EXPERIMENTAL STUDY

Estrogen responsiveness of bone formation *in vitro* and altered bone phenotype in aged estrogen receptor- α -deficient male and female mice

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Abstract

Objective: Although the beneficial effects of estrogen on bone are well known, the roles of estrogen receptors (ERs) in mediating these effects are not fully understood.

Methods: To study the effects of long-term ER α deficiency, bone phenotype was studied in aged ER α knockout (ERKO) mice. In addition, ERKO osteoclasts and osteoblasts were cultured *in vitro*.

Design and results: Histomorphometric analysis showed that the trabecular bone volume and thickness were significantly increased and the rate of bone formation enhanced in both male and female ERKO mice in comparison to the wild-type animals. In ERKO males, however, the bones were thinner and their maximal bending strengths decreased. Consistent with previous reports, the bones of knockout mice, especially of female mice, were shorter than those of wild-type mice. In addition, the growth plates were totally absent in the tibiae of aged ERKO females, whereas the growth plate cartilages were detectable in wild-type females as well as in all the males. Analysis of cultured bone marrow cells from 10- to 12-week-old mice demonstrated that 17 β -estradiol could stimulate osteoblastic differentiation of bone marrow cells derived from ERKO mice relatively to the same extent as those derived from wild-type mice. This was demonstrated by increases in synthesis of type I collagen, activity of alkaline phosphatase and accumulation of calcium in cultures. Total protein content was, however, reduced in ERKO osteoblast cultures.

Conclusions: These results show altered bone phenotype in ERKO mice and demonstrate the stimulatory effect of estrogen on osteoblasts even in the absence of full-length ER α .

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Introduction

Estrogens are key regulators of bone growth, maturation and metabolism both in females and males. In menopause, decreased estrogen leads to an increased rate of remodeling, which is caused by an imbalance between bone resorption by osteoclasts and new bone formation by osteoblasts. This condition is associated with a decrease in bone mineral density leading to an increased fracture risk. Lack of estrogen in aromatase deficiency (due to mutations in the cytochrome P450 subfamily XIX (CYP19) gene) results in decreased bone mineral density, delayed epiphyseal closure and tall stature in affected humans (1, 2). Therapeutic responses of these patients to estrogen, but not to androgen therapy, have been demonstrated (2). Bone loss was also demonstrated experimentally in aromatase knockout mice (3, 4) and in the mice treated with aromatase inhibitors (5). Male, but

not female, aromatase knockout mice have, however, a shortened femur length (3), which is opposite to what was found in the aromatase-deficient humans.

At cellular level 17 β -estradiol (E2), a biologically active form of estrogen, is known to stimulate osteogenic activity (6, 7) and to have anti-apoptotic effects in osteoblasts (7–9). It is also well demonstrated that osteoclast formation from mononuclear hematopoietic stem cells is inhibited by E2 treatment (10, 11). In addition, we have recently shown that bone resorption activity of mature osteoclasts is inhibited by E2, which leads to reduced depth of resorption lacunae (12). Most estrogen effects are thought to be mediated via nuclear estrogen receptors alpha (ER α) and beta (ER β), although non-receptor-mediated effects have also been suggested. Both ER α and ER β are expressed in bone marrow cells (13, 14), and in osteoblasts (15–17). Data on ER expression in osteoclasts remains

conflicting. ERs have been reported to be expressed in osteoclasts (18, 19) and in their mononuclear precursors (20, 21) whereas some reports claim that they are not expressed in osteoclastic cells at all (22).

Estrogen resistance due to a disruptive mutation in the ER α gene leads to an altered bone phenotype – as shown by a 28-year-old man who has a tall stature, an incomplete epiphyseal closure and increased bone turnover rate (23). In the *in vitro* cultures, mesenchymal cells of this patient were capable of differentiating to bone-forming osteoblasts, which secreted high levels of insulin-like growth factor-I (IGF-I) and interleukin-6 (24).

Transgenic animal models have provided excellent tools for studies on the role of ERs in bone tissue. These studies have emphasized a central role of ER α in bone physiology (25, 26). ER α deficiency (27) leads to marked consequences in bone as in various other tissues (28). Bone phenotype in ER α knockout (ERKO) mice, however, clearly differs from that in ovariectomized animals (29). Trabecular bone mineral density, for example, has been demonstrated to be increased in ERKO animals (30–32). Studies on ERKO mice have also shown that estrogen does not have a stimulatory effect on cancellous bone formation in male knockout mice (33). It also fails to prevent orchidectomy-induced bone loss in male ERKO animals (31, 34). In a recent study using another ER α -deficient mouse line (ER $\alpha^{-/-}$), E2 could partially protect female ER $\alpha^{-/-}$ mice against ovariectomy-induced bone loss, but no response to E2 was found in orchidectomized male ER $\alpha^{-/-}$ mice (26). Analysis of ERKO mice has also demonstrated that bone adaptation to mechanical loading requires ER α (35).

In this study we used aged (over 1 year old) female and male ER α knockout mice to analyse the effects of long-term ER α deficiency on bone. The long bones (tibiae and femora) were analysed by peripheral quantitative computed tomography, histomorphometry and strength measurements. We also cultured bone cells (osteoblasts and osteoclasts) in *in vitro* conditions to learn whether the presence of full-length ER α is needed for normal cell growth and function. The capacity of bone marrow cells of the ERKO vs wild-type mice to differentiate to osteoblasts and to respond to estrogen was studied in the cultures. We demonstrate in this work that estrogen can stimulate osteoblastic differentiation and bone formation *in vitro* in bone marrow cultures of ERKO mice in the absence of full-length ER α .

Materials and methods

Animals

ERKO mice with a C57Bl/6 genetic background generated as previously described (27) were a gift from Dr Kenneth Korach (National Institute of Environmental Health Sciences, National Institutes of Health,

Research Triangle Park, North Carolina, USA). They were transferred to and bred in the animal facilities of the University of Turku. PCR-based genotyping was performed on DNA extracted from mouse tail tips. Primers for determination of the wild-type ER gene were CGGTCTACGGCCAGTCGGGCACC and GTA-GAAGGCGGGAGGGCCGGTGTC (27). Primers for the knockout gene were TGTGGCCGGCTGGGTGTG and GGCGCTGGGCTCGTTCTC (36).

Mice were given standard commercial food (RM1, SDS Ltd, Witham, Essex, UK) and water *ad libitum*. The animals were maintained in accordance with the guidelines of the Turku University Ethical Committee for the use and care of experimental animals. Fifty-two ERKO or wild-type mice (over 1 year old) were used for histomorphometric analysis and peripheral quantitative computed tomography (pQCT). In order to study bone formation *in vivo*, mice were given intraperitoneal injections of tetracycline hydrochloride (15 mg/kg; Sigma) and calcein to label bone-forming surfaces (15 mg/kg; Sigma) at 17 and 3 days respectively before killing the animals.

pQCT

The mice were killed by CO₂ asphyxiation. Right tibiae and femora were excised and stored at –20 °C. For analysis, they were first thawed at room temperature for 1 h. After cleaning up soft tissues, the bone was fixed in a plastic tube (8 mm diameter) with a spring and scanned by pQCT (XCT 540; Stratec, Norland Medical Systems, Pforzheim, Germany). For tibial measurements, the reference line was placed at the proximal end of tibia. Three cross-sections, with a 0.5 mm distance between each other, were measured 1.5 mm from the reference line. Two sections with a distance of 0.5 mm, 3 mm above the reference line in the tibiofibular junction were also measured. In femur, three sections were measured starting 1.8 mm from the distal end of the femur, and another two sections were measured 5 mm from the distal end of the femur. Special Software version 5.40 was used for the images of each section with a voxel size of 0.10 mm. A standardized analysis (peel mode 2, cort mode 1, contour mode 1, threshold 0.25 g/cm³ for trabecular bone and 0.71 g/cm³ for cortical bone) was applied.

Mechanical testing of bones and determination of ash weight

After measuring the bone density and length, the maximal bending load was measured from the right tibiae and the right femora as described previously (37). The lateral surface of the tibia or posterior surface of the femur was put on the supports. The span of the supports was 8 mm. Finally, the fractured tibiae and femora were burned at 550 °C for 20 h to get the individual bone ash weight.

Bone histomorphometry

Left tibiae and femora were fixed in 40% ethanol. After dehydration, samples were embedded into methylmethacrylate as described previously (37). Longitudinal undecalcified sections (4 and 8 μ m thick) were prepared from tibia. Cross-sections of 80 μ m were harvested 6 mm above the distal end of the femur by a low-speed saw. Sections of 4 μ m thickness were stained using the Masson–Goldner trichrome method and the histomorphometric data of the trabecular bone in the proximal tibia were measured in a field of 0.75 mm² below the growth plate and locating the middle of the section. Because the growth plate disappeared in female ERKO mice, the measured field was 0.9 mm below the proximal end of the tibia. Unstained sections of 8 and 80 μ m thickness were used to measure the dynamic parameters of bone formation. All measurements and analyses were performed according to the report of the ASBMR Histomorphometry Nomenclature Committee (38), and using an Olympus microscope combined to a computer with the OsteoMeasure V 2.31-program (OsteoMetrics Inc., Atlanta, GA, USA).

Hormone measurements

Serum samples were collected by cardiac puncture, separated by centrifugation and stored at -20°C until used for testosterone and E2 measurements. Testosterone levels were measured by DELFIA after diethyl ether extraction, and the concentrations of E2 were measured using commercial RIA kits (Wallac Delfia; Perkin Elmer, Turku, Finland) as described previously (39). Testosterone levels were measured from serum samples of the same animals as were used for histomorphometric analysis. The amount of serum collected was not sufficient for estradiol measurements in all the animals. Thus, serum was collected for estradiol analysis also from other aged ERKO and wild-type animals. Estradiol data are a combination of original samples (from 12 mice) and new serum samples (from 21 mice).

Cell culture reagents

All cell culture reagents, if not otherwise mentioned, were obtained from Invitrogen. E2 (Sigma) and ICI 182,780 (a gift from Dr A E Wakeling; Zeneca Pharmaceuticals, Macclesfield, UK) were dissolved in ethanol as a 1 mM stock solution. Peroxidase-conjugated wheat germ agglutinin (WGA)-lectin, Hoechst 33 258, leukocyte acid phosphatase kit 387-A, dexamethasone, β -glycerophosphate and ascorbic acid 2-phosphate were purchased from Sigma.

Recombinant human bone morphogenetic protein-4 (BMP-4) was from R&D Systems Inc. (Minneapolis, MN, USA).

Osteoclast cultures

A mixed mouse bone cell population was cultured on bovine bone slices as described previously (40, 41). Briefly, 3-day-old mouse pups were killed by cervical dislocation and osteoclasts and other cells were scraped from tibiae, femora and humeri and allowed to attach to bone slices for 30 min, after which the non-attached cells were washed away. The cells on bone slices were cultured for 3 days in phenol-red-free minimal essential medium (MEM) α medium buffered with 20 mM HEPES and containing 100 IU penicillin/ml, 100 μ g streptomycin/ml and 10% heat-inactivated fetal calf serum (FCS) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After the culture period, bone slices with cells were fixed in 3% paraformaldehyde for 15 min. Cells were stained with tartrate-resistant acid phosphatase (TRACP) to detect osteoclasts, and with the DNA-binding fluorochrome Hoechst 33 258 to visualize all the nuclei. Multinucleated TRACP-positive cells were counted as osteoclasts and the average number of nuclei per osteoclast was quantified. In addition, bone slices were stained with TRITC-conjugated phalloidin (Sigma) to study actin ring formation in cultures (42).

After the cell parameters and the number of actin rings were determined, all cells were removed from bone slices. Resorption lacunae were visualized by WGA-lectin according to Selander and co-workers (43). Briefly, bone slices were incubated with peroxidase-conjugated WGA-lectin for 40 min, after which diaminobenzidine solution was added to the bone slices for 10 min. The number of resorption pits was quantitated under a Leica DMRB microscope (Leica, Wetzlar, Germany).

Osteoblast cultures

Osteoblasts were cultured in phenol-red-free MEM α medium supplemented with 10% FCS, 20 mM HEPES, 10 nM dexamethasone, ascorbic acid (50 mg/l), 10 mM sodium β -glycerophosphate and antibiotics. Bone marrow cells were obtained from the tibiae and femora of 10- to 12-week-old mice as previously described (6). Animals were killed by cervical dislocation, after which bones were removed and the soft tissues detached aseptically. Metaphyses from both ends were resected and bone marrow cells collected by flushing the diaphysis with warm culture medium repetitively. Nucleated cells were counted with a hemocytometer, and cells were plated in tissue culture flasks (Nunc, Roskilde, Denmark) at a density of 10⁶ cells/cm², and cultured for 7 days by replacing the medium after 3 days. On day 7, cells were washed with warm PBS and adherent cells were detached into the medium using a cell scraper. After centrifugation, cells were counted and plated in 24-well plates at a density of 10 000 cells/well. All

these subcultures were made in duplicate, since alkaline phosphatase (ALP) activity and calcium content of culture were measured from different cell lysates. ALP activity was measured on subculture day 9 (day 16) and calcium on subculture day 14 (day 21). N-terminal propeptide of type-I procollagen (PINP) was measured from culture medium on subculture days 3, 6 and 9.

Assay of the activity of cellular ALP

After the culture period, mouse bone marrow cells were washed twice with warm PBS, and extracted into 200 µl assay buffer containing 50 mM Tris-HCl, 0.1% Triton X-100 and 0.9% NaCl (pH 7.6). After adding the buffer solution to each well, culture plates were frozen. After thawing, cell lysate was rinsed out and enzyme activity measured using 0.1 M 4-*p*-nitrophenylphosphate (Sigma) as substrate. Absorbance was read at 405 nm in a plate reader (Victor 2, Wallac, Turku, Finland). Each sample was measured in duplicate. In parallel, the protein contents of the wells were determined by BIO-RAD protein assay (BioRad). Specific activity of ALP was expressed as absorbance per protein (mg/ml).

Determination of calcium content

Calcium content in the mouse bone marrow cell cultures was determined after 14 days in subculture (21 days in culture). Cells on 24-well plates were washed two times with Ca²⁺- and Mg²⁺-free PBS and incubated overnight in 200 µl 0.6 N HCl at +4 °C. Extracts were complexed with o-cresol-phthalein-complexon (Roche) and the colorimetric reaction was read at 570 nm in a plate reader. Calcium contents were determined by comparison with a calibrated standard (Roche).

RIA for mouse PINP

PINP was analysed as follows. A synthetic peptide derived from the aminoterminal propeptide of the mouse α1-prochain of type I collagen was manufactured by the Neosystems Laboratoire (Strasbourg, France). The sequence of eight amino acids from the aminoterminal end of the propeptide, QEDIPEVS, was selected and a YC-residue was added to the carboxy-terminal end to enable iodination. For polyclonal antibody production, the peptide was conjugated to 140 µl keyhole limpet and injected intradermally with Freund's adjuvant into two New Zealand white rabbits. Boosters were given at 3-week intervals until a good titer was reached.

The synthetic peptide was labeled with ¹²⁵I by the chloramine T method. The labeled peptide was separated from free iodine with the disposable Sep-Pak C18 reversed-phase cartridges (Waters, Milford, MA, USA) by elution with 11 ml of 0.1 mol/l acetic acid and

3 ml of 50% 2-propanol, 0.1 mol/l acetic acid. In the RIA, 100 µl aliquots of known standards based on the synthetic peptide or unknown samples were incubated with 200 µl tracer solution (~50 000 counts/min) and 200 µl diluted antiserum for 2 h at 37 °C. Then 500 µl of a PEG second antibody were added and the incubation continued at +4 °C for 30 min. The samples were centrifuged at 3000 r.p.m. for 30 min at +4 °C and the supernatants discarded. The radioactivity of the samples was counted with a 1470 Wizard counter (Wallac, Turku, Finland). The specificity of the antibody was assessed by testing the binding of a synthetic peptide of the mouse α1(III) collagen. Medium for RIA was collected from osteoblast cultures at subculture days 3, 6 and 9 (culture days 10, 13 and 16).

RNA isolation and RT-PCR

Mice were killed using CO₂, tissue samples were rapidly excised and frozen in liquid nitrogen. Bone marrow samples were taken from vertically split femurs and tibias with a needle. Total RNA was isolated from tissue samples by the single-step method (44). Complementary DNA synthesis was performed from 1–2 µg total RNA using 90 pmol oligo d(T)18 (New England Biolabs Inc., Beverly, MA, USA), 1 nM dNTPs (Finnzymes, Espoo, Finland), 20 units of RNase inhibitor (Promega) and 10 units of avian myeloblastosis virus (AMV) reverse transcriptase (Finnzymes) in the final volume of 20 µl. Sequences of the primers used for estrogen receptor-related receptor-α (ERRα) PCR were ERRαF: 5' CCAGGCTTCTCCTCACTGTC 3' (exons 5–6) and ERRαR: 5' GCCCCCTCTTCATCTAGGAC 3' (exon 6) resulting in an amplification product of 153 bp. Sequences for ERβ PCR were ERβF: 5' GAAGCTGGCTG-ACAAGGAAC 3' (exon 3) and ERβR: 5' AACGAGGTCTGGAGCAAGA 3' (exon 4) resulting in an amplification product of 187 bp. Sequences for ERRγ PCR were 5' GATGAGCCTCCTCCAGAGTG 3' and 5' TGCACAGCTTCCACATCTTC 3' resulting in an amplification product of 275 bp. Amplification parameters used for ERRα PCRs were: denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min and extension at 72 °C, for 35 cycles. For ERβ and ERRγ PCR, the conditions were the same except for an annealing temperature of 58 °C and a cycle number of 40 for ERβ and an annealing temperature of 60 °C for ERRγ. PCR products (20 µl) and 100 bp ladder (New England Biolabs) were run on a 1.5% agarose gel stained with ethidium bromide.

Statistical analysis

The statistical significance of differences between treatment groups was determined using ANOVA and the unpaired Student's *t*-test was used to compare means between groups. A *P* value of less than 0.05 was considered significant.

Results

Bone ash weight and bone length

Gross bone phenotype in old ERKO mice (average age 14 months) in comparison to wild-type controls was studied by determining bone ash weights and measuring bone lengths. As previously reported in younger mice (30), body weight was significantly increased in aged ERKO females compared with wild-type animals (Fig. 1A). Ash weights of tibiae were also higher in ERKO females (Fig. 1A). Interestingly, ash weights of tibiae and femora were not increased, but rather decreased, in ERKO males. The lengths of tibiae, femora and lumbar vertebrae (L1–L6) were measured and all the bones were shorter in ERKO males, and even more so in ERKO females (Fig. 1B).

Figure 1 (A) Total body weight and ash weight of tibia and femur. (B) Length of lumbar vertebrae (L1–L6), tibia and femur. Values are presented as means $\pm$ s.d. The *P* values from Student's *t*-test between wild-type and ERKO females, or wild-type and ERKO males, are shown as asterisks (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). The number of animals per group was: 11 (WT female), 14 (KO female), 16 (WT male) and 13 (KO male). WT, wild-type; KO, knockout.

pQCT measurements

Bone mineral density (BMD) was measured at the proximal tibia and distal femur of mice (Table 1). Both female and male ERKO mice showed an increased density of trabecular bone. The increase was more pronounced in females, trabecular BMD being even 97% (tibia) or 111% (femur) higher than in wild-type mice. Cortical bone density was slightly increased in the tibial, but not in femoral, shaft of ERKO females. The cortical BMD in ERKO males was similar to that of wild-type males. The tissue area of the proximal tibia and distal femur was markedly reduced in male ERKO mice, but in females it was similar to wild-type mice. Tissue area of bone shafts was significantly reduced (meaning thinner bones) in tibiae of both sexes of ERKO mice and in femora of male ERKO animals compared with wild-type animals. Despite decreased tissue area in tibial shafts, cortical bone area was even increased in tibiae and femora of female ERKO mice suggesting a narrowed bone marrow cavity. In contrast, cortical bone area was decreased in tibiae of ERKO male mice. The polar moment of inertia was significantly higher in the femoral shaft of ERKO females, but it was reduced in the long bone shafts of ERKO males when compared with the wild-type animals (Fig. 2A). This is in agreement with the results of maximal bending strengths (Fig. 2B). Femora of ERKO females had a higher maximal bending strength, whereas bones of ERKO males had a reduced bending strength compared with the controls.

Bone histomorphometry

The left tibia and the femur were used for the histomorphometric analysis. Longitudinal bone sections show the distribution of trabecular bone at the proximal tibia of wild-type and ERKO animals (Fig. 3A). They also demonstrate an increased amount of trabecular bone in both female and male ERKO mice (Fig. 3A). The most conspicuous finding was the total disappearance of the growth plates in aged female ERKO mice whereas they were still open in wild-type females as well as in wild-type and ERKO males. This observation is in accordance with reduced bone lengths in female knockout mice. Histomorphometric analysis of the proximal tibia confirmed that trabecular bone volume (TBV) and trabecular thickness (Tb.Th) were significantly elevated in ERKO females and males (Fig. 3B). The number of osteoclasts on the trabecular bone surface was decreased in ERKO females vs wild-type females (Fig. 3C).

The mineral apposition rate at the proximal tibia was higher in ERKO females and males compared with their wild-type controls (Table 2). Of other parameters for new bone formation only bone formation rate per trabecular bone surface (BFR/BS) and BFR per bone tissue volume (TV) showed statistically significant differences between wild-type and ERKO mice and this was demonstrated

Table 1 pQCT analysis of tibia and femur in wild-type and ERKO mice.

	WT female	ERKO female	WT male	ERKO male
Trabecular density (mg/cm ³)				
Tibia	77±10.8	152±24.2***	147±27.8	204±31.2***
Femur	71±11.3	150±23.5***	160±23.7	189±32.0*
Cortical density (mg/cm ³)				
Tibia	1089±41	1178±100.2*	1096±28.7	1085±23.4
Femur	1033±55	1049±39	1006±31	1024±41
Tissue area (mm ²)				
Proximal tibia	2.56±0.33	2.67±0.28	2.72±0.33	2.23±0.27***
Distal femur	3.42±0.35	3.42±0.23	3.62±0.28	3.10±0.27***
Tissue area of bone shaft (mm ²)				
Tibia	1.53±0.15	1.35±0.07**	1.63±0.15	1.35±0.09***
Femur	2.33±0.23	2.45±0.22	2.42±0.19	2.12±0.16***
Cortical bone area (mm ²)				
Tibia	0.71±0.06	0.80±0.08**	0.77±0.06	0.66±0.04***
Femur	0.82±0.08	0.94±0.11**	0.84±0.06	0.81±0.07

Bone mineral density of trabecular and cortical bone, tissue areas (T.Ar) and cortical bone areas (Ct.Ar) are presented. Data are expressed as means±s.d. The *P* values from Student's *t*-test between wild-type (WT) and ERKO females and wild-type and ERKO males are shown as asterisks (**P*<0.05, ***P*<0.01, ****P*<0.001).

Figure 2 Maximal bending strength for tibia and femur. Values are presented as means±s.d. The *P* values between wild-type and knockout animals are shown as asterisks (**P*<0.05, ***P*<0.01, ****P*<0.001). Numbers per group are the same as for Fig. 1.

only in females. Bone formation at periosteal and endosteal surfaces of the bone was studied by measuring the fluorescent labels from the cross-sections of the femoral

shaft. BFR/BS was significantly increased at the periosteum, but decreased at the endocortical surface of male ERKO mice compared with the wild-type males. No significant changes could be measured in ERKO females, but there was a trend towards increased bone formation at endosteal bone surfaces.

Serum sex hormone levels

Analysis of serum sex hormone levels in aged animals showed that E2 concentrations were significantly increased (approximately fivefold) in ERKO females compared with wild-type females (Table 3). In contrast, E2 levels were not altered in male knockouts. Testosterone levels were elevated in both female and male ERKO animals.

Osteoblast activity in vitro

Since the mineral apposition rate was enhanced and the trabecular bone volume increased in ERKO animals, we next studied the activity of bone cells *in vitro*.

The primary question was whether ERKO osteoblasts would respond to estrogen or not. Bone marrow cultures were performed using a previously described technique (10- to 12-week-old mice) since it was known that at this age it is possible to isolate sufficient amount of mesenchymal stem cells for cultures (6). Bone marrow cells were first cultured in cell culture flasks for 7 days and after that subcultured in multi-well plates. BMP-4 at 10 ng/ml was used as a positive control to stimulate osteoblast differentiation. Calcium content of cultures, used as a marker for the mineralization process, was significantly elevated by BMP-4 in both wild-type and ERKO cultures after 14 days in subculture (Fig. 4A and B). Calcium content was also increased in cultures grown in the presence of E2. The specific activity of ALP (i.e. ALP activity per total

Figure 3 Histomorphometric analysis of trabecular bone of proximal tibia. (A) Longitudinal sections show that ERKO animals have more trabecular bone than their wild-type controls. Note that the growth plate is not detectable in the sample from the ERKO female. (B) Trabecular bone volume (TBV; in %) and tibial thickness (Tb.Th; in μ m). (C) Number (Oc.N; per mm) and surface area of osteoclasts (Oc.S; in %) per bone surface (BS). Number of animals per group are the same as for Fig. 1.

Table 2 Bone formation in aged wild-type and ERKO mice.

	WT female	ERKO female	WT male	ERKO male
Trabecular bone				
MAR (μ m/day)	0.24 $\pm$ 0.12	0.50 $\pm$ 0.04***	0.26 $\pm$ 0.12	0.42 $\pm$ 0.11**
BFR/BS (μ m ³ /μm ² per day)	0.023 $\pm$ 0.024	0.049 $\pm$ 0.03*	0.018 $\pm$ 0.019	0.05 $\pm$ 0.07
BFR/BV (%/day)	0.19 $\pm$ 0.18	0.27 $\pm$ 0.21	0.15 $\pm$ 0.16	0.22 $\pm$ 0.23
BFR/TV (%/day)	0.0013 $\pm$ 0.001	0.030 $\pm$ 0.017***	0.011 $\pm$ 0.013	0.020 $\pm$ 0.021
Periosteum				
MAR (μ m/day)	0.31 $\pm$ 0.09	0.32 $\pm$ 0.10	0.37 $\pm$ 0.13	0.47 $\pm$ 0.18
BFR/BS (μ m ³ /μm ² per day)	0.08 $\pm$ 0.07	0.10 $\pm$ 0.09	0.12 $\pm$ 0.11	0.22 $\pm$ 0.13*
BFR/TV (%/day)	0.012 $\pm$ 0.011	0.016 $\pm$ 0.014	0.018 $\pm$ 0.015	0.033 $\pm$ 0.02*
Endosteum				
MAR (μ m/day)	0.57 $\pm$ 0.09	0.62 $\pm$ 0.10	0.58 $\pm$ 0.16	0.48 $\pm$ 0.19
BFR/BS (μ m ³ /μm ² per day)	0.12 $\pm$ 0.08	0.19 $\pm$ 0.10	0.21 $\pm$ 0.11	0.13 $\pm$ 0.09*

Bone formation in trabecular bone of proximal tibia and in periosteum and endosteum of femoral shaft. Mineral apposition rate (MAR) and bone formation rate (BFR) are expressed as means $\pm$ s.d. Statistical analysis was performed using Student's *t*-test between wild-type (WT) and knockout females and between wild-type and knockout males. The *P* values are shown as asterisks. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). BS, bone surface; BV, bone volume; TV, tissue volume.

protein content) was measured on subculture day 9 from wild-type and knockout mice cultures. Specific ALP activity was increased by 10 pM and 1 nM E2 (Fig. 4C and D). The effect of E2 on calcium and ALP stimulation was similar in female and male mice and thus combined data from female and male cultures are presented. Antiestrogen ICI 182,780 is capable of

preventing the stimulatory effect of E2 in bone nodule assay. This was demonstrated in osteoblast assays when measuring calcium content of the culture (3.6 mmol/l for control, 4.1 mmol/l for E2, 3.4 for ICI 182,780 and 3.4 for ICI + E2; *P* < 0.05 control vs E2 and for E2 vs ICI + E2). Similar results were obtained in three different cultures.

Table 3 Serum sex steroid levels in aged wild-type and ERKO mice.

Hormone	WT female	ERKO female	WT male	ERKO male
E2 (pg/ml)	4.27±1.84	22.73±3.45**	2.12±0.12	2.12±0.10
Testosterone (ng/ml)	0.23±0.11	1.82±0.21***	2.80±0.83	6.34±1.02*

WT, wild-type mice. Data are presented as means±s.e. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for WT vs ERKO mice.

To study type I collagen synthesis by osteoblastic cells, PINP concentrations were analyzed from culture media after 3, 6 and 9 days in subculture. After 6 days, both wild-type and ERKO cells were stimulated by E2 and BMP-4 (Fig. 5A). A similar result was obtained after a 9-day culture (data not shown).

However, basal PINP concentrations were much lower in knockout cultures. Figure 5B presents PINP levels from female cultures in each time point. It shows that PINP concentration levels in ERKO cultures do not rise to the same extent as in wild-type cultures.

The measurement of total protein content from the same cultures after 9 days shows that total protein level in ERKO mouse cultures was only 35±4% of the wild-type value, which could, at least partly, explain the lower PINP levels in knockout mice. When all the culture data were combined, it was demonstrated that the average PINP concentration, as well as total protein content, was indeed significantly lower in knockout cultures compared with wild-type cultures (Fig. 5C). Although total protein content was reduced, ALP levels were not significantly altered in knockout

Figure 4 Effect of BMP-4 (10 ng/ml) and E2 on the calcium content of culture and the specific activity of ALP in mouse bone marrow-derived osteoblast cultures. E2 concentrations are expressed as follows: E2 11 (10 pM), E2 10 (100 pM) and E2 9 (1 nM). Calcium content is measured on subculture day 14 from wild-type (A) and ERKO (B) cultures. Specific activity of ALP is determined as ALP activity per total protein concentration and is measured on subculture day 9 from wild-type (C) and ERKO (D) mice cultures. All results (A–D) are combinations of data from female and male cultures, and all the values are presented as a percentage of control. One ERKO and one wild-type mouse were used in each culture and cells were cultured on six parallel wells per group. All the experiments were repeated. Absolute values for ALP controls were 0.35±0.02 for wild-type and 0.63±0.11 for ERKO mice cultures. Calcium content in control groups were 0.28±0.09 mM (wild-type) and 0.05±0.01 mM (ERKO). Values are presented as means±s.e.m. The P values from Student's t -test between control and other groups are shown as asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

cultures (Fig. 5C). The specific ALP activity was thus even increased, which suggests that differentiation of bone marrow cells to osteoblasts is not disturbed in ERKO animals, although the *in vitro* proliferation rate might be lower.

Osteoclast resorption cultures

Histomorphometric analysis revealed that the number of osteoclasts was decreased in aged ERKO females. In order to find out whether ER α deficiency alters osteoclast function, a mixed bone marrow cell population

from 2- to 4-day-old mouse pups was cultured on bone slices. The number of osteoclasts (with three or more nuclei) or mono- and binuclear TRACP-positive cells was not significantly different when wild-type and knockout cultures were compared (Fig. 6A). The discrepancy between *ex vivo* and *in vitro* results in osteoclast number is not surprising since the age and developmental stage of the animals were totally different. The number of actin rings, resorption pits or nuclei per osteoclast were not significantly altered, either. The appearance of resorption lacunae in knockout mice was also similar to that in wild-type mice (Fig. 6B).

Expression of mRNA for ER β , ERR α and ERR γ in bone marrow cells

RT-PCR of the bone marrow cells showed expression of ER β in both wild-type and ERKO animals (Fig. 7A). In addition, all the wild-type and ERKO mouse bone marrow samples tested for the presence of ERR α (Fig. 7B) and ERR γ (Fig. 7C) mRNA showed a positive result in RT-PCR. Interestingly, RT-PCR produced stronger bands of ERR γ from the bone marrow sample of females than from males in both wild-type and ERKO mice. The possibility of amplification products arising from small amounts of genomic DNA in the isolated RNA was excluded by using mouse genomic DNA template or cDNA reactions without RT enzyme as negative controls in PCR reactions.

Discussion

Our results demonstrate a statistically significant increase in trabecular bone density, as well as in trabecular bone volume of aged ERKO mice, which is in accordance with previous studies using younger animals (30–32). The changes in trabecular bone were associated with an increase in mineral apposition rate and a slight decrease in the number of osteoclasts in the bone. Serum E2 levels are markedly elevated in female ERKO mice (28, 32) and ovariectomized

Figure 5 Analysis of PINP from culture medium. BMP-4 (10 ng/ml) and E2 had a stimulatory effect on PINP secretion after female cells had been grown for 6 days in subculture (A). Abbreviations are: B, BMP-4; C, control; 10, 100 pM E2; 9, 1 nM E2. Medium background levels have been subtracted from values presented. After 6 and 9 days in subculture, control PINP levels were significantly lower in knockout animal cultures compared with wild-type cultures (B). Combination of data and analysis of all cultures ($n = 7$) revealed that average medium PINP concentration and total protein content of cultures were reduced in knockout animals, whereas ALP activity was unchanged and specific ALP activity (ALP activity per protein content) even significantly increased in knockout animals (C). Values are presented as means $\pm$ S.E.M. The P values from Student's t -test between control and other groups (A), or between wild-type and knockout cultures (B and C), are shown as asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 6 Analysis of ERKO and wild-type osteoclasts cultured on bovine bone slices. The number of osteoclasts (NO OF OC), mono- and binuclear TRACP-positive cells (MONO/BI), actin rings and resorption pits (PITS) per bone slice plus nuclei per osteoclast (NUCLEI/CELL) were counted (A). Images of WGA-lectin stained resorption pits reveals that wild-type and knockout pits look similar (B). Values are presented as means $\pm$ S.E.M. Combined data from 16 cultures of wild-type and 11 cultures of ERKO pups are shown. Student's *t*-test did not reveal any statistically significant differences between groups.

female ERKO (carrying full knockout of ER α) mice have recently been shown to respond to E2 treatment (26); these facts suggest a role for ER β in maintaining bone mass in females. In males increased trabecular bone volume and bone formation rate are probably associated with the high serum levels of testosterone, since testosterone is capable of maintaining bone volume independently of ER α (26, 31, 33, 34). Serum testosterone levels are also elevated in female ERKO mice and they have been shown to respond normally to testosterone treatment (26). Furthermore, it has been demonstrated that antiandrogen, but not anti-estrogen, treatment reduces the amount of trabecular bone in ERKO females (26) suggesting that the bone phenotype observed in ERKO mice is largely elicited via androgen receptor (AR) in both sexes. Interestingly, the level of AR mRNA is elevated twofold in ERKO females, but not in males (26).

In addition to estrogen and testosterone, serum levels of some peptide hormones such as leptin and IGF-I are

markedly altered in ER α -deficient mice. After sexual maturation ERKO mice have enhanced serum leptin levels and increased total body fat content (45). This is in accordance with increased body weight in ERKO animals, which is demonstrated in our study and in the study by Lindberg *et al.* (30). It is also possible that elevated BMD in ERKO mice is, at least partly, a consequence of high leptin levels (46). Serum levels of IGF-I are decreased in ERKO animals (30, 47), which might partly explain the decreased bone length. In addition to decreased IGF-I levels, the decrease in bone lengths may be directly associated with high estrogen levels. Crown-rump length of ERKO mice has previously been reported to be normal (47), suggesting decreased bone growth only in the appendicular skeleton. However, our data demonstrate a significant decrease in length of lumbar vertebrae as well.

We also observed that growth plates are fused in bones of old ERKO females, whereas in wild-type females and in bones of ERKO males they are open, as in younger animals. These observations are consistent with recently reported results of Chagin *et al.* (48). This together with the reduced length of bones suggests that bone maturation and growth plate closure are induced or accelerated by ER β in the presence of high E2 in female ERKO mice. ER α and ER β are both expressed especially in the proliferation zone of the growth plate in human and rodents (49). However, lack of functional ER α in a 28-year-old man has been reported to result in severe osteoporosis and incomplete epiphyseal closure (23), which is opposite to our results with ER α knockout mice. However, diagnosis of osteoporosis in this case as well as in aromatase-deficient patients, has been strongly criticized (50). An increased rate of remodeling in these subjects has been suggested to be due to the fact that they do not reach skeletal maturity. Hence, an increased rate of remodeling in these patients, unlike in the mature skeleton where it is caused by a decrease of estrogens or androgen steroids, would be physiologic and appropriate. Differences in epiphyseal plate maturation between ERKO animals and ER α -deficient man are most obviously due to species differences in regulation of growth plate closure as is also seen in normal mice. Interestingly, serum IGF-I levels were elevated in ER α -deficient man (23), while decreased in ERKO animals.

Although full-length ER α is not present in our ERKO animals, expression of an ER α splicing variant has been reported at least in uterus (51) and bone (33). A corresponding 46 kDa isoform of ER α lacking the N-terminal activating function-1 (AF-1) domain has also been detected in human tissues including primary osteoblasts, as well as in bones of ERKO mice (52). This isoform can bind E2 and DNA and transactivate target genes via the AF-2 domain, but its importance in bone function remains unclear. However, neither bones of male mice carrying a total ER α knockout (26) nor male ERKO mice expressing the N-truncated

Figure 7 RT-PCR demonstration of expression of mRNA for ER β , ERR α and ERR γ in bone marrow. (A) ER β RT-PCR products on the agarose gel: lane 1, 100 bp ladder; lane 2, positive control (ventral prostate of wild-type mouse); lanes 3 and 4, wild-type female bone marrows; lanes 5 and 6, ERKO female bone marrows; lanes 7 and 8, WT male bone marrows; lanes 9 and 10, ERKO male bone marrows; lane 11, genomic DNA; and lane 12, blank. Expected PCR product size: 187 bp (upper band). (B) ERR α RT-PCR products on the agarose gel: lane 1, 100 bp ladder; lane 2, positive control (wild-type kidney); lane 3, ERKO female bone marrow; lanes 4 and 5, wild-type female bone marrows; lane 6, ERKO male bone marrow; lane 7, WT male bone marrow; lane 8, blank. Expected PCR product size: 153 bp. (C) ERR γ RT-PCR products on the agarose gel: lane 1, 100 bp ladder; lane 2, positive control (kidney); lanes 3 and 4, wild-type female bone marrow samples; lanes 5 and 6, ERKO female bone marrows; lanes 7 and 8, wild-type male bone marrows; lanes 9 and 10, ERKO female bone marrows; lane 11, blank; lane 12, genomic DNA. Expected PCR product size: 275 bp.

ER α isoform (31–33) responded to E2 administration *in vivo*. This suggests that the full-length ER α possessing AF-1 is required for most of the effects of E2 on mouse bones.

In vitro results are of special interest since no corresponding experiments have been reported previously. An obvious advantage of the *in vitro* model is the exclusion of systemic factors other than those of interest. Only one *in vitro* experiment with ERKO bone cells has been described previously (53). It reported differentiation of ERKO calvarial cells to osteoblastic cells, which formed mineralized nodules. We show here that E2 is capable of stimulating osteogenic differentiation of bone marrow cells derived from both male and female mice even in the absence of ER α . This was demonstrated by increased synthesis of type 1 collagen, increased ALP activity and increased accumulation of calcium in mineralizing bone nodules in cultures. These results suggest that the N-terminal-truncated ER α isoform, ER β or another yet unknown ER mediates the effects of E2 on osteoblast differentiation of ERKO bone marrow cells. Previous experiments with knockout mice have shown that ER β female knockout mice exhibit a higher bone mineral content or bone mineral density compared with control animals and the E2-induced increase in bone formation in ER $\beta^{-/-}$ mice is

equivalent to the increase in wild-type animals (32, 54–57). Taken together, the previous experiments with ER α and ER β knockout mice suggest that ER β plays a minor role in bone metabolism whereas ER α has been shown to have an important role in the mediation of estrogen effects in the skeleton (26, 34, 36). We describe a stimulatory effect of E2 in ERKO bone cells, but further experiments are needed to learn how E2 stimulates bone formation in ERKO animals. The fact that the pure antiestrogen ICI 162,780 opposed the stimulatory effect of E2 suggests that this is ER mediated. One possibility is that the ERRs are also involved since at least ERR α is known to regulate bone formation in calvarial cultures (58). As demonstrated in this work, the orphan receptors ERR α and ERR γ are expressed in the bone marrow of ERKO as well as wild-type mice. It is possible that these ERRs mediate or modulate effects of E2-activated ER β (59).

Despite a response of ERKO osteoblasts to E2, we found that the total protein content of cultures was markedly lower in ERKO than in wild-type cultures. One possible explanation for a difference between the groups might be an increased apoptosis rate of ERKO cells. However, it is more probable that cell proliferation is slowed down in the absence of ER α . This could be caused by altered interactions of ERR α or γ , or the

inability of the N-terminal-truncated ER α isoform to respond to growth factor-elicited stimuli. Several growth factors have been shown to cause ligand-independent activation of ERs via the mitogen-activated protein kinase (MAPK) pathway (60, 61). These estrogen-independent ER activation mechanisms have been recently demonstrated to be present in particular in non-reproductive tissues, such as bone (62). Bone cells produce different growth factors and FCS used in the culture mediums also contains them. It is thus possible that these factors stimulate cell proliferation especially in wild-type cultures where ER α is present. Whether reduced PINP levels in ERKO animals are a consequence of decreased protein levels or due to specific inhibition in collagen synthesis remains questionable. However, matrix maturation and mineralization phases occurred normally in ERKO cells, and specific ALP activity was even higher in ERKO cells compared with wild-type cells.

An interesting question is why the stimulatory effect of E2 has not been demonstrated in the *in vivo* experiments with ERKO males (26, 31, 33, 34), although E2 stimulates osteoblasts *in vitro* as shown here. One explanation for this discrepancy may be that the *in vivo* effects are much more complicated than those in the *in vitro* conditions. Osteoblasts and osteoclasts, for example, continuously communicate with each other and with other cells in the *in vivo* situation. Another reason may be that only trabecular bone parameters have been measured in several studies. Vandenput *et al.* (31) have provided an interesting report showing that although estrogen treatment did not prevent trabecular bone loss in orchidectomized ERKO mice, it stimulated bone formation at the endocortical bone surface suggesting that osteoblasts may respond to estrogen in the absence of ER α .

To summarize, we demonstrate in this work that E2 is able to stimulate osteoblastic differentiation and function *in vitro* in bone marrow cultures of ER α -deficient female and male mice. *In vivo*, aged ERKO animals were shown to have increased trabecular bone volume, which was associated with an enhanced rate of bone formation and a decreased number of osteoclasts. ERKO animals had shorter bones and, interestingly, growth plates were fused in aged ERKO females, although they were open in ERKO males and wild-type mice. These results suggest that ER β -mediated or non-receptor-mediated mechanisms are involved in E2 stimulation of bone formation even in the absence of the full-length ER α .

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