J Musculoskel Neuron Interact 2001; 2(2):143-151

Review Article

Hylonome

Oestrogen action on bone cells

G.E. Krassas and Ph. Papadopoulou

Department of Endocrinology & Metabolism, Panagia General Hospital, Thessaloniki, Greece

Abstract

Sex steroids have an important impact on bone physiology. Oestrogen (E) appears to be the most important sex steroid in preventing osteoporosis in women. Despite the overwhelming evidence that oestrogens modulate bone growth and turnover *in vivo*, oestrogen receptors (ER) were detected only recently. Two forms of ER have been discovered so far, ERα and ERβ. Both have been detected in osteoblasts and osteoclasts as well. A number of growth factors and cytokines appear to modulate bone resorption *in vitro* and *in vivo*. Among others, interleukin-1 and -6 and tumor necrosis factor alpha and beta were found to be extremely potent stimulators of bone resorption. Binding of different cytokines to their receptors in osteoblasts result in the release of soluble factors that act directly on osteoclasts to modulate their recruitment or activity. Thus, E, apart from the direct regulation of osteoclasts, which it achieves through its receptors, can inhibit the release of osteoclast stimulatory factors or enhance the release of osteoclast inhibitory factors. In general, E is an inhibitor of bone resorption that decreases both osteoclast numbers and activity. Recently, it has also been shown that it promotes apoptosis. Moreover, it also has anabolic effects on osteoblasts. However, E action on osteoclasts is superior in comparison with that on osteoblasts. Recent data have shown that transforming growth factor beta (TGFβ) mediates the actions of E in bone. Following the example of raloxifene it may be proved that the role of TGFβ in the actions of E in bone is central and has not only academic interest. More data are needed to elucidate this issue. Finally, recent data suggest the importance of E for bone maturation and development of peak bone mass in men. It seems likely that both E and androgens are required for the growth and maintenance of the adult male skeleton.

Keywords: Oestrogen, Osteoclasts, Osteoblasts, Oestrogen Receptors, Cytokines, Growth Factors

Bone differs from reproductive tissues in that many aspects of skeletal growth, turnover, and function occur in the absence of gonadal hormones. Nevertheless, sex steroids have an important impact on bone physiology: they participate in the sexual dimorphism of the skeleton, play a role in maintenance of mineral homeostasis during reproduction and are essential for maintaining bone balance in adults. Insufficient levels of certain sex steroids predispose the human skeleton to bone loss and to osteoporotic fractures. Oestrogen appears to be the most important sex steroid in preventing osteoporosis in women[1].

Despite the overwhelming evidence that oestrogens (E) modulate bone growth and turnover *in vivo*, oestrogen receptors (ER) were not initially detected in cultured bone cells[2] and E was not shown to affect bone cells in organ culture[3]. The early negative results led to the long-held belief that the effects of oestrogens on bone *in vivo* were entirely indirect and were mediated by calciotrophic hormones such as calcitonin, parathyroid hormone, and 1, 25 dihydroxyvitamin D3 or by some unidentified humoral factors.

In 1988 Komm et al.[4] and Eriksen et al.[5] simultaneously reported the detection of high-affinity, competable estradiol binding, ERα mRNA, and the induction of E responsive genes in cultured rat and human osteoblast-like cells, the bone-forming cell.

Oursler et al.[6] have since demonstrated ERα and oestrogenic activity in cultured avian osteoclasts, the bone-resorbing cell type, including oestrogen induction of the genes for c-fos and c-jun. Using *in situ* RT-PCR analysis, Hoyland et al.[7] demonstrated the presence of ERα mRNA in both osteoblasts and osteoclasts in bone grafts from human females. Immunocytochemical methods have also been used to demonstrate the presence of ERα in multiple bone cell lines[8]. Therefore, there is adequate experimental evidence to support the presence of a direct ERα mediated oestrogen-signaling pathway in bone.

Until recently, only one "classical" form of the ER was

Corresponding author: G.E. Krassas MD, Associate Professor of Medicine, Chairman, Department of Endocrinology & Metabolism, Panagia General Hospital, 22 N. Plastira, 55132, Thessaloniki, Greece. E-mail: krassas@the.forthnet.gr

Accepted 4 June 2001

known to exist. In 1996, Mosselman et al.[9] discovered a new form of the receptor named ERβ. The latter has been shown to be expressed in the human thymus, spleen, ovary and testis and recently its expression in osteoblasts from rat bone has been described[10]. Both ERα[11] and ERβ[12] have also been detected in human epiphyseal chondrocytes.

Several recent studies have reported the detection of ERβ in bone cells. Onoe et al.[13] demonstrated the presence of both ERα and ERβ mRNA in immortalized as well as primary osteoblast cell cultures from the rat. Similar to reports of ERα, both Onoe et al.[13] and Arts et al.[14] reported significant increases in ERβ mRNA levels during *in vitro* dexamethasone – induced differentiation of osteoblasts derived from the rat and human, respectively. Therefore, the generation of mice lacking ERα or ERβ will once again prove invaluable in delineating the roles of the two receptors in bone physiology.

The majority of animal studies concerning the role of E in bone morphology and metabolism have been carried out in the rat[1]. Ovariectomy in the rodent results in increased bone turnover similar to that seen in postmenopausal women; however, the mechanisms of action may differ between the species[1].

Oestrogens are thought to passively diffuse into all cells. In general, steroids are preferentially retained in target cells through the formation of complexes with specific intracellular receptor proteins that are both steroid- and tissue- specific[15]. It is generally accepted now that both osteoclasts and osteoblasts do express ER[13,14]. Evidence for receptors in bone cells was reported in mice and humans using binding assays[16,17], immunohistochemistry[18,19] and radioautography[20]. The mRNA for ER was reported in rat bone[21], providing additional evidence for receptors. The latter are typically present at a concentration of 10,000 – 100,000 molecules per target cell in reproductive tissues in mammals, but much lower levels, i.e. 200-1000 molecules per cell, have been reported in other tissues of mammals including the skeleton and in tissues of birds and lower vertebrates[22,23]. The low concentration of ER in osteoblasts relative to reproductive tissues is consistent with the hormone having a more limited number of direct actions on the skeleton[1].

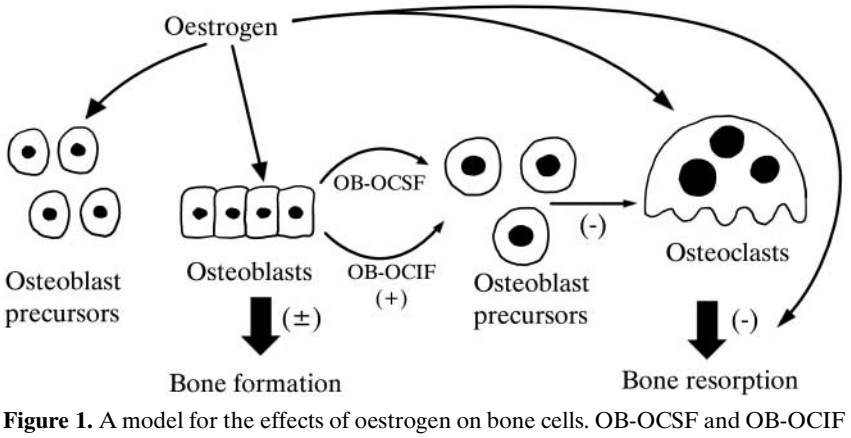

Figure 1. A model for the effects of oestrogen on bone cells. OB-OCSF and OB-OCIF indicate osteoblast-derived osteoclast stimulating and inhibiting factors, respectively[1].

Oestrogens, cytokine production and growth factors

The major effect of oestrogen on bone remodeling in adult humans may be to decrease bone resorption rather than to modulate bone formation. Despite the presence of ER in osteoclasts, it is possible that the binding of E to receptors in osteoblasts regulates osteoclast function indirectly, which would add a second layer of regulation to the direct regulation of osteoclasts by E and provide an opportunity for cell-cell interactions[1]. New studies suggest that many factors regulating bone resorption do so indirectly through an osteoblast – mediated mechanism[24-27].

Binding of different cytokines to their receptors in osteoblasts is hypothesized to result in the release of soluble factors that act directly on osteoclasts to modulate their recruitment or activity. Thus, E could inhibit the release of osteoclast stimulatory factors or, alternatively, could enhance the release of osteoclast inhibitory factors (Fig. 1)[1].

Based on their action in experimental animals, in organ cultures, or in cell cultures, a number of cytokines and growth factors appear to modulate bone resorption and could play roles in the coupling of bone formation to bone resorption. Cytokines that have been reported to increase osteoclastic activity include colony stimulating factor (GM-CSF)[28], macrophage-colong stimulating factor (M-CSF)[29], tumor necrosis factor-a (TNF-a)[30], interleukin-1 (IL-1)[31] and interleukin-6 (IL-6)[32].

In the mid-to-late 1980s the proinflammatory cytokines IL-1 and TNFs alpha and beta were found to be extremely potent stimulators of bone resorption *in vitro* and *in vivo*. Spontaneous release of these cytokines along with IL-6 by peripheral blood mononuclear cells was found to be increased after menopause or ovariectomy[33].

The following key data summarize the role of these cytokines in the pathogenesis of oestrogen-deficiency – related bone loss. First, blockade of IL-6 by infusion of a neutralizing antibody prevents postovariectomy bone loss in mice. Second, IL-6 knock out mice have been reported not to lose bone after ovariectomy. Third, simultaneous treatment of animals with IL-1 receptor antagonist and TNF binding protein prevents postovariectomy bone loss. Finally, the cytokines are enhancers of osteoclast function, IL-6 probably acting mainly at the levels of osteoclast generation and IL-1 and TNFs acting more (but not exclusively) at the level of the mature osteoclast, possibly via autocrine/paracrine signals and possibly by inhibition of apoptosis[33,34].

In several classical oestrogen-responsive tissues, such as the breast and uterus, insulin-like growth factor (IGF) -1 appears to mediate the E induction of cell proliferation and differentiation[35,36]. A number of studies suggest that E has similar

effects on the rodent osteoblastic system, e.g. in rat calvarial osteoblasts, E increases IGF-1 gene transcription[37] and oestrogen-induced increases in osteoblast proliferation are blocked by neutralizing anti-IGF-1 antibodies[38,39]. In contrast, E treatment did not affect gene transcription or protein production of IGF-2 in rat osteoblasts[40]. Thus, the data obtained in rodent models suggest that some of the E effects on bone cells are mediated through the IGF-1. It is not known, however, whether a similar action of E on IGFs occurs in human osteoblasts. This is important to clarify because differences between rodents and humans have been reported in the IGF system in bone[41,42]. Also, in contrast to the findings in rodent osteoblasts, IGF-2, rather than IGF-1, is the main IGF produced by human osteoblasts[41-43]. Whereas IGF-2 seems to be constitutively produced by rodent osteoblasts and is not regulated by osteotropic hormones[40], little is known about the regulation of IGF-2 in human osteoblasts.

In addition to growth factors, bone cells produce proteins that modulate the activity of growth factors either by binding to them and thereby preventing interaction with their receptors, by competing with the same receptors, or by promoting the activity of a particular factor. For example, osteoblasts produce several IGF-binding proteins (IGFBPs). It has been described that IGFBP-4 binds to IGF and blocks its action, whereas IGFBP-5 promotes the stimulatory effects of IGF on osteoblasts[44].

Mechanism of oestrogen action on bone

Osteoblastogenesis and osteoclastogenesis

Both osteoblasts and osteoclasts are derived from precursors originating in the bone marrow. The precursors of osteoblasts are multipotent mesenchymal stem cells, which also give rise to bone marrow stromal cells, chondrocytes, muscle cells, and adipocytes[45,46], whereas the precursors of osteoclasts are hematopoietic cells of the monocyte/macrophage lineage[47,48].

It has been found that even though millions of small packets of bone are constantly remodeled, bone mass is preserved thanks to a remarkably tight balance between the amount of bone resorbed and formed during each cycle of remodeling. In any established basic multicellular unit (BMU), bone resorption and formation are happening at the same time; new osteoblasts assemble only at sites where osteoclasts have recently completed resorption, a phenomenon referred to as coupling, and formation begins to occur while resorption advances. The whole process ends with the formation of a new bone, either a cylindrical osteon or Haversian system, or a plate-like hemiosteon, that has replaced the older bone which was removed[49].

The distinction between the cross-sectional and longitudinal events during BMU progression could be explained on the basis of two models of osteoblast recruitment, a serial and a parallel[50] (Fig. 2).

According to the serial model, factors released from resorbed bone stimulate osteoblast precursor, cell proliferation and differentiation[51,52]. According to the parallel model, osteoblast and osteoclast precursor, cell proliferation and differentiation occur concurrently in response to whatever signal conveys the need for initiation of new BMUs, and whatever hormone prolongs their progression[50,53]. At the end with either model, the new osteoblast must be directed to the right location.

The molecular mechanism of the dependency of osteoclastogenesis on cells of the mesenchymal lineage has been elucidated in recent years with the discovery of three proteins involved in the TNF-signaling pathway. Two of these proteins are membrane-bound cytokine-like molecules: the receptor activator of nuclear factor-κB (NF-κB) (RANK) and the RANK-ligand. RANK is expressed in hematopoietic osteoclast progenitors, while RANK-ligand is expressed in committed preosteoblastic cells and T lymphocytes[54-56]. RANK-ligand binds to RANK with high affinity. This interaction is essential and sufficient for osteoclastogenesis. Osteoprotegerin (OPG), the third of the three proteins, unlike the other two, is a secreted disulfide-linked dimeric glycoprotein. Unlike other members of the TNF receptor family, OPG does not possess a transmembrane domain. OPG has very potent inhibitory effects on osteoclastogenesis and bone resorption *in vitro* and *in vivo*[57]. OPG transgenic mice develop osteopetrosis, whereas OPG knock out mice exhibit severe osteoporosis[58], a finding which is in accord with the important role of OPG in the regulation of osteoclast formation. The antiosteoclastogenic property of OPG is due to its ability to act as a decoy by binding to RANK-ligand and blocking the RANK-ligand/RANK interaction. In addition to skeletal metabolism, the RANK/RANK-ligand/OPG circuit may regulate several other biological systems. Indeed, OPG, is produced by many tissues other than bone, including skin, liver, stomach, intestine, lung,

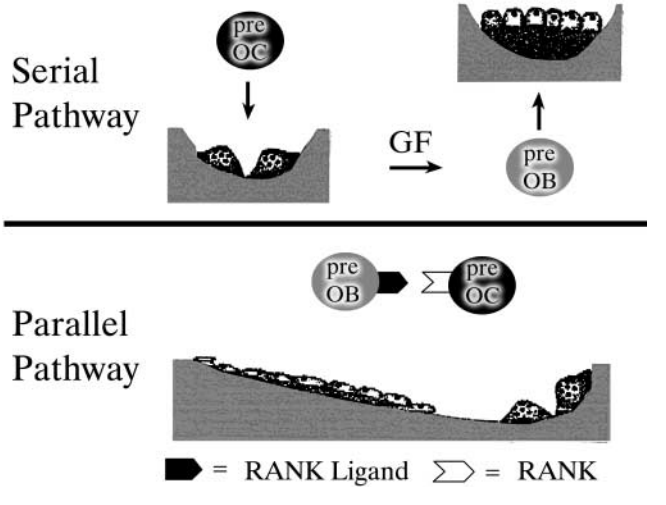

Figure 2. Serial and parallel models of osteoblast and osteoclast development. For an explanation, please see text. PreOC, Preosteoclast; GF, growth factors released from the matrix of resorbed bone; preOB, osteoblast progenitors. The expressions of RANK ligand and RANK on preosteoblasts and preosteoclasts, respectively, are depicted to indicate their critical contribution in osteoclastogenesis and thereby the dependency of osteoclastogenesis on preosteoblastic cells[50].

heart, kidney, and placenta as well as hematopoietic and immune organs. Mice deficient in RANK-ligand completely lacked lymph nodes as well as osteoclasts[59], a finding which supports the latter. Moreover, OPG is also a receptor for the cytotoxic ligand TRAIL (TNF-related apoptosis-inducing ligand) to which it binds with high affinity and inhibits TRAIL-mediated apoptosis in lymphocytes[60] and also regulates antigen presentation and T cell activation[61].

Evidence from *in vivo* studies. Apoptosis

Oestrogen is an inhibitor of bone resorption that decreases both osteoclast numbers and activity. The osteoclast is a fairly short-lived cell that appears to die by apoptosis during the reversal phase of bone remodeling. Thus, the reduction of osteoclast numbers seen *in vivo* could be due simply to reduced osteoclast generation. There is evidence to suggest that E does reduce formation of osteoclasts from their precursors. Moreover, Liu and Howard[62] demonstrated that E treatment caused osteoclasts to lose attachment to the bone surface and to undergo degenerative anatomical change, indicating that E treatment has effects on mature osteoclasts. More recently it has been shown that these morphological changes reflect induction of osteoclast apoptosis by E33.

The average lifespan of human osteoclasts is about 2 weeks, while the average lifespan of osteoblasts is 3 months. After osteoclasts have eroded to a particular distance, either from the central axis in cortical bone or to a particular depth from the surface in cancellous bone, they die and are quickly removed by phagocytes[63]. The majority (65%) of the osteoblasts that originally assembled at the remodeling site also die[64]. The remaining are converted to lining cells that cover quiescent bone surfaces or are emtombed within the mineralized matrix as osteocytes. Both osteoclasts and osteoblasts die by apoptosis or programmed cell death, a process common to several regenerating tissues[65].

Eventually, the cell breaks apart to form so-called apoptotic bodies. Osteoblast apoptosis explains the fact that 50-70% of the osteoblasts initially present at the remodeling site of human bone cannot be accounted for after enumeration of lining cells and osteocytes[66]. Moreover, the frequency of osteoblast apoptosis *in vivo* is such that changes in its timing and extent could have a significant impact on the number of osteoblasts present at the site of bone formation[67]. Osteocytes are long-lived but not immortal cells; some die by apoptosis[68,69]. Osteocyte apoptosis could be of importance to the origination and/or progression of the BMU.

Evidence from *in vitro* studies

Various effects of E on cells of the osteoclast lineage have been described in different species (calvariae, rodents, mice) which can be explained by recent evidence that E promotes apoptosis of both mature osteoclasts (thus reducing bone resorption) and their precursors (thus reducing osteoclast formation)[63]. As mentioned above, osteoblasts and osteoclasts do express ER. The latter have anabolic effects on osteoblasts in humans. There is a species difference, which makes it rather difficult to extrapolate data from animals to humans, at least at the level of bone formation.

The mechanism of action of the ER

The ER is a member of the steroid receptor (SR) superfamily of ligand-dependent transcription factors[70]. Within this

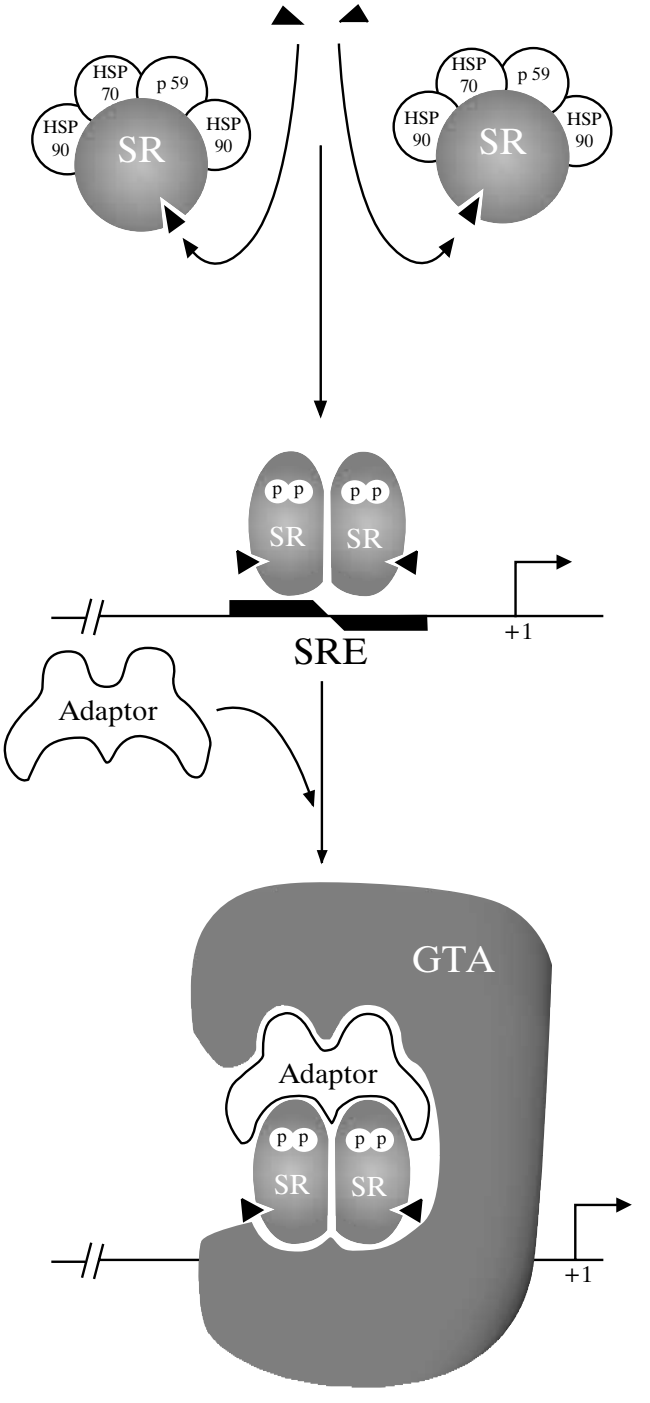

Figure 3. The mechanism of action of the oestrogen receptor[71].

superfamily, the sex steroid receptors are the most conserved both in primary sequence and mechanism of action.

Consequently, a composite model of the mechanism of action of this class of proteins has emerged. Specifically, ER agonists mediate their effect on gene transcription via specific intracellular receptor proteins located within target cell nuclei. Upon interaction with each cognate ligand the latent receptor becomes "activated". This event permits the displacement of heat-shock proteins (HSPs), facilitates receptor dimerization and promotes the interaction of the receptor with specific steroid response elements (SRE) located within the regulatory regions of target promoters. At this location, depending on the cellular and promoter context, the ligand – activated receptor can interact with the general transcription apparatus (GTA) directly or indirectly through adaptor proteins. Ultimately, these interactions stabilize the transcription preinitiation complex and enhance RNA polymerase activity. Although several rounds of phosphorylation of the receptor have been shown to occur, its in ER signaling has yet to be determined[71] (Fig. 3).

In general, E are "conditional" inhibitors of bone resorption, in contrast to other inhibitors of bone resorption, such as bisphosphonates and calcitonin, which have far more predictable and universal effects. Thus, E are potent inhibitors of bone resorption in the setting of oestrogen deficiency but are far less effective in the oestrogen – replete organism. *In vitro*, their action appears to be influenced by species, age and probably by the presence of other cell types. Taking these factors together, it would appear likely that E require the presence of co-factors, second messengers, or both and that the potency of their action depends on other stimuli to which the target cell is subjected[62].

The role of oestrogen on bone tissue in men

Recent data suggest the importance of E for bone maturation and development of peak bone mass in men.

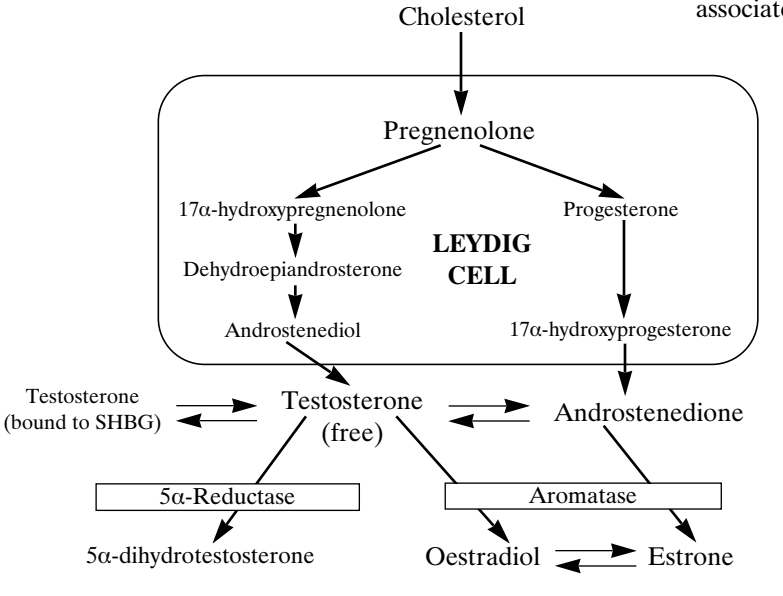

Figure 4. Sex hormone synthesis in men[72].

Oestradiol is detectable in the serum of healthy men at levels comparable to those in postmenopausal women. This is a result of peripheral conversion of testosterone by the enzyme aromatase, a member of the microsomal cytochrome P450 group (Fig. 4)[72].

Because these levels are rather low, they were not regarded as physiologically important until epidemiological research into heart disease risk suggested a protective effect of endogenous E in men[73].

A role for E in skeletal maintenance in the human male is supported by evidence at the cellular level by animal experiments and clinical findings. Osteopenia was reported in an aromatase-deficient young man whose oestradiol levels were below 26 pmol/l, but whose testosterone levels were high[74].

It was also reported in another case with non-functioning ER[75]. Serum testosterone and androgen receptors were normal or elevated. In both cases, bone mineral density (BMD) values were similar to those seen in the converse syndrome of genetic males with androgen insensitivity (androgen receptor defect but normal testosterone and oestradiol levels)[76]. Perhaps the most convincing evidence is from another case report, in which a 28-year-old man with an inactivating mutation of his aromatase gene presented with infertility and was found to have a eunuchoid habitus with nonclosure of the epiphyses, below-average BMD, and a bone age of 15 years. Treatment with regular intramuscular testosterone produced no benefit, but treatment with transdermal oestradiol, 50 μg twice weekly, resulted in skeletal maturation, a rapid increase in lumbar spine BMD, and epiphyseal closure[77].

Moreover, in a cross-sectional study in 37 healthy older men with no history of bone disease, Anderson et al.[78] found that BMD at the lumbar spine and hip correlated more closely with serum oestradiol ($r = 0.383$, $p<0.03$) than with testosterone ($r=0.245$, $p>0.15$). Also, in a prospective study of 93 healthy men aged over 65, serum oestradiol levels were positively associated with initial BMD values at all sites[79] and were associated with significantly lower rates of bone loss at the radius and hip on twice-yearly BMD measurements over a mean of 2 years ($p<0.05$, test for trend), whereas testosterone levels were not predictive[80]. In men with vertebral osteoporosis, oestradiol levels were found to be positively correlated with BMD at the femoral neck ($r= 0.41$, $p<0.02$)[81] and spine ($r= 0.29$, $p<0.03$)[82] and negatively correlated with markers of bone resorption such as hydroxyproline ($r=-0.57$, $p< 0.05$)[83].

It seems likely, on the basis of the somewhat limited evidence currently available, that both E and androgens are required for the growth and maintenance of the adult male skeleton.

TGFβ and bone

The concept that transforming growth factor beta (TGFβ) mediates the actions of E in bone is

supported by a growing body of literature[33,34].

The term TGFβ refers to a family of closely related proteins with similar amino acid sequence and similar biological activities.

TGFβ has biological effects on E responsive tissues, such as breast epithelium and endometrium and may have a role in the pathogenesis of breast and ovarian cancers[84].

In the mid-1980s, it was demonstrated that TGFβ is an abundant constituent of bone matrix where its concentration is higher than that of all other tissues, except platelets. TGFβ is produced by both osteoclasts and osteoblasts, the latter presumably being the source of the TGFβ present in bone matrix[85].

There are at least four main evidences that TGFβ mediates the action of E in bone. First, E increase TGFβ production in bone, second, TGFβ production in bone is reduced after E withdrawal, third TGFβ inhibits bone resorption in ovariectomized animals and finally, 17β E-mediated osteoclasts apoptosis is blocked by an anti-TGFβ antibody *in vitro*.

However, if there is real substance behind this theory, the evidence should fulfill a number of postulates such as: Oestrogens should increase TGFβ production and E deficiency should result in decreased TGFβ production. Blocking TGFβ should inhibit the action of E. TGFβ should be able to substitute for E in their absence. The *in vivo* and *in vitro* effects of TGFβ and E should be similar. Substances such as drugs that have oestrogen-like effects on bone are likely to have similar effects on TGFβ production and finally, factors mediating the effects of E deficiency should have opposite effects to TGFβ.

Most of these postulates are supported by some fairly strong evidence *in vitro* and *in vivo,* details of which are presented in references[63,85-90].

It has been suggested that the bone-selective effects of raloxifene may be due to its selective induction of TGFβ3. If this is true, influencing TGFβ3 production in bone may be a central therapeutic objective, thus facilitating selective drug development[33]. With that in mind, the relative roles of the different TGF family members in mediating the action of E may prove to be of great importance and not only of academic interest. Moreover, a more precise understanding of the actions of different E metabolites in bone is required, along with knowledge of how other endocrine factors (e.g. other sex steroids, corticosteroids and others) cooperate with E in exerting their actions.

In summary, oestrogen affects the skeleton in all species studied. They act on osteoblast precursors to increase or decrease proliferation and differentiation to osteoblasts. A pronounced inhibition of the resorptive activity of osteoclasts have also been demonstrated. Moreover, ER, ERα and ERβ, have been identified in bone cells in cultures. However, there is no definite evidence that these cells represent the target cells for E *in vivo*. Potential target cells in addition to osteoblasts, and perhaps osteoclasts, include lymphocytes, macrophages, mast cells, and stromal cells. Second messengers implicated in coordinating the sequence of events initiated by E include interleukins (IL-1, IL-6, IL-11), prostaglandins (PGE-2), TNF-α, the insulin-like growth factor (IGF) system and TGFβ. Specifically, TGFβ as outlined above mediates the actions of E in bone, at least to some extent. Oestrogens clearly can stimulate TGFβ gene transcription in bone *in vivo*, and E withdrawal has the opposite effect. However, there are a lot of observations, which still cannot be explained, as well as questions, which still cannot be answered. One of those is the observation that suggests that pituitary function is required for the effects of E on bone[91]. In hypophysectomized rats, E fails to reduce bone turnover and protect skeletal mass. It is obvious that further research is needed until all such issues can be solved.

References

1. Turner RT, Riggs BL, Spelsburg TC. Skeletal effects of estrogen. Endocr Rev 1994; 3:275-300.
2. van Paassen HC, Poortman J, Borgart-Creutzburg HC, Thijssen JHH, Duursma SA. Oestrogen binding proteins in bone cell cytosol. Calcif Tissue Res 1978; 25:249-254.
3. Caputo CB, Meadows D, Raisz LJ. Failure of estrogens and androgens to inhibit bone resorption in tissue culture. Endocrinology 1976; 98:1065-1068.
4. Komm BS, Terpening CM, Benz DJ, Grexeme KA, O' Malley BW, Haussler MR. Estrogen binding receptor mRNA and biologic response in osteoblast-like osteosarcoma cells. Science 1988; 241:81-84.
5. Eriksen EF, Colvard DS, Berg NJ, Graham MC, Mann KG, Spelsberg TC, Riggs BL. Evidence of estrogen receptors in normal human osteoblast-like cells. Science 1988; 241:84-86.
6. Oursler M, Osdoby P, Pyfferoln J, Riggs BL, Spelsberg TC. Avian osteoclasts as estrogen target cells. Proc Natl Acad Sci USA 1991; 88:6613-6617.
7. Hoyland JA, Mee AP, Baird P, Braidman IP, Mawer EB, Freemont AJ. Demonstration of estrogen receptor mRNA in bone using *in situ* reverse-transcriptase polymerase chain reaction. Bone 1997; 20:87-92.
8. Stulc T, Klement D, Kuasnicka J, Stepan JJ. Immunocytochemical detection of estrogen receptors in bone cells using flow cytometry. Biochem Biophys Acta 1997; 1356:95-100.
9. Mosselman S, Polman J, Dijkema R. ER-β: identification and characterization of a novel human estrogen receptor. FEBS Lett 1996; 392:49-53.
10. Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T. Expression of estrogen receptor-β in rat bone. Endocrinology 1997; 138:4509-4512.
11. Kusec V, Virdi AS, Prince R, Triffitt JT. Localization of estrogen receptor-a in human and rabbit skeletal tissues. J Clin Endocrinol Metab 1998; 83:2421-2428.
12. Nilsson LO, Boman A, Savendahl L, Grigelioniene G, Ohlsson C, Ritzen EM, Wroblewski J. Demonstration of estrogen receptor-β immunoreactivity in human growth plate cartilage. J Clin Endocrinol Metab 1999; 84:370-373.
13. Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T.

Expression of estrogen receptor-β in rat bone. Endocrinology 1997; 138:4509-4512.
14. Arts J, Kuiper GG, Janssen JM, Gustafsson JA, Lowik CW, Pols HA, van Leeuwen JP. Differential expression of estrogen receptors-α and-β mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 1997; 138:5067-5070.
15. Katzenellenbogan BS. Dynamics of steroid hormone receptor in action. Annu Rev Physiol 1980; 42:17-35.
16. Lopez A, Ventanas J, Burgos J. Oestradiol and testosterone binding sites in mice tibiae and their relationship with bone growth. Exp Clin Endocrinol 1986; 88:31-38.
17. Frenay M, Milano G, Formento JL, Francoval M, Moll JL, Namer M. Oestrogen and progesterone receptor status in bone biopsy specimens from patients with breast cancer. Eur J Cancer 1991; 27:115-118.
18. Ohashi T, Kushuhara S, Ishida K. Histochemical identification of oestrogen target cells in the medullary bone of laying hens. Br Poult Sci 1990; 31:221-224.
19. Ohashi T, Kushuhara S, Ishida K. Estrogen target cells during the early state of medullary bone osteogenesis: immunohistochemical detection of estrogen receptors in osteogenic cells of oestrogen treated male Japanese quail. Calcif Tissue Int 1991; 49:124-127.
20. Turner RT, Bell NH, Gay CV. Estrogen receptors and bone: evidence that estrogen-binding sites are present in bone cells and mediate medullary bone formation in Japanese quail. Poultry Sci 1993; 72:728-740.
21. Turner RT, Backup P, Kline BC, Colvard DS, Spelsberg TC. Evidence that the inhibition of osteoblast activity by estrogen is preceded by down regulation of IGF-1 gene expression and that these changes are estrogen receptor mediated. In: Spencer EM (ed) Molecular concepts of insulin-like growth factors. Elsevier, New York; 1991:143-153.
22. Turner RT, Eliel L. Nuclear estrogen receptor in the reproductive tract of laying Japanese quail. Gen Comp Endocrinol 1978; 34:141-148.
23. Turner RT, Dickhoff WW, Gorbman A. Estrogen binding to hepatic nuclei of Pacific hagfish, *Eptatretus stouti*. Gen Comp Endocrinol 1981; 45:26-29.
24. McSheehy PMJ, Chambers TJ. Osteoblastic cells mediate osteoclastic responsiveness to PTH. Endocrinology 1986; 118:825-828.
25. McSheehy PMJ, Chamberts TJ. 1,25-Dihydroxyvitamin D3 stimulates rat osteoblastic cells to release a soluble factor that increases osteoclastic bone resorption. J Clin Invest 1987; 80:425-429.
26. Jilka RL. Are osteoblastic cells required for the control of osteoclast activity by parathyroid hormone? Bone Miner 1986; 1:261-266.
27. Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC. Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 1992; 257:88-91.
28. Lorenzo JA, Sousa SL, Fonseca JM, Hock JM, Medlock ES. Colony stimulating factors regulate the development of multinucleated osteoclasts from recently replicated cells *in vitro*. J Clin Invest 1987; 80:160-164.
29. Corboz VA, Cecchini MG, Felix R, Fleisch H, van der Pluijm G, Lowik CW. Effect of macrophage colony-stimulating factor on *in vitro* osteoclast generation and bone resorption. Endocrinology 1992; 130:437-442.
30. Thomson BM, Mundy GR, Chambers TJ. Tumor necrosis factors alpha and beta induce osteoblastic cells to stimulate osteoclastic bone resorption. J Immunol 1987; 138:775-779.
31. Gowen M, Wood DD, Ihrie EJ, McGuire MKB, Russell RGG. An interleukin-1 like factor stimulates bone resorption *in vitro*. Nature 1983; 306:378-380.
32. Ishimi Y, Miyaura C, Him CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiki S, Matsude T, Hirano T, Kishimoto T, Suda T. Il-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990; 145:3297-3303.
33. Hughes DE, Boyce BF. Estrogen transforming growth factor-beta, and the regulation of bone metabolism in health and disease. The Endocrinologist 1998; 8:55-61.
34. Manolagas SC. Birth and death of bone cells: Basic regulatory mechanism and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 2000; 21:115-137.
35. Owens PC, Gill PG, De Young NJ, Knowles SE, Noyse KJ. Estrogen and progesterone regulate secretion of insulin-like growth factor binding proteins by human breast cancer cells. Biochem Biophys Res Commun 1993; 193:467-473.
36. Murphy LJ, Ghahary A. Uterine insulin-like growth factor-1: regulation of expression and its role in estrogen-induced uterine proliferation. Endocr Rev 1990; 11:443-453.
37. Ernst M, Rodan GA. Estradiol regulation of insulin-like growth factor-1 expression in osteoblastic cells: evidence for transcriptional control. Mol Endocrinol 1991; 5:1081-1089.
38. Ernst M, Heath JK, Rodan GA. Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-1 and parathyroid hormone. Stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. Endocrinology 1989; 125:825-833.
39. Ernst M, Schmid C, Froesch ER. Enhanced osteoblast proliferation and collagen gene expression by estradiol. Proc Natl Acad Sci 1988; 85:2307-2310.
40. McCarthy TL, Centrella M, Canalis E. Constitutive synthesis of insulin-like growth factor-II by primary osteoblast-enriched cultures from fetal rat calvariae. Endocrinology 1992; 130:1303-1308.
41. Okazaki R, Conover CA, Harris SA, Spelsberg TC, Riggs BL. Normal human osteoblast-like cells consistently express genes for insulin-like growth factor-I and II but

transformed human osteoblast cell lines do not. J Bone Miner Res 1995; 10:788-795.
42. Mohan S, Baylink DJ. Characterization of the IGF regulatory system in bone. Adv Exp Med Biol 1993; 343:397-406.
43. McCarthy TL, Centrella M, Canalis E. Cyclic AMP induces insulin-like growth factor 1 synthesis in osteoblast enriched cultures. J Biol Chem 1990; 265:15353-15356.
44. Canalis E. Skeletal growth factors in: Marcus R, Feldman D, Kelsey J (eds) Osteoporosis. Academic Press, San Diego, CA; 1996:P261-279.
45. Friedenstein AJ, Chailakhjan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. Transplantation 1974; 17:331-340.
46. Triffitt JT. The stem cell of the osteoblast. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, CA; 1996:39-50.
47. Roodman GD. Advances in bone biology: the osteoclast. Endocr Rev 1996; 17:308-332.
48. Suda T, Takahashi N, Martin TJ. Modulation of osteoclast differentiation. Endocr Rev 1992; 13:66-80.
49. Ott SM. Theoretical and methodological approach. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, CA; 1996:231-241.
50. Manolagas SC, Jilka RL, Bellido T, O'Brien CA, Parfitt AM. Interleukin-6-type cytokines and their receptors. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, CA; 1996:701-713.
51. Mohan S, Baylink DJ. The role of IGF-1 in the coupling of bone formation to resorption. In: Spencer EM (ed) Modern Concepts of Insulin-Like Growth Factors. Elsevier, New York; 1991:169-184.
52. Rodan GA. Coupling of bone resorption and formation during bone remodeling. In: Marcus R, Feldman D, Kelsey J (eds) Osteoporosis. Academic Press, San Diego, CA; 1996:28-299.
53. Parfitt AM. Skeletal heterogeneity and the purposes of bone remodeling: Implications for the understanding of osteoporosis. In: Marcus R, Feldman D, Kelsey J (eds) Osteoporosis. Academic Press, San Diego, CA; 1996:315-329.
54. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997; 390:175-179.
55. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998; 93:165-176.
56. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinoshaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 1998; 95:3597-3602.
57. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density: Cell 1997; 89:309-319.
58. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu WL, Lacey DL, Boyle WJ, Simonet WS. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998; 12:1260-1268.
59. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 1999; 397:315-323.
60. Degli-Esposti M. To die or not to die – the quest of the TRAIL receptors. J Leukoc Biol 1999; 65:535-542.
61. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC, Young PR. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem 1998; 723:14363-14367.
62. Liu CC, Howard GA. Bone-cell changes in estrogen-induced bone mass increase in mice: dissociation of osteoclasts from bone surfaces. Anat Rec 1991; 229:240-250.
63. Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. Nature Medicine 1996; 2:1132-1136.
64. Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. J Bone Miner Res 1998; 13:793-802.
65. Steller H. Mechanisms and genes of cellular suicide. Science 1995; 267:1445-1449.
66. Parfitt AM. Bone-forming cells in clinical conditions. In: Hall BK (ed) Bone. The osteoblast and osteocyte. Telford Press and CRC Press, Boca Raton, FL; 1990:vol 1,351-429.
67. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of

apoptosis of osteoblasts and osteocytes by glucocorticoids: potential mechanisms of their deleterious effects on bone. J Clin Invest 1998; 102:274-282.
68. Frost HM. *In vivo* osteocyte death. J Bone Joint Surg Am 1960; 42:138-143.
69. Noble BS, Stevens H, Loveridge N, Reeve J. Identification of apoptotic changes in osteocytes in normal and pathological human bone. Bone 1997; 20:273-282.
70. Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988; 240:889-895.
71. McDonnell DP, Norris JD. Analysis of the molecular pharmacology of estrogen receptor agonists and antagonists provides insights into the mechanism of action of estrogen in bone. Osteoporosis Int 1997; (suppl 1):S29-S34.
72. Anderson FH, Francis RM, Selby PL, Cooper C. Sex hormones and osteoporosis in men. Calcif Tissue Int 1998; 62:185-188.
73. Khaw KT, Barrett-Connor E. Endogenous sex hormones, high density lipoprotein cholesterol and other lipoprotein fractions in men. Arteriosclerosis Thromb 1991; 11:489-494.
74. Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 1995; 80:3689-3698.
75. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS. Estrogen resistance caused by a mutation in the estrogen-receptor gene in man. N Engl J Med 1994; 331:1056-1061.
76. Soule SG, Conway G, Prelevic GM, Prentice M, Ginsburg J, Jacobs HS. Osteopenia as a feature of the androgen insensitivity syndrome. Clin Endocrinol 1995; 43:671-676.
77. Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER. Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 1997; 337:91-95.
78. Anderson FH, Francis RM, Hindmarsh P, Fall C, Cooper C. Serum oestradiol in osteoporotic and normal men is related to bone mineral density. In: Papapoulos SE, Lips P, Pols HAP, Johnston CC, Delmas PD (eds) Osteoporosis 1996 – Proceedings of the 1996 World Congress on Osteoporosis. Elsevier, Amsterdam; 1996:377-381.
79. Slemenda C, Zhou L, Longcope C, Hui S, Johnston CC. Sex steroids, bone mass and bone loss in older men: estrogens or androgens? J Bone Miner Res 1995; 10 (suppl 1):S440.
80. Slemenda C, Longcope C, Hui S, Zhou L, Johnston CC. Estrogens but not androgens are positively associated with bone mass in older men. Osteoporosis Int 1996; 6 (suppl 1):279.
81. Selby PL, Braidman IP, Mawer EB, Freemont AJ. Hormonal influences in male osteoporosis. Osteoporosis Int 1996; 6(suppl 1):279.
82. Bernecker PM, Willvonseder R, Resch H. Decreased estrogen levels in patients with primary osteoporosis. J Bone Miner Res 1995; 10(suppl 1):T 364.
83. Selby PL, Braidman IP, Freemont AJ, Mawer EB. Is estrogen an important regulator of bone turnover in the male? Preliminary evidence from osteoporosis. J Endocrinol 1995; 1(suppl 1):O54.
84. Bonewald LF, Mundy GR. Role of transforming growth factor beta in bone remodeling: a review. Connect Tissue Res 1989; 23:201-208.
85. Ikeda T, Shigeno C, Kasai R, Kohno H, Ohta S, Okumura H, Konishi J, Yamamuro T. Ovariectomy decreases the mRNA levels of transforming growth factor-beta 1 and increases the mRNA levels of osteocalcin in rat bone *in vivo*. Biochem Biophys Res Commun 1993; 194:1228-1231.
86. Yang NN, Bryant HU, Hardikar S, Sato M, Galvin RJ, Glasebrook AL, Termine JD. Estrogen and raloxifene stimulate transforming growth factor-beta 3 gene expression in rat bone: a potential mechanism for oestrogen- or raloxifene-mediated bone maintenance. Endocrinology 1996; 137:2075-2084.
87. Yang NN, Vengopalan M, Hardikar S, Glasebrook A. Identification of an estrogen response element activated by metabolites of 17β-estradiol and raloxifene. Science 1996; 273:1225-1231.
88. Beaudreuil J, Mbalaviele G, Cohen-Solal M, Morieux C, De Vernejoul MC, Orcel P. Short-term local injections of transforming growth factor-beta 1 decrease ovariectomy-stimulated osteoclastic resorption *in vivo* in rats. J Bone Miner Res 1995; 10:971-977.
89. Wright CDP, Garrahan NJ, Stanton M, Gazet J-C, Mansell RE, Compston JE. Effect of long-term tamoxifen therapy on cancellous bone remodeling and structure in women with breast cancer. J Bone Miner Res 1994; 2:153-159.
90. Arnett TR, Lindsay R, Kilb JM, Moonga BJ, Spowage M, Dempster DW. Selective toxic effects of tamoxifen on osteoclasts: comparison with the effects of oestrogen. J Endocrinol 1996; 149:503-508.
91. Yeh JK, Chen MM, Aloia JF. Effects of estrogen and growth hormone on skeleton in the ovariectomized rat with hypophysectomy. Am J Physiol 1997; 273:E734-E742.