

# Estrogen receptors in bone

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The importance of estrogen to bone has been known for several decades, but its precise mechanism of action in skeletal tissue remains elusive. Based largely on corollary evidence from reproductive tissues, a picture has emerged for a general mode of action connecting estrogen to its receptor and ultimately to target genes within the nucleus. With new tools available, including specific antibodies and cDNA probes, the precise location of estrogen receptors has now been elucidated in the developing hard tissues of rats, rabbits, and humans. Cytokines and growth factors may be critical in the mechanisms of action and seem to interplay in a complex network with the receptor and the genes targeted for activation or repression. The discovery of a second estrogen receptor, ER- $\beta$ , adds another dimension to the picture and may shed light on the actions of selective estrogen receptor modulators in skeletal tissue. *Curr Opin Orthop* 1999, 10:361-366

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## Abbreviations

<b>BMD</b>	bone mineral density
<b>ER</b>	estrogen receptor
<b>IL</b>	interleukin
<b>TGF</b>	transforming growth factor
<b>HERKO</b>	human estrogen receptor knockout (of ER- $\alpha$ )
<b>ERKO</b>	(mouse) estrogen receptor knockout (of ER- $\alpha$ )

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Bone is a complex tissue consisting of many cell types, including osteoclasts, osteoblasts, osteocytes, and cells within the marrow, endosteum, and periosteum that are the source of their precursors. Bone tissue maintains its integrity with an intricate balance of bone formation and bone resorption in a process that is sensitive to nutrition, mechanical force, and hormonal status. Numerous hormones influence bone function, including vitamin D<sub>3</sub>, parathyroid hormone, thyroid hormone, calcitonin, the synthetic glucocorticoid dexamethasone, growth hormone, testosterone, and estrogen. This article focuses on the relationship of estrogen and its receptors to bone cell function.

## Estrogen production

Estrogen is made by the conversion of androgen in the gonads, brain, and adipose tissues through the actions of a microsomal P-450 superfamily member known as *aromatase* [1•]. Humans with mutations in the gene show defects in secondary sexual maturation and delayed bone age [2•]. A mouse model for aromatase deficiency has been generated by targeted disruption of the *cyp-19* gene [3]. Human and mouse models, like these with defined defects in the pathway of estrogen production, are critical for further assessment of estrogen action in bone.

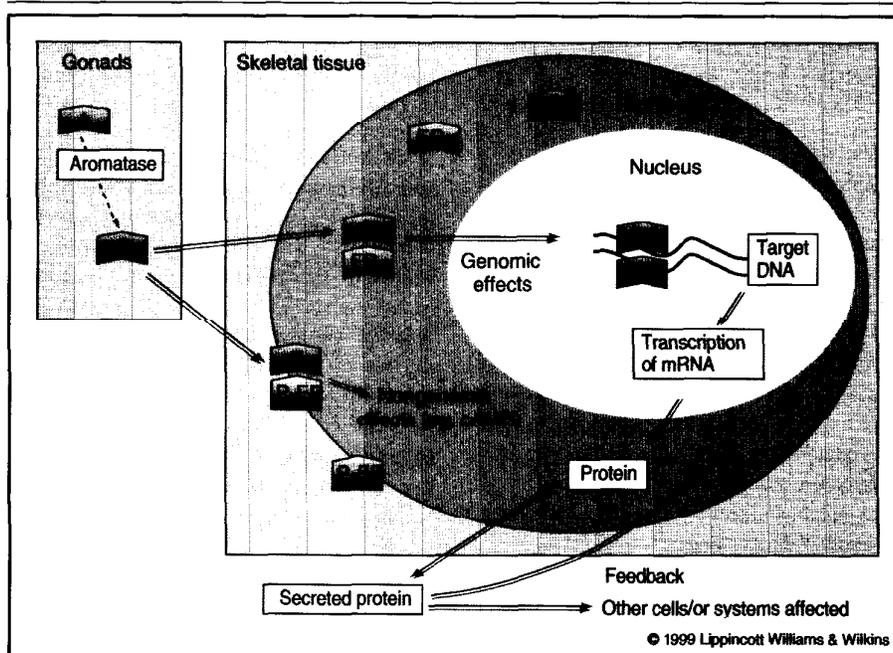
## "Nongenomic" estrogen receptors

After estrogen is produced, it must circulate to its target tissue, where it interacts with receptors found within the cell. A complex of estrogen and its receptor is formed and interacts with target genes and DNA within the nucleus to elicit a tissue response (Fig. 1). Although physicians generally accept that estrogen acts by nuclear receptors, "nongenomic" effects have been reported in human pre-osteoclastic-like cells FLG 29.1 [4]. In this study, binding sites for estrogen were discovered on the plasma membrane at the cell surface that, when activated by the active metabolite of estrogen (17 $\beta$ -estradiol 2) elicited increases in cellular pH, cyclic adenosine monophosphate, cyclic guanine monophosphate, and calcium concentration. Whether other skeletal cell types use similar nongenomic mechanisms in estrogen-signaling events is important to determine.

## Estrogen receptors in skeletal cells acting through the genome

Recent studies that localized the estrogen receptor (ER)  $\alpha$  (ER- $\alpha$ ) mRNA and protein in skeletal tissues indicate that estrogen interaction with "classic" nuclear receptors also occurs in bone. The earliest reports of the presence

Figure 1. Model of estrogen action in bone cells



Bone cells include the osteoclasts, osteoblasts, osteocytes, or precursors thereof. Estrogen binds to a receptor and can act at the plasma through a "nongenomic" pathway or, alternatively, by a "genomic" pathway by binding ER- $\alpha$ , ER- $\beta$ , or both [51], followed by transit to the nucleus. In the latter case, estrogen complexed with the receptor(s) locates a target gene and activates (or represses) its transcription. mRNA encoded by the target gene transits out of the nucleus and is translated into protein in the cytoplasm. The target protein is secreted and will affect other cells or systems in a paracrine manner or feeds back on the producing cell to affect its own activities in an autocrine fashion.

of the ER in human bone were documented independently by two separate groups in 1988 [5,6]. More recently, using a well defined rat model of osteoblast differentiation, ER- $\alpha$  mRNA expression was shown to be developmentally regulated with a 23-fold increase of gene expression coincident with the onset of alkaline phosphatase activity, an early marker of bone cell differentiation [7]. ER- $\alpha$  mRNA and protein also have been found in the cell line TCG-51, which is thought to be of the osteoclast lineage [8] in a pattern that decreased with maturation in culture. Other earlier studies also showed the presence of the receptor in osteoclasts isolated from chickens starved of calcium [9]. In long bones of rabbits and humans, ER- $\alpha$  mRNA was localized to the proliferative and early hypertrophic zone of the growth plate [10,11].

The necessity of the ER for normal skeletal development is shown in a case report of a naturally occurring mutation of the receptor gene, resulting in a loss of ER- $\alpha$  expression. The afflicted patient was a man who had incomplete epiphyseal closure and continued linear growth [12••]. In addition to his tall stature, the patient displayed low bone mineral density (BMD). Taken together, these data point to the concept that estrogen can act directly on cells within the growth plate and on the

osteoblasts and osteoclasts to regulate the growth and maintenance of skeletal tissue.

### Bone cell response to estrogen through the estrogen receptor

The effects of estrogen action on isolated bone cells have been explored using differentiating osteoblast precursors obtained from mouse marrow [13] and mature human trabecular bone immortalized and engineered to express high levels of the ER [14]. In cells derived from mouse marrow, estrogen caused an increase in cell proliferation, with a concomitant increase in the levels of mRNA for type 1 collagen, alkaline phosphatase, and osteocalcin markers of osteogenesis. In a human bone cell line [15] with high levels of ER- $\alpha$ , estrogen treatment caused a decrease in [3H] thymidine incorporation, reflecting a decrease in cell proliferation. Although alkaline phosphatase mRNA and protein levels were increased by estrogen action, other matrix proteins were either decreased (osteocalcin) or unaffected (type 1 collagen) in the human system [14]. Differences in cell or species origin may explain the variability in response to estrogen in these bone cell types.

### Cytokine and growth factor interplay

Although clear positive effects of estrogen on bone formation have been found *in vivo*, only a few modulations

have been found in isolated cells, suggesting that estrogen action is intertwined in a complex interplay with growth factors and cytokines exerting their action on the various cell types in bone. Using cultured fetal rat calvaria cells [16], investigators found that estrogen may inhibit insulin-like growth factor-1 promoter activity by an indirect mechanism involving cyclic adenosine monophosphate. In cultured human bone cells, in contrast, estrogen increased the levels of insulin-like growth factor-1 mRNA production. Differences in estrogen action on insulin-like growth factor-1 in these two systems could be attributed to the age or species of cell origin and further exemplify the complexity of estrogen action in bone-derived cells. The inhibiting effects of estrogen on osteoclastogenesis (or the antiosteoporotic effects of estrogen) by down-regulating interleukin (IL) 6 production is now well documented [17], and decreased sensitivity to this IL may be related to a decreased concentration of its receptor subunits gp80 and gp130 [18].

Another example of growth factor "interplay" involves the active metabolite of vitamin D, 1,24-dihydroxyvitamin D<sub>3</sub>. Investigators have shown that estrogen increased the number and activity of 1,24-dihydroxyvitamin D<sub>3</sub> receptors [19]. Vitamin D, in turn, up-regulated the levels of the ER in marrow derived stromal cells [20]. A major challenge will be to dissect out the intricate patterns of "cross-talk" and feedback among the growing list of cytokines and growth factors as they affect ER action in bone.

### **"Rescue" of estrogen receptor in bone cells with a nonfunctional gene**

In an attempt to clarify the relationship of growth factors to the function of the ER, human bone marrow stromal cells were cultured from a patient with two null alleles for the ER- $\alpha$  gene [12] and were called HERKO (human ER knockout) [21]. The afflicted patient presents with delayed bone age, low BMD, and continued linear growth, and stromal cells from the patient do not make ER- $\alpha$  mRNA or protein (Neal Fedarko, Personal communication). Proof that ERs could be genetically engineered into cells was previously demonstrated [15] by cotransfecting an ER- $\alpha$  gene under the control of the cytomegalovirus promoter, together with an estrogen response element connected to a luciferase reporter. Using this rescue system in HERKO, we showed ER- $\alpha$ -dependent gene activation by estrogen was possible (Fig. 2A) and was also influenced by treatment with transforming growth factor (TGF) B1 (Fig. 2B) [22]. IL-6 production in ER rescued HERKO was also influenced by treatment with TGF-B1 (Fig. 2C). Estrogen was previously shown to increase the expression of TGF-B activity in normal, nonmutated marrow-stromal-derived cells [13]. These data also suggest that feedback regulation involving TGF-B and estrogen may be important in controlling ER function in bone tissue.

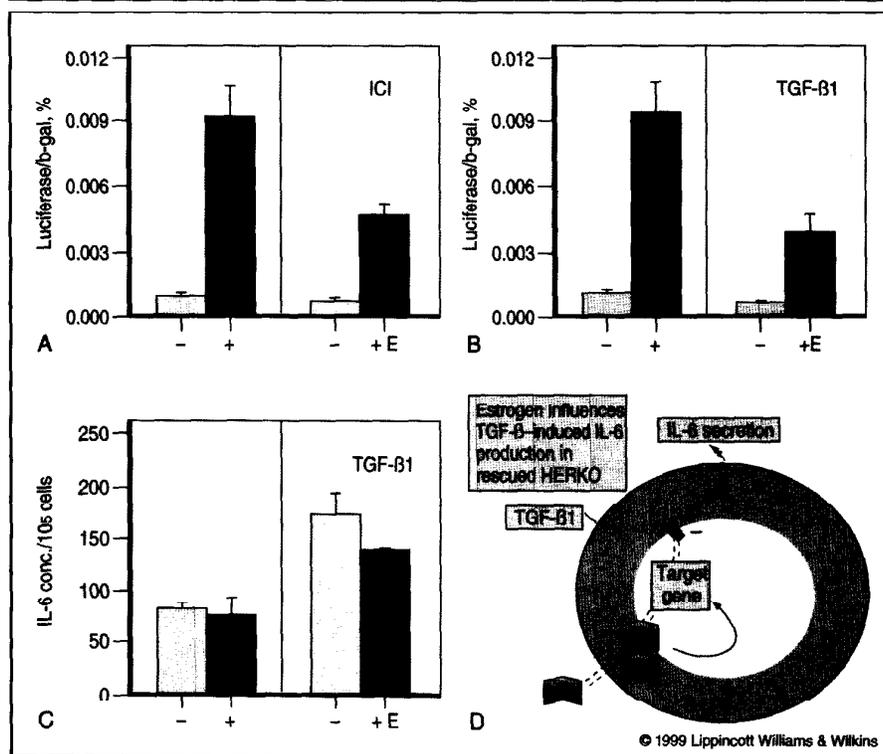
### **A novel estrogen receptor: estrogen receptor- $\beta$**

A novel gene was cloned using degenerative DNA polymerase chain reaction primers that was shown to activate an estrogen response element when transfected into Chinese hamster ovary cells [23]. The new receptor, called ER- $\beta$ , has structural similarities to ER- $\alpha$  and is coexpressed with ER- $\alpha$  in human hypertrophic epiphyseal chondrocytes [24] and in cancellous rat bone [25]. A recent report using murine and human long bone showed the expression of ER- $\beta$  in osteocytes and osteoblasts [26]. Although ER- $\alpha$  and ER- $\beta$  were found in cultured human osteoblast cells, their expression became divergent as the cultures matured *in vitro* [27]. Whether the two receptors share common functions and could "compensate" for each other's activity is unclear. Recently, mice that are unable to express the ER- $\beta$  gene were generated [28]. Previously, mice that are unable to make the ER- $\alpha$  gene (ERKO mice) were bred [29] and, surprisingly, seem to have little or no effect on BMD [30]. By breeding the two ER knockout strains together, investigators can now create "double-knockout" mice unable to make either receptor. Although similar genetic manipulation of genes cannot reasonably be done in humans, one must still consider the differences in bone structure, function, and development between mice and humans. ERKO mice are short, with mild or no effects on BMD, depending on the sex of the animal [30]. The human counterpart of this mutated gene (*ie*, HERKO), on the other hand, is abnormally tall, with continued linear growth and low BMD. Considering the high degree of expression of ER- $\alpha$  in the growth plate, this discrepancy may be caused by the fact that, in contrast to those of humans, mouse growth plates do not fuse. Nevertheless, growing bone cells from singly and doubly mutated animals will be critical for future studies aimed at defining the functional similarities and possible functional compensation of the ERs in skeletal tissue.

Three questions yet to be answered about the ER- $\beta$  function are: 1) Does the receptor form "homo" dimers of itself, or does it complex with ER- $\alpha$  and form heterodimers with ER- $\alpha$  to activate gene expression? 2) Could ER- $\beta$  "scavenge" ER- $\alpha$ , thereby controlling ER- $\alpha$  function? and 3) Does ER- $\beta$  activate genes independent of the ER- $\alpha$  receptor? Some of these hypotheses could be tested directly using cell lines, *eg*, the HERKO, or from skeletal tissues from the ERKO mice that specifically lack ER- $\alpha$  expression.

### **Selective estrogen receptor modulators**

Osteoporosis is a bone defect that is clearly related to estrogen deficiency and leads to fractures [31•]. A common therapy for patients with osteoporosis involves hormone replacement therapy [32•], but high levels of non-compliance have forced consideration of alternative therapies [33•]. Selective ER modulators is a new therapeutic [34•] that is thought to mimic estrogen action in a

**Figure 2. Rescue of estrogen response in human ER- $\alpha$  knockout cells (HERKO)**

**A.** Estrogen response can be "rescued" in HERKO by transfection with the estrogen receptor (ER). Cells were transfected with the ER- $\alpha$  gene along with an estrogen response element connected to a luciferase reporter. ICI 182,780 ( $1 \times 10^{-8}$ ) was added to show the specificity of the estrogen response. ICI, a synthetic compound, is an antiestrogen and is used to show the effects seen as working through the ER. Luciferase activity was used to estimate the rescue response in HERKO cells. +E = in the presence of  $10^{-9}$  M estradiol. ICI = Imperial Chemical Industries. **B.** Rescue with the ER is sensitive to TGF- $\beta$ . Cells engineered as described earlier were also treated with 1 ng/mL TGF- $\beta$ . Luciferase activity was used to estimate the rescue response in HERKO cells. **C.** Rescue with estrogen is sensitive to TGF- $\beta$  at the level of IL-6 production. ER was transfected into HERKO cells as described earlier and IL-6 production measured as described earlier [21]. **D.** Model of the interplay between estrogen, IL-6, and TGF- $\beta$ . The response of these cytokines and growth factors that are dependent on the actions of the estrogen receptor are depicted.

tissue-selective manner [35•]. Specifically, selective ER modulators can act as an agonist in bone to diminish bone loss and as an antagonist in other tissues, *eg*, uterus or breast, where estrogen activity is undesired. Some investigators have reported selective ER modulators action resulting in reduced lipids and lipoproteins, making them a potential "panacea" for women's health care [36•]. Although their efficacy has now been established, how they work it is still unclear. With the discovery of a second ER and other accessory factors that may synergize with the ERs [37,38], new hypotheses can be tested to unravel their mechanisms of action.

#### Further considerations in estrogen receptor action in bone: heterogeneity

The potential for heterogeneity of the ER exists at multiple levels in bone. In the breast and uterus, ER- $\alpha$  is transcribed from multiple promoters [39,40] resulting in multiple mRNA transcripts [40] that are expressed dif-

ferentially in different tissues. Multiple transcripts derived from alternative mRNA splicing have also been reported in bone tissue [41] that show differential transactivation capacity by estrogen. Additional considerations about heterogeneity arise from observations that the ER gene is expressed differentially during the cell cycle [42]. Heterogeneity also exists in the form of restriction fragment length polymorphisms in the ER- $\alpha$  gene; interestingly, a polymorphism an *Xba*I restriction site could be correlated to the accretion of BMD during young adulthood [43]. Finally, phosphorylation of the receptor may be critical to its activation function and dependent on a mitogen-activated protein kinase [44]. Such heterogeneity of ER may explain, for example, the differential responses of the mandibular condyle and femur to estrogen treatment *in vivo* [45]. One could also argue, on the other hand, that heterogeneity may not reside in the receptor but, rather, in other cell components of these two bone types. In either case, much work

still needs to be done to understand how the heterogeneity of the ER and complementary cell factors regulate estrogen action in bone.

### Future directions of estrogen receptor in bone and orthopedics

One of the key issues for the future will be to identify the target gene(s) of estrogen action in bone. In one novel approach, investigators expressed the receptor and looked for fragments of genomic DNA that bound to the receptor. By DNA cloning and sequencing, a new estrogen-responsive gene that contains a "ring finger" motif for DNA binding was identified [46]. In an *in vivo* model of fracture healing, increased ER mRNA was detected in callus tissue [47]. One way to broaden the scope of such experiments would be to use "laser capture" [48]. In this procedure, lasers are used to "capture" minute quantities of tissue so that mRNA can be isolated and characterized from a single cell. Recent experiments using primary cultures of rat long bone indicate that biomechanical strain stimulates mitosis through the actions of the ER [49]. To apply new microchip array technology [50] to assess the repertoire of gene activity that accompanies bone cells undergoing controlled biomechanical stress would be interesting. Similarly, one could further imagine examining either fracture healing or the response to mechanical strain in cells with mutated forms of the ER. Thus, by combining new biologic reagents with new technical tools, investigators will be able to define more "players" in the ER functional cascade. Such new information could, in turn, help in the design of new therapies for patients with acquired bone defects, *eg*, bone fracture or bone loss through estrogen-related deficiencies.

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- Of outstanding interest

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