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# Expression of Estrogen Receptor β in Rat Bone

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**Abstract.** A novel estrogen receptor, estrogen receptor β (ERβ), has recently been cloned from a rat prostate cDNA library. In bone, which is an important target tissue of estrogen, ERα has been reported to be present preferentially in osteoblasts, but the mechanism of action of estrogen in bone is still not known. In the present study, we examined expression of ERβ mRNA in bone. Expression of ERβ mRNA was evident in primary osteoblastic cells isolated from 1-day-old rat calvaria and rat osteosarcoma cells (ROS 17/2.8), and its level was higher than that of ERα mRNA. When osteoblastic cells were cultured for 28 days to induce differentiation into mature osteoblasts capable of forming bone nodules, ERβ mRNA was constantly and highly expressed during the entire culture period. In contrast, the level of ERα mRNA was very low at the beginning of culture and it gradually increased during the differentiation of osteoblastic cells. Various tissues including bone were isolated from 8-week-old rats of both sexes, and total RNA was extracted to compare the tissue distribution of expression levels of ERβ mRNA. In cancellous bone of the distal femoral metaphysis and lumbar vertebra, expression of ERβ mRNA was obvious, and its level was equivalent to those in the uterus and testis, but lower than those in the ovary and prostate. The level of ERβ mRNA in femoral cortical bone was lower than that in cancellous bone. There was no appreciable differences between female and male rats in the distribution and expression levels of ERβ mRNA in bone. These results indicate that ERβ mRNA is highly expressed in osteoblasts in rat bone, suggesting that there is a distinct mechanism of estrogen action mediated by ERβ in bone.

## Introduction

Estrogen affects bone metabolism, and its deficiency caused by ovariectomy (OVX) results in marked bone loss by stimulating osteoclastic bone resorption. Recent studies have focused on the possible involvement of bone-resorbing cytokines such as Interleukin-1 (IL-1), IL-6, and TNFα in the stimulation of bone resorption due to estrogen deficiency (1-4). It is likely that estrogen suppresses the production of these cytokines by osteoblasts and bone marrow stromal cells. We reported that OVX selectively stimulated B-lymphopoiesis which resulted in marked accumulation of pre-B cells in mouse bone marrow, and that the increased B-lymphopoiesis could induce osteoclastic bone resorption, leading to bone loss in vivo (5, 6). Estrogen suppresses stromal cell-dependent differentiation of B cells (5, 21). Taken together, estrogen appears to act on osteoblasts and bone marrow stromal cells to regulate bone metabolism and hemopoiesis. However, the mechanism of action of estrogen in bone is still a matter of controversy.

A novel estrogen receptor, estrogen receptor β (ERβ), has recently been cloned from a rat prostate cDNA library (7). A marked expression of ERβ has been shown in the prostate and ovary. There was a selective expression of ERβ mRNA in granulosa cells of follicles in ovary, suggesting that estrogen regulates the differentiation and maturation of follicles in an ERβ-dependent mechanism (8). Most estrogen and anti-estrogen compounds bind to both classical ER (ERα) and ERβ, and their binding affinity is almost identical between ERα and ERβ (9, 10). Although ERβ transactivates promoters containing estrogen responsive elements (ERE) in an estradiol-dependent manner, the molecular mechanism to regulate the transcriptional activity of ERβ appears to be distinct from that of ERα (11). Therefore, it is possible that estrogen exhibits its tissue-specific actions in an ERβ-dependent mechanism as well. In bone, ERα has been reported to be present preferentially in osteoblasts, but the mechanism of action of estrogen is still not known. In the present study, we demonstrate that expression of not only ERα but also ERβ is evident in rat primary osteoblastic cells and a rat established osteosarcoma cell line (ROS17/2.8).

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## Materials and Methods

### *Animals and drugs*

Male and female Wistar rats (newborn and 8-week-old) were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Dexamethasone was purchased from Sigma (St. Louis, MO), and ascorbic acid was from WAKO Pure Chemicals (Osaka, Japan). All other chemicals were of analytical grade.

### *Culture of primary rat osteoblastic cells and established rat osteosarcoma cells (ROS 17/2.8)*

Primary osteoblastic cells were isolated from 1-day-old rat calvaria after routine five sequential digestions with 0.1% collagenase (WAKO) and 0.2% dispase (Godo Shusei, Tokyo, Japan) as reported (12). Osteoblastic cells isolated from 3 to 5 fractions were combined and cultured in α-minimal essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ℃ in a humidified atmosphere of 5% $CO_2$ in air. To measure steady state levels of ERα and ERβ mRNAs, osteoblastic cells were cultured for 3 days.

For a long term culture, osteoblastic cells were cultured for 28 days in αMEM containing 1 nM dexamethasone and 50 μg/ml of ascorbic acid as reported (13). To detect calcified bone nodules, alizarin red-S staining was performed and the number of calcified nodules stained red was counted.

Rat osteosarcoma cells (ROS 17/2.8) were cultured in Ham's F12 medium (ICN, Costa Mesa, CA) containing

10% FBS, and confluent cells were used for the preparation of total RNA.

***RT-PCR analysis of osteoblastic cells and various rat tissues***

Male and female rats, 8-week-old, were killed by cervical dislocation, and various tissues were collected. Cancellous bone was surgically collected from the distal femoral metaphysis, and cortical bone was from the femoral diaphysis. The fifth lumbar vertebra (L5) was collected and ground into small pieces. Tissue samples and cultured osteoblastic cells were processed for total RNA isolation according to the acid guanidium-phenol-chloroform method (12).

cDNA synthesis was performed using random hexamers as primers. For PCR amplification, 5 % of the synthesized cDNA pool was added to reaction mixtures and amplified for 25-35 cycles by incubating it at 95℃ for 30 sec, 57℃ (ERα) or 66℃ (ERβ) for 15 sec, 72℃ for 60 sec, and by finally incubating at 72℃ for 3 min in a thermal cycler (DNA Thermal Cycler 480; Perkin-Elmer, Norwalk, CT). The oligonucleotide primers, 5'-AAAGCCAAGAGAAACG GTGGGCAT-3' (sense primer) and 5'- GCCAATCATGTGC ACCAGTTCCTT-3' (anti-sense primer), were used for amplification of a 203-bp fragment of the ERβ mRNA as previously reported (7). The oligonucleotide primers, 5'-AA TTCTGAC AATCGACGCCAG-3' (sense primer) and 5'-GT GCTTCAACATTCTCCCTCCTC-3' (anti-sense primer), were used for amplification of a 344-bp fragment of the ERα mRNA as previously reported (10). The oligonucleotide primers, 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' (sense primer) and 5'-CATGTAGGCCATGAGGTCCACCA C-3' (anti-sense primer), were used for amplification of a 983-bp fragment of G3PDH mRNA. After agarose gel electrophoresis, the gels were examined with an image analyzer (Micro Computer Imaging Device; Fuji-film, Tokyo, Japan) to determine the intensities of the signals derived from the respective DNA fragments.

## Results

***Expression of ERβ mRNA in rat osteoblastic cells***

To examine expression of ERβ mRNA in rat osteoblasts, total RNA was extracted from primary osteoblastic cells isolated from 1-day-old rat calvaria, and RT-PCR analysis was performed for various numbers of cycles. After 25 cycles, ERβ mRNA was slightly detected in osteoblastic cells and it was elevated with increasing number of cycles (Fig. 1). On the other hand, ERα mRNA was hardly detected in primary osteoblastic cells from 25-35 cycles. In rat osteosarcoma cells (ROS17/2.8), both ERα and ERβ were expressed, and the level of ERβ mRNA was higher than that of ERα (Fig. 1).

When primary osteoblastic cells were cultured for 28 days in the presence of ascorbic acid and dexamethasone, osteoblastic cells differentiated into mature osteoblasts and formed bone nodules (Fig. 2A). ERβ mRNA was constantly and highly expressed in osteoblastic cells until day 28 in culture (Figs. 2B and 2C). In contrast, the level of ERα mRNA was very low at the beginning of culture and it was gradually elevated during the differentiation of osteoblastic cells.

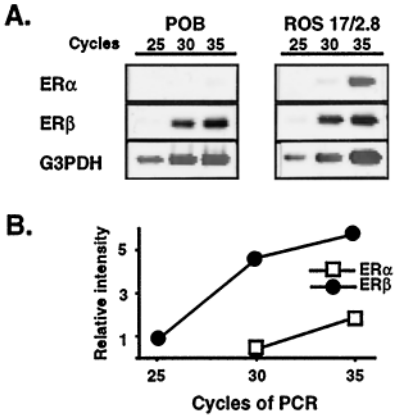


**Figure 1** Expression of ERα and ERβ mRNAs in rat osteoblastic cells. **A.** Total RNA was extracted from primary osteoblastic cells (POB) and rat osteosarcoma cells (ROS 17/2.8), and subjected to RT-PCR for ERα and ERβ mRNAs for 25-35 cycles. Agarose gel electrophoresis of products obtained by PCR was performed and an image analyzer was used to quantify the signals. **B.** Signals in the RT-PCR of primary osteoblastic cells shown in A were quantified and normalized relative to the signals of G3PDH to compare mRNA expression of ERα (□) and ERβ (●).

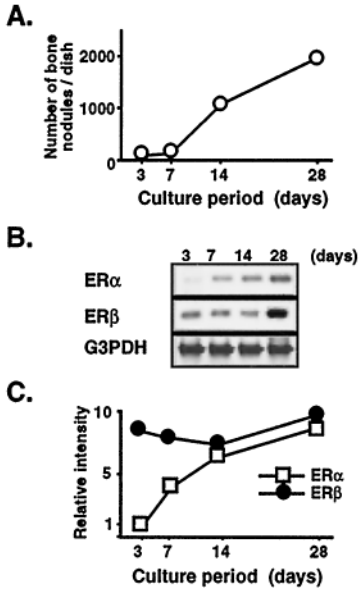


**Figure 2.** Time course of change in mRNA expression of ERα and ERβ in primary osteoblastic cells during the differentiation into mature osteoblasts. Primary osteoblastic cells were cultured for 28 days as described in Materials and Methods. **A.** The number of bone nodules was counted after alizarin red-S staining. **B.** Expression of ERα and ERβ mRNAs was analyzed by RT-PCR for 35 cycles. **C.** Signals in the RT-PCR shown in B were quantified and normalized relative to G3PDH using an image analyzer to compare mRNA expression of ERα (□) and ERβ (●).

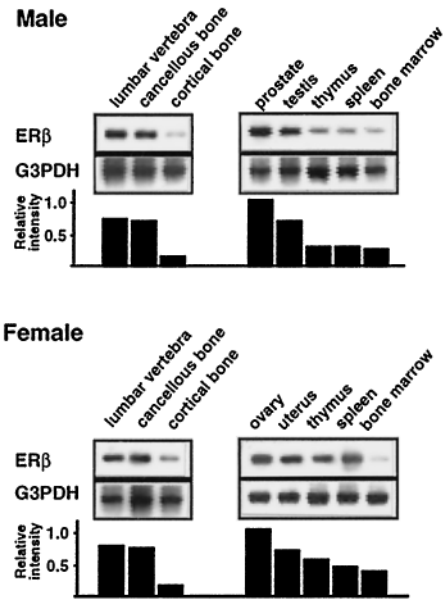


**Figure 3.** Expression of ERβ mRNA in bone in male and female rats. Cancellous bone of the distal femoral metaphysis, cortical bone of the femoral diaphysis, and lumbar vertebra (L5) were collected from 8-week-old male and female rats. Total RNA was extracted from respective bones and other tissues, and subjected to RT-PCR for 35 cycles to examine the expression of ERβ mRNA. Signals in the RT-PCR were quantified and normalized relative to G3PDH using an image analyzer.

### *Comparison of ERβ mRNA expression between bone and other tissues in female and male rats*

To compare expression of ERβ mRNA between bone and other tissues, tissues were isolated from 8-week-old rats of both sexes, and total RNA was extracted for RT-PCR analysis. For bone tissues, cancellous bone of the distal femoral metaphysis, cortical bone of the femoral diaphysis, and lumbar vertebra (L5) were collected. As reported, expression of ERβ mRNA was the highest in the ovary in female and prostate in male rats (7, 8, 10; Fig. 3). In male rats, the level of ERβ expression in the testis was lower than in prostate, but higher than in other tissues such as the spleen, thymus and bone marrow. In female rats, ERβ mRNA was similarly expressed in the uterus, spleen and thymus. In cancellous bone of the distal femoral metaphysis and lumbar vertebra, ERβ mRNA was highly expressed, and its level was equivalent to those in the uterus and testis (Fig. 3). The level of ERβ mRNA was much lower in cortical bone than in cancellous bone (Fig. 3), indicating the site-specific expression of ERβ mRNA in bone. The distribution and the expression level of ERβ mRNA in bone were similar between female and male rats.

## Discussion

The present study clearly demonstrates that ERβ mRNA is highly expressed in bone at levels similar to those in the uterus and testis but lower than those in the ovary and prostate. Expression of ERβ mRNA was higher in cancellous bone of the distal femoral metaphysis and lumbar vertebra than in cortical bone of the femoral diaphysis (Fig. 3). It is known that estrogen deficiency caused by OVX induces bone resorption preferentially in cancellous bone. Therefore, it is likely that estrogen regulates bone remodeling mainly in cancellous bone. In primary osteoblastic cells, ERβ mRNA was stably and highly expressed during the entire culture period, whereas the expression of ERα mRNA was very low at the beginning of culture and it increased gradually during the differentiation of osteoblasts into mature osteoblasts (Fig. 2). In rat osteosarcoma cells (ROS17/2.8), expression of both ERα and ERβ was detected, and the latter was higher than the former (Fig. 1). These results suggest that the relative level of expression of these two ERs changes during the differentiation of osteoblasts. Recently, Byers *et al.* (8) reported that ERβ mRNA is localized mainly in the granulosa cells of small, growing and preovulatory follicles, but ERα mRNA is expressed at a low level throughout the ovary with no particular cellular localization. Further studies are needed using in situ hybridization to examine the distribution of ERβ in bone.

Like estrogen deficiency, androgen deficiency also causes bone loss by stimulating osteoclastic bone resorption. Recent studies have focused on the role of estrogen in bone metabolism not only in females but also in males. In male rats and mice, bone loss induced by orchidectomy could be prevented by treatment with estrogen (14, 15). Morishima *et al.* (16) reported that a man with an aromatase gene mutation, in whom testosterone was not metabolized into estrogen, showed low bone mineral density. These results suggest that estrogen regulates bone metabolism in the male as well. ERβ mRNA in bone was expressed equally in female and male rats (Fig. 3). This is consistent with the recent findings which suggest an indispensable role of estrogen in the bone in males. Using ERα knockout mice, Korach and his associates reported that the estrogen action was greatly suppressed in reproductive tissues in both females and males (17-18). Furthermore, they reported that the development and maintenance of bone were not solely dependent on ERα (19). Recently, Iafrati *et al.* (20) reported that estrogen inhibits the injury-induced vascular lesions in ERα knockout mice. This indicates that there is an ERα-independent mechanism in estrogen action at least in blood vessels. Osteoblasts express both ERα and ERβ mRNAs. It is not known whether osteoclasts express ERα and/or ERβ, and whether estrogen directly acts on osteoclasts to suppress bone resorption. Further studies are needed to examine what action of estrogen is mediated by ERα and ERβ in bone.

Recent studies have focused on the possible involvement of bone-resorbing cytokines such as IL-1, IL-6, and TNFα in the stimulation of bone resorption due to estrogen deficiency (1-4). Estrogen may suppress the production of these cytokines by osteoblasts and bone marrow stromal cells. In addition, we have reported that OVX selectively stimulates B-lymphopoiesis which results in marked accumulation of pre-B cells in bone marrow. Estrogen suppresses stromal cell-dependent B cell differentiation in vitro (5, 21). Increased B-lymphopoiesis, not only by OVX but also by IL-7 treatment, caused osteoclastic bone resorption in vivo, resulting in a marked

bone loss (6). Estrogen may act on osteoblasts and stromal cells to regulate bone remodeling and hemopoiesis. In the present study, ERβ mRNA was detected in the thymus and bone marrow, which may indicate an ERβ-dependent action of estrogen in hemopoiesis and the immune system.

The present study has confirmed the tissue distribution of ERβ mRNA in male rats reported by Kuiper *et al.* (10). In addition, the levels of ERβ mRNA expression in various tissues including spleen, thymus, kidney and liver were higher in female than in male rats (Fig. 3, data not shown). ERβ mRNA was expressed at a low level in the stomach and small intestine in males, but was stably expressed at a higher level in females (data not shown). These results suggest that there is a sex-dependent expression of ERβ in some tissues.

In summary, ERβ mRNA was highly expressed in osteoblastic cells of rat bone. The level of expression of ERβ mRNA was much higher in cancellous bone than in cortical bone, and the level of ERβ was equivalent between female and male rats. These results suggest a distinct mechanism of action of estrogen regulated by ERβ in bone. Investigation of the ERβ-mediated mechanism of action of estrogen in bone is essential to understand the pathogenesis of bone loss in postmenopausal osteoporosis.

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