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Estrogen Receptors Alpha and Beta in Bone

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Abstract

Estrogens are important for bone metabolism *via* a variety of mechanisms in osteoblasts, osteocytes, osteoclasts, immune cells and other cells to maintain bone mineral density. Estrogens bind to estrogen receptor alpha (ERα) and ERβ, and the roles of each of these receptors are beginning to be elucidated through whole body and tissue-specific knockouts of the receptors. *In vitro* and *in vivo* experiments have shown that ERα and ERβ antagonize each other in bone and in other tissues. This review will highlight the role of these receptors in bone, with particular emphasis on their antagonism.

Keywords

Estrogen; estrogen receptor alpha (ERα, ESR1); estrogen receptor beta (ERβ, ESR2); bone

Estrogen Receptors

Estrogens have physiological functions in almost all tissues in the body in both males and females [1]. Estrogens, including the most abundant estrogen 17β-estradiol (E2), interact with estrogen receptors (ER) alpha and beta (ERα and ERβ) [2]. ERα was identified in the 1960s [3] (and cloned in 1986 [4]) and is best studied in the female reproductive system and in breast cancer. ERβ was identified and cloned in 1996 [5] and is less well characterized. ERα and ERβ are highly conserved in the DNA binding domain (95%) and ligand binding domain (60%), but the NH2-terminal domains, including the transcriptional activation domain AF-1, are only 20% conserved. While the role of estrogen in bone has been reviewed (for example, refs. [6–8],) this review will highlight the role of these *receptors* in bone, with particular emphasis on their antagonism.

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ERα and ERβ Antagonism

It is beginning to become clear that ERα and ERβ antagonize each other's actions in many tissues. In breast cancer cell lines, such as T47D cells, overexpression of ERβ inhibits E2-mediated proliferation and gene expression [9]. In the prostate, ERα also promotes cell proliferation and survival, whereas ERβ is protective and pro-apoptotic [10]. Another example of their antagonism *in vivo* is observed in behavior; compared to WT mice, ERβKO mice have an increase in sexual aggression, whereas ERαKO mice have a decrease in such behavior, compared to control WT mice [11]. At the molecular level, ERα and ERβ have been observed to signal in opposite ways (activation *vs*. repression of transcription) at an AP1 site [12]. However, the roles of ERα and ERβ in different tissues and under different conditions remain to be further elucidated, particularly in bone biology.

Estrogens in Bone

Estrogens are important for maintaining bone mineral density in both mice and humans. When women go through menopause estrogen levels decrease and there is a decrease in bone mineral density, along with an increased risk for fractures, particularly in the hip, vertebrae and wrist [13]. Treatment of women with hormone replacement therapy (HRT) (either estrogen alone or estrogen plus progesterone) has been shown to prevent this bone loss [14]. In 2002 the Women's Health Initiative (WHI) showed that HRT prevents bone fractures [14]. However, the routine use of HRT has diminished significantly due to the results of the WHI suggesting an increased risk of breast cancer, heart disease and stroke in women taking HRT. While we have long known the beneficial effects of estrogen in bone, surprisingly the molecular mechanism for the role of estrogen in bone cells is only beginning to be unraveled.

The skeleton is constantly being remodeled. Osteoblasts lay down the matrix for bone and osteoclasts degrade bone. If there is an increase in osteoblast number and/or activity, especially if coupled with a decrease in osteoclast activity, there is overall building of bone, such as occurs during E2-driven acquisition of bone mass during puberty. On the other hand, if there is a decrease in osteoblast number or activity and that is coupled to no change or an increase in osteoclast number or activity, a decrease in bone mineral density will occur. Thus, it is the balance between osteoblast and osteoclast numbers and activity that determines the quality and quantity of bone.

The protective effects of E2 in bone are due to many mechanisms. For example, repression of pro-osteoclastic cytokines, such as IL-1, IL-6, IL-7 and TNF, in T cells and osteoblasts have been well documented to promote increased bone mass [15–17]. The mechanism of E2 repression of proinflammatory cytokines in osteoblasts has not been as well characterized, but is thought to be *via* inhibition of the nuclear factor κB (NFκB) pathway and its multicomplex effects on a wide-variety of cellular and molecular processes [18].

E2 exposure not only represses pro-osteoclastic cytokines, but it induces apoptosis in bone resorbing osteoclasts [19, 20]. Mechanistically, E2, *via* ERα activation, induces transcription of Fas Ligand (FasL) in osteoblasts. FasL is cleaved from the cell surface by MMP3, and the

soluble FasL induces osteoclast apoptosis [21, 22]. A third mechanism of estrogen-mediated suppression of osteoclasts involves the regulation of the RANKL/(OPG) ratio. Receptor activator of nuclear factor κB ligand (RANKL) is an essential cytokine for osteoclast differentiation. The RANKL pathway can be inhibited by OPG, which acts as a decoy receptor for RANKL. Thus, the RANKL:OPG ratio is critical for osteoclastogenesis. E2 has been shown to increase the transcription of OPG [23] and to affect RANKL localization at the cell surface of osteoblasts [24].

Furthermore, E2 is pro-osteoblastic, leading to a net increase in bone building. E2 is anti-apoptotic in osteoblasts [25], and the anti-apoptotic protein Bcl2 is regulated by E2 in osteoblasts [26]. Ovariectomy leads to an increase in osteoblast and osteocyte apoptosis, and conversely E2 can prevent etoposide, dexamethasone or TNFα-induced apoptosis in osteoblasts [25]. E2 also induces the transcription of alkaline phosphatase, a marker of osteoblast differentiation [27]. There are very few other known direct targets of E2 in normal osteoblasts and little to no information on the targets of ERβ [7].

The Role of Estrogen Receptors in Bone *in vivo*

ERα and ERβ have been detected by immunohistochemistry in osteoblasts [28–30], osteocytes [28, 30], and osteoclasts [28–30]. ERα and ERβ are also expressed in immune cells, such as T cells and monocytes [31–33], which are important in bone regulation. By IHC, ERβ is expressed at higher levels in trabecular bone than in cortical bone. Interestingly, in this same study ERα was detected in the opposite pattern: higher in cortical bone than in trabecular bone [28].

Both ERα and ERβ polymorphisms have been shown to correlate with bone mass in humans [34]. The so-called “PvuII” and “XbaI” haplotypes of ERα are well-studied at the population level. More recently, two genome-wide association studies have correlated SNPs upstream of the ERα promoter with bone mineral density [35, 36]. However, these polymorphisms have no known function to explain their effects on bone mineral density. Similarly, three intronic variations in ERβ are associated with femoral bone mineral density, but the mechanism is unknown. While an ESR1 mutation in a man has been described in the literature [37], no known ESR2 mutations have been reported. The patient had an ERα truncating mutation that led to incomplete epiphyseal closure and low bone mineral density.

Mouse models of the function of ERα and ERβ have been generated by several labs and provided us with some insights to their individual functions (Table 1). For example, ERαKO mice were generated in the Korach and Smithies laboratories (K/G-ERαKO) [38] and Chambon laboratory (C-ERβKO) [39]. The K/G ERαKO is not a complete knockout and expresses some ERα due to splicing of the Neo cassette. The female and male C-ERαKO mice have a decrease in cortical bone mineral density, and an increase in trabecular bone mineral density [40].

Several ERβ knockout mice have also been created. ERβKO mice were first developed in the Korach, Gustafsson and Smithies laboratories (K/G-ERβKO) [41] and the Chambon laboratory (C-ERβKO) [39]. The female K/G-ERβKO mice have an *increased* cortical BMD

at 11 weeks of age [42] and both cortical and trabecular BMD increases by 12 months of age [43], whereas the BMD in the Chambon ERβKO female mice is unchanged compared to WT controls. The Chambon group claimed that the phenotype of the K/G-ERβKO knockout mouse is a result of the *neo* selection cassette inserted into the ERβ gene and is not due to loss of ERβ itself. Furthermore, these mice express truncations in the ERβ transcript that might contribute to the phenotypes [44]. In 2007 Antal, *et al.,* published an ERβ knockout ($ERβ_{ST}^{L-/L-}$) that is not thought to have any ERβ splice forms. This mouse had reproductive abnormalities, but no phenotype in the prostate or other tissues shown to have an effect in the other ERβKO strains [44]. However, the bone phenotype for the $ERβ_{ST}^{L-/L-}$ mouse has not been published.

The C-ERβKO mice have no difference in the femoral length, but the K/G-ERβKO mice have longer femurs than WT mice, whereas ERαKO mice have shorter femurs than WT mice. However, the male C-ERβKO mice do have an increase in femoral width compared to WT mice and the C-ERαKO mice have a decrease in femoral width [40]. Together, this data demonstrate opposing effects of ERα and ERβ on femoral size [42, 45].

Estrogen and estrogen receptors play a critical role in the normal adaptation of bone to loading. The C-ERαKO and C-ERβKO knockout mice show opposite osteogenic responses to loading in the cortical bone. Whereas the ERαKO mice had a lower response, the ERβKO had a higher response to loading when compared to their respective wildtype mice [46]. *In vitro*, ERα and ERβ also differentially regulate SOST, which is involved in the osteoblast response to strain [47]. Loading experiments have shown that the ulnae of female ERβKO mice bone were stiffer than those from WT mice when subjected to mechanical strain. This increase in stiffness was due to the increase in the periosteal bone formation per unit increase in strain; however, there was no difference in periosteal bone formation per unit increase in strain reported in male ERβKO mice and WTs [48]. These results suggest that ERβ reduces response to mechanical loading at periosteal bone surface; interestingly these results are opposite to the effect of ERα. Cultured bone cells from ERβKO mice subjected to mechanical strain resulted in an increase in the number of osteoblast-like cells, while cells from ERαKO did not increase in number in response to mechanical loading [49]. This suggests that signaling through ERα increases the bones' response to mechanical strain (increases cell number) while ERβ suppresses it.

In female mice there was no difference reported for trabecular bone mineral density between C-ERαKO and their age matched wildtype controls or C-ERβKO and their age matched wildtype controls, while the C-ERαβKO double knockout mice showed a decrease in trabecular BMD that mimics loss of estrogen in mice by ovariectomy or in humans by menopause [6, 40]. E2 can prevent ovariectomized trabecular bone mineral density loss in wildtype and ERβKO mice, and E2 can moderately rescue bone loss in ERαKO mice, indicating that ERβ can partially compensate for ERα [50]. Thus, it is thought that ERα and ERβ have redundant functions in trabecular bone, but opposing functions in femur length and response to mechanical strain.

The study of ERs in these KO mice is complicated by the facts that female ERαKO mice have high levels of serum estrogen and testosterone [51] and there are endocrine, paracrine

and autocrine effects of estrogen receptors. Therefore, in the past decade many labs have created cell type specific knockouts of ERs.

Osteoblast-Specific ERαKO Mice

The Cre-lox system has been extensively used to characterize temporal and cell-specific deletions of genes involved in osteoblastogenesis [52]. The PRX-1 promoter drives Cre recombinase in osteoblast progenitors (limb bud mesenchyme). The OSX1 promoter drives Cre recombinase in osteoblast precursors. The 2.3 kb promoter of Col1a1 drives Cre recombinase in osteoblasts precursors (but later than OSX1-Cre). The osteocalcin promoter drives cre-recombinase in mature osteoblasts. The DMP1 promoter expresses Cre recombinase in osteocytes and some bone lining cells [53]. Each of these has been used to characterize the role of ERα in osteoblasts at various stages in bone development to elucidate its function in bone biology.

ERα deleted from limb bud mesenchyme ($ERα^{f/f}$;PRX1-Cre) and cells expressing osterix1 ($ERα^{f/f}$;Osx1-Cre) (osteoblast progenitors) both revealed low femoral BMD in adult females measured by DEXA, as compared to wildtype mice [54]. The trabecular bone volume in the femur of both $ERα^{f/f}$;PRX1-Cre and $ERα^{f/f}$;Osx1-Cre were both indistinguishable from their littermate controls. In line with reduced femoral BMD, mice with $ERα^{f/f}$;PRX1-Cre and $ERα^{f/f}$;Osx1-cre both had reduced cortical thickness.

In contrast to the early deletion of ERα, no effect on trabecular bone volume or cortical thickness was reported when ERα was deleted from mature osteoblasts during the matrix maturation phase ($ERα^{fl/fl}$;Col1a1-Cre) [54]. These data suggest that ERα is responsible for maintaining optimal periosteal bone formation through osteoblasts progenitors and not via mature osteoblasts.

Two conditional models were described in which ERα was deleted by Osteocalcin-Cre. Osteocalcin is expressed by mature osteoblasts. Almeida and colleagues suggested that ERα does not play a role in mature osteoblasts, based on their $ERα^{fl/fl}$;Col1a1-Cre model. However, Melville, *et al.*, and Määttä, *et al.*, both showed that in $ERα^{fl/fl}$;OCN-Cre mice deletion of ERα led to a decrease in both cortical and trabecular bone parameters [55, 56], suggesting a role for ERα in mature osteoblasts

Deletion of ERα in osteocytes, using the DMP1-Cre mouse ($ERα^{fl/fl}$;DMP1-Cre), revealed a decrease in trabecular bone mass phenotype, but only in male mice [57]. Female $ERα^{fl/fl}$;DMP1-Cre mice did have a reduced response to E2 after ovariectomy. Interestingly $ERα^{fl/fl}$;DMP1-Cre mice had reduced expression of both early (*Runx2* and *Sp7 (osterix)*) and late (*Ibsp*) osteoblast markers, suggesting that osteocytes communicate with osteoblasts. Female ERαKO mice had a reduced response to mechanical loading [46], and since osteocytes are thought to respond to mechanical loading, it was thought that female $ERα^{fl/fl}$;DMP1-cre would have reduced bone mineral density.

Osteoclast-Specific ERα Knockout Mice

ERα has also been specifically knocked out of osteoclasts, using the promoter of the LysM gene that regulates cre expression in monocytes and macrophages. The bone phenotype of a female osteoclast specific ERαKO mice was similar to that of an osteoporotic woman with low trabecular bone mass due to high bone turnover rate and decreased apoptosis of osteoclasts [58]. This suggests that ERα is necessary in both osteoblasts and osteoclasts, although the protective effects of ERα in cortical bone is via osteoblasts while in trabecular bone it's through osteoclasts.

Other Models of ERα Function in Bone

Other interesting mouse models of ERα have been created to elucidate the molecular mechanism of ERα, especially to decipher the "classical" ERE-mediated signaling and "non-classical" non-ERE functions. Syed *et al.* described the consequences of either partial ($ERα^{+/NERKI}$) or complete ($ERα^{-/NERKI}$) loss of classical ERα signaling on the male and female skeleton due to substitution mutations (E207A/G208A) in the first zinc finger of the DNA binding domain. The NERKI (non-classical ERα knock-in) ERα can still regulate transcription through protein-protein interactions, for example at AP-1 elements, but cannot bind DNA at classical estrogen response elements (ERE). $ERα^{-/NERKI}$ mice had decreased cortical bone, but normal trabecular bone [59]. Interestingly, after ovariectomy the $ERα^{-/NERKI}$ mice gained bone, in contrast to wildtype mice that lose bone mass. These results not only suggest the importance of both ERα and the classical nuclear signaling pathway for bone homeostasis, but also indicate that classical and non-classical signaling have different effects of estrogen on bone cells.

Other mutations in ERα have been modeled in mice. A mouse in which ERα is transcriptionally constitutively active due to a substitution of tyrosine to serine at amino acid 537 reveals an increase in bone mineral density [60]. The membrane only estrogen receptor alpha (MOER) mouse has a mutant ERα that is localized to the cell membrane only [61], with no nuclear or cytoplasmic ERα detected. The nuclear-only ERα (NOER) mouse has a mutant ERα that has a mutation at amino acid 451 that prevents its palmitoylation and membrane localization [62]. The bone phenotypes of the MOER and NOER mice have not been analyzed yet.

In addition to the DNA binding domain and the membrane localization domain, ERα has other functional domains, including a ligand-independent activation function (AF)-1 domain near the N terminus and a ligand-dependent AF-2 domain near the C-terminus of the protein. The Ohlsson lab generated mice that are missing either the AF-1 or the AF-2 domain of ERα to determine the effects of ERα domains in male mice [63]. Wildtype mice and mice with a mutant AF-1 that were orchidectomized (orx) and treated with E2 had an increase in BMD compared to vehicle treated mice, but mice with a mutant AF-2 did not have an increase in BMD. In contrast, all three mutants (total ERα, $AF\text{-}1^{mut}$ and $AF\text{-}2^{mut}$) did not have an increase in trabecular bone volume after orx and E2 treatment. Thus, there are cell-type (location) specific effects of ERα functional domains.

ERα has also been specifically ablated from nestin-positive cells using the nestin –cre driver. ERα was demonstrated to be deleted from the brain, but not the bone [64], although one must consider that MSCs are nestin-positive [65]. The $ERα^{fl/fl}$;nestin-Cre mice showed an increase in both trabecular and cortical bone mineral density [64]. Leptin levels were higher in the serum of $ERα^{fl/fl}$;nestin-Cre mice, which could explain the high bone mass phenotype. Osteoblasts express the leptin receptor, and studies have shown that leptin signaling can increase bone mass [66–68].

Transplantation experiments using ERαKO or WT hematopoietic stem cells into either ERαKO or WT mice were employed to control for tissue specific cre deletion and incomplete deletion. E2 restored ovariectomy-induced cortical and trabecular bone loss in WT mice receiving WT bone marrow or ERαKO bone marrow, implicating a non-hematopoietic cell (most likely an osteoblast) [69].

Osteoblast-specific ERβ Knockout Mice

ERβ was recently deleted in osteoprogenitor cells using the Prx1-Cre [70]. Six and 12 week old female $ERβ^{fl/fl}$;PRX1-Cre had an increase in trabecular bone, compared to wildtype mice, while there were no changes in cortical bone properties, as is seen in aged ERβKO mice [43]. The expression of ERβ has been reported to be localized to trabecular bone [28], and thus the phenotype correlates with the expression pattern. The authors report a similar phenotype with the Col2.3kb-Cre driver. Together, these experiments are in line with the hypothesis that ERβ antagonizes ERα action. Loss of ERβ in other cell types and later in osteoblast differentiation, will be informative, and will probably follow a similar trend.

ERα-Regulated Genes in Bone

Many genes have been reported to be regulated by E2 [7], but the receptors responsible for up-regulating these genes have not been well characterized until recently. Roforth and colleagues performed RNA-sequencing in human fetal osteoblast (hFOB cells) overexpressing ERα [71]. They identified 4353 genes upregulated by E2. By also using mutant ERα constructs with nuclear only (NOER) functions or non-classical ERα knock in (NERKI) mutations they determined that 45% of the genes are nuclear ERE-independent, 27% are nuclear ERE-dependent and 28% are extra-nuclear. However, these experiments are not designed to determine the role of ERα vs. ERβ in regulating E2-mediated gene transcription.

One specific role for ERα is to induce Fas Ligand (FasL) in osteoblasts. ERαKO mice have an increase in the total number of osteoclasts due to the lack of E2-induced osteoclast apoptosis [72]. E2, *via* ERα and not ERβ, binds to enhancers near FasL and induces transcription of FasL in osteoblasts resulting in a paracrine signal to induce osteoclast apoptosis. Furthermore, E2 increases the transcription of MMP3, which cleaves FasL, creating a soluble form of FasL that is necessary for osteoclast apoptosis [21]. This work is supported by the fact that a non-hematopoietic cell is necessary for maintenance of bone mineral density [69].

ERβ-Regulated Genes in Bone

Specific ERβ ligands are valuable tools to dissect out the functions of ERα vs. ERβ. The soy isoflavone genistein binds with a 20-fold greater affinity for ERβ than ERα [73]. Soy supplements are marketed for bone health, and several trials have shown promising results with giving women soy isoflavones. However, a meta-analysis of randomized controlled trials showed no effect of soy isoflavones on bone mineral density [74]. In addition, genistein had no effects on the decreased bone mineral density of ovariectomized rats [75, 76]. Similarly, the ERβ ligand ERB-041, which has an over 200-fold affinity for ERβ, did not increase proximal tibial bone mineral density in ovariectomized rats [77].

The U2OS osteosarcoma cell line that stably over-expresses ERα, ERβ or both receptors are useful cell lines as a screen for osteoblast genes [27, 78]. Expression arrays show only 21% overlap between E2-regulated genes in U2OS-ERα and U2OS-ERβ cell lines [79], demonstrating that the two receptors have different functions in osteoblast-like cells. When ERα and ERβ are expressed together, a distinct set of E2-regulated genes was observed [80].

However, it may not be “different” functions, but that ERβ represses ERα gene transcription. Genes that are upregulated by E2 in WT bones, as determined by microarray analysis, are also increased by estrogen in ERβKO mice, but at an increased level, suggesting that ERβ is inhibitory to ERα [81]. And although with different genes, the same inhibitory pattern of ERβ was seen in ERβKO and WT liver cells after E2 treatment. Lindberg, *et al.* hypothesize that the inhibitory effect of ERβ might be explained mechanistically by the observation that ERβ does not contain a strong AF-1 domain, but, rather, contains a repressor domain [81]. They also demonstrate that in the absence of ERα, ERβ can partially replace ERα. ChIP-sequencing experiments could show if ERβ and ERα are binding at the same genomic locations.

Conclusions and Future Directions

Studies have confirmed the importance of estrogen and estrogen receptors, not only in cortical and trabecular bone, but also in different bone cell types. However, further work still needs to be done to identify different genes that are regulated by estrogen in each of these cell types during different stages of differentiation. The function of ERβ in different cell types and stages of differentiation can be elucidated with cell type specific knockouts, as has been done for ERα. The function of ERβ in bone cells may be to antagonize the effects of ERα, but this remains to be fully explained by molecular and cellular assays in bone cells.

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Highlights

- This review will highlight the role of estrogen receptors in bone.
- Estrogen receptors alpha and beta can partially compensate for each other
- Estrogen receptor beta antagonizes many effects of estrogen receptor alpha in bone

Table 1

Tissue specific ER knockout mice

Mice genotype	Cortical	Trabecular
Menopausal women	↓	↓
OVX in mice	↓	↓
C-ERαKO [40]	↓	↑
C-ERβKO [40]	NC	NC
C-ERαβKO [40]	↓	↓
ERα$^{f/f}$;Prx1-cre [54]	↓	NC
ERα$^{f/f}$;Osx1-cre [54]	females ↓	NC
ERα$^{f/f}$;Col1a1-cre [54]	NC	NC
ERα$^{f/f}$;OCN-cre [55]	females ↓	↓
ERα$^{f/f}$;OCN-cre [56]	females ↓	↓
ERα$^{f/f}$;DMP1-cre [57]	NC	males ↓
ERα$^{-/NERKI}$ [59]	NC	↓
ERα$^{f/f}$;LysM-cre [58]	NC	↓
ERα$^{f/f}$;Nestin-cre [58]	↑	↑
ERβ$^{f/f}$;PRX1-cre [70]	NC	↑