JOURNAL OF BONE AND MINERAL RESEARCH
Volume 16, Number 8, 2001

Female Estrogen Receptor β−/− Mice Are Partially Protected Against Age-Related Trabecular Bone Loss

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ABSTRACT

Recently, it has been shown that inactivation of estrogen receptor β (ER-β) by gene targeting results in increased cortical bone formation in adolescent female mice. To study the possible involvement of ER-β in the regulation of the mature skeleton, we have extended the analyses to include 1-year-old ER-β knockout mice (ER-β−/−). Male ER-β−/− mice did not express any significant bone phenotypic alterations at this developmental stage. However, the increase in cortical bone parameters seen already in the adolescent female ER-β−/− mice was maintained in the older females. The aged female ER-β−/− mice further exhibited a significantly higher trabecular bone mineral density (BMD) as well as increased bone volume/total volume (BV/TV) compared with wild-type (wt) mice. This was caused by a less pronounced loss of trabecular bone during adulthood in female ER-β−/− mice. The growth plate width was unaltered in the female ER-β−/− mice. Judged by the expression of the osteoclast marker tartrate-resistant acid phosphatase (TRAP) and cathepsin K (cat K; reverse-transcription–polymerase chain reaction [RT-PCR]) as well as the serum levels of C-terminal type I collagen cross-linked peptide, bone resorption appeared unaffected. However, an increase in the messenger RNA (mRNA) expression levels of the osteoblast marker core-binding factor α1 (Cbfa1) suggested an anabolic effect in bones of old female ER-β−/− mice. In addition, the mRNA expression of ER-α was augmented, indicating a role for ER-α in the development of this phenotype. Taken together, the results show that ER-β is involved in the regulation of trabecular bone during adulthood in female mice and suggest that ER-β acts in a repressive manner, possibly by counteracting the stimulatory action of ER-α on bone formation. (J Bone Miner Res 2001;16:1388–1398)

Key words: bone, estrogen receptor β, transgenic, osteoblasts, osteoclasts

INTRODUCTION

POSTMENOPAUSAL OSTEOPOROSIS is a condition primarily caused by the severe decrease of serum estrogen levels after cessation of ovarian function. The absence of estrogen results in an increase in bone turnover[(1)] and a negative bone-remodeling balance, leading to bone loss and an increased fracture risk. The decrease in bone mass can be prevented by treatment with estrogens.[(2)] Although the bone-preserving effect of estrogen replacement is indisputable[(1)] the cellular mechanism of action for this hormone effect is unclear.

The cloning of a novel estrogen receptor, estrogen receptor β (ER-β), suggested that there might exist alternative mechanisms of action for estrogen.[(2)] In general, ER-β is expressed in the same tissues as ER-α, but expression levels

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and cell type may vary.[3–5] Both ER-α and ER-β have been detected in human and rat osteoblast-like cell lines[6–11] as well as in osteoblasts and osteocytes in human and rodent bone tissue.[12–17] Both ER-α and ER-β also have been detected in chondrocytes in the epiphyseal growth plates in both humans and rodents.[16,18,19]

All stages of skeletal development and maturation appear to be influenced by estrogen in both males and females. Interestingly, neither the ER-α[20,21] nor ER-β[22] knockout animals show any significant alterations of bone phenotype in either sex before onset of puberty, indicating that although both ERs are expressed abundantly by osteoblasts in sexually immature rodents,[9,17] they probably are not activated until the estrogen levels increase during puberty.

Careful analysis of mice that have been genetically depleted of either ER-α or ER-β shows that lack of either ER will affect the adult bone in an opposite manner,[21,22] indicating distinct functions for the two ERs in bone development. In male mice, loss of ER-α is associated with decreased longitudinal and radial limb growth and cortical osteopenia as a result of decreased radial growth of the bones of adult mice.[20,21] Female ER-α−/− mice also exhibit shorter femoral bones, but interestingly, ovariectomy of female ER-α knockout mice results in a similar loss of trabecular bone compared with sham-operated wild-type (wt) mice.[23] Together, these results indicate that ER-α mediates the growth-promoting effects of estrogens during pubertal bone development in both males and females but does not seem to be required for the maintenance of the trabecular bone at this developmental stage.

With regard to the effect of ER-β on adult mouse bone, recent observations from analysis of female ER-β knockout mice[22] surprisingly show an opposite phenotype to the ER-α−/− mice with respect to longitudinal and radial bone growth, that is, an increased limb length and increased (20–30%) cortical bone mineral content (BMC) in adult (3 months old) female mice. Similar to the ER-α−/− mice, trabecular bone mineral density (BMD) was normal.[21,22] Interestingly, the homozygous mutant ER-β males exhibit no significant changes in their bone phenotype. Moreover, the sexual dimorphism of bone size in the ER-β knockout animals is eliminated because of increased bone growth in the females. These observations suggest that one role of ER-β during pubertal growth could be to stop the pubertal growth spurt in females to limit longitudinal and radial bone growth. However, because the control of bone mass by bone remodeling is more important later on in life, for example, during adulthood, we have extended the bone phenotypic analysis of ER-β−/− mice by studying 1-year-old male and female mice. The results show a previously unrecognized involvement of ER-β in the maintenance of the trabecular bone compartment in aging mice.

MATERIALS AND METHODS

Animals

The animals were maintained under standardized environmental conditions, with free access to food ("rat and mouse autoclavable diet"; Bantin & Kingman, Ltd., N. Humberside, UK) and water. Genotyping of tail DNA was performed at 4–5 weeks of age using polymerase chain reaction (PCR) as previously described.[22]

Histological examination and histomorphometry

Right femora were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (4-μm thickness), and stained with hematoxylin and eosin. The width of the growth plates was measured using an image-processing system (Easy Image; Bergströms Instrument, Stockholm, Sweden) coupled to a microscope. The average of 27 growth plate measurements (3 sections, 9 measurements/section) was calculated for each femur. For quantification, the areas of trabecular bone within a reference area were measured on printed copies using a semiautomatic interactive image analyzer (Videoplan; Zeiss, Oberkochen, Germany). Three fields of vision on four sections from each animal were used for the analysis. Data are presented as ratio of trabecular bone volume (BV) to total volume (TV).

Dual-energy X-ray absorptiometry

Areal BMD (BMC/cm^2) and BMC were measured with the Norland pDEXA Sabre (Norland, Fort Atkinson, WI, USA) and the Sabre Research software (version 3.6; Norland).

In vivo measurements of animals were performed to determine total body, spine, and cranium BMC **(**medium resolution scan with line spacing set at 0.05 cm). Three mice were analyzed at a time. A mouse, which was killed at the beginning of the experiment, was included in all the scans as an internal standard to avoid interscan variations. Ex vivo measurements of the left femur and tibias and vertebrae L5 were performed on excised bones placed on a 1-cm thick Plexiglas table (Norland). All bones compared were measured in the same scan (high-resolution scan with line spacing set at 0.01 cm).

Peripheral quantitative computerized tomography

Computerized tomography was performed with the Stratec peripheral quantitative computed tomography (pQCT) XCT Research M (software version 5.4B; Norland) operating at a resolution of 70 μm.[24]

Middiaphyseal pQCT scans of femora and tibias were performed to determine the cortical volumetric BMD (volumetric BMD), cortical thickness, and the cortical cross-sectional area. The middiaphyseal region of femora and tibias in mice contains only cortical bone.

Metaphyseal pQCT scans of left femora and tibias were performed to measure trabecular volumetric BMD. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 4% of the total length of the femur (an area containing cortical as well as trabecular bone). The trabecular bone region was defined by setting an inner area to 45% of the total cross-sectional area. The interassay CVs for the pQCT measurements were less than 2%. It should be emphasized that the dual-energy X-ray absorptiometry (DXA) technique gives the areal BMD whereas the pQCT gives the real/volumetric BMD.

Thus, the DXA technique gives the mineral content per area and not per volume. Therefore, a factor regulating the outer dimensions of a bone will affect the areal BMD (DXA) but not the volumetric BMD (pQCT).

Serum estradiol measurements

Serum was collected from anesthetized animals. A single determination on pools of 4 μl $\times$ 50 μl of each wt and ER-$\beta-/-$ female mouse concentrated to 100 μl using a Speedvac was performed by an estradiol radioimmunoassay (RIA; Immunotech, Marseille, France) according to the manufacturer's instructions.

RNA preparation and reverse-transcription–PCR

Total RNA was extracted from individual intact femoral bones of wt and ER-$\beta-/-$ animals after homogenization in denaturation buffer with a Polytron homogenizer using the kit ToTALLY RNA (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was done with 5 μg total RNA and 200 U Superscript Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA) at 42°C for 1 h, using oligo-$(dT)_{12-18}$ as the primer (Gibco, Paisley, Scotland). For the PCR amplification, 10% of the synthesized cDNA was added to a 20 μl PCR mix containing 130 ng of each oligonucleotide primer, 0.5 U *Taq* DNA-polymerase (Promega, Madison, WI, USA), and the corresponding buffer containing 15 mM $MgCl_2$. The cDNA was amplified by incubation at 94°C for 30 s, 55°C for 40 s, and 72°C for 50 s. Five different cycles, two cycles apart, were run to identify the linear phase of amplification. The oligonucleotide primers used for the amplification of α1(I) collagen (Col I), alkaline phosphatase (ALP), osteocalcin (OC), tartrate-resistant acid phosphatase (TRAP), cathepsin K (cat K), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as the primers for ER-α[25] were described previously.[22] The oligonucleotide primers used for the amplification of bone sialoprotein (BSP; GenBank accession no. L20232) were forward primer 5′-GTCAACGGCACCAGCACCC-3′, corresponding to the sequence from nucleotide 607–624, and reverse primer 5′-TAGCCTTCATAGCCATGC-3′, corresponding to the sequence from nucleotide 1007–990, product size 400 base pairs (bp); for osteopontin (OPN; GenBank accession no. AA138305) forward primer (mOPN15′) 5′-GACCCATCTCAGAAGCAG-3′, corresponding to the sequence from nucleotide 22–39, and reverse primer (mOPN13′) 5′-TCGGTACTCAGTTCAGTC-3′, corresponding to the sequence from nucleotide 528–511, product size 506 bp; and for the core-binding factor α1(Cbfa1/Osf2; GenBank accession no. AF010284) forward primer (Cbfa1) 5′-CCGCACGACAACCGCACCAT-3′, corresponding to the sequence from nucleotide 511–530, and reverse primer (Cbfa1R) 5′-CGCTCCGGCCCAC AAATCTC-3′, corresponding to the sequence from nucleotides 794–780, product size 283 bp.[27] The PCR products were run on a 2% agarose gel in the presence of ethidium bromide. The intensity of the bands was analyzed by GelPro 2.0 (Media Cybernetics, Inc., Madison, WI, USA). The PCR products were cloned into pGEM T-easy vectors (Promega). The identity of the bands was verified by sequencing.

Real-time PCR

The cDNA was prepared as mentioned previously. The oligonucleotide primers and probe for GAPDH (Rodent GAPDH Control Reagents, VIC Probe) were purchased from PE Applied Biosystems (Warrington, UK). The forward primer for ER-α was 5′-GTGCCTGGCTGGAGATTCTG-3′, corresponding to nucleotides 1349–1368, and the reverse primer 5′-GAGCTTCCCCGGGTGTCC-3′, corresponding to nucleotides 1414–1397. The ER-α TaqMan probe 5′-TGATTGGTCTCGTCTGGCGCTCC-3′, corresponding to nucleotides 1371–1393, was fluorescein labeled with the reporter dye FAM and quencher dye TAMARA. The 87.5-μl mastermix containing 200 nM primers, 100 nM TaqMan probe, and 1xTaqMan Universal Mastermix (PE Applied Biosystems) was added to 1.75 μl cDNA (10 ng/μl) before addition in triplicates to a 96-well microtiter plate. The cDNA was amplified using ABI PRISM 7700 (PE Applied Biosystems) under the following conditions: 1 cycle at 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 s and 60°C for 1 minute. The amount of GAPDH and ER-α messenger RNA (mRNA) was calculated using the Standard Curve Method (separate tubes, following the instructions in User Bulletin 2; PE Applied Biosystems). In short, control cDNA prepared as mentioned previously from control RNA from whole mouse (purchased from PE Applied Biosystems), was serially diluted and analyzed together with the sample cDNA. Fluorescence intensity was measured during the PCR run. A graph was drawn with the threshold cycle C_T value versus the logarithm of the amount of serially diluted control cDNA. Using this graph and the C_T value of GAPDH and ER-α in the wt and ER-$\beta-/-$ samples, the relative amount of ER-α mRNA adjusted for GAPDH could be calculated.

Serum measurements of C-terminal telopeptide fragments of type I collagen cross-links, OC, and insulin-like growth factor 1

Serum was collected and analyzed individually in duplicates with the Rat Laps kit (Osteometer Bio Tech A/S, Herlev, Denmark) by the manufacturers. Four animals of each wt and ER-$\beta-/-$ male and female mice were analyzed. For measurements of serum OC, serum was collected and analyzed individually with the OC kit (Osteometer Bio Tech A/S) according to the manufacturer's instructions. Four animals of each wt and ER-$\beta-/-$ male and female mice were analyzed. Serum insulin-like growth factor 1 (IGF-1) levels were measured by double antibody IGF binding protein–blocked RIA.[28] Four animals of each wt and ER-$\beta-/-$ female mice were analyzed.

Number of osteoclasts per trabecular length

For counting the number of osteoclasts, right femora were fixed, embedded in paraffin, sectioned as mentioned previ-

TABLE 1. BODY WEIGHT AND DIMENSIONS OF BONES

	Female		*Male*	
	WT	*ER-β −/−*	*WT*	*ER-β −/−*
Body weight (g)	31.0 ± 5.4	30.2 ± 5.6	38.6 ± 7.3*	35.5 ± 5.8
Tibia length (mm)	18.6 ± 0.5	18.7 ± 0.2	18.6 ± 0.6	18.4 ± 0.6
Femur length (mm)	16.4 ± 0.5	16.6 ± 0.5	16.5 ± 0.0	16.4 ± 0.4
Femur growth plate width (μm)	67.3 ± 6.3	67.1 ± 7.6	70.5 ± 6.4	70.4 ± 10.1

For female wt $n = 10$ and for ER-β −/− mice $n = 8$; $n = 7$ for male wt and $n = 7$ for ER-β −/− mice with the exception of tibia length in which $n = 3$ for wt and $n = 2$ for KO and femur length for males in which $n = 2$ for wt and $n = 7$ for KO. Femur growth plate width was measured in the distal femur. Values are given as means ± SD.

* $p < 0.05$ for wt males versus wt females as analyzed by two-way ANOVA. The tibia length was analyzed by a Kruskal-Wallis test.

ously, and stained for cat K. The sections were deparaffinized, hydrated, and treated with 3% H_2O_2 to block endogenous peroxidase activity and rinsed in Tris-buffered saline (TBS; 50 mM Tris, pH 7.4, and 150 mM NaCl). The sections were blocked for 20 minutes in normal goat serum (Dako, Copenhagen, Denmark), diluted 1:5 in TBS, exposed for 60 minutes to the primary rabbit polyclonal anti-rat cat K antibody (kindly provided by Dr. H. Sakai, Kyushu University, Japan[29]), diluted 1:500 in TBS, washed further in TBS, and exposed to the secondary biotin-labeled goat anti-rabbit immunoglobulin G (IgG) antibody (Dako). After a further wash in TBS and to enhance the signal, the sections were exposed to StreptABComplex/HRP (Dako) before the binding sites were visualized with 3,3′-diaminobenzidine (DAB) and 3% H_2O_2. Counterstaining was performed with Ehrlich's hematoxylin before dehydration in ethanol and mounting. The stained sections were used for light-microscopical identification of osteoclasts and quantification of the trabecular length. An osteoclast was defined as a cell stained for cat K and lining the trabecular surface. For quantification, the length of trabecular bone within a reference area was measured on printed copies using a semiautomatic interactive image analyzer (Videoplan; Zeiss). Three fields of vision on three sections from each animal were used for the analysis. Data are presented as ratio of the number of osteoclasts to trabecular length.

Statistical analysis

Data were presented as means ± SD. The significance of differences between groups was tested by two-way (gender and genotype) or three-way (age, gender, and genotype) analysis of variance (ANOVA), followed by post hoc comparisons of group means according to least significant difference methods (Statistica Software; Stat Soft, Scandinavia, Uppsala, Sweden). To stabilize variances, data were transformed logarithmically when a correlation between means and variances was found. Whenever the logarithmical transformation was unable to stabilize variables, Kruskal-Wallis ANOVA was performed. Then, comparison of group means was tested with the Mann-Whitney test after correction of the p value by Bonferroni.

RESULTS

Body weight, dimensions, and mineral content of bones

ER-β−/− and wt mice were analyzed at 1 year of age. Both wt and ER-β−/− male mice increased from 11 weeks to 1 year of age in body weight (the wt increased from 25.9 to 40.4 g corresponding to 56%, $p < 0.01$, and the ER-β−/− increased from 26.8 to 35.5 g corresponding to 32%, $p < 0.05$) as well as femur length (the wt increased from 15.0 to 16.5 mm, corresponding to 10%, $p < 0.001$, and the ER-β−/− increased from 15.2 to 16.4 mm, corresponding to 8%, $p < 0.001$; Table 1[22]). The wt males were, as expected, heavier than female wt mice (Table 1) but the ER-β−/− mice did not display this gender difference. During adulthood, wt female mice displayed general growth features including significant increases in body weight (the wt increased from 19.9 to 31.0 g, corresponding to 56%, $p < 0.001$, and the ER-β−/− increased from 23.6 to 30.2 g, corresponding to 28%, $p < 0.05$), femur length (the wt increased from 14.6 to 16.4 mm, corresponding to 12%, $p < 0.001$, and the ER-β−/− increased from 15.2 to 16.6 mm corresponding, to 9%, $p < 0.001$), and cortical area (the wt increased from 0.80 to 0.95 mm^2, corresponding to 19%, $p < 0.01$, and the ER-β−/− increased from 0.91 to 1.07 mm^2, corresponding to 18%, $p < 0.01$). In contrast to the situation at 11 weeks of age (in which ER-β−/− female mice were 18% heavier and had 4% longer femurs than their wt littermates[22]), the 1-year-old ER-β−/− female mice were not heavier nor did they have longer femoral bones than their wt littermates (Table 1). By using DXA, gender differences could be detected in total BMC and spine BMC but not cranial BMC when comparing male and female ER-β−/− and wt mice (Table 2). Interestingly, pQCT measurements showed that although the male ER-β−/− mice had similar total BMC to the wt mice (Table 3), the female ER-β−/− mice had significantly higher total BMC (35%, Table 3). This could be caused by a less pronounced decrease in BMC, because the wt femur decreased from 2.14 to 1.36 mg/mm^3, corresponding to −36% ($p < 0.001$), while the ER-β−/− femur decreased from 2.52 to 1.64 mg/mm^3, corresponding to −27% ($p < 0.001$), when comparing the 1-year-old mice with 11 week-old mice (Table 3).[22]

TABLE 2. DXA MEASUREMENTS OF BMC IN VIVO

	Female		*Male*	
BMC (mg)	*WT*	*βERKO*	*WT*	*βERKO*
Total body	905 ± 137	971 ± 65	1148 ± 129†	1178 ± 79*
Cranium	304 ± 30	312 ± 17	296 ± 26	309 ± 22
Spine	183 ± 27	183 ± 20	229 ± 19†	244 ± 26†

For female wt $n = 10$ and for ER-β $-/-$ mice $n = 8$; $n = 7$ for male wt and $n = 6$ for ER-β $-/-$ mice. Values are given as means ± SD.

The BMC of the cranium was analyzed by a Kruskal-Wallis test.

* $p < 0.01$; † $p < 0.001$ for males versus females of the same genotype as analyzed by two-way ANOVA.

TABLE 3. TRABECULAR VOLUMETRIC BMD AND CORTICAL BONE PARAMETERS AS MEASURED USING PQCT

	Female		*Male*	
	WT	*βERKO*	*WT*	*βERKO*
Tibia				
Total BMC (mg/mm)	1.61 ± 0.25	1.97 ± 0.29†	1.65 ± 0.15	1.79 ± 0.21
Trabecular density (mg/mm^3)	0.170 ± 0.023	0.256 ± 0.088†	0.208 ± 0,039	0.180 ± 0,041§
Cortical density (mg/mm^3)	1.194 ± 0.033	1.235 ± 0.050	1.215 ± 0.042	1.223 ± 0.037
Cortical area (mm^2)	0.67 ± 0.08	0.76 ± 0.06†	0.79 ± 0.08§	0.80 ± 0.07
Cortical BMC (mg/mm)	0.80 ± 0,11	0.94 ± 0,10†	0.96 ± 0.11§	0.98 ± 0.11
Cortical thickness (mm)	0.19 ± 0.01	0.22 ± 0.02†	0.21 ± 0.02	0.22 ± 0.02
Femur				
Total BMC (mg/mm)	1.36 ± 0.23	1.83 ± 0.37***	1.65 ± 0.17‡	1.56 ± 0.22
Trabecular density (mg/mm^3)	0.086 ± 0.031	0.169 ± 0.094†	0.165 ± 0.042§	0.148 ± 0.027
Cortical density (mg/mm^3)	1.224 ± 0.041	1.271 ± 0.056*	1.214 ± 0.047	1.230 ± 0.012
Cortical area (mm^2)	0.95 ± 0.13	1.07 ± 0.08*	1.08 ± 0.13‡	1.09 ± 0.12
Cortical BMC (mg/mm)	1.16 ± 0.18	1.37 ± 0.14*	1.32 ± 0.20	1.34 ± 0.15
Cortical thickness (mm)	0.20 ± 0.02	0.23 ± 0.02*	0.21 ± 0.02	0.21 ± 0.01

For female wt $n = 10$ and for ER-β $-/-$ mice $n = 8$; $n = 7$ for male wt and $n = 7$ for ER-β $-/-$ mice. Values are given as means ± SD. Trabecular and cortical densities of tibia were analyzed by a Kruskal-Wallis test. The trabecular densities were further analyzed by a Mann-Whitney test after correction of the p value according to Bonferroni.

* $p < 0.05$ and † $p < 0.01$ for ERβ $-/-$ mice versus wt; ‡ $p < 0.05$, and § $p < 0.01$ for males versus females as analyzed by two-way ANOVA; *** $p < 0.001$.

Trabecular BMD

Trabecular volumetric BMD was measured in the metaphysis of the distal femur and proximal tibia using the pQCT technique. In the males, a significant loss of trabecular volumetric BMD in the 1-year-old animals as compared with the adolescent 11-week-old mice was noted (the wt femur decreased from 0.331 to 0.171 mg/mm^3, corresponding to −43%, $p < 0.001$, and the ER-$\beta$$-/-$ femur decreased from 0.328 to 0.148 mg/mm^3, corresponding to −53%, $p < 0.001$, Table 3).[22] The female ER-$\beta$$-/-$ mice exhibited significantly higher trabecular volumetric density than their wt littermates (51%, $p < 0.001$; Table 2). The higher trabecular volumetric density noted in the ER-$\beta$$-/-$ females could be caused by a less pronounced decrease in density rather than an increase during aging, as can be seen when comparing the 1-year-old with the 11-week-old mice (the wt femur density decreased from 0.287 to 0.086 mg/mm^3 corresponding to −70%, $p < 0.001$, and the ER-$\beta$$-/-$ femur density decreased from 0.274 to 0.169 mg/mm^3 corresponding to −38%, $p < 0.001$; Table 3).[22]

Cortical bone parameters

As cortical bone parameters were altered in the 11-week-old female mice, the same parameters also were investigated by middiaphyseal pQCT sections of the femur in the 1-year-old mice. As in the young adults, male ER-$\beta$$-/-$ mice exhibited no change in their cortical bone as compared with their wt littermates. On the other hand, female ER-$\beta$$-/-$ mice still displayed an 18% increase in cortical BMC (the wt mice increased from 0.91 to 1.16 mg/mm, $p < 0.001$, whereas the ER-$\beta$$-/-$ mice increased from 1.07 to 1.37 mg/mm, $p < 0.001$) and a 13% increase in cortical area (the wt mice increased from 0.80 to 0.95 mm^2, $p < 0.01$, whereas the ER-$\beta$$-/-$ mice increased from 0.91 to 1.07 mm^2, $p < 0.01$), notable already at 11 weeks of age, as compared with their wt littermates (Table 3). At 1 year of

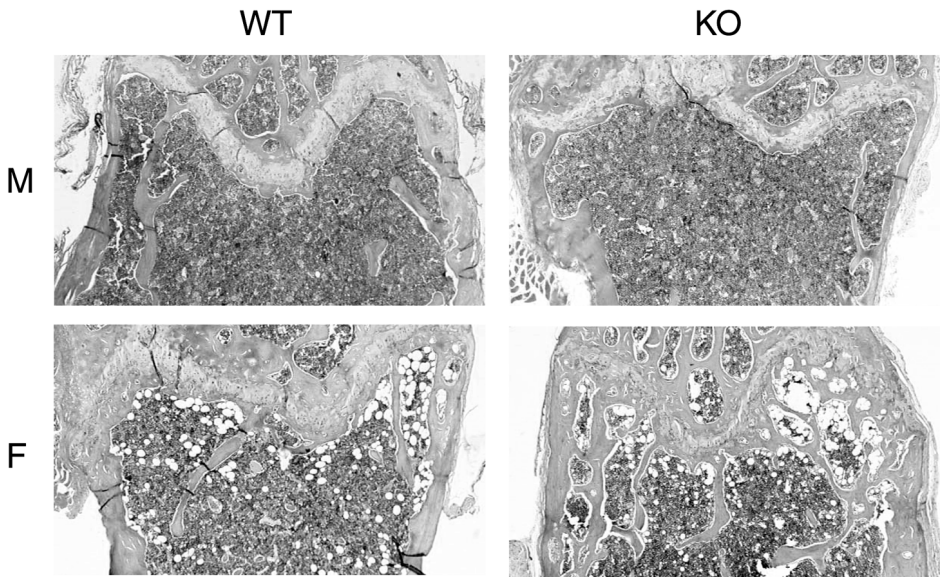

FIG. 1. Histological sections of 1-year-old male and female wt (WT) and ER-β−/− (KO) mouse bone. Femurs were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For quantification, the areas of trabecular bone within a reference area were measured on printed copies using a semiautomatic interactive image analyzer (Videoplan, Zeiss). Three fields of vision on four sections from each animal were used for the analysis. No apparent difference was observed in trabecular BV when the male wt (upper left) and the ER-β−/− (upper right) femurs were compared. Female wt mice (lower left) exhibit less trabecular BV than ER-β−/− females (lower right; magnification ×33).

TABLE 4. HISTOMORPHOMETRY OF TRABECULAR BONE

		Female		*Male*	
Age		*WT*	*ER-β −/−*	*WT*	*ER-β −/−*
11 Weeks	BV/TV	0.326 ± 0.042[§]	0.345 ± 0.071[‡]	0.363 ± 0.071[§]	0.363 ± 0.070[§]
1 Year	BV/TV	0.161 ± 0.038	0.248 ± 0.109*	0.175 ± 0.033	0.147 ± 0.028[†]

For 11-week-old animals, $n = 7$ for female wt and $n = 6$ for ER-β −/− mice; $n = 5$ for male wt and $n = 5$ for ER-β −/− mice. For 1-year-old animals, $n = 8$ for female wt and ER-β −/− mice; $n = 5$ for male wt mice, and $n = 4$ for ER-β −/− mice. Values are given as means ± SD. *$p < 0.05$ for female ER-β −/− versus female wt mice; [†] $p < 0.05$ for ER-β −/− males versus ER-β −/− females as analyzed by three-way ANOVA. [‡] $p < 0.01$ and [§] $p < 0.001$ for 11-week-old versus 1-year-old mice of same genotype and gender.

age, a 15% increase in cortical thickness (the wt mice increased from 0.18 to 0.20 mm^2, $p < 0.05$, whereas the ER-β−/− mice increased from 0.19 to 0.23 mm^2, $p < 0.05$) also could be noted.

Bone histology and morphometry

Histological sections showed that ER-β−/− mice exhibited overall normal bone tissue organization. The growth plate of ER-β−/− male and female mice displayed signs of normal aging, for example, fewer and shorter columns of chondrocytes[30] (Fig. 1). No significant changes could be seen in the growth plate width in either sex (Table 1). The histomorphometrical analysis of the trabecular bone confirmed the pQCT measurements showing that male ER-β−/− mice exhibit a normal age-related loss of their trabecular BV (60%, $p < 0.001$, vs. 52%, $p < 0.001$ in the wt mice; Table 4),[22] whereas the female ER-β−/− mice exhibit a reduced loss of their trabecular BV as compared with their wt littermates (28%, $p < 0.05$, vs. 51% in the wt mice, $p < 0.001$; Table 4).[22] Measurements of trabecular volumetric BMD as well as BV/TV by histomorphometry on all animals allowed us to correlate these parameters (Fig. 2). Linear regression analysis gave a correlation coefficient of $r = 0.68$ with $p = 0.0002$.

Estradiol and ER-α m RNA levels

To test whether the slower decrease in trabecular BV in the ER-β−/− females was caused by delayed menopause reflected by higher serum estradiol levels compared with the wt females, serum estradiol was measured but no differences in serum estradiol could be shown (Table 5). To investigate whether the depletion of ER-β would result in

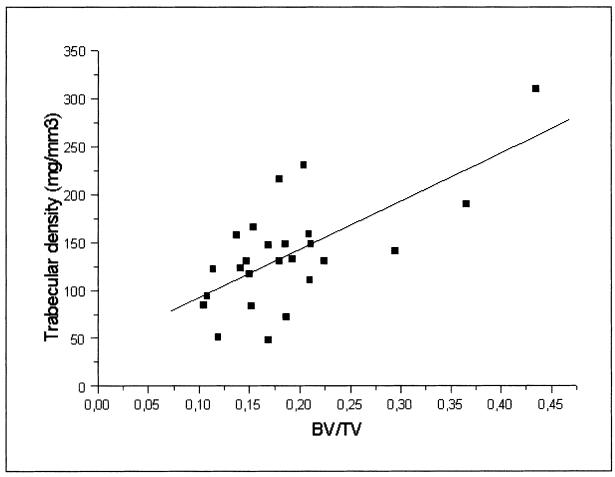

FIG. 2. Correlation between pQCT and histomorphometric measurements of femoral trabecular densities. The trabecular densities of eight wt and eight ER-$\beta-/-$ female and five wt and four ER-$\beta-/-$ male mice are plotted against the BV/TV values for the same mice ($R = 0.68$ and $p = 0.0002$).

TABLE 5. SERUM ESTRADIOL LEVELS

	Female		*Male*	
Age	*WT*	*ER-β −/−*	*WT*	*ER-β −/−*
8 Weeks	35	20	ND	ND
11 Weeks	48	57	ND	ND
9 Months	66	66	ND	ND
1 Year	21	16	ND	ND

Values are given in picograms per milliliter serum. Pools of 4 μl $\times$ 50 μl serum from wt or ER-β $-/-$ female mice were analyzed by an estradiol RIA according to the manufacturer's instructions.

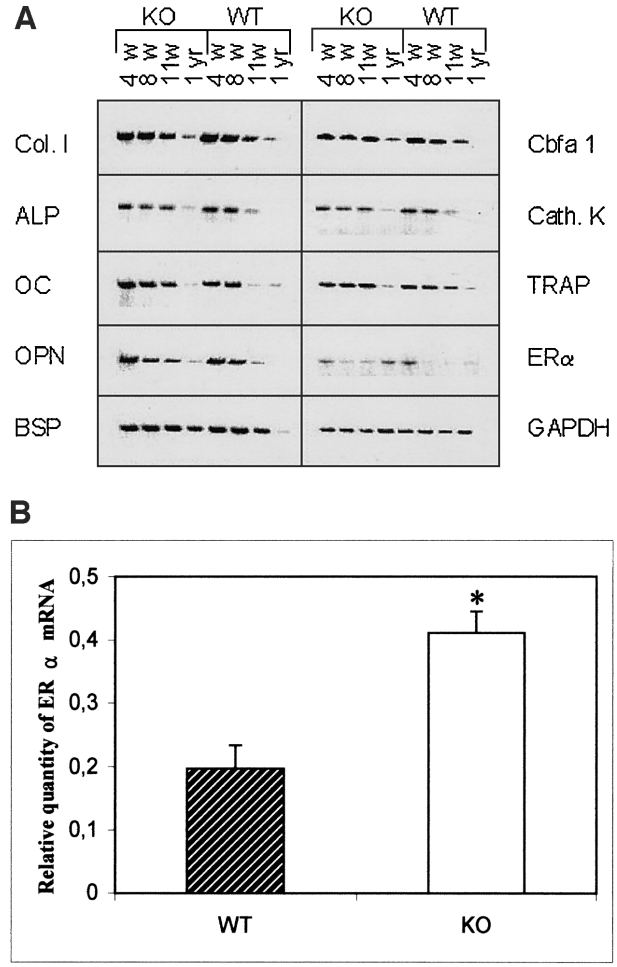

FIG. 3. Expression of osteoblast- and osteoclast-associated genes in long bones of prepubertal (4 weeks), pubertal (8 weeks), adult (11 weeks), and old (1 year) female wt (WT) and ER-$\beta-/-$ (KO) mice by RT-PCR. Total RNA was extracted and used for cDNA synthesis as described in the Material and Methods section. Five different cycles, two cycles apart, were run to identify the linear phase of amplification. In the experiment shown, the cDNA was amplified for 20 (OC, OPN, actin, and GAPDH), 25 (Col I, ALP, BSP, and TRAP), or 35 cycles (ER-α and Cbfa1). (A) RT-PCR analysis showing osteoblast markers Col I, ALP, OC, OPN, BSP, and Cbfa1; osteoclast marker TRAP and Cath. K; and other genes ER-α, and GAPDH. (B) Graphs visualizing the expression of ER-α mRNA in 1-year-old wt and ER-$\beta-/-$ females quantified by real-time PCR, using the Standard Curve Method. The graphs show the mean and SD of triplicates in a representative experiment.

changes in the ER-α expression, we performed reverse-transcription (RT)–PCR of ER-α in the female long bones. In the wt mice, ER-α was down-regulated after puberty (Fig. 3). This also could be seen in the ER-$\beta-/-$ females, although to a lesser extent (Fig. 3). Interestingly, at 1 year of age, the prepubertal levels of ER-α were restored in the ER-$\beta-/-$ females, suggesting that ER-α could be involved in the maintenance of trabecular bone seen in the old ER-$\beta-/-$ female mice. Indeed, by performing a real-time PCR, we were able to show that ER-α mRNA was increased by 2-fold in the ER-$\beta-/-$ females as compared with wt mice.

Expression of osteoblast- and osteoclast-associated genes

To investigate the molecular mechanisms underlying the bone phenotypic alterations in old female ER-$\beta-/-$ mice, the expression of osteoblast- associated (Col I, ALP, OC, OPN, BSP, and Cbfa1) and osteoclast-associated (TRAP and cat K) mRNAs was assessed by RT-PCR of RNA extracted from long bones from wt and ER-$\beta-/-$ mice at different ages (Fig. 3). As we have previously shown, the expression levels of these genes generally were higher in prepubertal mice compared with adult wt mice[22] This is consistent with the increased bone metabolism and turnover seen during rapid postnatal growth. The expression levels of Col I, ALP, OC, OPN, cat K, and TRAP were strongly down-regulated or undetectable in the 1-year-old females, irrespective of the genotype. Interestingly, ER-α and the osteoblast marker Cbfa1 appeared more highly expressed in

TABLE 6. BONE TURNOVER MARKERS

		Female		*Male*	
		WT	*ER-β −/−*	*WT*	*ER-β −/−*
Osteocalcin (ng/ml)	11 weeks	141 ± 66.2	100 ± 32.9	105 ± 25.0	84.7 ± 6.43
	9 months	62.2 ± 33.8	40.3 ± 14.0	20.2 ± 5.18†	21.1 ± 7.44*
C-terminal type I	8 weeks	94.6 ± 21.4	111.8 ± 37.3	82.0 ± 18.6	114 ± 25,3
Collagen (ng/ml)	11 weeks	50.7 ± 19.6	48.9 ± 14.0	72.2 ± 63.7	82.5 ± 32.9
	9 months	29.8 ± 10.2	22.1 ± 3.35	40.4 ± 6.52	34.3 ± 5.80
Osteoclast number (osteoclast/mm)	12 months	1.32 ± 0.75	2.05 ± 1.44	2.61 ± 0.54	3.88 ± 1.67*

For all serum measurements $n = 4$ for female wt and $n = 4$ for ER-β −/− mice; $n = 4$ for male wt and $n = 4$ for ER-β −/− mice. For the histomorphometrical analysis of osteoclast (no./mm), $n = 8$ for female wt and $n = 8$ for ER-β −/− mice; $n = 5$ for male wt and $n = 4$ for ER-β −/− mice. Values are given as means ± SD. Serum was collected and analyzed individually in duplicates with the Rat Laps kit by the manufacturers. A total of four animals of each wt and βERKO male and female mice were analyzed. For measurements of serum OC, the serum was analyzed individually with the osteocalcin kit according to the manufacturer's instructions. A total of four animals of each wt and ER-β−/− male and female mice were analyzed. Serum osteocalcin at 11 weeks of age and C-terminal collagen type I cross-links at 8 weeks of age were analyzed by Kruskal-Wallis test.

* $p < 0.05$ and † $p < 0.001$ for males versus females as analyzed by two-way ANOVA.

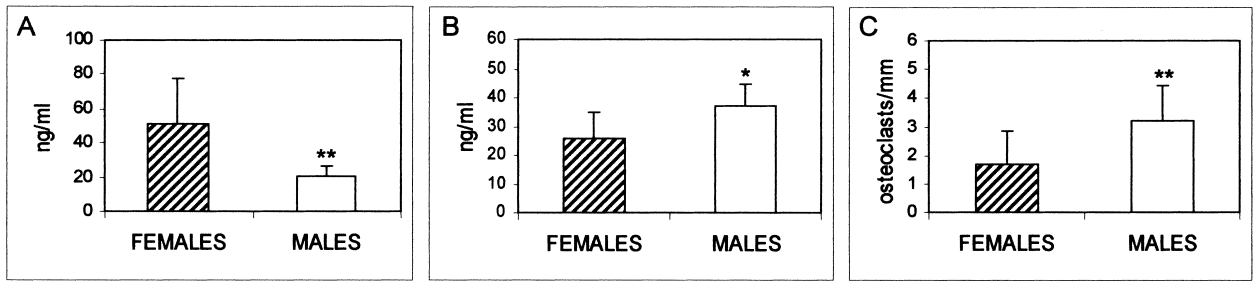

FIG. 4. Comparison of male and female bone turnover. (A) OC was measured in serum with a OC RIA. The graph shows the mean and SD of individual measurements of eight female and eight male mice, both genotypes combined. (B) C-terminal type I collagen cross-links were measured in serum with Rat Laps (Osteometer Bio Tech A/S). The graph shows the mean and SD of individual measurements of eight female and eight male mice, both genotypes combined. (C) The number of osteoclasts per trabecular length was measured by counting the number of cat K–stained cells lining the trabecular surface. For quantification, the length of trabecular bone within a reference area was measured on printed copies using a semiautomatic interactive image analyzer (Videoplan). Three fields of vision on three sections from each animal (9 males and 16 females, both genotypes combined) were used for the analysis. Data are presented as the ratio of the number of osteoclasts to trabecular length. *$p < 0.05$; **$p < 0.01$ versus females.

the 1-year-old ER-β−/− females as compared with their wt littermates.

Serum measurements of C-terminal telopeptide fragments of type I collagen cross-links, OC, osteoclasts, and IGF-1

To test whether the increased trabecular BMD in the ER-β−/− females was associated with a general decrease in bone turnover, serum OC was measured and found higher in the female wt and ER-β−/− mice than in the male mice of the same genotype. We then measured C-terminal type I collagen cross-links by the serum C-terminal type I collagen cross-linked peptide assay. This assay showed no significant increase in type I C-terminal collagen cross-linked peptides in the ER-β−/− mice, male or female, compared with their wt littermates (Table 6). We also counted the number of osteoclasts per millimeter trabecular surface but failed to measure any significant differences between the genotypes (Table 6). From these results and the RT-PCR results (mentioned previously), we conclude that the bone phenotype seen in the ER-β−/− females is not likely to be caused by a general effect on bone turnover. A comparison of the bone turnover markers between the two sexes did reveal sexual differences regarding the mechanism of bone loss caused by aging (Fig. 4). The males (wt and ER-β−/−) had significantly higher concentrations of C-terminal type I collagen cross-links (37.4 ng/ml vs. 26.0 ng/ml, corresponding to a 44% increase, $p < 0.05$) and number of osteoclasts (3.18/mm vs. 1.68/mm, corresponding to a 53% increase, $p < 0.01$) whereas the females (wt and ER-β−/−) had higher concentrations of serum OC (51.2 ng/ml vs. 20.6 ng/ml, corresponding to 40%, $p < 0.01$) than the males. Analysis of the serum IGF-1 levels in the female mice

showed no significant changes (228 ± 84 ng/ml for the wt and 216 ± 60 ng/ml for the ER-$\beta-/-$ mice, $p = 0.6$) indicating that the growth hormone (GH)/IGF-1 axis is not affected in the old female ER-$\beta-/-$ mice.

DISCUSSION

In this study, we showed, by pQCT and histomorphometry, that the 1-year-old female ER-$\beta-/-$ mice exhibited higher total BMC and trabecular BMD and that the increase in cortical area and BMC seen in the 11-week-old mice was preserved. The higher BMC and trabecular BMD seen in the 1-year-old female ER-$\beta-/-$ mice was caused by a less pronounced bone loss as compared with 11-week-old mice. A significant correlation between the two techniques measuring the trabecular density (pQCT and histomorphometry) could be shown. Furthermore, several parameters have been analyzed to delineate possible mechanisms underlying this phenotype.

First, we asked whether the decreased loss of the total BMC and trabecular BMD in the aged female ER-$\beta-/-$ mice could be caused by increased longitudinal growth. Thorough measurements of the growth plate width of animals from both sexes and genotypes were performed. These indicated that the epiphyseal cartilage was normal in the ER-$\beta-/-$ mice and that no significant changes in the widths of the growth plates could be found. In addition, although the female ER-$\beta-/-$ mice had significantly longer femora at 11 weeks of age as compared with their wt littermates, the old ER-$\beta-/-$ females did not exhibit longer femoral bones. Thus, we conclude that the phenotype seen in the old female ER-$\beta-/-$ mice was not caused by increased longitudinal growth.

The next question addressed was whether a relative decrease in bone turnover in the ER-$\beta-/-$ mice compared with the wt female mice could be associated with the bone-preserving effects. Osteoblast and osteoclast markers measured by RT-PCR did not indicate substantial effects on bone turnover. This was corroborated with the outcome of the serum C-terminal type I collagen cross-link and OC assays as well as the number of osteoclasts per millimeter trabecular surface. Interestingly, when comparing males with females, there was a significant difference between the sexes in the three assays. Although females had significantly higher serum OC, they had significantly lower serum type I collagen cross-links and number of osteoclasts than the males, indicating that the mechanism of bone loss in these aging mice differs between the sexes, for example, older males exhibit a significantly higher bone resorption compared with female mice of the same age.

Interestingly, although no general increase in expression levels of different osteoblastic phenotypic markers could be detected, one of the earliest markers for osteoblast differentiation, Cbfa1, was higher in female ER-$\beta-/-$ mice compared with their wt littermates and 11-week-old wt mice as shown by RT-PCR (Fig. 3B). Cbfa1 is necessary for osteoblast development in both man and mouse.[27,31,32] It has been shown that Cbfa1 binds to and regulates the expression of genes expressed in osteoblasts, for example, OC, BSP, and OPN,[33] though it is still unknown if any of these genes are more dependent on Cbfa1 than the others.

In contrast to the ER-α knockout mice, in which the serum estradiol levels were elevated by 186%[34] and IGF-1 levels were decreased by 26%,[21] we could not show any differences in serum estradiol or IGF-1 levels in the female ER-$\beta-/-$ mice as compared with their wt littermates. Interestingly, the RT-PCR analysis indicated an increase in the amount of ER-α mRNA in the female ER-$\beta-/-$ mouse bone. This was confirmed with real-time PCR, showing a 2-fold increase in ER-α mRNA in ER-$\beta-/-$ mice compared with wt mice. Thus, the positive effects on trabecular bone seen in the female ER-$\beta-/-$ mice potentially could be caused by increased estrogen responsiveness mediated by ER-α.

Because osteoblast populations are known to be heterogeneous and the osteoblasts express different markers depending on their status of differentiation and of their stage of functional activity,[35–37] it is possible that the absence of ER-β or increase of ER-α favors osteoblast activity by pushing the osteoblasts to a certain stage of differentiation leading to the bone-preserving effects seen in the female ER-$\beta-/-$ mice. Indeed, deletion of ER-α leads to cortical osteopenia in adult mice,[21] indicating that ER-α is the ER responsible for the anabolic effect at least in male mice. Moreover, ER-β appears to act as a modulator of the ER-α response in female rodents.[22,38] It can be noted that there are species differences between humans and rodents regarding the importance of the ERs. So far, only one man has been reported lacking functional ER-α protein (hERKO),[1] two other men have been reported with aromatase deficiency due to mutations in the aromatase gene,[39,40] no man has been reported lacking functional ER-β, and no females have been reported lacking either ER. In contrast to the male ER-$\alpha-/-$ mouse, which display shorter limbs, these human mutants all showed continued linear growth through adulthood because of unclosed epiphyses, low bone age, and severe osteoporosis associated with decreased trabecular BMD. Thus, growth plate fusion does not occur in humans with estrogen deficiency or estrogen resistance due to absence of ER-α. The possibility that differences in the action of ER-β between humans and rodents could explain the apparent phenotypic species-dependent differences with regard to inactivating mutations in the ER-α gene locus needs to be investigated further.

This study has revealed that deletion of ER-β not only results in increased cortical bone in female ER-$\beta-/-$ mice, but also provides a partial protection against the age-related trabecular bone loss. This effect is not likely to be caused by changes in serum estradiol levels or a general change in bone turnover or growth. It is possible that the bone-preserving effects seen in ER-$\beta-/-$ mice, at least in part, could be caused by an increase in ER-α mRNA associated with an increase in osteoblast differentiation and/or activity.

ACKNOWLEDGMENTS

We thank Dr. Paolo Parini, Department of Biosciences, Karolinska Institutet, Sweden, for helpful discussions con-

cerning the statistical analysis. This study was supported by the Swedish Medical Research Council, Swedish Foundation for Strategic Research, the Lundberg Foundation, and the Swedish Association Against Rheumatic Disease.

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Received in original form June 23, 2000; in revised form December 26, 2000; accepted January 16, 2001.