receptors such as the EGF- and IGF-I-receptor. Each of these factors involved in ER-mediated transactivation, i.e. ligand binding, dimerization, DNA binding and the assembly of ER-associated protein complexes offers potential targets for the therapeutic modulation of ER action and may play a role in the development of resistance to anti-estrogens (27).

Structure of the estrogen receptor

The definition of the functional domains of nuclear receptors was derived from amino acid sequence comparison between different members of this family as well as mutational analysis of individual receptors (42–44). The subdivision of six functional domains is outlined in Fig. 2.

The N-terminal A/B region is not well conserved among different nuclear receptors and extends in the case of ER from amino acid 1–180. The transactivation domain, AF-1, contained in this region is able to perform its transactivating function in the absence of ligand (43, 45–49). This region was shown to be a target of the MAP-kinase regulatory pathway, indicating a cross-talk between signals initiated by growth factors and steroid hormones (50) at the level of the receptor.

The C-region, also referred to as the DNA binding domain reveals a very high degree of conservation throughout the nuclear receptor family and allows

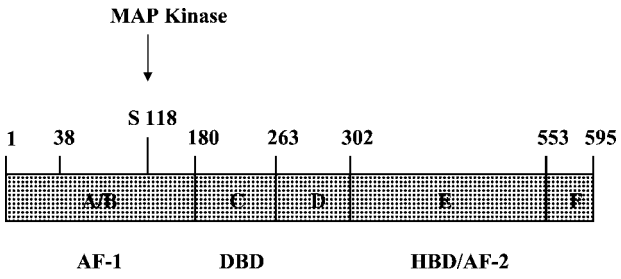

Figure 2 Structure of the estrogen receptor. Numbers represent amino acid positions; letters represent subdivided regions. AF-1, constitutive activation domain; DBD, DNA-binding domain; HBD, hormone-binding domain; AF-2, ligand-dependent activation domain; S118, serine 118.

the receptor to recognize DNA. ER expressed *in vitro* or expressed in recombinant systems is able to bind the ERE even in the absence of hormone, although *in vivo* studies suggest that hormone binding plays an important role in mediating DNA binding. The cause of this discrepancy is not well understood, but given the strong preference of ER to bind DNA as a homodimer it seems that the role of ligands in stabilizing dimerization is significant (51).

The hormone-binding domain (HBD; region E), which in the case of the estrogen receptor encompasses 251 amino acids is complex in function including ligand binding, heat-shock protein association, ligand-dependent transactivation and dimerization. From mutational analysis it is known that ligand binding involves almost all of this domain, since most of the mutations within this area compromise the ability of the mutated receptor to bind its hormone (19, 51). The ligand-dependent activation domain, AF-2 overlaps the HBD (52). The AF-2 domain is thought to activate transcription through the ligand-dependent association with a large complex of proteins that integrates and transmits transactivating signals to the basal transcription machinery (53, 54). Structural studies of the HBD of other nuclear receptors have shown that this region is composed of a series of α-helices and that the critical AF-2 region forms a terminal α-helix. The position of this helix relative to the structural ligand binding pocket is altered as a result of ligand binding (55, 56). This conformational change is believed to be required for the interaction between nuclear receptors and their coactivators. The crystal structures of the ligand binding domain (LBD) of ER in complex with the endogenous estrogen, 17β-estradiol, and the selective antagonist, raloxifene, provide a molecular basis for the distinctive pharmacophore of the ER and its binding properties. Agonist and antagonist bind at the same site within the core of the LBD but demonstrate different binding modes. In addition, each class of ligand induces a distinct conformation in the transactivation domain of the LBD, providing structural evidence of the mechanism of antagonism (57). The crystal structure of the human ligand binding domain bound to a peptide derived from the coactivator glucocorticoid receptor interacting protein (GRIP1) in the presence of either estrogen or tamoxifen revealed the structural requirements for anti-estrogen-mediated inhibition of coactivator binding to this domain (58).

The region responsible for ligand-dependent dimerization has been localized in the carboxy-terminal half of the HBD (59, 60). Both estradiol and partial agonists such as tamoxifen stabilize dimerization and subsequent DNA binding (45). Although pure anti-estrogens such as ICI 182,780 have been thought to prevent dimerization and DNA binding under certain conditions (61), there is controversy about this assumption, since Metzger *et al.* could demonstrate that ICI 164,384 does not prevent ER dimerization and DNA

binding (62). Unliganded steroid hormone receptors including the estrogen receptor are known to be associated *in vivo* with a large complex of heat shock proteins (hsp). This complex contains hsp90, hsp70 and hsp56. The domain that is required for binding of hsp90 to ER includes the LBD. This association is thought to be critical for proper folding to allow high affinity ligand binding. The binding of both estradiol and tamoxifen then promotes dissociation of the receptor from the heat shock protein complex (63–65).

The region F contains 42 amino acids and is found at the carboxy terminus of the estrogen receptor. It is not very conserved among different nuclear receptors and a specific function has not been identified as yet for this region. However, deletion of the F domain may alter tamoxifen's estrogenic effect in a cell type-dependent manner (66). More recent experiments demonstrated that this domain is essential for estradiol-stimulated transcription of target genes through Sp1-sites, suggesting that F domain interactions with nuclear cofactors are required for ERα/Sp1 action (67).

Identification of a novel estrogen receptor, ERβ

More recently, a new member of the nuclear receptor family with highest homology to the estrogen receptor has been cloned in rat (68), mouse (69) and human (70) and was therefore termed ERβ. The homology to the rat estrogen receptor protein was found to be 95% in the DNA-binding domain and 55% in the C-terminal ligand-binding domain. *In situ* hybridization studies in rat showed a prominent expression in the epithelial cells of the secretory alveoli of the prostate and the ganulosa cells in the primary, secondary and mature follicles of the ovary. ERβ binds 17β-estradiol with high affinity ($K_d = 0.6$ nmol/l). In transient transfection experiments the ability of ERβ to transactivate in an estrogen-dependent manner was also demonstrated. The identification of this new estrogen receptor may explain the residual estradiol binding which could be measured in some tissues of the ERα knockout mice (24). Mice lacking ERβ develop normally, but female knockout mice have fewer and smaller litters than wild-type mice while older males develop signs of prostate and bladder hyperplasia pointing to a role for this receptor in ovulation and prostate growth (71). Cell culture experiments revealed some interesting differences between ERα and ERβ. While both receptors bind estradiol and activate transcription through an ERE, they exert differential transcriptional actvities through AP-1 sites, pointing to differential gene regulation through these closely related receptors. In this field, recent progress in pharmaco-development has yielded the identification of selective ERβ-agonistic agents, adding yet further complexity to the modulation of ER-regulated transcription (72). More recently, it could be demonstrated that ERβ is essential for terminal differentiation of mammary gland epithelium in mice and it remains an important open question whether ERβ plays any role in breast cancer development or response to endocrine therapy (73).

Mutations and splice variants of ER

The phenomenon that ~35% of ER-positive tumors fail to respond to hormonal manipulations led to a search for mutations of ER in these tumors. These efforts resulted in the identification of numerous mutations and alternative splice variants of ER in tumors (for review see 74). A detailed discussion of each of these mutations exceeds the scope of this article, but mutations can be classified into three groups according to their functional significance. First, there are 'negative mutants' which lead to a complete or partial transcriptionally inactive receptor and whose expression does not influence the function of coexpressed wild-type receptor. These mutations probably resemble the majority of those found in tumors, with the only consequence that cells exclusively harboring the mutant receptor exhibit a reduced response to estrogen. This category also includes mutations in the C-terminal domain of the protein that reduce hormone binding. Secondly, there are those mutations which are transcriptionally inactive, but also render coexpressed wild-type receptors transcriptionally inactive and are therefore called 'dominant-negative' mutants. An example of this type is an exon 7 deletion (75). The third group of mutations include those which render the receptor transcriptionally active even in the absence of ligand, and are therefore called 'dominant-positive' mutations. These mutations compromise the integrity of the AF-2 domain of the receptor leaving the constitutive AF-1 activity unaltered. Examples include an exon 5 deleted mutant described by Fuqua *et al.* (76). Although mutations in the ER have been used to explain hormone resistance in ER-positive tumors and a variety of mutations with different functional consequences have been identified, it is not clear in what proportion of tumors these mutants are clinically important. However, at least one study (77) showed that all major estrogen receptor splice variants that were detected among the RNA population of the ER-positive breast cancer cell line MCF-7 are also present in normal tissue.

As ERβ has been identified more recently, it is still subject to ongoing research as to whether mutations and splice variants of this receptor play any role in breast cancer development and/or prognosis. At least ten different exon deletion variants of ERβ could be identified, out of which one (exon 5 deletion) correlated with the grade of the tumor (78). Interestingly, this variant exhibited transcriptional activity in the absence of estradiol and its expression correlated with a higher

grading of the tumors (78). Independently, Chi *et al.* demonstrated by immunohistochemistry that ERβ isoforms are differentially expressed in breast cancer cells and in benign epithelial and non-epithelial components of breast tissue, and suggested that ERβ isoform-specific agonists and antagonists are likely to have different biological effects on normal and cancerous cells and tissues (79). Following this line of research, Murphy *et al.* found evidence for a role of differential expression of ERβ isoforms in primary tumors of breast cancer patients who proved to have differential sensitivity to tamoxifen therapy (80).

In vitro mutagenesis of ER revealed interesting ER molecules which exhibit features explaining some characteristics of tamoxifen-resistant tumors. Mahfoudi *et al.* demonstrated that point mutations in the critical region for AF-2 activity between amino acids 538 and 552 changed tamoxifen's properties from antagonists to agonists (81). Whether mutations like the ones created *in vitro* exist in tamoxifen-resistant tumors remains to be examined.

Another important question is why not all breast cancers express ER. It has been shown that expression of ER is regulated at a trancriptional level in ER-positive and ER-negative tumors (29, 82, 83). One study identified a positive enhancer element at position −3778 to −3744 in the ER gene, which is active only in ER-positive breast cancer cell lines (84). These findings may allow the development of therapeutic approaches to induce ER expression in ER-negative tumors rendering them responsive to endocine treatment. Another possible mechanism regulating the ER status of breast cancer is the methylation state of the ER gene itself. It has been shown that ER-negative breast cancer cells display extensive methylation of CpG islands in the 5′ promoter region of the ER gene (85). Moreover, it has been demonstrated that treatment of ER-negative cells with inhibitors of DNA methylation such as 5-azacytidine partially induces ER expression (86). However, whether methylation of the ER gene is the consequence or the cause of decreased expression remains to be elucidated.

Ligand-dependent transcriptional activation – role of coactivators

Gene transcription by RNA polymerase II requires assembly of a large initiation complex at the TATA box. Both the AF-1 and the AF-2 domain of the estrogen receptor bind to the TATA-binding protein (TBP) *in vitro* (87). However, while TBP is necessary, it is not sufficient to mediate RNA polymerase II-dependent transcription in response to transactivators. This and other observations led to the hypothesis that additional factors, termed 'coactivators', are also required for efficient transcription (88). TBP-associated factors (TAFs) are thought to play a role in ER-mediated activation. For example, TAFII30 (TBP associated factor) interacts specifically with the AF-1, but not the AF-2 domain of the ER (89). In addition, it has been shown that TFIIB can associate with the AF-2 domain of the estrogen receptor (90). Since the binding of nuclear receptors to components of the basal transcription apparatus is necessary but did not appear to be sufficient for ligand-dependent transcriptional activation, further research has focused on the identification of other receptor-associated proteins whose binding to the receptors was both AF-2 and ligand dependent.

Members of the yeast mating type switching/sucrose non-fermenting (SWI/SNF) family of proteins were the first coactivators to be characterized for nuclear receptors (Table 1). These proteins are part of the pol II holoenzyme complex and probably act through the release of repressive effects of chromatin components (91–93). It has been reported that transcriptional activation by ER in yeast is dependent on the function of SWI1-3 (94). Moreover, their human homologs hSNF2α (hbrm) and hSNFβ (BRG1) enhance ER-mediated transactivation in cotransfection experiments (91, 92).

Putative ER coactivators were identified biochemically as estrogen receptor associated proteins (ERAP) 160 and ERAP 140 (95). These proteins were identified by their ability to interact with the bacterially expressed AF-2 domain of the human estrogen receptor in a ligand-dependent manner (95). This same approach led to the identification of receptor interacting protein (RIP) 160, 140 and 80 (96). The molecular cloning of ER-associated proteins has been pursued both by yeast two hybrid screening using nuclear receptors as a bait, and by expression cloning using radiolabeled nuclear receptor fusion proteins as probes. The first approach led to the molecular cloning of steroid receptor coactivator 1 (SRC1), which when cotransfected with nuclear receptors including ER was able

Table 1 Estrogen receptor coregulators.

Coactivator	Targeted nuclear receptor	Reference
SRC1 family*		
SRC1	ER, PR, TR, GR, RXR	97
TIF2	ER, RXR, RAR	100
mGRIP	ER, GR, AR	99
p/CIP	ER, TR, RAR	161
CBP family		
p300	ER, TR, GR, RXR, RAR	107
CBP	ER, GR, RXR, RAR	108
		98,109
SWI/SNF family		94
hSNF2a (hbrm)	ER, GR, RAR	91
hSNF2b (BRG1)	ER, GR, RAR	92
Others		
RIP140	ER, RAR	96
TIF1	ER, TR, RXR, RARα, VDR	
CARM-1	ER, PR, TR, GR, RXR	162

* Biochemically identified as ERAP160 and RIP160 (95, 96).

to augment ligand-dependent transactivation (97). Subsequently, other groups cloned different forms of SRC1. Antisera raised against the cloned SRC1 recognize the biochemically defined ERAP 160 suggesting that both proteins are related (98). Using the HBD of the glucocorticoid receptor as a bait, others cloned GRIP1, which also interacts with the liganded HBD of ERα (99). In addition, TIF2 was cloned by expression screening using radiolabeled ER-HBD as a probe (100). Sequence comparison of TIF2 with GRIP1 revealed GRIP1 to be the mouse homolog of human TIF2. This protein shares similarity to SRC1 (between 30–40% identity on an amino acid level) (100). Therefore, the biochemically characterized protein complex ERAP 160 is composed of at least two structurally related gene products, SRC1 and TIF2/GRIP1. More recently, p300/CBP interacting protein (p/CIP) (also RAC3 and ACTR) was shown to be a new member of this family (101). Interestingly, this ER coactivator was also identified as a gene amplified in breast cancer (AIB1) (102, 103). In these studies the investigators used chromosome microdissection and hybrid selection to identify expressed sequences which map to an amplicon on chromosome 20q found in certain breast cancers. Among three cDNAs identified in this way, AIB1 was found to map to a chromosome 20q12 amplicon which was found in several cancer cell lines. A further study then demonstrated that AIB1 amplification correlated in a large number of breast and ovarian tumors with estrogen receptor positivity and tumor size. On the other hand, high levels of AIB1 correlated with worse disease-free survival, when patients received tamoxifen as an adjuvant agent, indicating that AIB1 might be involved in a molecular mechanism leading to tamoxifen resistance (104). These data are of potential significance but it has still to be proven whether AIB1 plays a causal role in the development of ER-dependent breast cancer. Moreover, recent work demonstrated that expression of SRC1 and ER are segregated in different cell types of the mammary epithelium and estradiol-stimulated expression of the progesterone receptor occurs in the absence of immunologically detectable SRC1 (105). These data indicate that SRC1 might act in a cell type-specific manner rather than as a general steroid receptor coactivator.

More recently, a novel ER-interacting protein could be identified as proline glutamic acid and leucine-rich protein (PELP)-1. Interestingly, PELP-1 overexpression hypersensitized breast cancer cells for estradiol signaling and enhanced progression of these cells to S-phase, leading to persistent hyperphosphorylation of the retinoblastoma protein, Rb (106). These findings offer a unique crosstalk between nuclear receptor coactivators and the regulation of cell cycle control.

The phospho-CREB-binding protein, CBP, and the related p300 have been implicated as proteins associated with ER and involved in ligand-dependent transactivation (98, 107–109). In contrast to the SRC1 family coactivators mentioned above, these proteins are targets of signals mediated by a variety of distinct pathways and are felt to function to integrate signals from these diverse pathways by interacting with components of the basal transcription machinery (Fig. 3).

More recently, it could be shown that protein methylation plays a central role in the modulation of chromatin structure and coactivators, leading to either gene silencing or activation. Methylation of arginine residues of histones by protein arginine methylases (PRMT/PAM) leads to transcriptional activation (110). PRMT/PAM methylases recognize RGG/GRG motifs; beside histones, their substrates include e.g. STAT1 in interferon signaling, CBP/p300, and several spliceosomal assembly factors and snRNP/hnRNP proteins. As PRMT1 and CBP/p300 form a chromatin modulatory/coactivator complex, methylation of histone H4 by PRMTs (PRMT1 and CARM1/PRMT4) and acetylation of other subunits of histone core (as well as p300-binding nuclear receptors) are coordinately regulated (110). While protein arginine methylases such as PRMT1 and CARM1 act as coactivators of nuclear hormone receptors including the ER through histone methylation, they can also act as negative regulators of transcription: methylation of the CREB-binding domain of CBP results in abrogation of CREB/CBP-interaction thereby inhibiting CREB-mediated transcription (111). Therefore, methylation through PRMT/CARM1 represents a unique positive and negative regulatory mechanism for transcription through cofactor-methylation. Also the identification of template-activating factor (TAF)Iβ as an ER-interacting protein underlines the

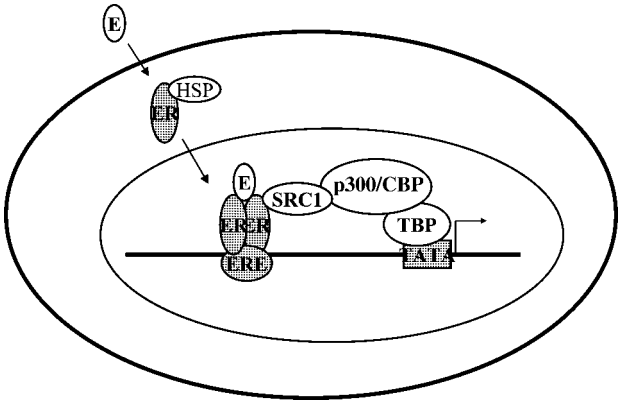

Figure 3 Model of the potential role of p300/CBP and ERAP 160/SRC1 in the integration of steroid hormone signals with other pathways. SRC1 is in a complex with p300 in the absence of exogenous signals. Binding of estradiol (E) to ER promotes the recruitment of this complex through a direct interaction with SRC1 and perhaps p300. p300 directly interacts with transcription factors that are activated by diverse cellular signaling pathways such as the cAMP signals mediated by phospho-CREB and with components of the basal transcription machinery (160). Thus, p300 may play a role as an integrator of signals from multiple pathways including ER TBP, TATA binding protein.

importance of methylation and acetylation in the regulation of gene transcription via nuclear hormone receptors (112). It could be demonstrated that TAF-Iβ binds to the unliganded ERα and represses p300-mediated acetylation of histones and decreases acetylation of ERα (113). Therefore, elucidating the exact molecular mechanisms regulating acetylation and methylation might offer a complete new target for pharmacological intervention in ER-mediated transcription and breast cancer therapy.

This emerging picture of a number of proteins assembling with liganded ER to mediate transcriptional activation, offers potential new candidates to explain resistance to estrogen action even in the presence of unaltered ER expression. Therefore, these proteins deserve careful study as potential targets in tamoxifen-resistant tumors. Studies with several nuclear receptors have shown the existence of the corepressors nuclear receptor corepressor (N-CoR) and silencing mediator for retinoic and thyroid receptor (SMRT), whose association with these receptors was shown to be destabilized by ligand (114, 115). Interestingly, it could be demonstrated that low expression of N-CoR was associated with significantly shorter relapse-free survival in ERα-positive tumors (116). These findings point to N-CoR as a promising independent predictor of tamoxifen resistance in patients with ERα-positive breast tumours. Future studies will also aim to clarify the role of corepressors in ER-mediated regulation, with the possible perspective of identifying anti-estrogens which might lead to corepressor association with the estrogen receptor.

Ligand-independent transactivation by ER

The nuclear receptors including the ER are phosphoproteins (for review see 117). The finding that growth factors such as EGF and IGF-I as well as stimulators of the protein kinase A pathway could activate ER-mediated transcription in the absence of the bona fide ligand, i.e. estrogen, as well as synergizing with the effects of estrogens to stimulate ER-mediated transactivation, led to the notion that post translational modifications of nuclear receptors by phosphorylation offered an alternative pathway of transactivation. The exact function of each potential phosphorylation site is not yet clear. This finding is complicated by the fact that the effects of ER phosphorylation might depend strongly on the individual cellular context (118). Serine 118 was identified as the major growth factor-stimulated phosphorylation site in the ER (119), whereas serine 167 is the major phosphorylation site upon estrogen stimulation (120). Two studies identified serine 118 as a target of the growth factor-stimulated MAP kinase (50, 121). Furthermore, it was demonstrated that the growth factor-stimulated AF-1 activity of ER depends on serine 118. Interestingly, this study demonstrated that phosphorylation on serine 118 can synergize with the partial agonist effect of tamoxifen through the AF-1 domain of ER. This might explain why tumors expressing oncogenes such as HER2/neu can promote tamoxifen resistance and negatively affect prognosis. HER2/neu is a member of the EGF receptor family of transmembrane tyrosine kinase receptors. Upon ligand-induced activation these receptors activate the ras/raf MAP-kinase pathway. This may induce ligand-independent or augment tamoxifen-induced transcription through the ER. Recently, it has been demonstrated that growth factor-stimulated phosphorylation of ERβ and its AF-1 domain can result in SRC1 recruitment even in the absence of ligand (122, 123). In summary, posttranslational modification of ER on serine residues can influence essential steps in receptor-mediated transactivation such as dimerization, DNA binding and transcriptional activation. These steps may become targets of novel anti-estrogenic agents (Fig. 2).

Rapid, non-genomic actions of estradiol

Besides the classical action of estrogen receptors as ligand-regulated transcription factors, it could be demonstrated that estradiol also exerts early signaling events in target cells within minutes after stimulation, making it unlikely that these effects are regulated through the transcriptional rate (124). The existence of a membrane-associated ER was hypothesized based on the finding that these early signaling events occur after stimulation with albumin-conjugated estradiol, which does not enter the cell (125). Moreover, antibodies against different epitopes for ERα detected membrane-associated ER in different cell lines derived from various tissues (126), indicating a high degree of similarity between ER detectable in the plasma membrane and the nucleus. Indeed, expression of the cloned ERs in CHO cells results in the detection of both nuclear and plasma membrane ER (127). A very recent study identified a structural determinant necessary for the localization and function of ERα at the plasma membrane. Razandi *et al.* demonstrated that serine 522 in ERα is required for its localization to the plasma membrane, and that mutation of this residue abrogated the rapid effects of estradiol without affecting its classical functions as a nuclear hormone receptor (128). Taken together, there is growing evidence for the association of ERs with the plasma membrane, mainly in caveolae (129, 130), to initiate signals distinct from their nuclear action.

The rapid non-genomic actions of estradiol include the activation of the MAP-kinase cascade, the activation of phosphatidylinositol-3-kinase (PI-3K) signaling and the rise in intracellular calcium concentrations (131, 132). Based on the assumption that the plasma membrane ER is structurally related to the nuclear receptor,

it presumably lacks catalytic or kinase domains. Therefore, the question arises as to how kinase cascades are initiated through ER action. Some of the molecular mechanisms leading to PI-3K activation and MAP-kinase activation could be identified, since it could be demonstrated that both pathways require Src-activation and that the latter engages the adaptor-protein Shc (133). On the other hand, membrane-associated ER has been demonstrated to activate the EGF-receptor through release of heparin binding EGF (134). Similarly, estradiol has been shown to stimulate the release of IGF-I thus activating the IGF-I-receptor and its downstream targets (135). More recent data demonstrated that estradiol can activate the Akt pathway through activation of ErbB2 signaling, adding yet another mechanism for the interaction of estradiol and protein tyrosine kinase signaling (136).

Additional complexity to the understanding of estradiol action has been added by the finding that these early signaling events also target the regulation of transcription. In vascular endothelial cells, estradiol activates several kinase cascades, including PI-3K/Akt. Short term estradiol stimulation significantly increased transcription of 250 genes in vascular endothelial cells, up-regulation that was substantially prevented by the PI-3K inhibitor, LY294002 (137). Taken together, the non-genomic actions of ER add yet another degree of complexity to estradiol-regulated responses (for review see 138) and even growing evidence for the interaction of classical and non-classical effects of estradiol might offer new molecular targets for the treatment of gynecological malignancies.

Anti-estrogen therapy for breast cancer

Attempts to pharmacologically block estrogen's effects led to the development of a variety of agents targeting ER function. Tamoxifen is the most widely used anti-estrogen for the treatment of breast cancer. It belongs to the family of triphenylethylenes, and besides its anti-estrogenic effects it also exhibits partial agonistic properties (139). This can be explained by the fact that tamoxifen, like estrogen, allows ER dimerization and binding of the ER homodimer to the ERE. The antagonistic effect is then thought to be mediated by inhibiting ER's ligand-dependent AF-2 activity. One potential mechanism by which tamoxifen achieves this effect is its ability to prevent coactivators such as SRC1 from interacting with the receptor. A major toxicity associated with the estrogenic effects of tamoxifen is increased risk of endometrial carcinoma (140). In addition, other side effects of tamoxifen include increased risk of thrombosis, hot flashes and depression (140). However, tamoxifen retains some of the beneficial effects of estrogen on bone and lipids (141, 142).

Recent attempts have been directed to the development of new anti-estrogens reducing tamoxifen's potential side effects while retaining the beneficial effects. The finding that tamoxifen can form DNA adducts led to the development of the second generation anti-estrogen, toremifene (Fareston) (143–145). In advanced disease, toremifene has been shown to be comparable to tamoxifen in terms of response rates as well as toxicities. However, no major benefit for toremifene over tamoxifen has been shown (143).

Another new generation anti-estrogen of the tamoxifen family is droloxifene (3-OH-tamoxifen). Compared with tamoxifen, droloxifene has a tenfold higher binding affinity for the ER, and a shorter half life. Droloxifene acts anti-estrogenically in a rat uterine model (146). Phase I and II trials suggest droloxifene may be a useful treatment for breast cancer (147). Further studies will be required to determine whether estrogenic effects in the uterus will be clinically relevant.

Raloxifene (Evista), another tamoxifen-like ER ligand, is approved for the prevention of osteoporosis. In laboratory animals raloxifene greatly enhances bone density (148), reduces circulating cholesterol levels and lacks estrogenic activity in the uterus (149). The multiple outcomes of raloxifene evaluation (MORE) trial suggested that, like tamoxifen, raloxifene may be able to prevent breast cancer (150). The study of tamoxifen and raloxifene for the prevention of breast cancer (STAR) trial is currently testing which component is more effective and causes fewer side effects (151).

In contrast, pure anti-estrogens such as ICI 182,780 (Faslodex) may play a role in women in whom tamoxifen has failed. In contrast to anti-estrogens like tamoxifen, ICI 182,780 does not exhibit estrogenic effects, since it completely inhibits assembly of an active transcriptional complex at the ERE. Early clinical trials investigating ICI 182,780 use in patients with primary breast cancer (152) showed no unanticipated toxicities and demonstrated antitumor effects in women with advanced breast cancer that had been proven resistant to tamoxifen (153–155). Its pure anti-estrogenic properties make it unlikely to be a candidate for the treatment of women with early stage disease.

The current availabilty of ER crystal structures when bound to estradiol or different anti-estrogens has added signficantly to the understanding of the mechanism of anti-estrogen action. Ligands of ERα can confer two different conformations to the receptor complex. Planar estrogens such as estradiol are class I, while angular estrogens such as tamoxifen and other triphenylethylenes are class II estrogens. The first class interacts with the A2 coactivator site. Class II estrogens, on the other hand, render the AF-2 function unaccessable for coactivators, resulting in a so called AF2b-conformation which depends on interaction with AF-1 for estrogenic effects (156). For a detailed review on the structure/function relation of anti-estrogens and the most recent development in the field of estrogen receptor modulators also see (157).

Another approach to modify hormone resistance is based upon the finding that the action of SERMs (selective ER modulators) such as tamoxifen is dependent on the cellular context mainly influenced by AF-1 and AF-2 dependence of target gene transcription. By primarily inhibiting AF-2 activity, as outlined above, tamoxifen acts as an ER antagonist in an AF-2-dependent context, while it will act as an agonist in an AF-1 dominant environment. Recently, it has been suggested that tamoxifen may exert its agonist activity through the recruitment of an as yet unidentified coactivator (158). Hormone resistance of a tumor might then result from epigenetic changes of tumors so as to change their phenotype to an AF-1 dominant one or to alter the levels of this tamoxifen-specific coactivator. These findings offer a promising approach to the development of novel therapies to overcome hormone resistance (159).

While it is clear that estrogen receptor expression will predict significantly the clinical response to endocrine therapy in breast cancer, the mechanisms leading to anti-estrogen resistance in ER-positive tumors are only now being elucidated. In most tumors which become tamoxifen resistant, ER expression does not change. While the identification of ER mutations as the cause of hormone resistance in ER-positive tumors has yielded somewhat disappointing results, the identification of multi-protein complexes involved in ER-mediated transactivation offers a wide field of potential molecular players for the development of hormone resistance, and as potential targets for new therapies.

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