Estrogen Signaling via Estrogen Receptor β*

Published, JBC Papers in Press, October 18, 2010, DOI 10.1074/jbc.R110.180109
Chunyan Zhao†, Karin Dahlman-Wright‡, and Jan-Åke Gustafsson§

From the †Department of Biosciences and Nutrition, Novum, Karolinska Institutet, S-141 57 Huddinge, Sweden and the ‡Center for Nuclear Receptors and Cell Signaling, Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204

Estrogens act by binding to and activating two estrogen receptors (ERs), ERα and ERβ. Transcriptional regulation by ERs is controlled by a complex array of factors such as ER-ligand binding, the DNA sequence bound by ERs, ER-interacting cofactors, and chromatin context. This minireview will provide an overview of the most recent advances in the identification of ERβ-regulated target gene networks and ERβ DNA-binding sites. We also highlight the recent work establishing new roles of ERβ signaling, including protective functions in the epithelial-mesenchymal transition and in atherosclerosis, as well as regulation of cell proliferation in the colon.

Estrogens, the main female sex steroids, control many cellular processes, including growth, differentiation, and function of the reproductive systems. In the non-pregnant female, estrone and estradiol (E2) are the two main forms of estrogens, whereas estriol is the main estrogen in pregnancy. Estrogens interact with two estrogen receptors (ERs), ERα and ERβ, and exert their effects through diverse signaling pathways that mediate genomic and nongenomic events, resulting in tissue-specific responses. Estrogen-regulated gene expression is controlled by a complex array of factors such as ER-ligand binding, the DNA sequence bound by ERs, ER-interacting cofactors, and chromatin context. The transcriptional responses to estrogen signaling depend on ligand identity and availability, the cellular concentration and localization of ERs, levels of various coregulator proteins and other signal transduction components, and the chromatin state (1). The discovery of a second ER, ERβ, in 1996 (2) prompted renewed efforts to investigate the mechanisms of action of estrogenic molecules. There is now compelling evidence that ERβ is involved in various types of cancer (breast, ovarian, colorectal, prostate, and endometrial), in bone and brain physiology, and in the cardiovascular system and inflammation (3, 4). Recently, global analysis of gene expression profiles and identification of protein-DNA interactions have begun to reveal the molecular architecture of ERβ binding to DNA and the subsequent effects on gene regulatory networks. In this minireview, we will discuss the current knowledge of gene regulatory networks influenced through ERβ, as well as several novel discoveries pertaining to roles of ERβ in epithelial-mesenchymal transition (EMT), atherosclerosis, and the colon.

ERβ Gene and Its Protein Variant ERβ2

ERβ is a member of the nuclear receptor superfamily and shares common structural characteristics with the other members of this family, including five distinguishable domains denoted A–F (Fig. 1) (5). The human ERβ gene (ESR2) is located on chromosome 14q23.2, spanning ~61.2 kb. The ERβ protein is produced from eight exons. Additionally, there are two untranslated exons, ON and OK, in the 5′-region and an exon at the 3′-end that can be spliced to exon 7 to produce the alternative ERβ isoform, ERβ2 (also called ERβcc) (6). Thus, ERβ2 has a unique C terminus, where the amino acids corresponding to exon 8 are replaced with 26 unique amino acids. The full-length human ERβ (also named ERβ1) protein includes 530 amino acids with an estimated molecular mass of 59.2 kDa, whereas ERβ2 encodes a protein of 495 amino acid residues with a predicted molecular mass of 55.5 kDa. ERβ2 has undetectable affinity for E2 and other tested ligands. ERβ2 was suggested to be a dominant-negative inhibitor of ERα (6). Further mechanistic study revealed that ERβ2 induces proteasome-dependent degradation of ERα, leading to suppression of ERα signaling (7). Although additional mRNA isoforms of ERβ arising from differential splicing have been described, only ERβ2 has been identified at the protein level (8, 9).

Mechanisms of ER Signaling

Upon ligand activation, ERs can regulate biological processes by divergent pathways (Fig. 2) (1). The so-called classical signaling occurs through direct binding of ER dimers to estrogen-responsive elements (EREs) in the regulatory regions of estrogen-responsive genes, followed by recruitment of coregulators to the transcription start site. The consensus ERE consists of a 5-bp palindrome with a 3-bp spacer: GGTCAnnnTGACC. However, many natural EREs deviate substantially from the consensus sequence (10). Estrogen also modulates gene expression by a second mechanism in which ERs interact with other transcription factors such as AP-1 (activating protein-1) and Sp-1 (stimulating protein-1) through a process referred to as transcription factor cross-talk. Furthermore, estrogen may elicit effects through nongenomic mechanisms, which occur much more rapidly. This action has been shown to involve the activation of downstream cascades such as PKA, PKC, and MAPK via membrane-localized ERs. Recently, an orphan G protein-coupled receptor (GPR30) in the cell membrane was reported to mediate nongenomic estrogen signaling. Subsequent studies by others demonstrated that the activities of GPR30 in response to estrogen were through its ability to induce expression of ERα36, a novel variant of ERα, and that in turn, ERα36, acted as an extranuclear ER to mediate nongenomic estrogen signaling (11). It is still possible that addi-
tional membrane receptors for estrogen are involved in mediating nongenomic estrogen action. The mechanistic details of activation through nongenomic pathways such as target genes remain to be characterized.

ERs can also be activated by extracellular signals in the absence of ligand. Growth factor signaling or stimulation of other signaling pathways leads to activation of kinases that can phosphorylate and thereby activate ERs or associated coregulators in the absence of ligand. As an example, the HER2 downstream signaling molecules ERK1 and ERK2 can phosphorylate ER, leading to ligand-independent receptor activation (12). The biological significance of this ER signaling remains unclear.

**Genome-wide Profiling of ERβ Gene Expression Programs**

There have been a number of studies in the past few years aimed at comprehensively unraveling the complete estrogen-regulated gene expression programs in cancer cells. These reports can be attributed to the introduction of microarrays for global gene expression profiling. DNA microarray technology allows quantitative monitoring of changes in the expression of thousands of genes simultaneously and has been described in several configurations, including oligonucleotide arrays and microarrays of cDNAs spotted on glass slides. During the past few years, the development of high-throughput DNA sequencing (HTS) methods for global gene expression profiling, also known as “RNA-Seq,” has challenged microarray technology because of its superior capability for detection of genes expressed at low levels, alternative splice variants, and novel transcripts (13). However, to our knowledge, no studies that explore HTS to assay genome-wide transcriptional regulation by estrogen have been reported.

Several reports have described global gene expression profiles in ERα-expressing breast cancer cell lines in response to E2 treatment (14). The available studies have reported different numbers of E2/ERα-regulated genes in MCF-7 breast cancer cells, ranging from ~200 to ~1500. These discrepancies may be attributed to differences in the length of the E2 treatment, application of different microarray platforms, and different analysis strategies (14). Two studies that aimed to identify E2/ERα direct targets by short-term E2 treatment (3 h) in

---

**FIGURE 1. Genomic organization of the human ERβ gene, protein, and functional domains.** For the gene, exons are indicated with boxes and introns with lines. 0k, exon 0k; 0N, exon 0N. The numbers above each box indicate the size of the exons in base pairs; the numbers below each line designate the size of the respective introns in base pairs. The diagonal lines between the gene and protein point to protein domain junctions. For the protein, numbers indicate the total size of the protein in amino acids (aa). The dark purple bar shows the divergent C-terminal regions between the isoforms.

**FIGURE 2. Four different pathways of ER action.** Upper left, in the classical signaling, ER dimers directly bind to EREs following ligand (L) activation. Upper right, upon ligand binding, ERs interact with other transcription factors (TF) such as AP-1 and Sp-1 through a process referred to as transcription factor cross-talk. Lower left, estrogen may elicit effects through nongenomic mechanisms via ERα or GPR30 in the cell membrane, involving interactions with cytoplasmic signal transduction proteins. Lower right, ER activity can be regulated through a ligand-independent pathway in which ERs are phosphorylated by activated kinases.
MCF-7 cells identified similar numbers of E₂ target genes. In one of the studies, 122 genes were identified as stimulated by E₂, and 95 genes were identified as inhibited by E₂ (15). In the other study, 134 genes were up-regulated, and 141 genes were down-regulated after E₂ treatment (16). However, a comparison of E₂-regulated genes between the studies has not been reported. Overall, gene expression profiling and candidate gene analysis have identified several well-known estrogen-regulated genes in breast cancer cells such as TFF1, CCND1, IGFBP4, C3, ADORA1, GREB1, and MYC. Furthermore, gene expression profiling has identified categories of ERα-regulated genes, including those that modulate the cell cycle, transcriptional regulation, morphogenesis, and apoptosis, compatible with a role of estrogen in inducing ERα-expressing breast cancer cell proliferation and survival (17).

With regard to identification of genes regulated by ERβ, gene expression studies have been performed mainly in cancer cell lines stably expressing ERβ either alone or together with ERα. This is due to the lack of immortalized cell lines expressing high levels of endogenous ERβ. The literature is still contradictory regarding which cell lines express endogenous ERβ mRNA and/or protein. For example, although MCF-7 cells are generally considered to be ERβ-negative, some studies reported that MCF-7 cells express endogenous ERβ (18, 19). Thus, the challenge now is to identify cell lines widely accepted to express endogenous ERβ also in the absence of ERα. Studies examining gene expression profiling in ERα-positive breast cancer cell lines stably expressing ERβ have provided insights into the interplay between ERα and ERβ for gene regulation (20–23). In these studies, several common features were observed: (i) ERα and ERβ share some target genes, although each receptor also appears to have distinct sets of downstream target genes; (ii) coexpression of ERβ with ERα significantly impacts the E₂-induced transcriptional response by ERα; and (iii) expression of ERβ inhibits E₂/ERα-induced cell proliferation. These studies have also provided new mechanistic insights into the suppressive effect of ERβ on estrogen-stimulated cell proliferation. For example, Williams et al. (24) reported that, of the categories of genes down-regulated by ERβ, the “regulation of cell proliferation” category was the most overrepresented one. Chang et al. (20) showed that ERβ regulated multiple components of TGFβ signaling, consistent with the observations that TGFβ is normally associated with the suppression of breast cancer cell proliferation. Additional experiments such as time course studies of ERβ-induced gene combinations with examination of ERβ-DNA binding by ChIP will help to identify direct ERβ target genes.

ERβ-specific effects on gene expression have been investigated in three different cell lines lacking expression of endogenous ERα and ERβ, namely U2OS (25), HEK293 (26), and HS578T (23) cells. Of the 76 ERβ-regulated genes, only 17 genes were commonly regulated by both ERα and ERβ, suggesting that the transcriptional effects of E₂ via ERα or ERβ, respectively, are largely distinct in U2OS cells. After a 24-h E₂ treatment, 61 and 95 genes were identified as ERβ-regulated genes in HEK293 and HS578T cells, respectively, as judged by a >2-fold induction in response to ERβ activation. However, there were only three genes (PTGER4, ENPP2, and DKK1) commonly regulated in both HEK293 and HS578T cells, suggesting that ERβ evokes distinct gene responses in different types of target cells. However, some of these discrepancies may be attributed to different expression levels of ERβ and differences in the array designs. Despite the differences, both studies reported inhibition of cell proliferation by ERβ expression independently of ERα, suggesting a similar function of ERβ in different cell types. Further studies are needed, however, to clarify molecular mechanisms by which ERβ elicits inhibitory effects on cell proliferation.

Global Identification of ERβ DNA-binding Regions

ChIP is a powerful method for studying protein-DNA interactions in vivo. In recent years, the development of whole-genome analyses by combining the ChIP assay with high-throughput genomic technologies has enabled researchers to gain new insight into interactions between ERs and regulatory networks contributing to gene regulation. The currently available ChIP-based methods for examining ER binding include ChIP-chip and ChIP-DSL (DNA selection and ligation), based on hybridization, or ChIP-PET and ChIP-Seq, based on HTS. These global studies of ER-DNA binding have revealed that ER-binding sites can be located at a large distance from the proximal promoter region of genes. Based on these findings, an enhancer-promoter looping mechanism has been proposed for transcriptional regulation by ERs (27). Very recently, Fullwood et al. (28) used ChIA-PET (chromatin interaction analysis by paired-end tag sequencing) to demonstrate that most distal ERα-binding sites are anchored at gene promoters through long-range chromatin interactions, suggesting that chromatin interactions constitute an important mechanism for gene regulation.

Although ERα DNA-binding regions have been extensively profiled, ERβ DNA-binding regions have been less well characterized. To date, three studies examined ERβ-binding sites in ERα-positive cells engineered to express ERβ (29–31). All of these studies used the MCF-7 cell line and the ChIP-chip platform. Charn et al. (29) examined the localization of ERα and ERβ DNA-binding regions in MCF-7 cells engineered to express one or both ERs and in response to E₂. They identified a higher number of sites bound by ERα (4405 sites) than by ERβ (1897 sites), but the majority of the ERβ sites (73%) also bound ERα when each of the two ER subtypes was present alone, consistent with a model in which ERα and ERβ can recognize the same ERE motif. However, fewer sites (33%) were shared when both ERs were present, suggesting a competition between the ER subtypes in their selection of DNA-binding sites.

Recently, our group (31) described 1457 high-confidence ERβ-binding sites on a genome-wide scale in MCF-7 cells using the ChIP-chip approach. Interestingly, ~60% of the genomic regions bound by ERβ contained both AP-1-like binding regions and ERα-like sites, suggesting a functional interaction between AP-1 and ERβ signaling. Furthermore, we demonstrated co-occupancy of ERβ and AP-1 on chromatin and that siRNA-mediated knockdown of c-Fos or c-Jun expression decreased ERβ recruitment to chromatin. These results suggest that the AP-1 transcription factor collaborates with ERβ in mediating estrogen responses in breast cancer cells.
MINIREVIEW: Estrogen Receptor β Signaling

ERα and ERβ exhibit distinct as well as overlapping functions at the level of DNA binding. In a study by Liu et al. (30), a high degree of overlap between ERα- and ERβ-binding sites was found. However, the regions bound by ERα had distinct properties in terms of genome landscape, sequence features, and conservation compared with regions that were bound by ERβ. For example, ERβ-bound regions included GC-rich motifs, whereas ERα-bound regions had an overrepresentation of TA-rich motifs, including forkhead-binding sites. Differences in the properties of bound regions might explain some of the differences in gene expression programs and physiological effects exerted by the two ER subtypes.

We compared the ERβ-binding sites described in our study (31) with those described by Charn et al. (29) and found only a limited overlap (170 sites) between the two data sets. The discrepancies can be attributed to many factors, including differences in (i) MCF-7 cell stocks, (ii) the ratio of ERα and ERβ levels, (iii) antibodies, (iv) data analysis protocols, and (v) biological handling of cell lines. In addition, the arrays used by Charn et al. (29) to identify binding regions cover only selected regions of the genome, whereas we have used arrays covering the complete genome. Notably, in the case of the ERα binding data, considerable variation in the number of binding sites and a limited overlap between studies have also been reported (14).

Overall, several common features of ERβ-DNA binding are revealed by these studies: (i) ERβ shares, to a large extent, common binding regions with ERα; (ii) ERβ-binding sites are enriched for ERE-like sites and AP-1 sites; and (iii) ERβ-binding sites are preferentially located at long distances from the proximal promoter region. Importantly, the profiling of ERβ-binding sites in vivo, such as in mouse and mammary tumor tissues that express ERβ, remains to be determined.

Novel Discoveries Pertaining to ERβ

Regulation of EMT—EMT is an essential process for normal development and is implicated in cancer progression to an invasive state (32). A recent article by Mak et al. (33) reported that ERβ can regulate EMT in prostate cancer. Using the PC3 prostate cancer cell line that endogenously expresses both ERα and ERβ, they showed that induction of EMT by treatment with TGFβ or exposure to hypoxia was paralleled by a reduction in ERβ, suggesting that loss of ERβ promotes EMT in prostate cancer cells. Under these conditions, ERα levels were not affected, implying an ERβ-specific function. Loss of ERβ led to increased VEGF-A production, which drove EMT by enhancing nuclear localization of Snail1. The investigators also demonstrated that decreased ERβ expression was correlated with a higher Gleason grading for prostate tumors. It is suggested that ERβ functions as a “gatekeeper” of the epithelial phenotype in the prostate gland. Intriguingly, a protective role of ERβ against the induction of EMT in laryngeal carcinomas was also suggested by studies showing a positive correlation between the expression of ERβ and the maintenance of the EMT marker E-cadherin in the plasma membrane of tumor cells (34). Moreover, a recent study by our group (35) revealed that EMT is involved in the progression of benign prostatic hyperplasia and is associated with high levels of ERβ2. We propose that ERβ2 can suppress expression of ERβ1, leading to EMT. Thus, ERβ may impede EMT in different types of tumors.

Regulation of Cell Growth in Nontransformed Colonocytes—Both clinical and animal studies show that estrogen replacement therapy reduces the risk of colon tumor formation (36, 37). ERβ is the predominant ER in the colonic epithelium (38), suggesting that effects of estrogen in the colon are mediated by ERβ. Additionally, ERβ expression has been shown to be inversely associated with colon tumor incidence (39). Using a nonmalignant cell line originally isolated from young adult mouse colonocytes, Weige et al. (40) showed that E2 treatment reduced cell growth and induced apoptosis. Furthermore, an ERβ-mediated mechanism was shown to be required for colonic cell growth control in mice in that E2-treated ovarietomized wild-type mice exhibited significantly fewer aberrant crypt foci and increased apoptotic activity in colonic epithelia compared with ERβ knock-out mice. This study indicated that E2 treatment protects colonocytes from malignant transformation by increasing apoptotic activity through an ERβ-mediated mechanism, consistent with other findings indicating ERβ to be protective against tumor formation (37).

Protective Role against Atherosclerosis—Atherosclerosis is a complex progressive disease characterized by alterations in endothelial function, smooth muscle cell proliferation, coagulation, and inflammation. Epidemiological and animal studies have suggested that E2 protects against development of atherosclerosis (41). ERα and ERβ are expressed in cells predominantly in vascular tissues such as endothelial cells (42), vascular smooth muscle cells (43), and macrophages (44). Using ERα- and ERβ-null mice, both ERs have been shown to be necessary and sufficient for estrogen-mediated protection against vascular injury (45). In apoE-deficient mice crossed with ERα−/− and ERβ−/− mice, respectively, ERα, but not ERβ, is a major mediator of the atheroprotective effects of E2 (46). In contrast, using wild-type and ERα-deficient mice consuming an atherogenic diet, Villablanca et al. (47) showed that the protection from development of early atherosclerotic lesions is dependent on estrogen but independent of ERα, indicating an ERβ-mediated process. HSP27 (heat shock protein of 27 kDa) prevents many processes associated with the formation of atherosclerotic plaques. Rayner et al. (48) reported that treatment with diarylpropionitrile, an ERβ-selective agonist, induced an extracellular release of HSP27 in macrophages and an increase in serum HSP27 levels in a mouse model of atherosclerosis, suggesting a novel mechanism regulating atherosclerosis specifically through ERβ. Clearly, there is continued need to define the individual contribution of ERα and ERβ in atherosclerosis, the stage of atherosclerosis when estrogen is atheroprotective, and the target genes involved in the atheroprotective effects of estrogen.

Summary and Perspectives

Recent genomic mapping of ERβ DNA-binding chromatin regions and gene expression profiling have provided a global view of estrogen signaling via ERβ. ERβ binds mainly outside of the proximal promoter regions, suggesting that long-range chromatin interactions may constitute an important mecha-
nism for transcriptional regulation of ERβ target genes. It is now evident that other DNA-binding transcription factors such as AP-1 collaborate with ERβ in mediating its actions. Future studies need to correlate ERβ-binding regions with genes regulated by ERβ. To understand how these general patterns of ERβ genomic localization direct physiological responses to ERβ ligands and to what extent location of ERβ determines transcriptional responses remains a great challenge. Importantly, further studies are required to elaborate on the role of ERβ in vivo, including profiling of ERβ-binding sites in mouse and mammalian tumor tissues. We have just begun to recognize newer aspects of ERβ function, for example its roles in EMT and atherosclerosis. Further development of better and specific anti-ERβ antibodies and ERβ-selective agonists will contribute to increasing our knowledge of the molecular basis of ERβ signaling and is likely to uncover hitherto unknown physiological functions of ERβ. Better insights into the physiology and molecular biology of ERβ-mediated signaling may lead to identification of novel targets for effective therapeutic intervention against estrogen-related diseases.

REFERENCES

1. Marino, M., Galluzzo, P., and Ascenzi, P. (2006) Curr. Genomics 7, 497–508
2. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 5925–5930
3. Derou, B. J., and Buensuceso, A. V. (2010) Mol. Endocrinol. 24, 1703–1714
4. Zhao, C., Dahlman-Wright, K., and Gustafsson, J. A. (2008) Nucl. Recept. Signal. 6, e003
5. Gronemeyer, H., and Laude, V. (1995) Protein Profile 2, 1173–1308
6. Ogawa, S., Inoue, S., Watanabe, T., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998) Nucleic Acids Res. 26, 3505–3512
7. Zhao, C., Matthews, J., Tuigaque, M., Wan, J., Ström, Å., Toresson, G., Lam, E. W., Cheng, G., Gustafsson, J. A., and Dahlman-Wright, K. (2007) Cancer Res. 67, 3955–3962
8. Fujimura, T., Takahashi, S., Urano, T., Ogawa, S., Ouchi, Y., Kitamura, K., Clark, A. G., and Kraus, W. L. (2007) Annu. Rev. Physiol. 191–218
9. Carson, A. C., Tenwolde, A., Lee, M., Huck, M., Mumenthaler, S., Andricopoulos, P., Varakis, I., Sotiropoulou-Bonikou, G., and Papavassilopoulos, P. (2009) Mammary Gland Biol. Neoplasia 29, 1063–1068
10. Konstantinopoulos, P. A., Kominea, A., Vandoros, G., Sykiotis, G. P., Andricopoulos, P., Varakis, I., Sotiropoulou-Bonikou, G., and Papavassilopoulos, P. (2009) Eur. J. Cancer 45, 1251–1258
11. Cho, N. L., Javid, S. H., Carothers, A. M., Redston, M., and Bertagnolli, M. M. (2007) Cancer Res. 67, 2366–2372
12. Weige, C. C., Allred, K. F., and Allred, C. D. (2009) Cancer Res. 69, 9118–9124
13. Arnal, J. F., Scarabin, P. Y., Trémolières, F., Laurell, H., and Gourdy, P. (2007) Curr. Opin. Lipidol. 18, 554–560
14. Lindner, V., Kim, S. K., Karas, R. H., Kuiper, G. G., Gustafsson, J. A., and Mendelsohn, M. E. (1998) Circ. Res. 83, 224–229
15. Karas, R. H., Patterson, B. L., and Mendelsohn, M. E. (1994) Circulation 90, 1943–1950
16. Guilshan, S., McCruden, A. B., and Stimson, W. H. (1990) Scand. J. Immunol. 31, 691–697
17. Couse, J. F., and Korach, K. S. (1999) Endocr. Rev. 20, 358–417
18. Hodgkin, J. B., and Maeda, N. (2002) Endocrinology 143, 4495–4501
19. Villablanca, A. C., Tenwelde, A., Lee, M., Huck, M., Mumenthaler, S., and Rutledge, J. C. (2009) J. Cardiovasc. Transl. Res. 2, 289–299
20. Rayner, K., Sun, J., Chen, Y. X., McNulty, M., Simard, T., Zhao, X., Wells, D. I., de Bellerose, J., and O’Brien, E. R. (2009) Arterioscler. Thromb. Vasc. Biol. 29, 1751–1756