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Estrogen Receptor Alpha: Molecular Mechanisms and Emerging Insights

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ABSTRACT

Estrogen receptor alpha (ERα) is a cellular receptor for the female sex hormone estrogen and other natural and synthetic ligands and play critical roles in normal development and physiology and in the etiology and treatment of endocrine-related diseases. ERα is a member of the nuclear receptor superfamily of transcription factors and regulates target gene expression in a ligand-dependent manner. It has also been shown to interact with G-protein coupled receptors and associated signaling molecules in the cytoplasm. Transcriptionally, ERα either binds DNA directly through conserved estrogen response element sequence motifs or indirectly by tethering to other interacting transcription factors and nucleate transcriptional regulatory complexes which include an array of co-regulator proteins. Genome-scale studies of ERα transcriptional activity and localization have revealed mechanistic complexity and insights including novel interactions with several transcription factors, including FOXA1, AP-2g, GATA3, and RUNX1, which function as pioneering, collaborative, or tethering factors. The major challenge and exciting prospect moving forward is the comprehensive definition and integration of ERα complexes and mechanisms and their tissue-specific roles in normal physiology and in human diseases. J. Cell. Biochem. 114: 2203–2208, 2013.

KEY WORDS: ESTROGEN RECEPTOR; TRANSCRIPTION FACTOR; COREGULATORS

The female steroid hormone estrogen has pleiotropic effects on human development, physiology, and a number of endocrine-related conditions and diseases [Gruber et al., 2002; Nilsson and Gustafsson, 2002; Burns and Korach, 2012]. Initially, estrogen was thought to be involved in cellular redox reactions through interactions with coenzymes [Talalay and Williams-Ashman, 1958]. A competing hypothesis suggested that cells in target tissues may harbor receptor molecules whose presence would dictate tissue-specific responses [Szego, 1957]. Jensen, who passed away recently, and Jacobson developed a method to tritiate estradiol, the predominant form of estrogen, and showed that radiolabeled hormones were functional, accumulated in estrogen-responsive reproductive tissues, and were not chemically altered [Jensen and Jacobson, 1960]. Studies by Gorski et al. and also Jensen et al. went on to demonstrate that estrogen was bound to proteins in the cytoplasm which subsequently localized to the nucleus of target cells and activated the synthesis of specific transcripts [Toft and Gorski, 1966; Toft et al., 1967; Jensen et al., 1968; O'Malley and Means, 1974]. Jensen termed the estrogen-binding protein estrophilin, whereas Gorski referred to the protein as the estrogen receptor (ER) [Gorski et al., 1968; Jensen et al., 1974]. Molecular characterizations of ER became possible when the ER gene was cloned by the Chambon group [Green et al., 1986]. Mutagenesis studies showed that the receptor consists of a DNA-binding domain containing zinc finger motifs and a ligand-binding domain, key structural elements of a ligand-dependent transcription factor [Kumar et al., 1986]. Other related receptors, including the glucocorticoid receptor, the thyroid hormone receptor, and the progesterone receptor, were also cloned and characterized around the same time [Hollenberg et al., 1985; Conneely et al., 1986; Jeltsch et al., 1986; Weinberger et al., 1986]. They, along with ER, became the founding members of the nuclear receptor superfamily of transcriptional regulators [Gronemeyer et al., 2004]. A second closely related ER with similar affinity for estradiol but distinct tissue specificity and affinity for other estrogenic compounds was discovered by Gustafsson and Kuiper and was subsequently named ERβ, and the original ER was renamed ERα [Kuiper et al., 1996]. The roles of ERα and ERβ in mediating estrogen response in normal physiology and in diseases have been subjected to intense investigation. An orphan G protein-coupled receptor (GPR30) was also reported to function as an ER, but these observations have been disputed by more recent hormone binding and genetic studies [Langer et al., 2010]. This prospect focuses on ERα and the mechanistic insights obtained since its discovery.

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TRANSCRIPTIONAL REGULATORY MECHANISMS

Upon activation, ERα dimerizes and binds directly to *cis*-regulatory regions of target genes via conserved estrogen response elements (EREs; consensus 5′-GGTCAnnnTGACC-3′) [Klinge, 2001]. ER can also be tethered to other transcription factors such as AP-1, Sp1, and NFκB to indirectly influence gene expression [Duan et al., 1998; Petz et al., 2002; Chadwick et al., 2005]. Structural changes induced by ligand binding to ERα facilitate the formation of nuclear receptor co-regulator binding surfaces which then complex SWI/SNF ATP-dependent nucleosome remodeling enzymes that enable necessary changes to histone spatial position and co-activators which include histone modifying enzymes such as histone acetyl-transferases (HATs: P300/CBP, P/CAF, SRC-1, and p/CIP/AIB1), histone methyl-transferases (HMTs: CARM1 and PRMT1), and histone ubiquitin ligases (RPF1 and E6-AP) [2,4-6] [Hall and McDonnell, 2005; Lonard and O′Malley, 2005]. Co-repressors, such as NCoR, NRIP1, and SMRT recruit histone deacetylases (HDACs) and have been shown to bind to ERα in the presence of antagonists or at specific *cis*-regulatory regions of repressed target genes following hormone activation [Stossi et al., 2006; Merrell et al., 2011; Watson et al., 2012]. These interactions and mechanisms are summarized in Figure 1.

Once the proper histone modifications are made at the target gene promoter orchestrated by ERα-bound coactivators, TFIID/TBP is stabilized at the promoter by TFIIA. TFIIB then binds and positions RNA polymerase II at the correct initiation site [Emerson, 2002]. Signaling of TFIIH by the TRAP/mediator complex stimulates phosphorylation of the C-terminal domain of the polymerase leading to transcription initiation and elongation. SWI/SNF is typically maintained at the promoter as the cells utilize this complex to modify histone spatial configuration in preservation of active transcription in the presence of ERα, but also to discontinue that configuration once estrogen response is uncoupled [Metivier et al., 2003]. After co-activator assembly, members of the ubiquitin proteasome pathway regulate degradation of portions of the preinitation complex so that elongation is possible [Lonard and O′Malley, 2009]. Treatment of estrogen-responsive cells with proteasome inhibitors interrupts the cyclic association of co-regulator proteins with the ERα complex, which eventually results in the loss of phosphorylated polymerase II. ERα itself is subjected to a variety of post-translational modifications which result in changes in receptor stability and activity in response to specific ligands and signaling events in the cell [Anbalagan et al., 2012].

NON-GENOMIC EFFECTS OF ER ACTIVATION

ERα is predominantly studied as a ligand-dependent transcription factor, but early work demonstrated that estrogen has rapid effects on cell signaling [Pietras and Szego, 1975; Levin, 2005; Levin and Pietras, 2008; Tian et al., 2012]. These effects suggested the existence of signaling pathways not mediated by transcriptional regulatory mechanisms. Estrogen rapidly signals ERα/G protein complexes through secondary messengers calcium and cAMP, activated PI3K, and activated RAS [Levin and Pietras, 2008]. Some ERα appear to localize to the cytoplasm and interact with growth factor receptors (GFRs) such as EGFR and IGF-1R. Activation of these receptors through ERα is thought to stimulate GFR effector kinases and their downstream targets such as PI3K and ERK. Activation of PI3K by GFR/ERα signaling results in the inhibition of GSK-3β, which is known to phosphorylate ERα S118 and inhibit its activity. This allows for amplified ERα transcriptional activity in the nucleus [Levin, 2005]. It is through these GFR effector signals that estrogen is able to upregulate cyclin D1 expression, thereby promoting G1/S transition through the cell cycle.

ERα is believed to be tethered to the cytoplasmic membrane through one or a combination of different mechanisms, such as association with lipid-raft proteins like caveolin-1 or by direct interaction with GFR complexes [Razandi et al., 2002; Tian et al., 2012]. Interaction of ER with caveolin-1 and the cell membrane requires palmitoylation of ERα [Marino et al., 2006]. Interestingly, this modification is required for signaling ERK and PI3K pathways. Recent experiments have attempted to characterize a separate membrane bound receptor for estrogen, as almost all ER is found in the nucleus. In fact, membrane bound ER was thought to be GPR30. However, knockdown experiments failed to prevent ERK activation after estrogen stimulation, raising questions about the significance of this finding [Otto et al., 2009]. Several lines of evidence suggest that membrane-bound ER appears to be the same ERα and ERβ found in the nucleus [Levin and Pietras, 2008]. The non-genomic mechanisms of ERα action are depicted in Figure 1.

ERα IN THE GENOMIC ERA

As genome sequencing picked up pace in the 1990s, microarray technologies were developed to simultaneously monitor the expression levels of large number of transcripts. High-density microarrays made it possible to measure the expression of nearly all of the protein-coding genes in large genomes. Prior to microarrays, only a handful of estrogen responsive and ERα target genes were known [Klinge, 2001]. The first microarray studies examining estrogen-responsive transcriptomes were carried out in ER-positive human breast cancer cell lines. In their study of ER-status associated genes in breast tumor samples, Gruvberger et al. [2001] treated MCF-7 cells with estradiol and measured changes in gene expression profiles and compared responsive genes with those that were associated with ER-status. A similar study was carried out by Finlin et al. [2001] to correlate estrogen-responsive gene expression with genes whose expression in breast tumors were associated with poor prognosis. The first microarray study to specifically identify ERα-regulated genes was performed by Soulez and Malcolm in ZR75-1 cells using Affymetrix HuGene FL arrays which contained probe sets for 5600 human genes [Soulez and Parker, 2001]. They identified 53 responsive genes which were sensitive to inhibition by ER antagonists and insensitive to translation blockage by cycloheximide. Inoue et al. [2002] utilized custom spotted arrays with probes for 8500 human genes to identify 286 estrogen responsive genes in MCF-7 cells. With advances in microarray technology and greater probe density, Frasor and Katzenellenbogen carried out the first genome-scale analysis of estrogen responsive gene expression profiling and showed that hormone treatment affected genes involved in cell proliferation and

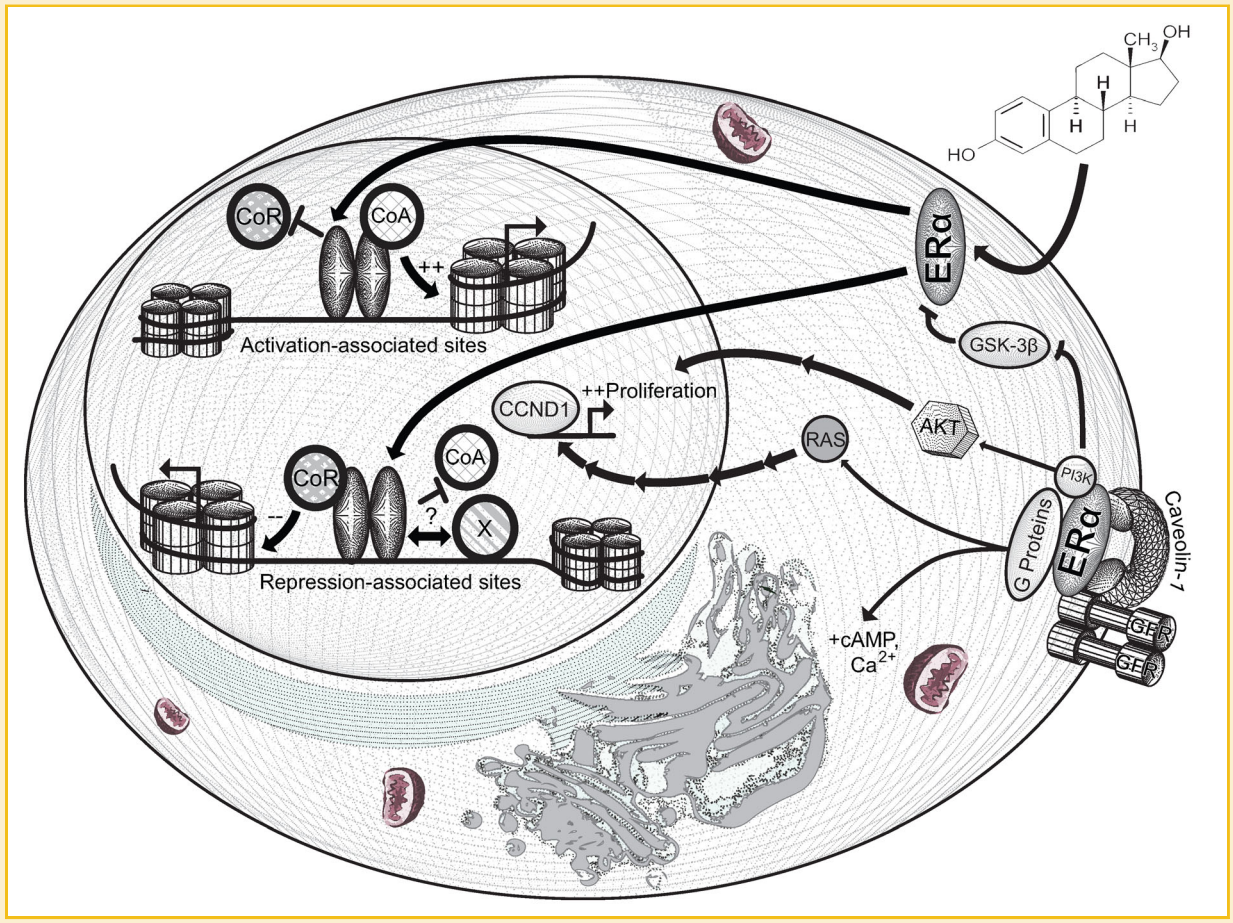

Fig. 1. Mechanisms of action of ERα in the nucleus and cytoplasm of target cell.

survival and, surprisingly, estradiol down-regulated the expression of the majority of responsive genes [Frasor et al., 2003]. A detailed time-course study in ER-positive T47-D cells was performed by the corresponding author and Liu and provided the first glimpse of the temporal dynamics and regulatory hierarchy of ER target genes and their downstream effects [Lin et al., 2004]. Using a technique combining nuclear run-on and next generation sequencing (GRO-seq), Hah et al. [2011] generated a genome-wide map of immediate transcriptional responses to ERα activation and showed transient transcriptional activities associated with nearly 23,000 transcripts, including both protein-coding and non-coding genes and those which originate from intergenic regions adjacent to mapped ERα binding sites. These and many other studies which cannot be referenced completely due to space constraints clearly demonstrate the profound direct and indirect impact of ERα on the transcriptomes of target cells and suggest that much remain to be done in order to fully understand the functions and mechanisms of action of these gene networks.

The completion of the first complete draft of the human genome and further innovations in microarray technology also enabled the development of whole-genome tiling arrays. Coupled with chromosome immunoprecipitation (ChIP), the so-called ChIP-on-chip experiments were instrumental in comprehensively mapping transcription factor binding sites. Carroll et al. [2005] carried out the first chromosome-wide ChIP-on-chip study of ERα binding sites using tiling arrays with probes for human chromosomes 21 and 22. They followed up with the first genome-wide mapping of ERα binding sites when whole-genome tiling arrays became available and identified over 3000 ER binding sites in MCF-7 cells [Carroll et al., 2006]. In a study by the corresponding author and Liu, using ChIP coupled with paired-end ditag sequencing (ChIP-PET), 1200 ERα binding sites were identified in MCF-7 cells following estrogen treatment, with over half of the sites also identified in the Carroll and Brown study [Lin et al., 2007]. The most comprehensive whole-genome ERα binding site mapping study to date, with over 10,000 binding sites reported, was performed by Stunnenberg and his group using ChIP followed by high-throughput sequencing with next generation sequencers (ChIP-seq) [Welboren et al., 2009]. These studies and the publicly available data not only represent a valuable resource for investigators studying ERα regulated target genes and potential transcriptional regulatory mechanisms but also provide a global perspective and mechanistic insights regarding ERα function. One of the surprising observations from these studies was that the vast majority of ERα binding sites are outside of the proximal promoter region of target genes and suggests that ERα bound to distal enhancers may form long distance looping structures with promoters

of target genes. This is in contrast to the then prevailing view that most transcription factor binding sites in general and EREs specifically are located in the promoter regions of target genes. Using chromosome conformation capture (3C) coupled with paired-end ditag sequencing, Fullwood et al. [2009] mapped these looping structures involved in distal interactions (interactome) between ERα and promoters of target genes across the genome. Another interesting and potentially important finding from both the binding site mapping and gene expression studies is that ligand-activated ERα can also repress a significant subset of target genes. Characterization of repression-associated ERα binding sites has shown that rather than recruiting co-activators, co-repressors are recruited to the receptor complex following estrogen treatment [Stossi et al., 2006; Merrell et al., 2011]. The significance of the repressed target genes in normal and pathological ERα functions remains to be determined.

NOVEL TRANSCRIPTION FACTOR PARTNERS

The genome-scale ERα binding site mapping studies yielded a large amount of positional and sequence data and provided rich datasets for computational modeling and analysis of sequence motifs associated with ERα binding. For example, motif analysis confirmed the preponderance of the previously defined ERE consensus sequence motif [Carroll et al., 2005, 2006; Lin et al., 2007]. Furthermore, other sequence motifs are enriched in the proximity of ERα binding sites and suggest potential physical and functional interactions between ERα and other transcription factors. The first chromosome-wide mapping studies revealed an enrichment of binding site motifs for Forkhead factors [Carroll et al., 2005]. This enrichment was subsequently confirmed by genome-wide mapping studies [Carroll et al., 2006]. Forkhead family member FOXA1was previously identified as an ERα interacting protein and was shown by Carroll et al. [2005] to be the Forkhead factor which localize to the proximity of ERα binding sites and serve as a pioneering factor which potentiate ERα binding and transcriptional activity. FOXA1 appears to play key roles in hormone-dependent tumor growth and response to endocrine therapy in breast cancer and has similar roles in prostate cancer through its interactions with the androgen receptor [Augello et al., 2011; Hurtado et al., 2011]. In another study of binding site motifs associated with ERα binding sites, Kong et al. [2011] identified the transcription factor GATA3 as a component of an enhanceosome which includes both ERα and FOXA1, and this complex is involved in optimization of transcriptional responses to estrogen treatment. Interactions between GATA3 and ERα also involve a cross-regulatory mechanism by which each of these factors regulates the expression of the other through direct binding of *cis*-regulatory regions of their partners [Eeckhoute et al., 2007]. Related to these molecular interactions, GATA3 has established roles in mammary gland development and its expression is highly correlated with ER-status in breast tumor tissues [Asselin-Labat et al., 2007; Wilson and Giguere, 2008]. In a follow-up study to the ERα-promoter interactome mapping paper, Tan et al. [2011] identified AP-2 binding site motifs in ERα binding sites which are involved in long distance looping structures and showed that AP-2γ is a collaborative factor in interactions involving ERα, FOXA1, and promoter regions of target genes. AP-2γ functions in hormone response in breast cancer cells and its expression levels are elevated in tumors with poor clinical outcomes [Woodfield et al., 2007; Gee et al., 2009].

Adding to the rapidly accumulating datasets and insights, Stender et al. [2010] carried out a genome-wide ERα binding site study using an ERα construct with mutations in the DNA-binding domain specifically designed to detect tethering mechanisms and factors. They showed that in addition to the binding site motif of the AP-1 complexes, known to tether ERα to DNA, there is an enrichment of binding site sequence motifs for runt-related transcription factors. They also provided evidence that RUNX1, specifically, binds ERα and regulates the expression of a subset of estrogen-responsive target genes. RUNX1 has been well studied for its roles in hematopoiesis and regulation hematopoietic stem cell differentiation. RUNX1 is implicated in leukemogenesis and is frequently mutated in malignant myeloid cells, and there is increasing evidence for its role in breast cancer [Ito, 2004; Janes, 2011]. Interestingly, a genetic study in mice examining strain-specific uterotropic responses to estrogen treatment identified quantitative trait loci which included the locus for the *Runx1* gene and showed a correlation between *Runx1* transcript and protein expression levels and response to hormone treatment [Wall et al., 2013].

UNRAVELING THE COMPLEXES AND THE COMPLEXITY OF MECHANISMS

The examples provided in the previous section illustrates a strategy for using ChIP-on-chip and ChIP-seq sequence data to model and predict putative partnering transcription factors for further functional characterization of ERα complexes and mechanisms of action. Given the advances and increased availability of mass spectrometry technology, it is now more feasible than ever to directly purify and characterize protein complexes. Carroll et al. devised an approach termed rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME), and identified GREB1, the product of an ERα direct target gene with previously unknown function, as a component of the ERα complex and a co-activator of transcriptional activity [Mohammed et al., 2013]. It is likely that similar approaches will yet yielded many more candidate transcription factors and co-regulators which interact with ERα. The emerging picture of ERα and its mechanisms of action suggests greater complexity than previously appreciated or currently depicted in working models. Furthermore, this emerging complexity will also likely be viewed as overly simplistic in the near future as the field continues to progress. It should be noted that the vast majority of the discoveries regarding ERα function were made in breast cancer cells, with nearly all of the observations coming from MCF-7 cells which represent a distinct genetic and epigenetic background and harbor-specific somatic mutations and chromosomal aberrations. Whether the same factors, interactions, and mechanisms are involved in estrogen responses in other cell and tissue types remain to be determined. Comparisons of estrogen responsive genes and ERα binding sites in osteoblast-like U2OS cells expressing ERα to those identified in MCF-7 cells indicate vast differences in the repertoire of binding sites, responsive genes, and histone marks and the requirement for FOXA1 as a pioneering

factor [Krum et al., 2008]. These initial observations indicate that significant differences are expected. These and future findings will need to be integrated with non-genomic mechanisms, cross-talk with signal transduction pathways, interactions with non-coding RNAs, and possible post-translational modifications of ERα and interacting proteins. A full mechanistic and functional understanding of ERα will require additional technological advances and application of systems biology principles and approaches. These types of studies, however, are greatly needed in order to further our understanding of tissue-specific effects and mechanisms of ERα function and will certainly yield important insights and corresponding biological and clinical implications.

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