[Cell Cycle 7:10, 1348-1352; 15 May 2008]; ©2008 Landes Bioscience

Perspective

Unraveling estrogen action in osteoporosis

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Key words: estrogen, estrogen receptor, osteoporosis, bone, osteoclast, osteoblast, Fas ligand (FasL)

A decrease in estrogen levels at menopause leads to a rapid loss of bone mineral density and an increase in fracture risk. For over ten years it has been known that the beneficial effects of estrogen are due in part to the ability of estrogen to suppress osteoclastogenic cytokine production in T-cells and osteoblasts. In addition to suppressing these cytokines, estrogen has been shown to induce the apoptotic death of osteoclasts. A variety of different mechanisms have been suggested to explain the estrogen regulation of osteoclast survival. One hypothesis is that estrogen, via rapid non-genomic signaling, induces apoptosis without the need for direct binding of estrogen receptor α (ERα) to DNA. A second hypothesis proposes that estrogen-stimulation of ERα in osteoclasts induces the expression of Fas Ligand which in turn leads to cell death via an autocrine mechanism. In contrast, recent work from our lab has led to a genomic model of estrogen action in which estrogen acts to induce ERα binding to transcriptional enhancers in the Fas Ligand gene leading to its upregulation in osteoblasts which through a paracrine mechanism induces apoptosis in osteoclasts. Here we will focus on these differing models of the mechanism of estrogen-mediated osteoclast apoptosis.

Introduction

During menopause, as estrogen levels decrease sharply, bone mineral density also decreases. Treatment of women with hormone replacement therapy (either estrogen alone or estrogen plus progesterone) has been shown to prevent this bone loss.[1] Recently, several papers have suggested different molecular mechanisms to explain this effect of estrogen in bone.[2-4]

Estrogen signals through two nuclear receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα and ERβ are both expressed in many tissues including bone, though usually at lower levels than those found in reproductive tissues.[5] ERα and ERβ have been localized by immunohistochemistry and PCR in osteoblasts, osteocytes and osteoclasts.[6,7] ERα and ERβ can bind to DNA at specific DNA motifs called estrogen response elements (EREs). In addition, ERα can indirectly activate transcription by binding to other DNA binding proteins such as Sp1 and c-fos or c-jun.[8] Furthermore, estrogen can have non-genomic effects, or rapid signaling effects, inducing the phosphorylation of components of various signaling pathways (e.g., the MAPK pathway) or calcium regulation (reviewed in ref. 9).

Estrogen is important in the development of bone in both mice and humans. A case report of a man with a mutation in ERα described a lack of full bone maturation with a failure of epiphyseal closure and osteoporosis at age 28.[10] Several men lacking aromatase (the enzyme that converts testosterone to estrogen) have also been reported. These men also did not have closed epiphyses and were osteopenic; however treatment with estrogen was able to revert the phenotype.[11,12] These mutations demonstrate that estrogen and ERα are critical to bone maturation. It is interesting to note that the bone age in these men was reported to be analogous to that of early puberty, indicating that estrogen is not necessary for early bone formation. Mice lacking ERα (ERαKO), ERβ (ERβKO) or both ERα and ERβ (ERαβKO) are also viable and have fairly normal bone development (no gross skeletal abnormalities), although bone mineral density is lower[13] by eight weeks of age. As in the man without ERα, the ERαKO, ERβKO and the ERβKO have normal bone mineral density before puberty (0–4 weeks of age of the mice).

In 2002 the Women's Health Initiative (WHI) showed that hormone replacement therapy (HRT) prevents bone fractures.[1] 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. The clinical implications of the WHI have been an area of controversy and it has been suggested that perhaps lower doses of hormones and/or beginning HRT at peri-menopause rather than later in life may reduce the risks of HRT.[14] Alternatively, selective estrogen receptor modulators (SERMs) can be used as ERα agonists in the bone. Raloxifene is currently approved for the prevention and treatment of osteoporosis. Tamoxifen, a SERM that is used as a treatment for ERα-positive breast cancers, is an ERα antagonist in the breast, but is an ERα agonist in the bone. The NSABP Study of Tamoxifen and Raloxifene (STAR) Trial showed no difference in the number of osteoporotic fractures in the patients treated with either tamoxifen or raloxifene however raloxifene was felt to be superior on the basis of a decreased risk of endometrial cancer compared with tamoxifen.[15] The differential recruitment of nuclear receptor coactivators is thought to be responsible at least in part for the tissue specificity of tamoxifen and raloxifene in the breast and uterus.[16] However, the contribution to SERM action in bone has not yet been explored. Recent progress on the potential mechanisms of action of both estrogen and SERMs in the bone will be discussed below.

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Submitted: 02/29/08; Accepted: 02/29/08

Previously published online as a *Cell Cycle* E-publication: http://www.landesbioscience.com/journals/cc/article/5892

The Regulation of Osteoclastogenic Cytokines by Estrogen

For many years it has been assumed that the bone protective effects of estrogen are due to estrogen's ability to decrease osteoclastogenic cytokines. A decrease in estrogen levels at menopause has been associated with an increase in serum interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 7 (IL-7) and Tumor Necrosis Factor α (TNFα).[17-20] These cytokines have been shown to be made in osteoblasts, bone marrow stromal cells and T-cells[5,21] and subsequently increase the number of osteoclasts. After ovariectomy of mice or rats, the decrease in bone mineral density can be prevented by blocking TNFα or IL-1, and the most favorable outcome is seen when both TNFα or IL-1 are blocked.[22] In contrast, blocking of IL-6 has no effect on bone mineral density.[23] These experiments highlight the many factors and the interplay between cell types that regulate osteoclasts.

Several hypotheses have been presented on how estrogen can repress the osteoclastogenic cytokines. The first is that ERα binds to NFκB preventing NFκB binding to enhancers and/or promoters of the cytokines.[24] A second hypothesis is that estrogen leads to the recruitment of GRIP1, which acts as a co-repressor on the TNFα promoter.[25] Alternatively, ERα has also been shown to sequester coactivators from NFκB[26] to prevent transcription of the cytokines. Other possibilities include the ability of ERα to inhibit IKK activity or degradation of IkB, thereby preventing NFκB transcriptional activation.[27]

Is Apoptosis in Osteoclasts Induced by a Non-Genomic Pathway?

In addition to decreasing cytokine levels, estrogen was shown to induce apoptosis in osteoclasts.[28] This groundbreaking observation led the way for the exploration of the mechanism of how estrogen caused apoptosis. Kousteni, et al., suggested that apoptosis in osteoclasts was induced via a non-genomic pathway[3]. Estren (4-estren-3, 17-diol) is an estrogen receptor agonist that was thought to only have non-genomic activity. It was shown to induce apoptosis in osteoclasts, forming the hypothesis that estrogen-mediated apoptosis of osteoclasts does not require ER binding to DNA.[3] However, recent papers have shown that estren does have transcriptional effects.[29,30] Furthermore, estren is thought to have androgenic effects mediated by the androgen receptor.[30,31] Therefore, the conclusion that estrogen-mediated apoptosis of osteoclasts is non-genomic may need to be reexamined. While non-genomic signaling may contribute to apoptosis of osteoclasts, this is not the sole mechanism of action and additional experiments need to be conducted to determine the relative contribution of genomic vs. non-genomic mechanisms.

ERα Knockout Mice have Low Cortical Bone Mineral Density

ERαKO, ERβKO and ERαβKO mice have been generated; the bone phenotypes of these have been described,[13] and the differing phenotypes provide clues to the roles of the different receptors in the bone. Two different ERα knockout mice strains have been generated. The first, developed in the laboratories of Korach and Smithies,[32] deletes exon 2 of ERα, but this results in an incomplete knockout of ERα. The second ERαKO mouse was generated in the laboratory of Pierre Chambon and deletes exon 3 of ERα, which encodes the first zinc finger of the DNA binding domain. This has been described as a complete knockout,[33] and so only the bone phenotype of this mouse will be discussed here. As with the original knockout of ERα, the ERβKO and the ERαβKO mice may not be complete knockouts, as these mice express truncated forms of ERβ.[34] Furthermore, different labs with the same line of ERβKO mice have described differing phenotypes. A mouse line with a complete knockout of ERβ (named $ERβ_{ST}^{L-/L-}$) was recently described, but an analysis of the bone has yet to be performed.[34]

Table 1 **The bone phenotypes of female mice and menopausal women**

	$ERα^{ΔOc/ΔOc}$	ERαKO	ERβKO	ERαβKO	OVX (mice)	Menopausal women
Cortical BMD	NC	⇓	NC	⇓	⇓	⇓
Cancellous BMD	⇓	NC	NC	⇓	⇓	⇓

⇓ = decrease, NC = no change, OVX = ovariectomy, BMD = bone mineral density.

Female and male ERα null mice have a decrease in cortical bone mineral density, whereas ERβKO mice have normal cortical and cancellous bone mineral density (Table 1). The ERαβKO mice have both decreased cortical and decreased cancellous bone mineral density, suggesting that ERα and ERβ can replace each other in cancellous bone, but they also have distinct roles. It will be interesting to analyze the $ERβ_{ST}^{L-/L-}$ line to determine if it has a bone phenotype. Usage of the splice variants expressed in the ERβKO compared with the $ERβ_{ST}^{L-/L-}$ may provide important biochemical information about a function for ERβ in bone.

Is FSH, not Estrogen, Directly Responsible for Bone Loss after Menopause?

Ovariectomy of mice leads to a greater bone loss than either the ERα or ERβ single knockouts or the ERαβKO. Thus, it is thought that the loss of estrogen has an effect independent of ERα or ERβ. One hypothesis is that follicle stimulating hormone (FSH) is responsible for the bone loss after loss of ovarian hormones.[35] During menopause, as estrogen levels decrease, serum levels of FSH increase. FSHβ and FSH receptor (FSHR) knockout mice do not undergo bone loss after ovariectomy,[35] nor do rats that have been both hypophysectomized and ovariectomized,[36] supporting the idea that FSH is mainly responsible for ovariectomy-induced bone loss. However, complicating the in vivo analysis, the FSHR knockout mice have an increased level of testosterone,[37] and testosterone (via aromatization to estrogen or testosterone itself) is protective of bone.[38] Despite the effects of testosterone, FSH acts, in part, directly on osteoclasts, as osteoclasts express the FSH receptor, and respond to FSH in vitro to activate osteoclastogenesis.[35]

While FSH may play an important role in regulating bone resorption, the suggestion that the main role estrogen plays is to regulate FSH cannot be supported. Estrogen plays an important role in the transcriptional regulation of cytokines and induces apoptosis of osteoclasts in the absence of FSH. Further studies must be done in vivo and in vitro to dissect the potential contribution of FSH.

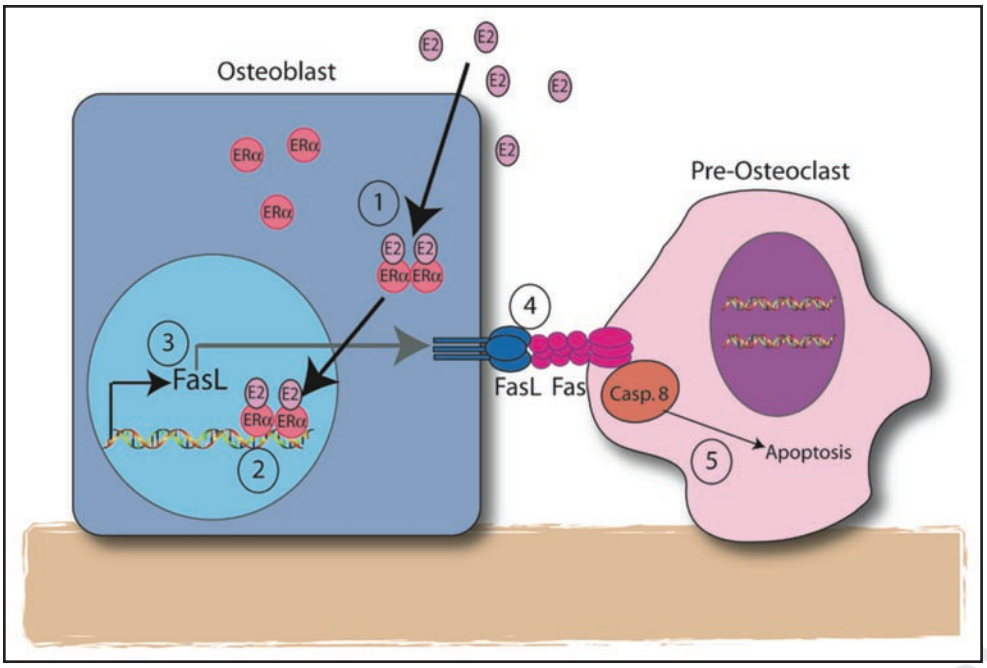

Figure 1. Estrogen upregulates FasL in osteoblasts to induce apoptosis in osteoclasts. (1) Estrogen binds to ERα in the cytoplasm, leading to nuclear localization of ERα. (2) ERα binds to enhancers 86 kilobases and 326 kilobases downstream of the FasL transcriptional start site. (3) Transcription of FasL is induced, and then FasL (4) binds to the Fas receptor. Ligand activation of the Fas receptor leads to (5) caspase 8 cleavage and subsequent apoptosis of the pre-osteoclast.

ERα Knockout in Osteoclasts Leads to Low Cancellous Bone Mineral Density

Because of the complexity of the different ERKO phenotypes ERα was specifically knocked out in osteoclasts ($ER\alpha^{\Delta Oc/\Delta Oc}$) using a cathepsin K-cre recombinase to determine the effect of ERα only in osteoclasts.[4] The female $ER\alpha^{\Delta Oc/\Delta Oc}$ mice have a decrease in trabecular bone, but not in cortical bone, as do the ERαKO mice (Table 1). After menopause, women have a decrease in both cortical and trabecular bone density,[39] mimicking the knockout of ERα and ERβ in all tissues and that of ovariectomized mice (Table 1). This is in contrast to the phenotype of $ER\alpha^{\Delta Oc/\Delta Oc}$; the difference in phenotype between the ERαKO and the $ER\alpha^{\Delta Oc/\Delta Oc}$ bones remains to be explained.

In $ER\alpha^{\Delta Oc/\Delta Oc}$ mice, apoptosis did not occur in osteoclasts when treated with estrogen, suggesting that ERα is necessary in osteoclasts.[4] Fas Ligand (FasL) was identified by microarray analysis as a gene that was no longer upregulated upon treatment of whole bones with estrogen.[4] It was hypothesized that because only the osteoclasts are missing ERα, that estrogen induces FasL in the osteoclasts, suggesting an autocrine stimulation of cell death. However, because whole tissues were used it could not be determined where FasL is being upregulated. It is possible that ERα is needed in the osteoclasts to induce FasL in osteoblasts (see below). The data of Nakamura, et al., is in contrast to our data,[2] where we find that estrogen induces FasL in osteoblasts, not in osteoclasts (see below).

One possibility for the difference is that loss of ERα in the $ER\alpha^{\Delta Oc/\Delta Oc}$ is not limited to osteoclasts. Other cell types, namely osteoblasts, were not tested for the presence of ERα in these studies. In addition, the use of lacZ as a reporter in bone is problematic as osteoclasts stain positive for lacZ.[40]

Estrogen Induces FasL in Osteoblasts to Kill Osteoclasts

Recent work from our lab shows that estrogen, via ERα, induces transcription of FasL in osteoblasts resulting in a paracrine signal to modulate osteoclast survival[2] (Fig. 1). Thus, the key difference between the Nakamura[4] paper and our model is the origin of FasL expression—osteoblasts or osteoclasts.

We performed several experiments to show that FasL is transcriptionally upregulated by estrogen in osteoblasts. The first is that FasL is induced by estrogen in purified osteoblasts. Estrogen induces FasL in the cell lines MC3T3, which is a calvarial-derived osteoblast cell line, and in U20S osteosarcoma cells that stably overexpress ERα. In addition, primary calvarial osteoblasts also express higher levels of FasL after treatment with estrogen. Second, estrogen induces FasL specifically in osteoblasts (as demonstrated by co-localization with RUNX2) using in vitro bone marrow differentiation assays and in vivo at the growth plate and endosteal surface, and not in osteoclasts at either location. Furthermore, antibody-purified osteoclasts do not undergo estrogen-induced apoptosis unless osteoblasts are added in a co-culture system. Finally, co-cultures of MC3T3 osteoblasts and ERαKO bone marrow derived osteoclasts demonstrate that ERα in osteoblasts is sufficient for osteoclast apoptosis.

To further demonstrate the cell-type specificity, estrogen was added to MCF7 breast cancer cells and primary osteoclasts in addition to osteoblasts. Estrogen only upregulates FasL mRNA in osteoblasts. Then, to understand the molecular mechanism of FasL upregulation, chromatin immunoprecipitation (ChIP) was performed in two cell types: MCF7, as a model for the lack of FasL upregulation, and osteoblasts. U20S-ERα cells were used as a model of an osteoblast-like cell line. ERα was shown to bind to an enhancer 86 kilobases downstream of the FasL gene[2] correlating with the upregulation of FasL. In MCF7 cells ERα does bind to the +86 kb FasL enhancer, however the level of binding is significantly less than in U20S-ERα cells.[2] In addition, we have recently identified a second enhancer ~240 kilobases downstream of the first enhancer by ChIP-on-chip analyses in U20S-ERα cells. This enhancer is specific to ERα in U20S-ERα cells (Fig. 2). The direct regulation of FasL mediated by the binding of ERα to chromatin argues that non-genomic signaling alone is unlikely to be responsible for the ability of estrogen to induce osteoclast apoptosis, as had been suggested.[3]

Importantly, selective estrogen receptor modulators (SERMs) also stimulate FasL expression in osteoblasts to induce apoptosis in osteoclasts. Tamoxifen and raloxifene recruit ERα to the +86 kb FasL enhancer, as does estrogen.[2] This agonist activity is critical for the development of SERMs as a treatment for osteoporosis.

Conclusions

Estrogen regulates bone mineral density acting via a variety of mechanisms and cell types. The effect of estrogen is both pro-osteoblastic and anti-osteoclastic leading to maintenance of bone. In addition, estrogen represses osteoclastogenic cytokine production from T-cells, bone marrow stromal cells and osteoblasts.

An unanswered question is what the roles of ERα and ERβ are in each bone cell type. ERα and ERβ are observed by immunohistochemistry in osteoclasts, osteoblasts and osteocytes. ERβ was not found at the FasL enhancer in osteoblasts and estrogen-induced apoptosis was observed in osteoclasts differentiated from ERβKO bone marrow,[2] suggesting that ERβ is not involved in the FasL induction in response to estrogen. The phenotypes of the ERαKO, ERβKO and ERαβKO suggest that ERα and ERβ both have functions in the bone, with both overlapping functions and distinct functions. Thus, the roles for ERα and ERβ in the osteoclast, in addition to other possible roles for ERα and in the osteoblast, remain to be fully understood.

At present, these different hypotheses have been proposed to explain the role of estrogen in maintaining bone mineral density. Our data strongly support the role of direct ERα-mediated FasL transcriptional induction in osteoblasts to promote apoptosis in osteoclasts. Further studies are needed to sort out the potential direct role of ERα in osteoclasts. What is clear is that a fuller understanding of the role of estrogen (and SERMs) in bone biology will lead to the development of improved therapies for the prevention and treatment of menopause associated bone loss.

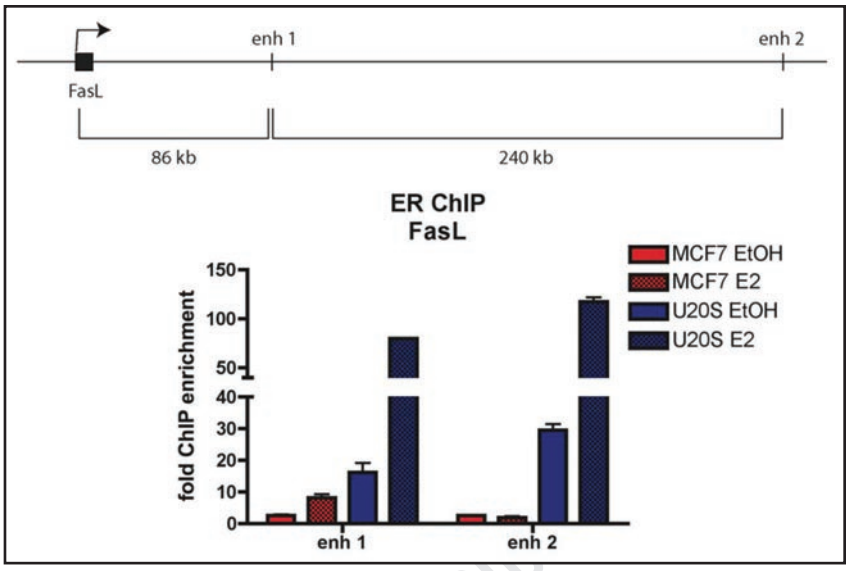

Figure 2. ERα is recruited to two enhancers downstream of the Fas Ligand gene. U20S-ERα cells and MCF-7 cells were deprived of estrogen for 3 days in phenol red-free media containing 5% CDT-FBS. They were then treated with 10 nM E2 for 45 minutes. ERα was immunoprecipitated, DNA was isolated and quantitative PCR was performed at the indicated sites of the FasL locus. Each PCR signal was normalized to input and is represented as fold enrichment over a control genomic locus.

Acknowledgements

The authors wish to thank Drs. G. Miranda-Carboni, M. Torres-Arzayus and J. Cook for helpful review of the manuscript. This work was supported by a post-doctoral fellowship from the Susan G Komen Breast Cancer Foundation to SAK. This work was also supported in part by a grant from Wyeth Research where M.B. serves as a scientific advisor.

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