Journal of Cellular Biochemistry Supplements 32/33:123–132 (1999)

Molecular and Cellular Mechanisms of Estrogen Action on the Skeleton

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Abstract The many recent and exciting advances that have taken place in the field of estrogen action on the skeleton are the subjects of this review. Leading these new developments is the discovery of alternative estrogen receptors that exhibit differential mechanisms of transcriptional control of estrogen-responsive promoters, thereby broadening both the ranges of possible target cells and their responses. More potentially important genes under estrogenic control have been identified in vitro, and the skeletal phenotypes caused by disruption of estrogen signaling due to mutations in humans and mice have been described. Lastly, clinical studies in humans have revealed a greater appreciation for the importance of estrogen in bone mass maintenance in both sexes. J. Cell. Biochem. Suppls. 32/33:123–132, 1999. © 1999 Wiley-Liss, Inc.

Key words: estrogen action; bone cells; estrogen analogues

Estrogen is the most important sex steroid for maintenance of skeletal homeostasis, as reflected in the widespread and beneficial use of estrogen replacement therapy to combat postmenopausal osteoporosis in women. Estrogen functions by binding to and activating nuclear receptors, which then act as transcription factors that induce or repress target gene expression. Early research into the action of estrogen on bone metabolism was largely focused on identifying indirect effects such as modulating the levels and bone sensitivity to calcitropic hormones. At that time, investigations of indirect effects were driven by the inability to detect estrogen binding sites (receptors) in bone tissue or to measure an anti-resorptive effect in bone organ culture. Subsequent advances in bone tissue and cell culture techniques were principally responsible for the identification of estrogen receptors initially in the bone-forming osteoblast, but later in the bone-resorbing osteoclast, and for the demonstration of reduced bone resorption in estrogen-treated bone cell and organ cultures. These findings led to a complete overhaul of the mechanisms of estrogen action on bone, with an emphasis now on bone cells as direct targets. These concepts are once again undergoing dramatic modifications at all levels— tissue, cellular, and molecular.

Clinical studies have highlighted the importance of estrogen deficiency in not only causing the rapid and transient bone loss that accompanies menopause in women, but also in contributing to the slower, sustained age-related bone loss in elderly women and men [Riggs et al., 1998]. At the cellular and molecular levels, the discovery of a second estrogen receptor (ERβ) with tissue distribution, ligand binding, and transcriptional regulatory properties distinct from the classical ER (ERα), has widened the potential cell and gene targets for estrogen, and has provided new explanations for the tissue/cell type-specific effects of partial estrogen agonists. Finally, the importance of estrogen to skeletal growth and metabolism has been able to be tested in vivo using genetically mutated (knockout) mice specifically deficient in estrogen receptors or estrogen biosynthesis.

MULTIPLE ESTROGEN RECEPTORS

Undeniably one of the most exciting recent advances in the study of estrogen action was the identification of a second estrogen receptor, ERβ, that exhibits a distinct tissue distribution profile to ERα. ERα, and ERβ share a highly conserved DNA binding domain, a moderately conserved ligand binding domain but a diver-

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Received 24 August 1999; Accepted 30 August 1999

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gent N-terminal A/B domain that contains a transactivation activity (AF-1) responsible for interacting with proteins of the core transcriptional machinery [Kuiper and Gustafsson, 1997]. The two isoforms can bind consensus estrogen response elements (ERE) in DNA; at this site, they may act as either homodimers or α/β heterodimers to stimulate transcription. Although they bind many natural estrogens, selective estrogen receptor modulators (SERMs, or partial agonists) and anti-estrogens with comparable affinity, there are some differences, but these are not dissimilar enough to indicate that ERβ uses a unique physiological ligand [Kuiper et al., 1997]. Importantly, estrogen and the partial estrogen agonist tamoxifen, differentially regulate transcription from two types of inducible promoter (containing an ERE or an AP-1 site) depending on the ER isoform expressed [Paech et al., 1997]. Since ERα and ERβ adopt distinct conformations upon binding the various ER modulators it is hypothesized that this will determine the repertoire of protein cofactors interacting with each ligand-activated, DNA-bound receptor and thus participating in the transcription initiation complex [Paige et al., 1999]. The tissue- and cell type-specific effects of SERMs on a given promoter may therefore be explained by the ratio of ERα to ERβ and the particular cofactors present. This mechanism may in reality be even more complex because of the identification in the rat of functional variants of ERβ.

EXPRESSION OF THE ER ISOFORMS IN SKELETAL CELLS IN VIVO AND IN VITRO

ERα and ERβ mRNA and protein have been localized to epiphyseal growth plate chondrocytes in humans and rodents although the relative abundance of the two isoforms in the growth plate is unknown. Detection of estrogen receptors in bone by conventional methods (e.g., immunohistochemistry and in situ hybridization) has proved more difficult requiring methods with greater sensitivity or enrichment and expansion of particular cell types in culture before detection. In bone, ERα transcripts were localized by in situ polymerase chain reaction (PCR) predominantly to trabecular osteoblasts and lining cells with lower levels in osteocytes, osteoclasts, and bone marrow [Hoyland et al., 1997]. The cellular distribution of ERβ in bone is unknown, but higher ERβ mRNA levels have been reported in trabecular compared with cortical bone. The earliest discovery of ER in the skeleton involved human osteoblasts in vitro [Eriksen et al., 1988]. More recent in vitro studies of differentiating osteoblasts showed a progressive increase in expression from very low levels for both ER isoforms with differentiation [Bodine et al., 1998; Arts et al., 1997]. It should be remembered, however, that expression levels measured in cultured cells—especially primary cultures—represent the average for all the cells present and that it is possible that only a subset of cells actually express ER but at a higher level. Confirmation that ERα is expressed and functional in at least some osteoblasts has been demonstrated by the ability to isolate ERα-positive and estrogen responsive cell lines from immortalized human bone-derived cultures [Bodine et al., 1997]. In a comparison of the relative mRNA levels for the two ER isoforms, ERα was the predominant form expressed in both osteoblast precursors (bone marrow stromal cells) and trabecular bone [Lim et al., 1999]. Taken together, these findings suggest that the concentrations of ERα are higher than ERβ in bone and in osteoblasts at all stages of differentiation.

Although osteoclasts possess ERα and respond to estrogen treatment in vitro [Oursler et al., 1991, 1994], it is unknown at this time whether osteoclasts also express ERβ. Furthermore, since a variety of cells in the bone microenvironment (immune cells, marrow adipocytes, and vascular endothelial cells) affect bone cell function, it will be important to determine the relative level of expression of the two ER isoforms in these cells. What factors regulate ERα and ERβ expression and whether their expression is altered in different pathological conditions is also unknown for any cell type associated with bone.

EFFECTS OF ESTROGEN ON SKELETAL GROWTH AND BONE REMODELING

The circulating level of estrogen is a more important determinant of bone mass and bone resorption than either progesterone or testosterone in early postmenopausal women and in elderly men and women [Greendale et al., 1997]. In addition, estrogen, but not testosterone, increased spinal bone density and returned serum markers of bone remodeling (bone formation and resorption) to normal in a man with aromatase deficiency—the enzyme responsible for converting androgens to estrogens [Carani

et al., 1997]. Hence the rapid and transient postmenopausal bone loss (type I osteoporosis) and the slower, age-related bone loss occurring in the elderly (type II osteoporosis) may both be caused by reduced estrogen [Riggs et al., 1998]. Early postmenopausal bone loss affects mostly trabecular bone and is the result of increased bone remodeling where the rate of bone resorption out-paces the rate of bone formation, whereas age-related bone loss is caused by indirect consequences of estrogen deficiency including increased renal calcium excretion, decreased intestinal calcium absorption, elevated secretion of parathyroid hormone and a diminishing bone formation capacity.

The effects of estrogen deficiency on bone growth and remodeling have been examined in the ovariectomized (OVX) rat model for postmenopausal osteopenia [for a review, see Turner et al., 1994]. In the adult skeleton, there is loss of both trabecular and cortical bone due to increased remodeling and increased resorption at the endosteal, but not at the periosteal bone surface, respectively. In growing rats, estrogen deficiency increases both radial and longitudinal bone growth by stimulating periosteal bone formation and delaying ossification of the growth plate. Although the anti-resorptive effect of estrogen is well recognized, it remains controversial whether estrogen directly stimulates bone formation or if effects on formation occur secondary to the effects on resorption as a result of the normally tight coupling between bone formation and resorption. In the OVX rodent model of osteopenia, most studies have shown reduced bone turnover (i.e., a reduction in both formation and resorption) after estrogen treatment, but some evidence suggests that resorption is inhibited to a greater extent than formation at both pharmacological and physiological estrogen concentrations [Turner et al., 1994]. By analyzing markers of bone formation within hours of estrogen treatment of OVX rats, possible inhibitory effects on osteoblast metabolism have been measured that cannot be explained as occurring secondary to decreased bone resorption [Turner et al., 1999]. However, it is difficult to reconcile the inhibitory effects of estrogen on bone formation in vivo with the response to estrogen of osteoblasts cultured in vitro in which the production of particular bone matrix proteins and growth factors are stimulated (see below and Table I).

TABLE I. Effects of Estrogen Treatment on Bone Cells Cultured In Vitro*

Cell type	Parameter
Primary osteoblast	
Rodent	↕ proliferation; ↑ Col I; ↑ IGF-1; ↓ OC; ↑ IGFBP-3; ↓ IGFBP-2; ↓ PGE
Human	↔ proliferation; ↑ Col I; ↑ PR; ↔ OC; ↔ AP; ↑ fos/jun; ↑ TGF-β; ↔ IL-1; ↔↓ IL-6; ↓ TNF
Immortalized osteoblast/ osteosarcoma	↓ proliferation; ↑ Col I; ↑ PR; ↔ OC; ↑ AP; ↑ fos/ jun; ↔↑ TGF-β; ↓ IL-6; ↓ PGE
Marrow stromal cell	↔ proliferation; ↔ AP; ↔↓ IL-6
ER-transfected osteoblast	↔↓ proliferation; ↔ Col I; ↔↑ PR; ↔↓ OC; ↕ AP; ↔↑ TGF-β; ↑ BMP-6; ↑ IGFBP-4; ↑ OPG
Osteoclast (human/ avian)	↓ resorption; ↓ acid; ↑ fos/jun; ↓ TRAP; ↓ lysozyme; ↓ cath B/D/L; ↑ apoptosis

Col I, type I collagen; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; OC, osteocalcin; AP, alkaline phosphatase; PR, progesterone receptor; IL, interleukin; TGF, transforming growth factor; BMP, bone morphogenetic protein; PGE, prostaglandin E_2; OPG, osteoprotegerin; acid, production of acid; TRAP, tartrate-resistant acid phosphatase; cath, cathepsin; ER, estrogen receptor.
*Parameters have been demonstrated to be increased (↑), decreased (↓), or unaffected (↔), or show conflicting reports (↕).

SKELETAL CONSEQUENCES OF PARTIAL ESTROGEN RESISTANCE AND ESTROGEN DEFICIENCY

The detection of estrogen receptors in growth plate chondrocytes and osteogenic cells of trabecular and cortical bone is consistent with the occurrence of abnormalities at these sites in man and laboratory animals with mutations either in the gene encoding ERα (causing partial estrogen resistance) or aromatase P-450 (causing complete estrogen deficiency). Males with either an aromatase deficiency [Carani et al., 1997] or an inactivating ERα gene mutation [Smith et al., 1994], exhibit delayed ossification of the growth plate, resulting in continued linear bone growth. Additionally, there is marked osteopenia of the lumbar spine. Similarly, growth plates fail to close, and trabecular bone

density is slightly reduced in knockout mice possessing a null mutation of the ERα gene [Korach et al., 1996]. Increased longitudinal bone growth also resulting from delayed epiphyseal cartilage closure has recently been reported in ERβ null mutant mice, indicating that both ERα and ERβ regulate chondrocyte maturation and mediate similar responses [Windahl et al., 1999]. In contrast to the ERα mutant mouse, trabecular bone density was unaffected by deletion of the ERβ gene, whereas cortical bone density was increased but only in post-pubertal female mice, thereby demonstrating greater importance for ERα in trabecular bone remodeling and pointing to a role for ERβ in establishing gender differences in radial bone growth. Since prenatal skeletal development is normal in both ER mutant mice, this suggests that either estrogen is dispensable for bone and cartilage formation or that either receptor alone is sufficient. Unfortunately the skeletal effects of a complete loss of estrogen action in mice, generated by knockout of the aromatase gene and possibly by ERα/β double gene knockout, have not been reported. Also not demonstrated is whether osteoblast and osteoclast development from their respective progenitor cells is perturbed in the ER mutants. Major goals of future research will be to determine whether ERα and ERβ are co-expressed and active or expressed at different stages of differentiation for each cell type in bone and subsequently to examine if ERα homodimers elicit different estrogen responses to ERβ homodimers or ERα/β heterodimers.

IN VITRO EFFECTS OF ESTROGEN ON OSTEOBLASTS AND OSTEOCLASTS

A variety of cell culture systems have been employed to determine the cellular targets for estrogen. There is extensive evidence that estrogen directly modulates osteoblast activity and indirectly regulates osteoclast formation and activity via the production of osteoblast-derived soluble and cell surface-associated cytokines [Spelsberg et al., 1999]. However, osteoclasts have also been shown to be immediate estrogen targets, and the possibility remains that a crosstalk ("coupling") exists between osteoblasts and osteoclasts in both directions. Furthermore, cellular interactions between the progenitors of these two cell types— mesenchymal bone marrow stromal cells and hematopoietic monocyte/macrophage lineage cells—are critical for osteoclast development, thus representing coupling at the progenitor cell level. The progenitors may also be important estrogen targets.

Despite exhaustive attempts, it has proved difficult to demonstrate consistent effects of estrogen on primary, immortalized and transformed osteoblastic cells, particularly those of human origin (Table I). This can be explained in part by the generally low and variable levels of ER in osteoblasts that may further diminish on culture, and by differences in cell source, methods of isolation, and culture. The most consistently observed estrogen responses are increased type I collagen and transforming growth factor-β (TGFβ) production, with effects on several bone-resorbing cytokines, including interleukin 6 (IL-6), IL-1, and tumor necrosis factor (TNF), varying with the particular culture system used. Since the expression level of endogenous ER in osteoblasts is limiting for estrogen responses [Ernst et al., 1991], osteoblast lines expressing artificially high levels of the receptor have been generated by stable transfection of the ERα gene. In these cell lines, the estrogen response is quantitatively and qualitatively dependent on the ER level, with a wider spectrum of responses produced by cells expressing the higher levels. Such cells have been valuable in identifying potential new estrogen regulated genes and the molecular mechanisms underlying the regulation. For example, two genes important to bone biology and recently shown to be induced by estrogen in ER-transfected osteoblasts are BMP-6 (a TGFβ-related, bone-inductive, and differentiation factor expressed by hypertrophic growth plate chondrocytes) and osteoprotegerin (OPG, a newly identified member of the TNF receptor family that potently inhibits bone resorption by neutralizing the osteoclastogenic factor OPG ligand) [Simonet et al., 1997; Hofbauer et al., 1999]. However, caution must be exercised when extrapolating the responses observed in ER transfected cells to bone physiology because various cell types expressing exogenous ER above their normal level have frequently shown paradoxical responses to estrogen and atypical gene activation [Levenson and Jordan, 1994). These spurious effects are believed to be due to the sequestering of common transcription factors by the exogenous receptor proteins. However, our and other laboratories have shown

that immortalized human fetal osteoblast cell lines stably transfected with ERα elicit many of the responses to estrogen reported for osteoblasts with endogenous ER [Harris et al., 1995; Robinson et al., 1997; Bodine et al., 1997].

Compared with osteoblasts, estrogen effects on osteoclasts have been much less intensively investigated, due mostly to their relative scarcity in bone and greater difficulty in obtaining sufficient numbers of viable cells for experimentation. Estrogen responses and ERα have been identified in isolated avian and human osteoclasts (Table I). Inhibition of the synthesis and/or secretion of matrix-degrading lysosomal enzymes may in part underlie the anti-resorptive action of estrogen [Oursler et al., 1994]. Estrogen may also inhibit resorption by inducing osteoclast apoptosis, an effect mediated by TGFβ, which in turn could be osteoclast or osteoblast-derived, and therefore constitute an autocrine or a paracrine effect on the osteoclast [Hughes et al., 1996]. As for osteoblasts, osteoclast responsiveness to estrogen has been shown to be correlated with the level of ERα.

It is uncertain whether osteoprogenitor cells including bone marrow stromal cells are targets of estrogen action but, since ERα and ERβ expression appears to increase during the course of osteoblast differentiation, the progenitor would be expected to exhibit a low level of ER. Only a few modest effects of estrogen on marrow stromal cell cultures have been reported, most notably an inhibition of IL-6 production in cells of murine but not of human origin. Osteoblasts and adipocytes are derived from a common mesenchymal progenitor, and thus estrogen could determine of the numbers of uncommitted progenitors available for osteogenic differentiation by actually regulating the opposing pathway of adipocytic lineage commitment. Commitment to the adipocyte lineage involves activation of lineage-restricted genes by the "master" regulatory transcription factors PPARγ (an eicosanoid-activated nuclear hormone receptor) and CCAAT/enhancer binding proteins (C/EBP)-α and β. Since ER can regulate gene expression by interacting with other transcription factors, including C/EBPs, it is tempting to speculate that estrogen may control adipogenesis—and, indirectly, osteogenesis—by modulating the activity of C/EBP or PPARγ, or both.

ESTROGEN REGULATION OF BONE-RESORPTIVE CYTOKINE PRODUCTION BY OSTEOBLASTS

Perhaps the best understood mechanism of estrogen action on bone is the regulation of bone-resorptive cytokine synthesis by cells of the osteogenic and monocyte/macrophage lineages. Estrogen has been reported to inhibit synthesis of IL-1, IL-6, IL-11, TNF, macrophage-colony-stimulating factor (M-CSF), and granulocyte/macrophage colony-stimulating factor (GM-CSF) in one or both of these cells types, but the debate is ongoing as to which is the dominant cytokine(s) regulated by estrogen and thus responsible for the increased bone resorption in estrogen-deficient conditions [for a review, see Spelsberg et al., 1999]. According to one hypothesis, loss of the estrogen-mediated inhibitory effect on osteoblastic IL-6 synthesis causes stimulation of an early stage in osteoclastogenesis and, by an unknown mechanism, osteoblastogenesis, resulting in elevated bone resorption and formation characteristic of postmenopausal bone loss [Manolagas and Jilka, 1995]. An alternative but related hypothesis claims that increased synthesis of IL-1 and TNF by marrow monocytes and macrophages is primarily responsible for the elevated bone resorption in estrogen deficiency. IL-1 and TNF then act synergistically to stimulate the release of M-CSF, GM-CSF, and IL-6 by hematopoietic and/or osteoblastic cells. Importantly, M-CSF is indispensable for osteoclast formation. In support of this theory, simultaneous blockade of both IL-1 and TNF activity by an IL-1 receptor antagonist and the inhibitory TNF binding protein (TNFbp) completely prevents OVX-induced bone loss in rats [Kimble et al., 1995]. However, these two mechanisms are not mutually exclusive because IL-1 and TNF separately and synergistically induce IL-6 secretion by many cell types; therefore, all three cytokines may be involved in the cascade of events culminating in elevated bone resorption.

As mentioned above, OPG is produced by osteoblasts and is a major inhibitor of osteoclastogenesis. If the estrogen stimulation of OPG synthesis observed in ER-transfected osteoblasts [Hofbauer et al., 1999] is also found to occur in vivo, then inhibition of bone resorption by estrogen may in large part be mediated by a down-regulation of the two cytokines known to

be essential for osteoclast formation; M-CSF and OPG ligand.

TRANSCRIPTIONAL CONTROL OF CYTOKINE PROMOTERS BY ER

In the classical model of ER modulation of promoter activity, the ligand activated receptor dimer binds to an ERE comprising two inverted hexanucleotide repeats in the gene regulatory region, resulting in activation or repression of transcription. However, several estrogen-regulated genes have since been identified whose promoters lack ERE-like sites and for which binding of the receptor to DNA is not required for transcriptional control. Some of the best-characterized alternative mechanisms of transcriptional control by estrogen are represented by cytokine gene promoters. The activity of these promoters is dependent upon the occupation of DNA regulatory sites by their respective transcription factor(s), the binding of which is enhanced or inhibited via protein-protein interactions with the ligand-activated ER. In the insulin-like growth factor (IGF)-1 promoter, estrogen stimulates transcription because interaction between the estrogen-ERα complex and fos/jun heterodimers enhances binding of fos/jun to an AP-1 site [Umayahara et al., 1994]. By contrast, estrogen suppresses IL-6 promoter activity because the interaction between ligand activated ERα and the transcription factors NF-κB and C/EBPβ prevents their binding to a C/EBP-NF-κB motif in the IL-6 promoter [Stein and Yang, 1995]. In both instances, transcriptional regulation by ERα is independent of receptor binding to DNA, but the receptor's DNA binding domain is still necessary for the interaction.

Repression of the M-CSF promoter by estrogen appears to occur by a more indirect mechanism whereby estrogenic control of Egr-1 phosphorylation determines the ability of Egr-1 to bind the transcriptional activator Sp-1 and hence achieve promoter activation through Sp-1 sites [Srivastava et al., 1998]. It will now be important to establish whether ERβ homodimers and ERα/β heterodimers differentially regulate these promoters compared with ERα. Of note, several members of the nuclear receptor transcription factor family including the vitamin D, thyroid hormone, androgen, and retinoic acid receptors, interact with fos/jun proteins and AP-1 sites to modulate promoter activity. Competition between the different receptors for fos/jun and hence binding to AP-1 sites may be one mechanism for the functional integration of different receptors on the activity of a given promoter [Uht et al., 1997].

CO-REGULATORY FACTORS FOR ESTROGEN RECEPTORS

The transactivating activity of the ER, as for other nuclear hormone receptors, is regulated by numerous co-activator and co-repressor proteins that enhance or suppress estrogen-induced gene expression [Shibata et al., 1997]. Whereas few if any of the cofactors are receptor-specific, the interaction of these cofactors with ERα and ERβ is governed by the conformation of the receptor's ligand binding domain, and consequently whether the ER is unliganded or complexed with agonist, antagonist, or a mixed agonist/antagonist such as tamoxifen and raloxifene. Co-activators bind to agonist-ER complexes in a ligand-dependent manner and are believed to enhance the AF-2 transactivation activity of ER by acting as a bridge between the ER and the basal transcriptional machinery and stabilizing the pre-initiation complex. In addition, some co-activators are histone acetylases and promote transcription by modifying chromatin structure. Co-repressors bind either unliganded receptor (for thyroid, retinoid, and vitamin D receptors), or in the case of ER, to an antiestrogen-ER complex. Such receptor-co-repressor interactions destabilize the binding of co-activators, generate a nonproductive transcription initiation complex and decrease gene expression. Importantly, ER bound to mixed agonists/antagonists adopt "intermediate" conformations, allowing interaction with both co-activators and co-repressors, and consequently the relative level of co-activator to co-repressor within a cell can determine whether an estrogen-responsive promoter is upregulated or downregulated [Smith et al., 1997]. Therefore the agonist behavior of tamoxifen and raloxifene on bone may be explained by the particular ratios of ERα to ERβ and co-activators to co-repressors that exist in the various target cells.

NONGENOMIC EFFECTS OF ESTROGEN

Nuclear hormone receptors including ERα and ERβ mediate their effects by regulating gene transcription, and consequently there is a delay of several minutes to hours to days between the administration of hormone and the response. For many years it has been known

that many steroids can produce responses in a variety of cells and tissues that are too rapid (from milliseconds to minutes) to be explained by a genomic mechanism. The characteristics of such nongenomic signaling pathways are slowly coming to light, and typically they involve rapid and transient changes in ion fluxes across the plasma membrane. For example, in neurons estrogen modulates glutamate channel activity thereby changing membrane potential and nerve cell excitability, in osteoblasts estrogen stimulates calcium ion influx and phosphatidyl inositol bisphosphate metabolism [Lieberherr et al., 1993], and in osteoclasts and osteoclast-derived plasma membrane vesicles, estrogen antagonizes PTH and cAMP-stimulated acid production. The nongenomic actions of estrogen require subnanomolar steroid concentrations, exhibit ligand stereospecificity, are unaffected by transcriptional inhibitors and anti-estrogens, and appear to activate intracellular second messengers via G-proteins [Moss et al., 1997]. Most intriguingly, the ineffectiveness of anti-estrogens to block this action, together with the demonstration of plasma membrane-associated, high-affinity estrogen binding sites suggests the existence of a distinct nongenomic estrogen receptor. The physiological significance of nongenomic estrogen effects remains obscure and it will be important to determine whether cross-talk exists between the nongenomic and the ERα/β-mediated genomic actions.

FUTURE DIRECTIONS.

Recent advances in our understanding of the molecular mechanisms of estrogen action have demonstrated a higher level of complexity due to the identification of multiple ER isoforms and the ability of these isoforms to differentially modulate the activities of different estrogen responsive promoters. The differential promoter regulation occurs through interactions between the receptor and other families of transcription factors, enabling additional regulation of ERE-less promoters, and by interaction with co-activators and co-repressors that modify the transactivation activity of the ER. All these processes may widen the spectrum of estrogen responses in target cells. A model simplifying the sequential steps of estrogen action in cells is shown in Figure 1.

With respect to future investigation of estrogen control of bone metabolism, it will be impor-

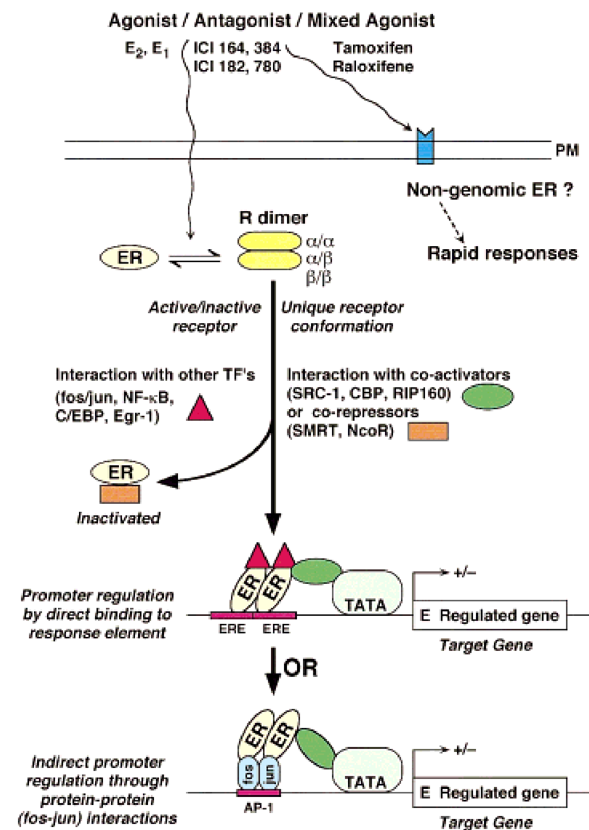

Fig. 1. Model of the sequential steps of estrogen action. Ligand binds to estrogen receptor isoforms (ERα and ERβ) inducing receptor dimerization and conformational changes allowing for protein-protein interactions with co-regulators (co-activators and co-repressors) and other families of transcription factors (e.g., fos/jun, C/EBP, NF-κB). These multi-protein complexes stabilize binding to DNA response elements located within the promoter regions of target genes either enhancing or inhibiting transcription initiation through the formation of productive or nonproductive interactions with the basal transcription machinery. Consequently, estrogen can exert a wide spectrum of effects on promoter activity depending on a combination of variables including the ER isoforms present, the nature of the ligand, the balance between co-activators and co-repressors, and finally, the type of DNA response element. Estrogen also induces rapid and transient nongenomic responses in certain types of cell by binding to a putative plasma membrane receptor.

tant to clarify the expression patterns of the different ER isoforms in osteoblast and osteoclast lineage cells, including their progenitors. It is possible that other cell types that constitute the bone microenvironment, such as vascular endothelial cells and bone marrow adipocytes, also express one or both ER isoforms and represent potential estrogen targets. This is noteworthy because both of these cell types influence bone formation. In embryonic bone formation and fracture repair in adults, the onset of ossification is intimately associated, by

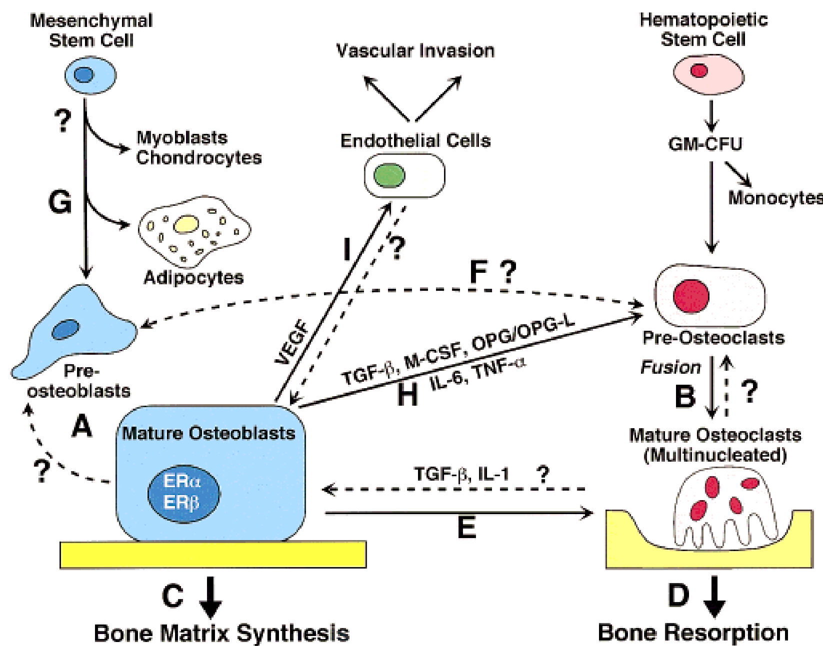

Fig. 2. Known and potential sites of estrogen action within the bone microenvironment. Estrogen may control each of the following processes: proliferation/differentiation of preosteoblasts (**A**) and preosteoclasts (**B**); the activity of mature osteoblasts (**C**) and osteoclasts (**D**); the "coupling" between mature osteoblasts and osteoclasts (**E**) and between preosteoblasts and preosteoclasts (**F**); the lineage commitment of mesenchymal progenitor cells to either osteoblasts and adipocytes (**G**); the production of bone-resorbing cytokines by osteoblasts (**H**) and osteoclast/monocyte/macrophage lineage cells (not shown for clarity); and the production of angiogenic factors and hence vascularization by osteoblasts (**I**). Solid lines, known cellular interactions mediated by cell-cell contact or soluble factors, dashed lines, putative intercellular communications.

means of unknown mechanisms, with vascular invasion, and the fat cell content of bone marrow is inversely related to the extent of new bone formation attributable to osteogenic and adipocytic differentiation from a common progenitor. Estrogen could potentially coordinate the activities of these cell types with bone cells by regulating production and/or responsiveness to cytokines. The range of cell processes and intercellular communications that may be affected by estrogen is depicted in Figure 2.

The isolation and/or generation of bone cell lines derived from laboratory mice harboring inactivating mutations of ERα, ERβ, and ERα and β combined, will be valuable for further defining the relative importance of each ER isoform in the development and function of the osteoblast and osteoclast lineages. Therefore, in a bone cell context it will be possible to address some of the many important questions that remain. For example, (1) which ER isoform mediates the known cellular responses to estrogen?, (2) do ERα homodimers, ERβ homodimers, and ERα/β heterodimers produce similar or distinct effects in the same cell type?, (3) does signaling through each receptor isoform influence the same panel of genes?, and (4) what critical cytokines does estrogen regulate to ultimately affect osteoblastogenesis and osteoclastogenesis, and osteoblast-osteoclast coupling?

Knowledge of the relative level of co-activators and co-repressors present in bone cells may help to rationalize the tissue-specific effects of selective estrogen receptor modulators such as tamoxifen and raloxifene, as well as environmental estrogens which display mixed agonist/antagonist effects. Such information should prove valuable in the identification and generation of more effective bone-sparing estrogen analogues that pose a negligible risk for reproductive cancer.

ACKNOWLEDGMENTS

We thank Dr. Katrina Waters for insightful discussions and advice.

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