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Perspective Article

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# Overview of estrogen action in osteoblasts: Role of the ligand, the receptor, and the co-regulators

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## Introduction

Osteoporosis is believed to be largely due to post-menopausal estrogen deficiency and is associated with an increase in osteoclast (OCL) activity without an adequate compensatory increase in osteoblast (OB) activity. Bone cell metabolism is a complex function involving many agents which mediate the tight coupling between bone resorbing OCLs and bone forming OBs that is necessary for maintaining skeletal mass[1-3]. While the cause(s) of the disease and the uncoupling between the actions of the OB and OCL are not clear, estrogen replacement therapy appears to reinstate the homeostasis between the OB and OCL and prevents bone loss[1-3]. Despite intensive studies, controversies remain as to the exact effects of estrogen on bone cells, including their differentiation, and homeostasis. Estrogens and other sex steroids play important roles in bone cell metabolism, including the regulation of OB and OCL activities and the reinforcement of the coupling between the OB and OCL via the regulation of the production of paracrine factors primarily by the OB[1-3]. Recent studies of the negative side effects of estrogen replacement therapy in females has increased the interest and need for selective estrogen receptor (ER) modulators (SERMs) as hormonal replacement therapies.

Nuclear receptors (NRs) are transcription factors that regulate the expression of target genes in response to steroid hormones and other ligands[4-6]. Their common structure is defined by a series of functional domains[7,8]. Beginning at the N-terminal end, the NRs contain 1) an amino-terminal activation function, termed activation function AF-1 (also termed the A/B domain); 2) a DNA-binding domain (the C domain); 3) a hinge region (the D domain); 4) a carboxy-terminal ligand-binding domain (LBD) containing a second activation function, AF-2 (the E domain); and 5) a carboxy end-heterogenous domain (the F domain) of undefined function. The classical view of ER-dependent transcriptional regulation involves direct ER binding to gene promoters via specific estrogen responsive elements (e.g., ERE) or through association with other transcription factors (AP-1, SP-1, etc.). Subsequently, ERs form a complex with co-regulators, either co-activators which open the histone-DNA complexes and connect with the transcriptional machinery, or corepressors, which reverse the chromatin remodeling and block the connection to the basal transcription machinery[4-6]. The tissue specific distribution of the NRs and their co-regulators determines the tissue specific responses to 17β-estradiol (E2) and SERMs. The non-classical view of ER regulation of gene transcription uses cell membrane bound "receptor-like proteins" and other signaling pathways to induce specific biological responses. [For a review see Monroe and Spelsberg[9]].

## ER isoform function

A second molecular species (isoform) of ER, termed ERβ, has been discovered in humans and other vertebrates that is transcribed from a distinct gene. ERβ has partial homology to the extensively studied ERα[10-12]. Both receptors consist of the five functional domains (described above) which are universally found in members of the nuclear hormone receptor superfamily[13]. Both ERα and ERβ bind E2 with similar affinity and both share a similar DNA binding domain; however, their tissue distribution, molecular size, and affinities for agonists and antagonists differ significantly[14]. It has been demonstrated in transient transfection assays that ERα activates, whereas ERβ inhibits, transcription at AP-1 regulatory elements. These responses are critically dependent on the ligand (E2 or SERM) bound to the ER[15]. The differential tissue distribution of the two ER isoforms could explain part of the differential effects of E2 and SERMs, such as tamoxifen or

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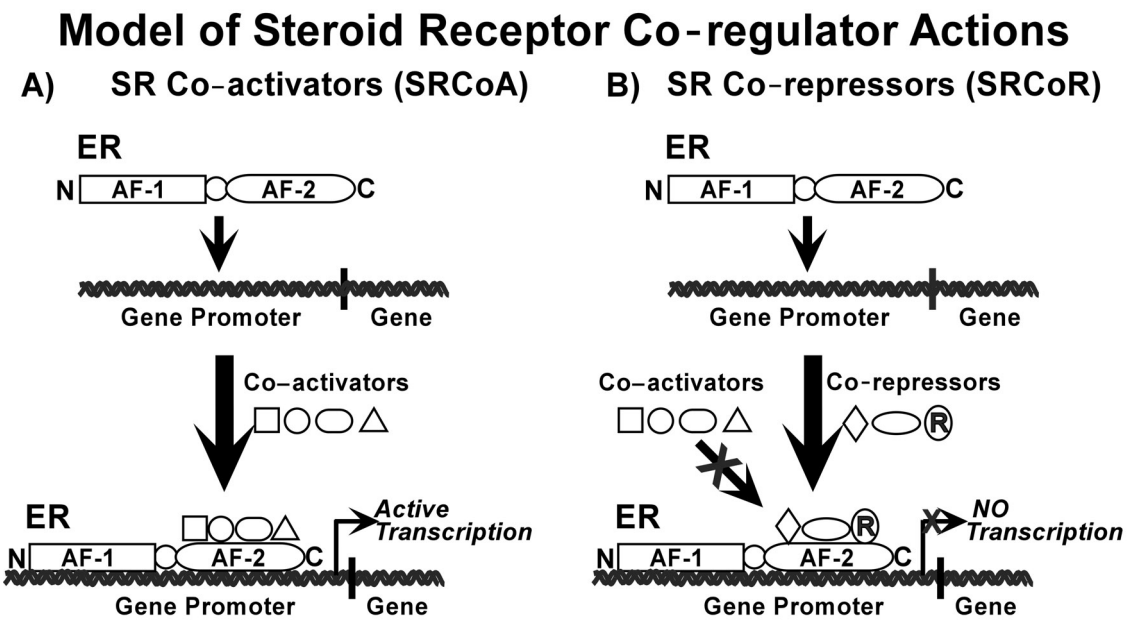


**Figure 1.** Schematic diagram of how transcriptional co-regulators are involved in estrogen receptor (ER)-dependent signaling. A) Steroid receptor co-activators (SRCoA) associate with the receptor following ligand binding and can activate transcription through recruitment of the basal transcriptional machinery. B) Steroid receptor co-repressors (SRCoR) can associate with the receptor molecule when bound with antagonist or in the absence of hormone. It is thought that the co-repressors compete with co-activators for binding the receptor molecule, which inhibits the recruitment of the basal transcriptional machinery and thus inhibits transcription.

raloxifene, in bone versus reproductive tissues. Recent *in situ* studies demonstrated that both ERα and ERβ are expressed in human OB cells, and that the two isoforms display different levels during OB differentiation, i.e., ERβ concentrations increase almost 10-fold with the ERα increasing only slightly during OB cell differentiation into mature human OB cells[16]. In contrast, Onoe et al.[17] recently reported that the erα levels in rat OB are very low in undifferentiated OB but significantly increase as the cells differentiate. ERβ remained high at all stages of differentiation and was higher in cancellous bone than in cortical bone. erα and ERβ are capable of forming heterodimer complexes both *in vitro* and *in vivo* with some evidence that ERβ inhibits ERα activity. Currently, there remain many unknowns in the ER isoform actions, including the effects of changing ratios of these receptors on the pattern of gene expression.

## The nuclear steroid receptor co-regulators

**General Properties:** NR co-regulators can be broadly defined as cellular factors recruited by NRs that complement their function as mediators of the cellular response to endocrine signals. They are generally divisible into co-regulators that promote transcriptional activation when recruited (co-activators), and those that attenuate promoter activity (co-repressors) (for reviews see references[18-21]). Co-regulatory complexes are differentially utilized in both a cell- and promoter-specific fashion to activate or repress gene transcription. The recruitment of co-regulators to nuclear receptors requires ligand-dependent allosteric alterations in the AF-2/LBD domains of the receptor. These co-regulatory components are also targets of diverse intracellular signaling pathways (i.e., phosphorylation, methylation) and may provide the mechanism for tissue- and gene-specific responses to the ligand.

**The Nuclear Receptor Co-activators:** Co-activators are generally defined as proteins that can interact with activated nuclear receptors and enhance their transcriptional function through recruitment of general co-activators (i.e., CBP/p300). Some of these co-activators contain histone acetyltransferase (HAT) activity that serve to loosen the histone's "grip" on DNA to allow efficient transcription[18]. The interaction of activated NRs with some of these Co-activators occur through a centrally located NR box in the co-activator molecule, comprised of an α-helical LXXLL motif[20,22]. Crystallographic evidence has demonstrated that a ligand-dependent shift in the position of several critical helices in the LBD of the receptor, especially helix 12, creates a thermodynamically secure nonpolar groove for binding the co-activator[23,24]. It is these conformational changes in the LBD that is responsible for recruitment of at least some of the primary co-activator proteins that are required for transcriptional activation by the receptor[19,21]. The p160 family of co-activators include: 1) SRC-1 (also called NCoA-1), 2) SRC-2 (also called TIF-2, NCoA-2, or GRIP-1), and 3) SRC-3 (also called p/CIP, AIB-1, TRAM-1, RAC-3, or ACTR)[20,25-28]. All the p160 co-activators contain three NR boxes arranged in tandem which bind to the LBD of the

NR[22,27]. Similar NR boxes have also been identified in other classes of coactivator molecules including p300, CBP, TRAP 220, DRIP 205, PBP, PGC-1, TRBP, ASC-2, and RAP250[29-31].

**The Nuclear Receptor Co-repressors:** The repression by NR co-repressors (i.e., REA, SMART, and N-CoR) in many ways closely mirrors the manner in which co-activators affect transcriptional activation[20]. The recognition of transcriptionally inert NRs by co-repressors is mediated by amphipathic helical peptides called "CoRNR box domains" in the co-repressors[32]. These sequences are similar to the previously characterized NR boxes in co-activators. In addition to their structural similarities, co-repressors functionally mirror co-activators, as some co-repressors contain histone deacetylase (HDAC) activity that deacetylates histones. This deacetylation reinstates the histone-DNA complex to inhibit efficient transcription. Co-repressors that lack intrinsic HDAC activity require the recruitment of factors such as Sin3, which contain HDAC activity, to achieve repression[21]. The LBD region of ER undergoes a marked shift in conformation in an ER-antiestrogen complex which shifts the LBD structure to favor co-repressor binding[20,21,24]. In addition, it appears that the ratio of the co-activators to co-repressors in a cell also determines the ultimate actions of E2 and SERMs (e.g., partial agonists).

## Mechanism of co-activator action

There are two categories of co-regulator actions. These are:

**Chromatin Remodeling (Acetyltransferases):** A critical aspect of gene activation involves nucleosomal remodeling[21,33,34]. Two general classes of chromatin remodeling factors appear in co-regulators and play critical roles in transcriptional activation by nuclear receptors. These are: 1) ATP-dependent nucleosome remodeling complexes, and 2) factors that contain HAT activity.

**Coupling to the Transcriptional Apparatus:** Many, if not all, of the co-activators serve as "couplers" to the nuclear receptors to the transcriptional machinery (binding to factors such as TF-IIB, STATs, AP-1, c-myb, CREB) and to the RNA polymerase complex itself, to complete the necessary requirements for the initiation of transcription[17-21,25]. Co-repressors block this coupling by competing with the co-activators for binding to the receptor. The p160 family and the p300/CBP co-activators thus have NR box domains to bind to the receptors but also contain other domains for binding transcription factors and other proteins of the transcription complex. The fact that many of these co-activators are used by many different signaling pathways besides steroid receptors, has led to their labeling as, "co-integrases" since they serve to integrate the signaling by different pathways.

## Action SERMs and anti-estrogens

The structural analyses of the LBDs of several NRs suggest that the binding of a partial agonist/antagonist ligand results in a realignment of helix 12 to create a hydrophobic groove for co-activator binding[21,35]. The binding of the anti-estrogen antagonists, ICI 182,780 and 4-hydroxytamoxifen (4-OHTam), as well as partial anti-estrogens raloxifene and tamoxifen, leads to a complete/partial misalignment of helix 12 blocking co-activator binding, but often encouraging the co-repressor (REA, SMRT, N-CoR) binding and ultimately inhibit ER-mediated gene transcription[21,24,35-37].

| | SRC1 | SRC2 | SRC3 |
|---|---|---|---|
| Estrogen | | | |
| ERα | ↑ | ↑↑↑↑ | ↔ |
| ERβ | ↑↑↑↑ | ↑↑ | ↔ |
| ERα/β | ↑↑↑↑ | ↑↑ | ↔ |
| | | | |
| 4OH-Tam | | | |
| ERα | ↓↓ | ↓↓ | ↔ |
| ERβ | ↔ | ↔ | ↔ |
| ERα/β | – | – | – |

**Figure 2.** Comparison of the estrogen receptor isoform (α, β, and α/β) transcriptional response to SRC-1, -2, and –3 overexpression in hFOB cells. This schematic diagram describes the activation of an ERE-driven reporter construct by various ER combinations when co-expressed with either SRC-1, -2, or –3 and treated with either 10 nM E2 or 4OH-tamoxifen. The direction of the arrows designates either activation (up arrows) or repression (down arrows) of the construct, and the number of arrows designates the relative magnitude of the response. A sideways arrow designates no response. The E2 data was taken from Monroe et al.[38].

## Recent studies in our laboratory with human osteoblasts

Differential actions of ERα and ERβ isoforms in human osteoblasts: Action of estrogen and SERMs on gene expression by ERα or ERβ in osteoblasts:

Although osteoblasts have been shown to express both isoforms of the estrogen receptor (ERα and ERβ), it is documented that the ratio of the two change during OB differentiation. However, the roles that each isoform plays in osteoblast cell function, differentiation, and in response to SERMs, are unknown. The two ER isoforms are known to differentially regulate transiently transfected estrogen-inducible promoter-reporter gene constructs in a ligand-specific manner, but their individual effects on endogenous gene expression in osteoblasts have not been clearly defined. Minimal information exists on the differential regulation of transcription by ERα and ERβ homo- or hetero-dimers.

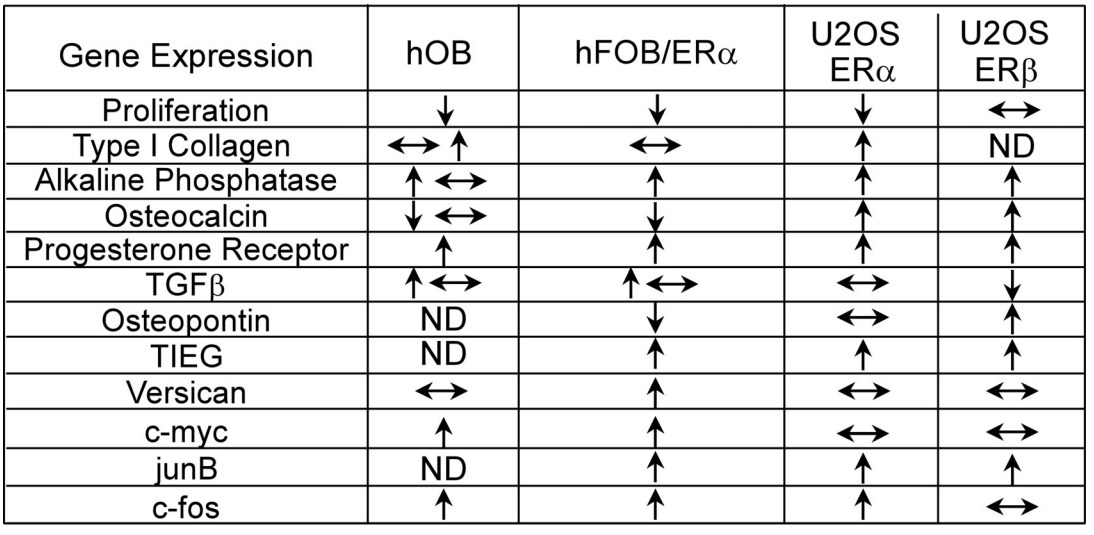


**Figure 3.** Comparison of estrogen (E2)-regulated genes in primary osteoblasts, hFOB/ERα, U2OS-ERα, and U2OS-ERβ cells. This schematic diagram compares the E2-responses of various bone markers primary among primary osteoblasts[2], hFOB/ERα9[39] and the U2OS-ERα[40] and U2OS-ERβ[40] lines. The direction of the arrows designates either upregulation (up arrows) or downregulation (down arrows) of the response (ND = not determined). A sideways arrow designates no response.

We have recently shown that ERα and ERβ co-expression decreases the transcriptional capacity (relative to each ER isoform alone) on an ERE-dependent reporter gene in OBs[38]. This mutual antagonism is not observed in non-osteoblastic cell lines[38]. These data suggest that ERα and ERβ co-expression can differentially influence the degree of transcriptional activation in certain cell types. Interestingly, the overexpression of the steroid hormone receptor co-activator-1 (SRC-1) results in preferential transcriptional enhancement by ERβ as well as co-expressed ERα and ERβ, whereas SRC-2 overexpression appears to preferentially enhance ERα transactivation (Figure 2). SRC-3 overexpression fails to enhance estrogen-dependent transcription of any ER combination in OBs (Figure 2). Subsequent studies have demonstrated that SRC-3 mRNA is low or absent in osteoblastic cells, suggesting that SRC-3 may have only a minor role in these cells. Similar overexpression experiments in COS-7 cells demonstrated preferential enhancement of ERa function with all SRCs, including SRC3. These data suggest that the transactivation capacity of various ER isoforms is both SRC-species- and cell-type-dependent.

We have also compared the effects of E2 and tamoxifen (TAM) on gene expression and matrix formation during the differentiation of normal human osteoblast cell lines, stably expressing either ERα (hFOB/ERα9) or ERβ (hFOB/ERβ6)[39]. The data demonstrate that: 1) in both the hFOB/ER cell lines the transcriptional responses of certain genes to E2 or TAM (including AP, IL-6 and IL-11 production) are more pronounced at the late mineralization stage of differentiation compared to earlier stages; 2) E2 exerts a greater inhibition of bone nodule formation and matrix protein/cytokine production in the ERα cells than in ERβ cells; and 3) the regulated expression of select genes differs between the ERα and ERβ cells. However, these hFOB/ER cell lines were handicapped by our inability to accurately quantitate the ERβ in the OB cells.

Thus, we recently developed and described new stable cell lines, which contain doxycycline (Dox)-inducible ERα and ERβ gene constructs stably introduced in the U2OS human osteosarcoma[40]. These constructs contain FLAG-epitope tags for accurate quantitation of the ER. These lines were shown to contain equivalent numbers of ERα and ERβ receptors (~8000 molecules each). These lines allow the assessment of gene regulation in the absence of the receptor, as well as in the presence of the ER. The regulation of classical OB estrogen-responsive genes in these U2OS-ERα and U2OS-ERβ cells compared to primary OBs and FOB/ERα9 cells is shown in Figure 3.

We then determined the global transcriptional profile of ERα and ERβ regulation of endogenous gene expression[40]. The U2OS-ERα and U2OS-ERβ cell lines were treated with Dox and 24 hours later with either vehicle control or E2 for 24 hours. Gene expression analysis was performed using a microarray containing ~6800 full-length genes. We detected 63 genes that were regulated solely by ERα and 59 genes that were regulated solely by ERβ. Of the ERα-regulated genes, 81% of the genes were upregulated and 19% were inhibited. Similarly 76% of the ERβ-regulated genes were upregulated whereas 24% were inhibited by E2. Surprisingly, only 17 genes were induced by both ERα and ERβ. Real-time PCR was employed to confirm the expression of a selected number of genes. The distinct patterns of

E2-dependent gene regulation in the U2OS cells by ERα and ERβ shown here suggest that during OB differentiation, when either isoform dominates, a unique pattern of gene responses will occur, partially due to the differentiation state and the ER isoform present.

GST-pulldown studies using GST-ERα ligand binding domain fusion protein to analyze osteoblast co-regulator binding. Our laboratory has initiated studies to determine the effect of E2 and various SERMs on the ERα LBD structure and on the resulting association of transcriptional co-regulators (e.g., co-activators, co-repressors) isolated from osteoblast nuclear extracts. A construct containing the glutathione S-transferase (GST) gene fused to the ERα LBD (amino acids 282-595) was provided by Dr. Geoff Greene. The construct was bacterially expressed and the resulting fusion protein purified using Sepharose 4B glutathione agarose beads. An aliquot of the GST-ERα(LBD) beads was prebound with the appropriate ligand (ethanol vehicle, E2, 4-OHTam, genistein, or ICI 182,780) for one hour. Five hundred μg of protein from the nuclear extract prepared from human fetal osteoblast cells (hFOB) was incubated overnight with each of GST-ERα(LBD) fusion protein resins bound with each of the different ligands. The GST-ERα(LBD) beads were extensively washed and subjected to SDS-PAGE. Western blot analysis was performed using an anti-SRC-1 antibody and visualized using enhanced chemiluminescence (ECL).

When the GST-ERα(LBD) fusion protein was treated with vehicle, minimal association of SRC-1 was observed. However, a robust association of SRC-1 with the E2-bound GST-ERα(LBD) was observed. The phytoestrogen genistein, a soy isoflavanoid that exhibits weak E2-like effects on target cells (described above for OB cells), was also capable of inducing the association of SRC-1 with the GST-ERα(LBD) but at a reduced level. The partial agonist/antagonist, 4-OHTam, and the pure antagonist, ICI 182,780, failed to induce the association of SRC-1 with the GST-ERα(LBD). These results have been repeated to validate the consistency and experimental method. Similar studies are planned with the ERβ(LBD).

Since the discovery of ERβ and of the steroid receptor co-regulators, information into their function and importance has been collected from various sources and from tissues such as the breast, uterus and brain. However, the understanding of how these co-regulators function in bone, and more specifically osteoblasts, is just beginning to be appreciated. Future studies into the function of ER and co-regulators will advance our basic understanding of not only these fundamental processes in osteoblasts, but will also aid in treatment of bone diseases such as osteoporosis.

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