Genomic actions of estrogen receptor $\alpha$: what are the targets and how are they regulated?

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Abstract

The estrogen receptor $\alpha$ (ER$\alpha$) is a ligand-dependent transcription factor that regulates a large number of genes in many different target tissues and is important in the development and progression of breast cancer. ER$\alpha$-mediated transcription is a complex process regulated at many different levels. The interplay between ligand, receptor, DNA sequence, cofactors, chromatin context, and post-translational modifications culminates in transcriptional regulation by ER$\alpha$. Recent technological advances have allowed the identification of ER$\alpha$ target genes on a genome-wide scale. In this review, we provide an overview of the progress made in our understanding of the different levels of regulation mediated by ER$\alpha$. We discuss the recent advances in the identification of the ER$\alpha$-binding sites and target gene network and their clinical applications.

Introduction

Estrogens regulate many cellular processes in a wide variety of target tissues during growth, development, and differentiation. Estrogens are mainly involved in the regulation and development of the female reproductive tract but also play a role in the central nervous system, cardiovascular systems, and in bone metabolism (Katzenellenbogen 1996). In addition to their role in physiology, estrogens are also associated with the development and progression of breast cancer (Anderson 2002). In 1896, Beatson discovered that removal of the ovaries resulted in breast cancer remission, connecting for the first time hormones with breast cancer, decades prior to the discovery of estrogens or estrogen receptors (ERs). Seventy years later, O’Malley observed changes in hybridizable RNA upon estrogen stimulation of the chick oviduct, indicating that estrogens regulate transcription (O’Malley et al. 1968). Again several years later a specific estrogen-binding protein was discovered that was present in breast tumors and its expression level could predict the response to endocrine disruption, thereby making the link between cancer and estrogens that was described almost a century before (Jensen et al. 1971, McGuire 1973). The subsequent cloning of the ER$\alpha$ gene and the identification of specific domains demonstrated that ER$\alpha$ functions as a ligand-dependent transcription factor (Green et al. 1986, Greene et al. 1986, Kumar et al. 1987). The structures of ER$\alpha$ and its nongenomic regulation have been reviewed extensively (Ruff et al. 2000, Warner & Gustafsson 2006). This review will focus on the regulation of ER$\alpha$-mediated transcription and on the advances made in the elucidation of its target gene network. Furthermore, the clinical significance and implications of ER$\alpha$ expression and genome-wide chromatin immunoprecipitation (ChIP) profiling is discussed.

Estrogen receptor

The ER$\alpha$ (NR3A1) is a member of the super family of nuclear receptors, which are ligand-dependent transcription factors. In addition to ER$\alpha$, the family includes other steroid hormone receptors such as the androgen receptor (AR, NR3C4), the glucocorticoid
receptor (GR, NR3C1), and other nuclear receptors such as the retinoic acid receptor (RAR, NR1B), retinoid X receptor (RXR, NR2B), and peroxisome proliferators-activated receptor (PPAR, NR1C). Estrogens are small lipophilic molecules that traverse the cell membrane and bind to cytoplasmic ER\(\alpha\) associated with chaperone proteins such as hsp90. Upon binding a cascade of events occur; the receptor dissociates from the chaperone proteins, dimerizes and associates with chromatin. ER\(\alpha\) can either bind directly to DNA (classical pathway) or indirectly via protein–protein interactions (nonclassical pathway). In the classical pathway, ER\(\alpha\) homodimers bind to a specific DNA sequence motif, the estrogen response element (ERE). The ERE is a 15 bp palindrome consisting of two PuGGTCA half sites separated by a 3 bp spacer. ER\(\alpha\) is also able to bind to imperfect EREs. Recent genome-wide studies show that the ERE is the most predominant motif in ER\(\alpha\)-binding sites (Carroll et al. 2006, Lin et al. 2007, Welboren et al. 2009). In the nonclassical pathway, the ER\(\alpha\) binds indirectly to the DNA via tethering to other transcription factors such as specificity protein 1 (Sp1), activating protein 1 (AP-1), or nuclear factor kappa b (NF-\(\kappa\)B), and regulates transcription in an ERE-independent manner. Sp1 family of transcriptional factors plays an important role in proliferation, differentiation, survival, and angiogenesis (Kaczynski et al. 2003, Safe & Abdelrahim 2005). Sp1 can bind to GC-rich regions, which are present in many estradiol (E2) responsive promoters. For example, mutational analysis revealed that the GC-rich region in the promoter of the transforming growth factor \(\alpha\) (TGF\(\alpha\)) gene is required for E2-mediated gene activation (Vyhlidal et al. 2000). Several other genes have been identified that are activated by the ER/Sp1 pathway including e.g. the c-myc and progesterone receptor genes (Miller et al. 1996, Petz & Nardulli 2000). The transcription factor AP-1 is a complex containing fos, jun, and other family members. Several E2-regulated genes are dependent on AP-1, such as insulin-like growth factor I (IGF-I), ovalbumin, progesterone receptor, and pS2/TFF1 (Gaub et al. 1990, Savouret et al. 1994, Umayahara et al. 1994, Barkhem et al. 2002). The AP-1 complex binds to promoters of genes involved in growth, differentiation, and development. Many ER/AP-1-responsive genes have been identified using microarray studies, indicating that E2-mediated regulation via the nonclassical pathway occurs frequently (DeNardo et al. 2005, Glidewell-Kenney et al. 2005). The NF-\(\kappa\)B family of transcriptional factors are involved in the immune and skeletal systems and inflammatory response (Kalaitzidis & Gilmore 2005). NF-\(\kappa\)B binds to \(\kappa\)B elements and regulates the expression of target genes. ER\(\alpha\) has been shown to inhibit NF-\(\kappa\)B in an E2-dependent manner. The ER\(\alpha\) can directly inhibit the binding of NF-\(\kappa\)B transcription factors to the DNA, but the mechanism is poorly understood.

**Target gene network**

**Single gene analysis**

To fully elucidate ER\(\alpha\) function, the identification of its target gene network is essential. Many strategies have been employed to gain insights into the target gene network governed by the ER\(\alpha\). Classically, target genes have been identified using ‘single gene’ experiments. The egg-white proteins in the chicken oviduct and the *Xenopus laevis* vitellogenin gene are among the first ER\(\alpha\) target genes to be identified (Hayward et al. 1982, Lai et al. 1983, Chambon et al. 1984, Jost et al. 1984). Later, by comparing cDNA libraries of nontreated and E2-treated MCF-7 human breast cancer cells several other ER\(\alpha\)-responsive genes were identified such as the classical and intensively studied ER\(\alpha\) target gene pS2/TFF1 (Brown et al. 1984, Jakowlew et al. 1984). ER\(\alpha\)-regulated genes identified using differential cloning are e.g. the cell cycle regulators c-myc and cyclin D1, connecting proliferation with E2 signaling (Dubik et al. 1987, Altucci et al. 1996).

**Estrogen response elements**

The first ERE was identified in 1986 in the promoter of the *Xenopus* vitellogenin gene. Transfection experiments showed that this element also functions in human cells, and that the core ERE could be defined as a 13 bp palindrome (GGTCANNNTGACC; Klein-Hitpass et al. 1986). The availability of the entire human genome sequence allowed the computational search for EREs and ER\(\alpha\) target genes. This approach led to the interesting observation that ~70 000 EREs are present in the human genome, i.e. one in every 43 kb of DNA (Bourdeau et al. 2004). By comparing these with the merely 9944 EREs identified in the mouse genome, a total of 660 evolutionary conserved elements were found. The large number of EREs present in the genome and limited conservation between human and mouse indicates that a large fraction of the EREs may not be ‘true’ ER\(\alpha\)-binding sites. These in *silico* studies suggested that actual binding by ER\(\alpha\) is dependent on more factors than just the DNA sequence. The hypothesis was put forward that the chromatin structure and hence the accessibility of binding sites plays a major role. Furthermore, the
expression profiles show that E2 activates as well as represses a large variety of targets including genes involved in cell cycle regulation and proliferation (e.g. cyclin D1 and cyclin G2), apoptosis (bcl-2 and survivin), and transcriptional regulation (c-fos and c-jun). Upregulated genes include among others activators of proliferation and downregulated genes include negative proliferation regulators.

Frasor et al. (2003) observed that at early time points following E2 induction a greater proportion of genes are upregulated, while at later time points more genes are downregulated. This was also observed by Carroll et al. (2006), who postulated that early ERα-mediated downregulation may be due to squelching while the increase in the number of downregulated genes at later time points may depend on the upregulation of the corepressor nuclear receptor interacting protein 1 (NRIP1) which mediates the repression of ERα target genes.

The number of E2-responsive genes identified by these transcriptome studies differs significantly even though the majority were performed using the MCF-7 cell line. The platform and specific conditions used certainly will have contributed to these differences, but other factors may also play a role. First of all, the experimental or technical differences between the profiling studies such as platforms, ligand concentration, treatment time, and statistical thresholds used. Secondly, the biological differences, i.e. the handling of the cells and the differences between MCF-7 sublines. MCF-7 cells have been in culture for many decades and as a result the cell lines used in different laboratories may not be the same anymore. Differences in karyotype, hormone receptor content, and growth rate have been observed although morphologically the cells look(ed) identical (Osborne et al. 1987, Bahia et al. 2002, Burdall et al. 2003).

It is important to note that a disadvantage of expression profiling is that indirect effects that affect the mRNA level are also measured which could be a major source of the observed differences. Lin et al. used the translation inhibitor cyclohexamide combined with the ERα antagonist ICI 182,780 to show that only a relatively small number (23%) of E2-responsive genes are actually direct targets. More recently changes in RNA polymerase II (RNAPII) occupancy over the gene body has been used as a direct measure of gene activity (Nielsen et al. 2008, Welboren et al. 2009). We have used this method to identify 596 genes directly responding to 1 h of E2 treatment (Nielsen et al. 2008, Welboren et al. 2009). Profiling RNAPII occupancy has the advantage that direct effects on target genes are observed, whereas transcriptome profiling is the sum of changes in transcriptional activity and regulation at mRNA level as observed by mRNA profiling. Both methods are complementary.

### ChIP analysis

ChIP assesses the binding of chromatin interacting proteins such as a transcription factors, cofactors, or histone modifications at a specific genomic position in living cells. Proteins and DNA are cross-linked with formaldehyde and the chromatin is fragmented by sonication. The protein of interest is precipitated using a specific antibody and the DNA to which it is bound is co-precipitated. In both the input and the precipitated fraction the relative concentration of specific DNA fragments is determined by qPCR and from this the occupancy of the protein of interest at any specific locus can be calculated. ChIP thus allows the identification of transcription- and cofactor recruitment to a specific locus upon ligand induction, as well as the order of recruitment. Furthermore, specific histone modifications present at a locus can be measured by ChIP, providing information on the local chromatin structure and epigenetic state. Shang et al. (2000) conducted early ERα ChIP experiments assessing ERα and cofactor occupancy at the cathepsin D (CATD), c-Myc, and pS2/TFF1 gene over time. They observed a stepwise recruitment of cofactors and found that the ERα transcriptional complex repeatedly cycled on and off promoters. Métivier et al. (2003) used ChIP to extensively analyze the occupancy of a large panel of transcription factors and histone modifications at the pS2/TFF1 promoter over time at 5 min intervals.
A cyclic recruitment of cofactors, histone modifications, histone acetyl transferases (HAT), histone methyl transferases, and chromatin remodelers was observed. The authors concluded that ERα-mediated transcription occurs in ‘waves’ that allow the cell to respond to environmental and other cues by continuously adjusting the rate of transcription of a gene.

**ChIP-chip**

Single ChIP, using sequence-specific primer sets combined with qPCR, is obviously not suited to gain genome-wide insight into transcriptional factor binding or histone modifications. By combining ChIP with microarray platforms (ChIP-chip), binding sites can be identified at a much broader scale. In ChIP-chip, the precipitated DNA is amplified, fluorescently labeled, and directly hybridized to a microarray or mixed with differential labeled input DNA. Initially, promoter arrays were used that contain the upstream regions of known genes including CpG islands that are often overlapping with promoter regions. These custom arrays have been used successfully by several laboratories resulting in the identification of many ERα-binding sites and in some cases histone modifications or cofactor occupancy (Jin et al. 2004, Laganière et al. 2005, Cheng et al. 2006, Kininis et al. 2007, Kwon et al. 2007). Microarrays comprising the entire nonrepetitive human genome enabled a true genome-wide identification of ERα-binding sites. The first study has been performed on Affymetrix arrays covering chromosome 21 and 22, and resulting in the identification of 57 ERα-binding sites (Carroll et al. 2005). The majority of these sites turned out to be located in introns or distal to genes and only a small percentage were located in promoter regions. The authors showed that the distal ERα-binding sites functioned as enhancers as demonstrated for the pS2/TFF1 gene and that the enhancer interacted with the promoter via looping. Sequence analysis of the ERα-binding sites also revealed an enrichment of the FOXA1 motif and it was postulated that FOXA1 might act as a pioneering factor that facilitates ERα binding. More recently, the same investigators published a genome-wide ERα and RNA polymerase II (RNAPII) ChIP-chip analysis using Affymetrix arrays identifying 3665 ERα and 3629 RNAPII-binding sites (Carroll et al. 2006). Re-analysis of the ERα ChIP-chip data revealed that the number of ERα sites was as high as 5782 (Lupien et al. 2008). As observed for chromosome 21 and 22, the number of ERα sites that is located in promoter regions is as low as 4%. Sequence analysis of all binding sites revealed several enriched motifs and showed that the co-occurrence of the ERE and the AP-1 motif is negatively correlated, suggesting the AP-1 and ERE motif occur mutually exclusive while the Oct, FOXA1, and C/EBP motif are positively correlated. In addition, a role for the nuclear receptor corepressor NRP1 in ERα-mediated repression was reported.

In a later study, the ERα ChIP-chip experiment was repeated and 8525 sites were identified, of which 86% overlapped with the dataset that was previously published (Hurtado et al. 2008). A newly identified site was present in the ERBB2 gene. Tamoxifen resistant tumors have increased ERBB2 expression levels and cell lines overexpressing ERBB2 acquire resistance to tamoxifen. Binding site sequence analysis showed an enrichment of the PAX transcription factor binding motif. PAX2 is expressed in a subset of breast tumors and is regulated by tamoxifen in endometrial cancer cells. ChIP-qPCR showed that PAX2 was recruited to ERα-binding sites upon tamoxifen but not upon E2 treatment. However, PAX2 recruitment to the ERBB2 gene was observed in the absence of tamoxifen as well as E2. Furthermore, the authors show that PAX2 competes with the co-activator AIB-1 for binding at the ERBB2 gene. Increasing levels of AIB-1 block PAX2 binding and result in upregulation of ERBB2 and subsequent cell proliferation, reversing the antiproliferative effects of tamoxifen. Interestingly, in tamoxifen resistant cells PAX2 levels are decreased, resulting in increased expression of ERBB2. Re-introduction of PAX2 restored the ability of tamoxifen to inhibit cell growth indicating PAX2 is an important factor in mediating selective ER modulator (SERM) action.

A more recent ChIP-chip paper investigated the tissue-specific behavior of estrogens. Krum et al. (2008) compared the expression and ERα-binding site profile in the breast cancer cell line MCF-7 with that of exogenous ERα in the U2OS osteosarcoma cell line to determine how estrogens exert this tissue specificity. Strikingly, <10% of the genes responding to E2 in MCF-7 are responsive in U2OS cells. Furthermore, ChIP-chip analysis on chr1 and chr6 showed that less than 15% of the ERα-binding sites were common between both cell types. To unravel the cell type-specific-binding of ERα, the presence of specific chromatin modifications at putative enhancers was determined. The authors showed that before E2 treatment some enhancers contained the active mark H3K4me2 while others contained the heterochromatic mark K3K9me2, and that these marks correlated with the binding of ERα. Interestingly, FOXA1 does not seem to play a role in the U2OS cells.
ChIP-chip has also been used to assess the effect of post-translational modifications of ERα on genome-wide binding and target gene profile (Bhat-Nakshatri et al. 2008). The authors focused on the serine/threonine kinase AKT and compared ERα binding in parental MCF-7 cells with AKT overexpressing MCF-7 cells. More than half of the ERα-binding sites are present in both datasets and gene expression profiling showed that AKT increased the number of E2 responsive genes from 833 to 1063. Analysis of the target genes revealed that AKT induces changes in the TGF-β, NF-κB/TNF, RA, and E2F pathways. Besides directly influencing ERα binding, AKT also induces secondary effects due to changes in the expression of E2F2 and E2F6, which subsequently change the expression of estrogen-induced or estrogen-repressed secondary target genes either in the presence (E2F6) or absence of E2 (E2F2). In conclusion, the authors postulate that AKT alters ERα and/or coactivator-binding resulting in changes in binding occupancy and target gene expression.

Liu et al. (2008) compared the binding of ERα and ERβ using ChIP-chip and an inducible ERβ system. Although there was a high degree of overlap between both profiles, a group of sites showed selective binding by either ERα or ERβ. Interestingly, ERβ-binding sites were located closer to the transcription start site compared to ERα. In addition, ERβ-binding site sequences included CG-rich motifs, while ERα sites were enriched for TA-rich motifs. Together these differences could explain the different expression profile of ERα and ERβ (Liu et al. 2008).

ChIP-deep sequencing

With next generation high throughput sequencing platforms millions of fragments can be sequenced simultaneously. DNA fragments can be identified at significantly decreased costs and with much higher sensitivity, resolution, and accuracy as compared to microarrays. Lin et al. used a ChIP-paired end diTag cloning and sequencing strategy (ChIP-PET) to map ERα-binding sites. A total of 635 371 tags have been sequenced (454 platform), of which 361 241 (57%) could be unambiguously mapped to the human genome. In total, 1234 high-confidence ERα-binding sites were identified. Of these, 71% contained an ERE-like sequence and many other transcription factor motifs were enriched, including Sp1, AP-1, and FOXA1. Illumina high throughput sequencing has also been successfully combined with ChIP for the identification of precipitated fragments. Recently, we identified 10 205 ERα-binding sites in MCF-7 cells using ChIP-Seq and assessed the effect of tamoxifen and fulvestrant on ERα binding. Fulvestrant is a full E2 antagonist that increases ERα protein turnover and results in degradation of ERα, although at the 1 h time-point no degradation was observed. We showed that both tamoxifen as well as fulvestrant affect but do not abolish ERα binding (Welboren et al. 2009). Also, the effect of different ligands on the RNAPII occupancy over target genes was investigated using ChIP-Seq. Upon E2 treatment, 596 genes show changes in RNAPII occupancy. Tamoxifen and fulvestrant treatment abolished RNAPII occupancy over E2-upregulated target genes. On E2-repressed genes tamoxifen acts as an agonist, downregulating these genes while fulvestrant antagonizes the E2-induced repression and often increases the RNAPII occupancy. Thus, both antagonists act differentially on E2-induced and E2-repressed genes.

Comparison of ChIP-profiles

Different ERα ChIP-profiling studies, i.e. ChIP-chip, ChIP-PET, and ChIP-Seq, have identified a multitude of ERα interaction sites. However, the number of sites detected in each study is significantly different and the profiles show a limited overlap, even though all studies used the MCF-7 breast cancer cell line and performed a similar E2 treatment.

Clearly, ChIP profiling is a relatively new development and it is likely that as the approach matures the variation between experiments and laboratories will disappear over time. In this light, the transition from microarray to sequencing-based detection of ChIP DNA fragments seems to be an important step in that direction.

Comparing the existing ChIP-chip and ChIP-Seq ERα sets shows that around half of the binding sites overlap and that both sets contain a considerable number of specific sites. Both sequence-based profiles (ChIP-Seq and ChIP-PET) show a larger overlap, although one has to keep in mind that the ChIP-PET set is much smaller (Fig. 1). One obvious factor that will certainly contribute but is unlikely to be the main course is the biological variation in the MCF-7 (sub)lines used and/or handling of the cells. One of the main differences between these profiles, however, is that different antibodies against the ERα were used, which is likely to affect the ChIP and thus the ERα-binding profiles. Different antibodies, or even antibody batches, will have different specificities and avidities for the target protein. Antibodies raised against different peptides will recognize different epitopes. As a consequence, epitope masking due to
conformational shielding or binding and cross-linking of an interactor may block or hinder the binding of some but not other antibodies. For polyclonal antibodies, epitope masking is less likely to affect the outcome as multiple epitopes can be recognized. On the downside, polyclonal antibodies may display more cross-reactivity.

Another factor contributing to the differences in ERα-binding profile is the platform used to identify the precipitated DNA fragments. Microarray based detection has been widely used and provides the first genome-wide views of transcriptional factor binding and histone modifications. However, microarray approaches do have intrinsic disadvantages related to the sequence composition of probes, differences in melting temperature, annealing efficiency, and cross-hybridization which are discussed in detail elsewhere (Buck & Lieb 2004, Hanlon & Lieb 2004, Bulyk 2006, Wu et al. 2006, Gräf et al. 2007). ChIP-Seq, in which co-precipitated fragments are identified by high throughput sequencing have important advantages over microarrays. First, ChIP-Seq is unbiased and enables a higher resolution and accuracy. Furthermore, the fabulous sequencing depth allows the identification of binding sites that are moderately enriched, that probably would not have escaped detection using an array approach. Because sequencing-based methods are not dependent on hybridization the ‘background’ is significantly lower when compared to microarrays. An additional important advantage of ChIP-Seq is that information on repetitive sequences can be obtained which is not possible on microarrays, because these contain only nonrepetitive regions (Marks et al. 2009).

Notwithstanding the differences, the combined ERα-binding site profiles identify a large number of stable, high-affinity ERα interaction sites and a set of more transient ERα interaction sites. High affinity sites are more likely to be detected by all techniques, while lower affinity sites may only be detected when using the more sensitive and accurate sequencing-based approaches, i.e. ChIP-Seq.

Collectively, ChIP profiling studies have revealed that the number of ERα interaction sites is much larger than the number of E2-responsive genes hitherto identified by gene expression profiling. The biological implications of the discrepancy remain as one of the questions to be answered. One assumption is that a large number of ERα-binding sites may not be functional, i.e. ERα binding has no consequence on the transcription of closely positioned genes in the particular cell investigated. It is likely that conditions for transcriptional regulation at these sites are not favorable, i.e. cofactors or transcription factors are not co-recruited or a specific post-translational modification of the chromatin is absent and hence the level of transcription from the gene is not affected. An alternative hypothesis is that multiple ERα sites cooperate and interact via looping to regulate expression of one or more target genes. More research is necessary to elucidate this aspect of ERα-mediated regulation. A recently developed technique, chromatin interaction analysis using paired-end tag sequencing (ChIA-PET), may shed light on this issue (Fullwood & Ruan 2009).

Cofactor choice

ERα mediated gene regulation is controlled by the interplay of ligand, receptor, DNA (ERE), and cofactors. Cofactors interact with the receptor in a ligand-dependent manner and are often part of large multiprotein complexes that regulate transcription by recruiting components of the basal transcription machinery, regulating chromatin structure and/or modifying histones (reviewed by Klinge 2000). Coactivators are required for transcriptional activation, while corepressors decrease the transcriptional activity. The presence and levels of cofactors will, to a large part, determine the transcriptional outcome and

Figure 1 Comparison of the ChIP-Seq (10,205), ChIP-chip (8,525), and ChIP-PET (1,234) ERα-binding site profile. The ChIP-Seq data is compared with the new ChIP-chip data from Carroll et al. (Hurtado et al. 2008) and the ChIP profile from Lin et al. The overlap between all profiles is limited.
are vital in ERα-mediated regulation. Protein–protein interaction studies have revealed a multitude of nuclear receptor cofactors.

The best characterized are the p160 family of steroid receptor cofactors; steroid receptor coactivator 1 (SRC-1), SRC-2 (TIF2/GRIP1), and SRC-3 (AIB1/RAC3/ACTR). An increase in the level of SRC-3/AIB1 has been observed in breast and prostate tumors (Anzick et al. 1997, Gnanapragasam et al. 2001). A limited number of SRC-binding sites have been identified using a ChIP-cloning approach (Labhart et al. 2005) while most recently ChIP-chip has been used (Kininis et al. 2007). Both studies show that the binding of SRC cofactors correlated very well with ERα binding. The p160 family and many other nuclear receptor cofactors contain an LXXLL (NR-box) motif, which facilitates the interaction of the cofactor with the AF-2 domain of the ERα. One function of these cofactors is to recruit other proteins such as CREB-binding protein (CBP)/p300 and pCAF that have HAT activity. The CBP is not only a coactivator of ERα but also of other nuclear receptors and many other transcription factors such as p53 and NF-κB (Chakravarti et al. 1996, Kamei et al. 1996, Avantaggiati et al. 1997, Perkins et al. 1997, Froïnsdal et al. 1998). p300 shares many functional properties with CBP, nevertheless, CBP and p300 are not completely redundant as shown by targeted deletion studies (Yao et al. 1998). Another group of factors recruited by the p160 family are the protein arginine methyl transferase (PRMT) family of proteins that are able to methylate histone H3 and H4. The first PRMT family member to be discovered, coactivator associated arginine methyltransferase 1 (CARM1), or PRMT4; Chen et al. 1999), enhances ERα-mediated transcriptional activity by interacting with GRIP1, a p160 family cofactor and p300 (Chen et al. 2000). CARM1 methylates several arginines in histone 3, i.e. H3R2, H3R17 and H3R26, upon nuclear receptor-mediated activation (Ma et al. 2001, Schurter et al. 2001). Another family member, PRMT1, methylates H4R3, which in turn facilitates the acetylation of H4 by p300 (Wang et al. 2001). PRMT1 is an essential cofactor for ERα-mediated pS2/TFF1 regulation (Wagner et al. 2006). Yeast two-hybrid studies have shown that PRMT2 can also interact with the ERα and that it enhances ERα transcriptional activity upon ligand binding (Qi et al. 2002). Other cofactors that possess chromatin remodeling activity are for example Brahma (BRM) and Brahma related gene-1 (BRG-1). They are subunits of an ATP-dependent chromatin remodeling complex and are recruited to E2 responsive promoters upon induction (DiRenzo et al. 2000, Belandia et al. 2002). These remodelers alter the local chromatin structure to a more open conformation, permitting transcription as is elegantly shown for GR (John et al. 2008).

Nuclear receptors that bind DNA in the absence of ligand, antagonist loaded receptors or receptors causing gene repression interact with corepressors proteins such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT). Corepressors interact with helix 3 and 5 of the ERα via so called CoRNR boxes (LXXI/HIXXI/L) (Hu & Lazar 1999). NCoR and SMRT in turn recruit large repressor complexes including histone deacetylases (HDAC) that repress gene activity by maintaining or reinforcing a repressive chromatin state. Antagonist loaded ERα does bind regulatory regions (Welboren et al. 2009) and recruits corepressor complexes, as shown for the pS2/TFF1 and c-myc genes where NCoR, HDAC3 and the nucleosome remodeling complex NURD are recruited, leading to a repressive chromatin state (Liu & Bagchi 2004). Low levels of NCoR expression have been shown to have a predictive value in tamoxifen resistance and NCoR levels decrease during progression of breast cancer (Kurebayashi et al. 2000, Girault et al. 2003). Another factor involved in ERα-mediated regulation is the histone demethylase LSD1. Using ChIP-DSL Garcia-Bassets et al. (2007) showed that LSD1 is recruited to a subset of E2-responsive genes upon treatment and subsequently demethylates H3K9, hence counteracting the repressive effect of methylation.

To gain insight into cofactor choice on ERα-mediated regulation genome-wide cofactor profiling will be essential. Hitherto, data obtained at a single gene level has been reported and has been extrapolated to hold true for all or a large number of similarly regulated genes. Genome-wide profiling of nuclear receptor cofactors will show whether or not the model of regulation that was determined on single or at best a handful of genes does apply genome-wide.

Epigenetic marks

Genome-wide epigenetic profiling studies have revealed that the chromatin structure and epigenetic marking of a promoter, enhancer, and coding body of a gene correlates with the transcriptional state. Epigenetic marks consist of covalent post-translational modifications of N-terminal histone tails (Roh et al. 2004, Schübler et al. 2004, Bernstein et al. 2005, Pokholok et al. 2005, Barski et al. 2007). Epigenetic marks such as acetylation, phosphorylation, methylation, and ubiquitination are deposited and removed by
coregulatory complexes in a serial and combinatorial manner. Modification of histone tails results in dynamic changes in the chromatin structure and restrict or permit binding of transcription factors (Jenuwein & Allis 2001, Rosenfeld et al. 2006). Several studies have been performed investigating the role of epigenetics in ERz-mediated regulation. Bauer et al. (2002) showed that the arginine methyltransferase CARM1 is recruited to the pS2/TFF1 promoter upon E2 induction, and that this event correlates with the methylation of arginine 17 of histone H3 and transcriptional activation. Kwon et al. (2007) profiled ERz binding, histone methylation, and acetylation. Histone H3K9 acetylation, which is associated with an open chromatin structure and increased transcriptional output, was observed at the promoters and enhancers of the active pS2/TFF1 and GREB1 gene. In addition, the promoter regions contained H3K4 tri-methylation, a mark that is widely associated with active promoter regions. H3K4 mono-methylation was associated with active genes, while H3K4 di-methylation was observed primarily on the promoters of active genes and to a lesser extent on enhancers. Both H3K27 di- and tri-methylation showed distinct patterns over the GREB1 and pS2/TFF1 gene (Kwon et al. 2007). Kininis et al. (2007) assessed histone H3 and H4 acetylation using a custom promoter array and revealed a correlation between histone acetylation and RNAPII occupancy, reinforcing the notion that histone acetylation correlates with gene activity. Métivier et al. (2003) performed a detailed time-course analysis of the pS2/TFF1 promoter and showed that H3 and H4 acetylation and di-methylation are deposited and removed in a cyclical fashion. The recruitment of cofactors, ERz, and RNAPII also occurred in a cyclical manner, producing transcriptional ‘waves’. Interestingly, two recent publications showed that besides histone modifications DNA methylation also takes place in a cyclical fashion at the pS2/TFF1 promoter (Kangaspeska et al. 2008, Métivier et al. 2008). The authors observed DNA methylation at the end of each productive transcription cycle. DNA methylation correlated with the occurrence of the methylated Cpg-binding proteins MeCP2, DNMT3a/b, DNMT1 and the chromatin remodeler SWI/SNF. Furthermore, the authors suggest that DNMT3a/b is involved in both methylation and demethylation of the promoter. These data suggest that both histone modifications and DNA methylation may be intricate parts of the ‘normal’ transcriptional cycle.

Histone methylation has been reported to prevent gene activation by unliganded receptors (Garcia-Bassets et al. 2007). Using ChIP-DSL and a promoter array the authors show that the histone demethylase LSD1 is recruited to a subset of ERz target genes upon E2 treatment. In the absence of ligand these genes show H3K9 methylation. The H3K9 methylation prevents the effective binding of unliganded ERz. The binding of liganded ERz permits LSD1 recruitment and the removal of the inhibitory marks allowing transcription to occur. Most current models on ERz-mediated regulation, however, are based on analysis of single genes and the question arises as to whether these reported deposition of marks and the inferred mechanisms are the rule or the exception in regulation by ERz.

Yet another intriguing layer of complexity to ERz target gene regulation has been recently reported. The occurrence of controlled and local DNA damage and repair has been shown to play a role in the ERz-mediated regulation of bcl-2 (Perillo et al. 2008). E2 treatment results in the demethylation of H3K9 by LSD1, methylation of H3K4 and the formation of a loop between the enhancer and promoter of bcl-2. Demethylation of lysine H3K9, in this case by LSD1, is an oxidative process that produces H2O2. H2O2 causes local DNA damage by formation of 8-oxo-guanine. The 8-oxo-guanine is removed via base excision repair by 8-oxo-guanine DNA glycosylase 1 (OGG1). In addition, topoisomerase IIβ, which is capable of repairing single stranded breaks in double-stranded DNA, is also recruited. These data show that local DNA damage and repair induced by E2-dependent demethylation of H3K9 may also play an important role in ERz signaling. Double strand DNA breaks, induced by topoisomerase IIβ, have been previously reported in the regulation of transcription of the pS2/TFF1 gene (Ju et al. 2006). The question is whether transient DNA breaks and the DNA repair machinery plays a general role in transcriptional regulation. The plethora of post-translational modifications, the vast number of histone modifying enzymes and the serial and combinatorial fashion in which these modifications are applied, points to a very complex process to ensure timely and tight regulation.

**Looping**

Genome-wide ERz ChIP profiling studies hitherto revealed that only a minor fraction of ERz-binding sites are located in promoter regions and that the vast majority are located at great distances (>20 kb) from genes (Carroll et al. 2006, Lin et al. 2007, Wellboren et al. 2009). Because of these large distances to genes, assignment of binding sites to target genes is often rather arbitrary. Moreover, the number of identified ERz-binding sites is much larger than the number of
E\textsubscript{2}-regulated genes identified by expression or RNAPII profiling, indicating that many binding sites are idle or that multiple ER\(\alpha\)-binding sites cooperate to regulate transcription. Using 3C it has been shown that at the classical ER\(\alpha\) target genes pS2/TFF1, GREB1, and bcl-2, multiple ER\(\alpha\)-binding sites interact via looping to regulate transcription (Carroll \textit{et al.} 2005, Deschênes \textit{et al.} 2007, Perillo \textit{et al.} 2008). Pan \textit{et al.} (2008) further investigated the interaction between the TFF1 promoter region and the upstream enhancer. They show that both the enhancer and the promoter region are occupied by the same suite of transcription factors consisting of ER\(\alpha\), cofactors and RNAPII. Furthermore, the promoter and upstream enhancer interact via looping in an E\textsubscript{2}-dependent manner and the transcriptional output of the interaction is dependent on the ERE sequence.

Long-range chromosomal interactions are probably a general mechanism in transcriptional communication and regulation by ER\(\alpha\) and many other transcription factors. Recently, a combination of 3C with ChIP-DSL, the 3D assay (deconvolution of DNA interactions by DSL), was used to identify regions that interact with the enhancer of the TFF1 gene on a genome wide scale (Hu \textit{et al.} 2008). Strikingly, the GREB1 promoter and enhancer on chr2 interacted with the TFF1 enhancer on chr21. Subsequent FISH analysis showed that indeed both loci interacted upon E\textsubscript{2} induction. The knockdown of CBP/p300 or SRC1 abolished the GREB1:TFF1 interaction. Furthermore, inhibition or knockdown of nuclear myosin-I abolished the interaction, as did the knockdown of dynein light chain-1 and the chromatin remodeler BAF53. The histone demethylase LSD1 has previously been shown to be essential for E\textsubscript{2} mediated regulation. Interestingly, LSD1 knockdown had little effect on interchromosomal interaction of GREB1:TFF1. Furthermore, FISH analysis showed that the sites of the interchromosomal interactions are spatially related to nuclear speckles, regions that are enriched for key transcriptional elongation factors, chromatin remodelers, and splicing factors. LSD1 knockdown prevented the interaction of the GREB1:TFF1 loci with these nuclear speckles. Liganded ER\(\alpha\) thus initiates interchromosomal interactions that are important for enhancement of ligand-dependent transcription (Hu \textit{et al.} 2008).

High throughput alternatives of the 3C method have been developed to study interactions on a genome-wide scale. Circular chromosome conformation capture (4C) identified more than a hundred regions that interacted with the H19 imprinting control region (Zhao \textit{et al.} 2006). An alternative high throughput technique named carbon copy chromosome conformation capture (5C) has been used to identify looping in the \(\beta\)-globin locus (Dostie \textit{et al.} 2006). More recently a new approach has been announced, ChIA-PET (Fullwood & Ruan 2009). Using this technique all chromatin interactions can be detected in one experiment. In ChIA-PET, chromatin is crosslinked and sheared in small fragments. A linker sequence is introduced in the junction of two DNA fragments that are in close proximity due to chromatin interaction. The linker-connected ligation products are subsequently extracted and sequenced. Mapping the two paired fragments onto the genome reveals the interacting regions.

Applying these technologies to ER\(\alpha\) will enable the detection of ‘all’ chromatin interactions taking place upon ligand or antagonist binding and may shed light on the difference in the number of ER\(\alpha\)-binding sites and number of genes changing upon E\textsubscript{2} treatment.

**FOXA1**

Recently, the forkhead box protein FOXA1 was identified as a factor intimately associated with ER\(\alpha\)-mediated transcriptional regulation. The FOXA1 motif was found to be highly enriched in ER\(\alpha\)-binding sites (Carroll \textit{et al.} 2005, 2006, Laganière \textit{et al.} 2005). ChIP-chip of FOXA1 showed that 50–60\% of the FOXA1-binding sites overlap with ER\(\alpha\)-binding sites. The authors postulated that FOXA1 acts as a pioneering factor that binds H3K4me1/2-rich and H3K9me2-poor regions and facilitates ER\(\alpha\) binding (Lupien \textit{et al.} 2008). ER\(\alpha\) ChIP-Seq data from our laboratory, however, shows that only a small number of ER\(\alpha\)-binding sites (7\%) contain the FOXA1 motif (Welboren \textit{et al.} 2009). In addition, in the ChIP-DSL study by Kwon \textit{et al}. no significant association of ER\(\alpha\)-binding sites and the FOXA1 motif was observed. However, only promoter regions were assessed by ChIP-DSL, which could contribute to the lack of FOXA1 enrichment because only a relatively small fraction of ER\(\alpha\)-binding sites are located in promoter regions (Kwon \textit{et al.} 2007). Taken together the question remains to what extent FOXA1 plays a role in ER\(\alpha\)-mediated regulation.

**ER\(\alpha\) antagonists**

The growth of many breast tumors is E\textsubscript{2} dependent, and hence blocking E\textsubscript{2} binding to ER\(\alpha\) by antagonists is used as a therapeutic treatment. The successful application of tamoxifen has triggered vivid interest in the development of many (partial) antagonists, so called SERMS. SERMs bind in the same pocket as E\textsubscript{2}, but the compound loaded ER\(\alpha\) ligand binding domain...
adopts a different conformation. The crystal structure of the compound loaded ligand-binding domain has been solved and shows that binding of an antagonist elicits a re-positioning of helix 12 that prevents/ disrupts the transcriptional activity of AF-2 and inhibits interaction with coactivators (Brzozowski et al. 1997). SERMs operate either as agonist, antagonist or mixed agonist/antagonist based on a set of variables such as type of drug or target tissue. The transcriptional outcome of SERM loaded ERα is mostly determined by the conformation of ERα upon ligand binding, the presence or absence of coregulatory proteins and the effector site/promoter. The most successful and widely used SERM is tamoxifen, which is applied in the clinic for the treatment and prevention of breast cancer. Notwithstanding its great beneficial effects, tamoxifen has some disadvantages, namely induction of resistance. Tamoxifen resistance will eventually occur in all cases of advanced breast cancer and enables the tumor to grow in the presence of tamoxifen. The mechanism by which this occurs is still not clear; it has been reported that growth factor signaling might play an important role. Changes in the levels of epidermal growth factor receptor (EGFR), human EGFR type 2 (ERBB2/HER2) and IGF receptor type 1 (IGF-IR) and their downstream signaling pathways are associated with tamoxifen resistance (Massarweh et al. 2008). Recently, PAX2 has been shown to mediate repression of ERBB2 upon tamoxifen treatment, connecting ERα with the ERBB2 pathway (Hurtado et al. 2008). An additional disadvantage of tamoxifen is its tissue specific effect; tamoxifen acts as an antagonist in breast, but as an agonist in the uterus, resulting in an increased risk of endometrial cancer. The expression pattern and abundance of activated cofactors in the different tissues may very well determine the tissue specific effects of SERMs (Smith & O’Malley 2004).

Interestingly, our recent ChIP-Seq data (Welboren et al. 2009) shows that tamoxifen induces ERα binding to a subset of the E2-induced ERα-binding sites, indicating that the DNA sequence or chromatin structures may in part determine the tamoxifen response. We have also shown that tamoxifen has a repressive effect on a large set of genes that appear not to respond to E2. The observed repression by tamoxifen may therefore be independent of the presence of ERα protein.

**ERα target genes and breast cancer**

Considerable effort has gone into gene expression profiling to diagnose, monitor and to predict disease progression and response to chemo- and endocrine therapy. Routine pathological tumor assessment of lymph node status, tumor size and histological tumor grade do not accurately predict the response to therapy or determine whether or not metastasis will occur. Based on histological and clinical guidelines, almost 90% of lymph node negative patients now receive systemic adjuvant treatment. The use of a tumor type specific molecular signature is generally seen as of great benefit to tailor treatment. Gene expression profiling studies have aided the selection of breast cancer patients that would not need adjuvant chemotherapeutic and/or endocrine therapy (van’t Veer et al. 2002). The gene expression signature predicts that 70–80% of these patients are unlikely to develop metastases, so these patients are currently overtreated. Several other gene expression profiles predict recurrence and overall survival and/or tamoxifen resistance (Ma et al. 2004, Paik et al. 2004, Jansen et al. 2005, Wang et al. 2005, Frasor et al. 2006, Oh et al. 2006, Gräf et al. 2007, Chanrion et al. 2008, Kok et al. 2009, Lippman et al. 2008, Zhang et al. 2009).

Epigenetic silencing of loci is frequently observed in cancer. HDAC inhibitors can alleviate the epigenetic repression of genes and has been shown to inhibit cell growth and activate apoptosis in the MCF-7 breast cancer cell line, indicating that epigenetic silencing plays an important role in breast cancer (Pledger-Tracy et al. 2007, Im et al. 2008). Alterations in the DNA methylation profile are observed in many cancers (Baylin 2005). Genome-wide profiling of DNA methylation, e.g. methylated DNA immunoprecipitation using a specific antibody (MeDIP) or methylated DNA precipitation using protein affinity columns (MethylCap), provides detailed information on the methylation state of loci and can identify epigenetically silenced loci (Wilson et al. 2006). So far, gene expression profiles are based on the expression level of a small group of genes. However, a wealth of information on ERα regulation and the network of target genes governed by ERα have become available in recent times and await its translation into clinic applications. Furthermore, several genomic regions important for the development of breast cancer have been identified recently using genome-wide association studies (Ahmed et al. 2009, Thomas et al. 2009, Zheng et al. 2009). These regions identify new pathways contributing to the development of breast cancer and can be combined with profiling data. Gene expression profiling using high throughput sequencing of the transcriptome (RNA-Seq) has much improved sensitivity, resolution and accuracy. Sequencing of the transcriptome has the big advantage that mutations and translocations can be detected because the sequence of
‘each’ transcript is determined. Furthermore, RNA-Seq is much more quantitative as compared to array based gene expression profiling. RNA-Seq can distinguish/detect different isoforms and can determine transcript boundaries. In addition, tumor specific and driver mutations can be determined (Nagalakshmi et al. 2008, Sultan et al. 2008, Wilhelm et al. 2008). The ever increasing sequencing capacity and decreasing costs hold great promises for the application of RNA/ChIP-Seq profiling to assist in diagnosis and prognosis. The presence or absence of genomic alterations such as copy number variation, inversions and deletions, the gene expression profile and the epigenetic state of loci are likely to be of high predictive value. Combined ChIP, epigenetic, RNA profiles or signatures and genome-wide association studies are likely to improve the monitoring of disease progression and may lead to a more tailored and personalized approach in endocrine treatment selection of breast cancer patients.

**Future directions and conclusions**

A comprehensive and detailed picture of ERα-mediated transcriptional regulation is beginning to emerge (Fig. 2). Several regulatory and signaling pathways converge to ultimately regulate expression of an ERα target gene. Genome-wide ChIP and RNA profiling combined with deep sequencing has greatly increased our knowledge of the ERα target gene network. Hitherto the majority of data has been generated using one cell line and rather similar conditions. The effect of signaling pathways, the presence and levels of cofactors on the global target gene network can now be determined with relative ‘ease’. In addition, the role of the ERα in different cell lines and tissues can be studied and most importantly, the application of the profiling approaches to tumors may provide the real *in vivo* picture. The sequencing of tumor genomic DNA and the determination of DNA methylation and RNA profile, as planned in the

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**Figure 2** Overview of ERα-mediated regulation of transcription. Activation of genes by an unliganded receptor is prevented due to methylation of histone tails by H3K9-specific histone methyl transferases. Ligand, e.g. estradiol (E2) or the SERM tamoxifen, diffuses through the cellular membrane and binds cytoplasmic ERα. Upon ligand binding ERα dissociates from chaperone proteins such as hsp90, translocates to the nucleus and dimerizes. The ERα binds chromatin and in a subset of genes recruits the histone demethylase LSD1. LSD1 removes the methyl mark that prevents gene activation by the unliganded receptor and also plays a role in the interaction of distal enhancer regions via looping. At upregulated genes, cofactors such as AIB1 (p160 family) are recruited and a complex containing histone acetyl transferase activity is assembled. The N-terminal histone tails are acetylated, resulting in an open chromatin conformation. In addition, cofactors with nucleosome remodeling activity (BRG1/BRM) are recruited. Finally, RNA polymerase II and the general transcription machinery assemble at the promoter and the gene is transcribed. At repressed genes, either E2-repressed genes or genes repressed upon tamoxifen treatment, the corepressors NCoR or SMRT are recruited and a histone deacetylase complex is assembled. Acetyl groups are removed from histone tails and the chromatin is in a closed conformation, which is not permissive for transcription to occur.
International Cancer Genome Consortium (ICGC), will provide invaluable insight in tumor development and progression. With the decreasing cost, and increasing accuracy and capacity of high throughput sequencing platforms, the amount of data is explosively increasing. The expectation is that our understanding of ERα-mediated regulation will greatly increase and will ultimately lead to new diagnostic/prognostic tools, to therapeutic targets and a more tailored treatment. The integration of all this genomic data and its clinical application will be the challenge of the near future.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported in part by the Radboud University Nijmegen Medical Centre, Radboud University Nijmegen Science Faculty and by HEROIC, an Integrated Project funded by the European Union under the 6th Framework Program (LSHG-CT-2005-018883) EU-FP6 Integrated Project.

Acknowledgements
The authors would like to thank Dr G Veenstra for critical reading of the manuscript.

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