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Original Article

Hylonome

The role of estrogen receptor-β in the early age-related bone gain and later age-related bone loss in female mice

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Abstract

The molecular and cellular mechanism of estrogen action in skeletal tissue remains unclear. The purpose of this study was to understand the role of estrogen receptor-β (ERβ) on cortical and cancellous bone during growth and aging by comparing the bone phenotype of 6- and 13-month-old female mice with or without ERβ. Groups of 11-14 wild-type (WT) controls and ERβ knockout (BERKO) female mice were necropsied at 6 and 13 months of age. At both ages, BERKO mice did not differ significantly from WT controls in uterine weight and uterine epithelial thickness, indicating that ERβ does not regulate the growth of uterine tissue. Femoral length increased significantly by 5.5% at 6 months of age in BERKO mice compared with WT controls. At 6 months of age, peripheral quantitative computerized tomography (pQCT) analysis of the distal femoral metaphysis (DFM) and femoral shafts showed that BERKO mice had significantly higher cortical bone content and periosteal circumference as compared with WT controls at both sites. In contrast to the findings in cortical bone, at 6 months of age, there was no difference between BERKO and WT mice in trabecular density, trabecular bone volume (TBV), or formation and resorption indices at the DFM. In 13-month-old WT mice, TBV (-41%), trabecular density (-27%) and cortical thickness decreased significantly, while marrow cavity and endocortical circumference increased significantly compared with 6-month-old WT mice. These age-related decreases in cancellous and endocortical bone did not occur in BERKO mice. At 13 months of age, BERKO mice had significantly higher total, trabecular and cortical bone, while having significantly lower bone resorption, bone formation and bone turnover in DFM compared with WT mice. These results indicate that deleting ERβ protected against age-related bone loss in both the cancellous and endocortical compartments by decreasing bone resorption and bone turnover in aged female mice. These data demonstrate that in female mice, ERβ plays a role in inhibiting periosteal bone formation, longitudinal and radial bone growth during the growth period, while it plays a role in stimulating bone resorption, bone turnover and bone loss on cancellous and endocortical bone surfaces during the aging process.

Keywords: Estrogen Receptor-β, Bone, Osteoporosis, Bone Remodeling, Uterus

Introduction

Estrogen is an important regulator in skeletal growth, development and maintenance[1]. The decline in estrogen level in postmenopausal women is, at least in part, responsible for the decrease in bone mass and architectural abnormalities which lead to the increased risk of osteoporotic fracture[2]. Although it has been clearly demonstrated that the decrease in bone mass and increase in skeletal fractures in postmenopausal women can be prevented by treatment with estrogen, the mechanism of action of estrogen in bone remains unclear[1,3,4]. Since the recent cloning of a novel estrogen receptor[5,6], estrogen receptor-β (ERβ), research efforts have focused on understanding the relative importance of the ERs now denoted as ERα, and ERβ, in mediating the action of estrogen in skeletal tissue. Although both ERα and ERβ are expressed in osteoblast and bone tissues in mice and humans[7-9], their physiological roles require further characterization.

Generations of knockout mice lacking ERα, ERβ, or both have provided an important tool for understanding the physiological role of each receptor subtype in the reproductive

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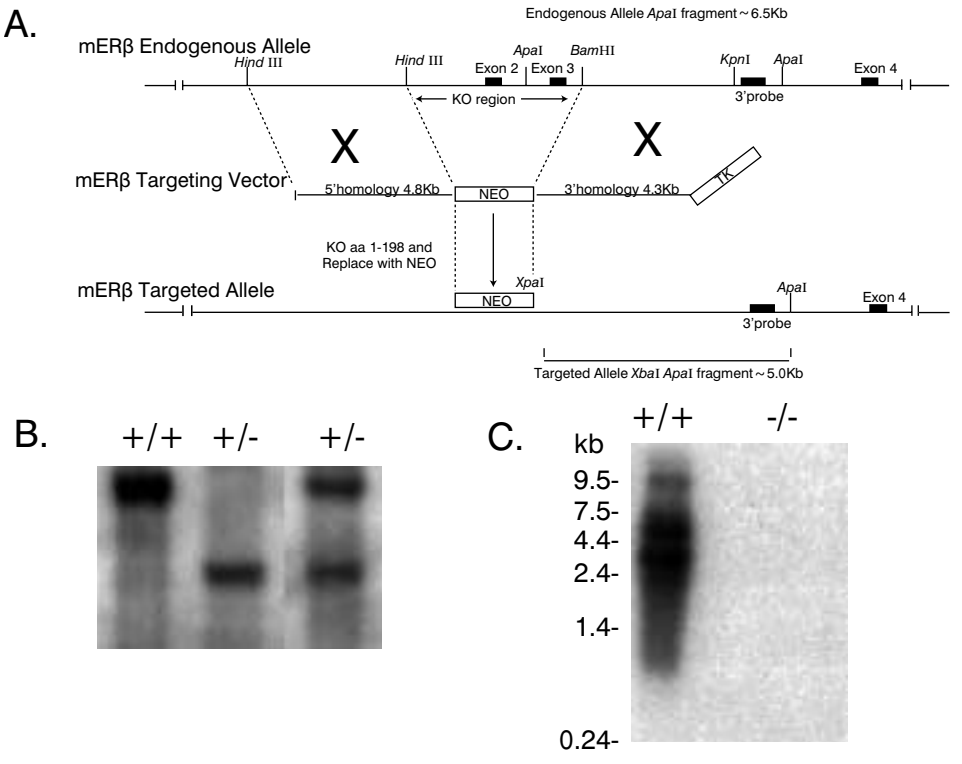

Figure 1. A) Schematic representation of genomic organization and targeting strategy, including targeted locus. Exon sizes and intron/exon boundaries of the murine ERβ gene are diagrammed, exons are indicated by gray boxes. Approximately 5.6 Kb of endogenous genomic sequence, including exons 2 and 3, was replaced by the neomycin (NEO) gene. This creates a deletion encoding from methionine 1 to glycine 198 within the native ERβ protein. B) Southern blot detection of successful ERβ targeting. Hybridization of genomic DNA with an external probe identified a predicted RFLP of ~5.0Kb, created as a result of the introduction of a novel XbaI restriction site in the targeting vector. C) Northern blot analysis of 5 μg poly A+ RNA prepared from ovary of wild-type and knockout animals. Note the absence of ERβ expression in the RNA from knockout mice.

system and in skeletal growth and development[10-16]. Recently, Vidal and colleagues[11] reported that the ERα-knockout (ERKO) and double ERα/β knockout (DERKO), but not ERβ-knockout (BERKO), decreased longitudinal as well as radial bone growth in male mice. Thus, ERβ is not essential for skeletal growth and development in male mice. In female ERKO mice, a decreased[12,13] or unchanged[16,17] longitudinal bone growth and an increased bone mineral density or trabecular bone volume was observed when compared with their wild-type littermates[12,13,16]. The most recent report by Sims et al.[16] demonstrated that only ERα was shown to regulate bone remodeling in males, whereas in females both ERα and ERβ influenced bone remodeling. Further study indicated that both wild-type and ERKO female mice lost cancellous and cortical bone following ovariectomy (OVX)[13]. However, it was found that ERKO female mice have 10-fold higher serum levels of both estradiol and total testosterone than in WT controls[10,16,17], and these hormones decreased to the levels of WT controls following OVX in ERKO mice. Therefore, it is difficult to discern the effects of testosterone from the effects of ERα knockout in these OVX mice. Using BERKO female mice, Krege et al. found that ERβ is essential for normal ovulation efficiency but is not essential for sexual differentiation, fertility, or lactation[14]. It has been reported that BERKO female mice had increased cortical bone mineral content with normal trabecular bone mineral density[15]. However, the roles of ERβ in the early age-related bone gain during the growth period and later age-related bone loss during the aging process on different bone surfaces in females have not been completely elucidated. The aim of the present study was to evaluate the role of ERβ on both cortical and cancellous bone surfaces during growth and aging by comparing the bone phenotype of 6- and 13-month-old female mice with or without ERβ. Our data demonstrate that ERβ plays an important role in the regulation of skeletal tissue in females during the growth and aging process.

Materials and methods

Gene Targeting, ES cell culture and microinjection. A 139-bp murine ERβ cDNA PCR fragment was used as a probe to identify genomic clones from a murine129SvJ genomic phage library (Stratagene). The mERb targeting vector (Figure 1A) was constructed by cloning 4.8 Kb of 5'homology and 4.3 Kb of 3'homology into a pJNS2 (PGK-

	6-month-old (adult)		13-month-old (aged)	
	WT	BERKO	WT	BERKO
Body weight (g)	26.9±0.88	34.3±1.49 ^{b}	30.2±0.78 ^{A}	34.8±2.18
Femoral length (mm)	15.95±0.10	16.83±0.08 ^{b}	16.33±0.12	16.81±0.10
Uterine weight (g)	0.131±0.013	0.170±0.026	0.310±0.036 ^{B}	0.312±0.045 ^{B}
Uterine epithelial thickness (μm)	22.68±13.8	21.73±1.74	28.73±1.73 ^{B}	25.43±1.86 ^{B}

Mean±SEM; WT: wild-type controls; BERKO: estrogen receptor-β knockout.
a: $p < 0.05$, b: $p < 0.01$ vs. WT at the same age;
A: $p < 0.05$, B: $p < 0.01$ vs. 6-month-old at the same genotype.

Table 1. Changes in body weight, femoral length, uterine weight and uterine histology.

NEO/PGK-TK) backbone vector[18]. The targeting vector replaced ~5.6 Kb of genomic locus with PGK-NEO. The deleted region included exon 3 as described by Krege et al.[14] and encoded the translated region from methionine at position 1 to the glycine at position 198 (numbering according to the start codon of the 549 amino acid mERb protein described in GenBank AF0667422). Electroporation, selection, expansion, and microinjection of ES cells into 2.5 day C57BL/6 embryos were as described[19]. Chimeric males were mated with C57BL/6 females. Mice were genotyped at 4 – 5 weeks of age using Southern blot analysis.

Animals. Deleting amino acids 1 – 134 and replacing with neomycin gene in estrogen receptor-β gene created the ERβ knockout mouse. Genotyping of tail DNA was performed at 4-5 weeks of age using PCR with primers of mERb 5'-CGGTAACCTGGAAGGTGGGCCT-3' and 5'-CACACCAAGGACTCTTTTGAGGTTC-3'; b-actin, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3'. Northern Blot analysis of ovary tissue confirmed that the ERβ knockout mice do not express ERβ mRNA. Further, Genomic Southern Blot analysis confirmed that ERβ knockout mice do not contain ERβ protein.

All mice were of mixed C57/BL6 background. The mice were housed 5 per cage in 20 x 32 x 20 cm^3 cages at local vivarium conditions (24ºC and 12 h/12 h light-dark cycle). All animals were allowed free access to water and a pelleted commercial diet (Agway ProLab 3000, Agway County Food, Inc., Syracuse, NY), containing 0.97% calcium, 0.85% phosphorus, and 1.05 IU/g of Vit.D3. The experiments were conducted according to Pfizer Animal Care approved protocols and animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

A total of fifty female estrogen receptor-β knockout (BERKO) mice (n=26) and wild-type (WT) littermates (n=24) were used in this study. Ten WT and 12 BERKO mice were necropsied at 6 months of age for determination of the effects of ERβ knockout on skeleton and uterine tissue during the growth period. Another 14 WT and 14 BERKO mice were necropsied at 13 months of age for determination of the effects of ERβ knockout on skeletal and uterine tissue during the aging process. All mice were given subcutaneous injections of 10 mg/kg of calcein (Sigma Chemical Co., St. Louis, MO), a fluorochrome bone marker, at 13 and 3 days before necropsy in order to determine dynamic changes in bone tissues[20]. The mice were necropsied under anesthesia by intraperitoneal (ip) injection of a mixture of ketamine/xylazine (57 mg per 0.86 mg/ml solution per kilogram body weight). The following endpoints were determined.

Body weight, femoral length, uterine weight, and uterine histology. The body weight was obtained before necropsy. Right femoral length was determined for all animals in excised femora using an electronic digital caliper. The uterine wet weight was determined immediately at necropsy. Five micron, paraffin embedded, hematoxylin and eosin stained uterine sections were prepared to determine the luminal epithelial thickness (the average thickness at 0.2 mm intervals along the uterine luminal epithelial layer) using an Image Analysis System (Osteomeasure, Inc., Atlanta, GA) as previously described[21].

Peripheral quantitative computerized tomography (pQCT). The right femur from each mouse was analyzed at two bone sites, the distal femoral metaphysis, a site containing both cancellous and cortical bone, and the femoral shaft diaphysis, a site containing only cortical bone. Excised femora were scanned by pQCT (Stratec XCT Research M, Norland Medical Systems, Fort Atkinson, WI) with software version 5.40. A 1 mm thick cross section of the distal femur metaphysis was taken at 2.5 mm proximal from the distal end, and a 1 mm thick cross-section of the femoral shaft diaphysis was taken at 6 mm proximal from the distal end with a voxel size of 0.10 mm. Total bone parameters were defined and analyzed using contour mode 2, and cortical bone parameters were defined and analyzed using cortical mode 4. An outer threshold setting of 340 mg/cm^3 was used to distinguish the cortical shell from soft tissue and an inner threshold of 529 mg/cm^3 to distinguish cortical bone from trabecular bone. Trabecular bone was determined using peel

	6-month-old (adult)		13-month-old (aged)	
	WT	BERKO	WT	BERKO
Total bone content (mg/mm)	1.60±0.04	1.99±0.09 [a]	1.56±0.09	2.03±0.15 [b]
Total bone density (mg/cm3)	555±25.2	597±21.8	484±23.9	602±40.5 [b]
Total bone area (mm2)	2.92±0.09	3.33±0.08 [b]	3.23±0.08 [A]	3.37±0.08
Trabecular bone content (mg/mm)	0.42±0.02	0.48±0.03	0.37±0.04	0.40±0.02
Trabecular bone density (mg/cm3)	245±11.2	261±13.8	178±17.8 [A]	263±28.3 [b]
Marrow cavity area (mm2)	1.75±0.10	1.84±0.07	2.07±0.07 [B]	1.73±0.16 [a]
Cortical bone content (mg/mm)	1.53±0.05	1.83±0.06 [a]	1.44±0.07	1.90±0.16 [b]
Cortical bone density (mg/cm3)	885±20.5	912±11.8	920±14.3	895±20.1
Cortical bone area (mm2)	1.73±0.03	2.00±0.06 [b]	1.56±0.07 [A]	2.12±0.17 [b]
Cortical Thickness (mm)	0.35±0.01	0.38±0.01	0.29±0.01 [B]	0.44±0.06 [a]
Periosteal circumference (mm)	6.05±0.09	6.47±0.08 [b]	6.36±0.08 [A]	6.50±0.08
Endocortical circumference (mm)	3.84±0.15	4.08±0.08	4.57±0.10 [B]	3.74±0.38 [b]

Mean±SEM; WT: wild-type controls; BERKO: estrogen receptor-β knockout.
a: $p < 0.05$, b: $p < 0.01$ vs. WT at the same age;
A: $p < 0.05$, B: $p < 0.01$ vs. 6-month-old at the same genotype.

Table 2. Changes in pQCT parameters of distal femoral metaphysis in adult and aged female mice.

mode 4 with a threshold setting of 655 mg/cm^3 to distinguish cortical and subcortical from cancellous bone. An additional concentric peel of 1% of the defined cancellous bone was used to ensure cortical and subcortical bone was eliminated from the analysis. Volumetric bone content, density and area were determined for total, trabecular and cortical bone[15,22]. In addition, cortical periosteal and endocortical circumferences were determined. Using the above setting, we have determined that with repositioning, the *ex vivo* precision of volumetric content, density and area of total, trabecular and cortical regions ranged from 0.99% to 3.49%.

Cancellous bone histomorphometry of distal femoral metaphysis. Undecalcified, methyl methacrylate embedded longitudinal sections of distal femoral metaphysis at 4 and 10μm thickness were prepared (Leica RM 2165 Microtome, Heidelberg, Germany) for histomorphometry as described previously[23-25]. The 4μm sections were stained with modified Masson's Trichrome stain, while the 10μm section remained unstained[23]. A video Image Analysis System (Osteomeasure, Inc., Atlanta, GA) was used for the static and dynamic histomorphometric measurements of cancellous bone of the distal femoral metaphysis. The area between 0.375 and 0.875 mm proximal to the growth plate-epiphyseal junction, and extending to the endocortical surface in the lateral dimension was selected for histomorphometric measurements. The indices of bone mass (trabecular bone volume), bone structure (trabecular thickness, number, and separation), bone resorption (osteoclast number per mm bone surface, percent osteoclast surface), bone formation (mineralizing surface, mineral apposition rate), and bone turnover (bone formation rate/bone surface referent) were determined as described previously[24,25].

Statistics. Data are expressed as mean ± SEM. Statistics were calculated using StatView 4.0 packages (Abacus Concepts, Inc., Berkeley, CA). The analysis of variance (ANOVA) test followed by Fisher's protect least significant difference (PLSD) was used to compare the differences between each group[26]. $P < 0.05$ was considered a significant difference.

Results

Gene Targeting. The targeting strategy resulted in the elimination of the DNA sequence encoding exons 2 and 3 of the mERβ gene (Figure 1A). The deletion encompassed the DNA sequence encoding the initiating methionine through the glycine residue at position 198. This targeting strategy deleted the translation start site, the entire A/B domain and the first Zn2+ finger of the DNA binding domain. Wild-type, heterozygous and knockout mice were readily identified by Southern blot analysis (Figure 1B). As shown in Figure 1C, targeting was confirmed by the absence of ERβ mRNA by Northern blot analysis.

Body weight. Body weight was significantly higher (+28%) in BERKO mice than in WT controls at 6 months of age (Table 1). At 13 months of age, body weight remained higher (+13%) in BERKO mice compared with WT controls. However, this difference was not statistically significant ($P > 0.05$, Table 1).

Femoral length. Similar to the findings for body weight,

	6-month-old (adult)		13-month-old (aged)	
	WT	BERKO	WT	BERKO
Total bone content (mg/mm)	1.37±0.04	1.60±0.05 [b]	1.51±0.06	1.80±0.11 [a]
Total bone density (mg/cm^3)	787±29.4	862±8.44 [a]	799±22.9	845±30.9
Total bone area (mm^2)	1.76±0.09	1.86±0.06	1.88±0.04	2.12±0.09 [a,A]
Cortical bone content (mg/mm)	1.49±0.05	1.72±0.06 [b]	1.66±0.06	1.95±0.10 [a]
Cortical bone density (mg/cm^3)	1073±17.9	1132±6.34 [b]	1154±13.5 [B]	1165±18.9
Cortical bone area (mm^2)	1.39±0.05	1.52±0.05	1.44±0.04	1.66±0.06 [b]
Cortical Thickness (mm)	0.41±0.02	0.44±0.01	0.40±0.01	0.45±0.02 [a]
Periosteal circumference (mm)	4.70±0.12	4.82±0.08	4.86±0.06	5.15±0.10 [a,A]
Endocortical circumference (mm)	2.10±0.21	2.03±0.08	2.35±0.09	2.33±0.15

Mean±SEM; WT: wild-type controls; BERKO: estrogen receptor-β knockout.
a: $p < 0.05$, b: $p < 0.01$ vs. WT at the same age;
A: $p < 0.05$, B: $p < 0.01$ vs. 6-month-old at the same genotype.

Table 3. Changes in pQCT parameters of femoral shafts in adult and aged female mice.

femoral length was significantly increased (+5.5%) in BERKO mice over WT controls at 6 months of age (Table 1). By 13 months, WT mice had increased their femoral length non-significantly over that measured at 6 months, while BERKO mice maintained the same femoral length during the study period. This results in a non-significant difference in femoral length between BERKO and WT at 13 months of age (Table 1).

Uterine weight and histology. There was no significant difference in uterine weight or uterine epithelial thickness between BERKO and WT mice at either 6 or 13 months of age, although there was a strong trend for increased uterine weight in BERKO mice at 6 months of age (Table 1). Uterine weight and uterine epithelial thickness increased with age in both the WT and BERKO mice between 6 and 13 months of age.

pQCT analysis of distal femoral metaphysis. At 6 months of age, BERKO mice had significantly higher total bone content (+24%), total bone area (+14%), cortical bone content (+19%), cortical bone area (+16%), and periosteal circumference (+7%) than WT mice (Table 2). Trabecular bone content, density and marrow area did not differ significantly between BERKO and WT mice at this age. There were significant age-related increases in total bone area (+11%), and age-related decreases in trabecular bone density (-27%), cortical bone area (-10%) and cortical thickness (-19%) in WT mice between 6 and 13 months of age. Similarly, age-related increases in marrow cavity area (+19%), periosteal circumferences (+5%) and endocortical circumferences (+19%) were found in WT mice at 13 months of age as compared with 6 months of age (Table 2). These age-related changes in WT mice did not occur in BERKO mice between 6 and 13 months of age. All the parameters listed in Table 2 did not differ significantly in BERKO mice between 13 months and 6 months of age. At 13 months, BERKO mice had significantly higher total bone content (+30%), total bone density (+24%), trabecular bone density (+48%), cortical bone content (+32%), cortical bone area (+36%) and cortical thickness (+54%) than WT mice. Further, BERKO mice had significantly lower marrow cavity area (-17%) and endocortical circumference (-18%) than WT mice at 13 months of age (Table 2).

pQCT analysis of femoral shafts. At this cortical bone site, there was a significant increase in total and cortical bone content and density (+6% to +17%) in BERKO compared with WT mice at 6 months of age (Table 3). In WT mice, cortical bone density was the only age-related change at 13 months (+8%) as compared with 6 months of age. In BERKO mice, an age-related increase in total bone area (+14%), and periosteal circumference (+7%) was found in 13 months as compared with 6 months. Compared with WT mice, BERKO mice had significantly higher total bone content (+19%), total bone area (+13%), cortical bone content (+17%), cortical bone area (+16%), cortical thickness (+12%) and periosteal circumference (+6%) at 13 months of age (Table 3).

Cancellous bone histomorphometry of distal femoral metaphysis. At 6 months of age, no significant difference was found in cancellous bone mass, structural indices, bone formation and bone resorption parameters as listed in Figures 2 and 3 between BERKO and WT mice. In WT mice, age-related decreases in trabecular bone volume (-41%) and trabecular number (-37%), and an age-related increase in trabecular separation (+173%) were identified in 13-month-old mice as compared with 6-month-old mice. Osteoclast number, osteoclast surface, mineralizing surface, mineral apposition rate and bone formation rate/BS were maintained at the levels of 6-month-old mice in 13-month-old WT

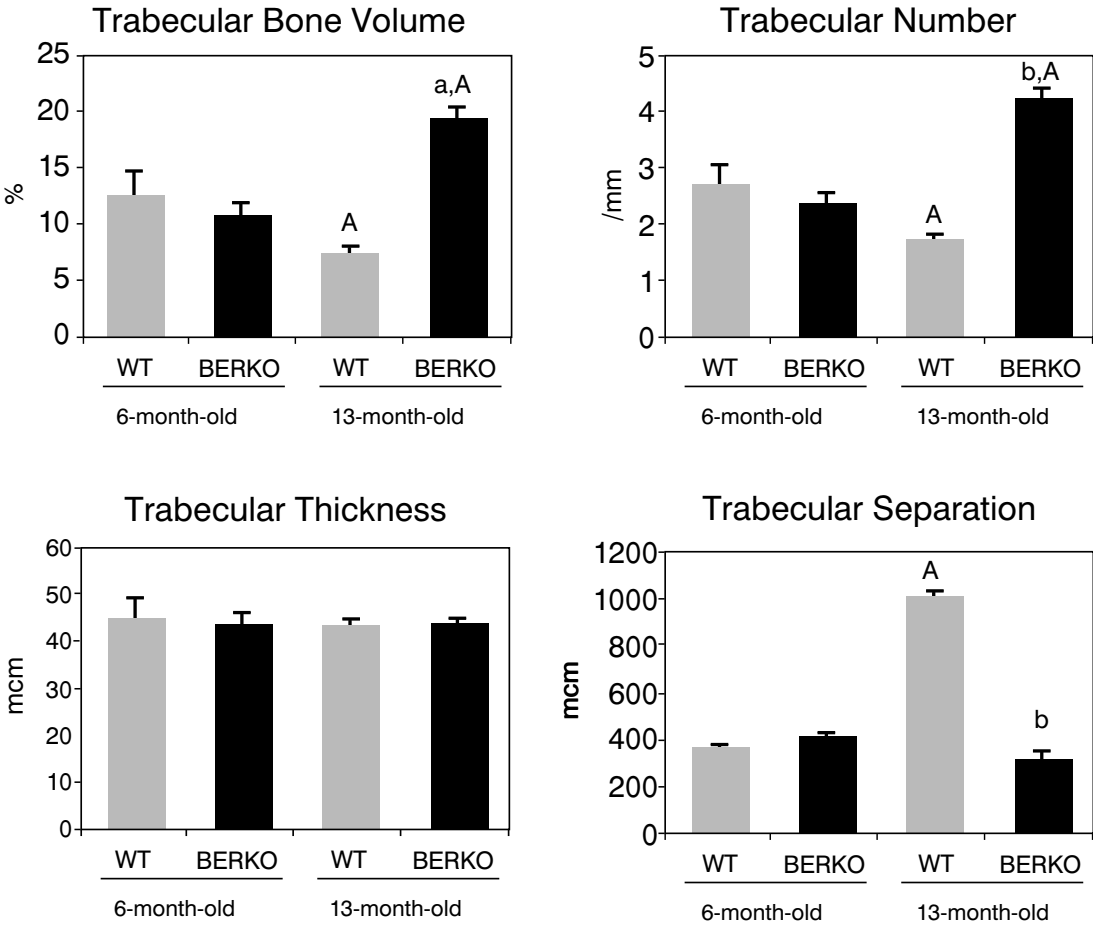

Figure 2. Trabecular bone volume, number, thickness and separation at distal femoral metaphysis of wild-type littermates (WT) and estrogen receptor-β knockout (BERKO) mice at 6 and 13 months of age. Values are given as mean ± SEM. a: $p < 0.05$, b: $p < 0.01$ vs. WT at the same age; A: $p < 0.05$, B: $p < 0.01$ vs. 6-month-old at the same genotype.

mice. In BERKO mice, there was a significant increase in trabecular bone volume (+81%) and trabecular number (+77%), and a significant decrease in osteoclast number (-55%), osteoclast surface (-54%), mineralizing surface (-65%), mineral apposition rate (-72%) and bone formation rate/BS (-90%) in 13-month-old as compared with 6-month-old mice. At 13 months, BERKO mice had significantly higher trabecular bone volume, trabecular number, and significantly lower trabecular separation, osteoclast number, osteoclast surface, mineralizing surface, mineral apposition rate, and bone formation rate/BS than WT mice (Figures 2 and 3).

Discussion

Decreased estrogen levels associated with menopause result in an increase in bone resorption and bone turnover, leading to bone loss and skeletal fractures[2]. These events can be prevented by estrogen replacement therapy[1,3,4]. Since both estrogen receptor subtypes, ERα and ERβ, have almost identical DNA-binding domains[5,27] and are expressed in both human and murine osteoblast and bone tissues[7,8], it is reasonable to hypothesize that estrogens may act via both ERα and ERβ in the skeleton. In this study, we documented the changes on cancellous and cortical bone surfaces in BERKO female mice as compared with WT controls at both 6- and 13-months of age. The results from the current study illustrate that ERβ plays an important role in regulation of female skeletal tissue during the growth and aging process.

In this study, we found that mice lacking ERβ had greater periosteal circumference, cortical bone mineral content and area in the distal femoral metaphysis (DFM) and greater cortical content and density in the femoral shaft compared with the wild-type littermates at 6 months of age. These data demonstrate that ERβ may play an inhibitory role in the cortical bone; specifically affecting bone formation on the periosteal surface of growing bone, which contributes to the attainment of peak bone mass during maturation. The cortical bone mass in 13-month-old BERKO mice was higher than in WT mice. We also found that femoral length was significantly higher in 6-month-old BERKO mice than in WT

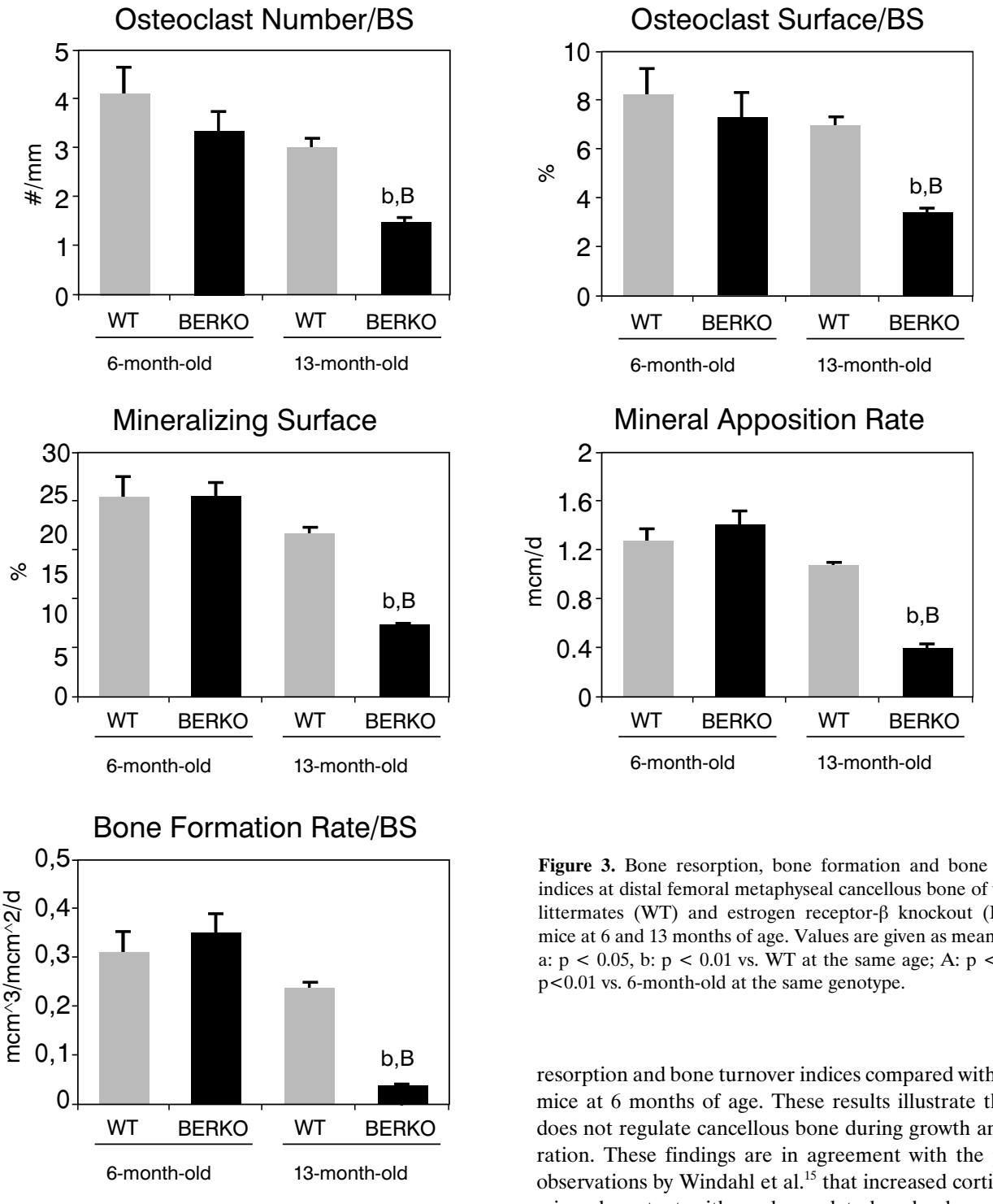

Figure 3. Bone resorption, bone formation and bone turnover indices at distal femoral metaphyseal cancellous bone of wild-type littermates (WT) and estrogen receptor-β knockout (BERKO) mice at 6 and 13 months of age. Values are given as mean ± SEM. a: $p < 0.05$, b: $p < 0.01$ vs. WT at the same age; A: $p < 0.05$, B: $p<0.01$ vs. 6-month-old at the same genotype.

mice, indicating that ERβ plays a inhibitory role in longitudinal bone growth during maturation. Endosteal circumference and marrow cavity area of DFM were not affected in BERKO mice, indicating that ERβ does not play a role in the regulation of endocortical modeling during maturation. On the trabecular surfaces of DFM, BERKO mice had normal trabecular bone content, trabecular density, trabecular bone volume, structural parameters, bone formation, bone resorption and bone turnover indices compared with the WT mice at 6 months of age. These results illustrate that ERβ does not regulate cancellous bone during growth and maturation. These findings are in agreement with the previous observations by Windahl et al.[15] that increased cortical bone mineral content with unchanged trabecular bone mineral density were found in female mice lacking ERβ at 11 weeks of age. Since BERKO female mice had normal cortical bone mineral content and density at 4 weeks of age[15], ERβ might not play a role in the early cortical bone growth and development due to the extremely low estrogen levels in prepubertal mice. Taking these data together, we find that ERβ begins to play an inhibitory role on cortical bone accumulation, specifically bone formation on the periosteal surface, after 4 weeks of age as circulating estrogen levels rise

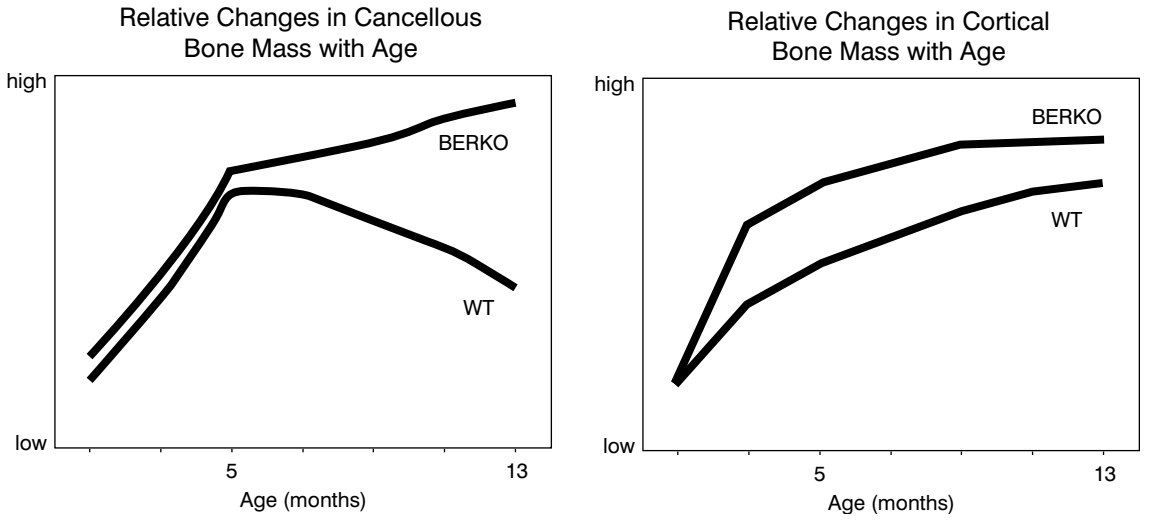

Figure 4. Illustration showing the effects of estrogen receptor-β knockout (BERKO) on relative changes in cancellous and cortical bone mass with age as compared with the wild-type littermates (WT) during growth and aging.

through puberty. However, ERβ does not play a role in the regulation of cancellous bone before 6 months of age, since no differences were found in cancellous bone parameters between BERKO and WT mice before 6 months of age.

In this study, we found that WT mice significantly increased body weight and non-significantly increased femoral length between 6 to 13 months of age, while these parameters plateaued in the BERKO mice after 6 months of age. This results in a non-significant difference in body weight and femoral length between WT and BERKO at 13-month-old, although BERKO had increased body weight and femoral length at 6 months. It has been reported[14] that mice lacking ERβ had normal body weight and femoral length at 4 weeks of age but had increased both parameters by 11 weeks of age. Together with the findings from the current study, we conclude that ERβ plays inhibitory roles in body weight gain and longitudinal bone growth during puberty in female mice. Deleting this receptor led to an early increase in overall growth of the whole body and long bone. However, by 13 months, mice with ERβ (WT) gradually reach the body weight and femoral length levels of mice lacking ERβ (BERKO).

The differential aging response of cancellous and endocortical bone between mice with or without ERβ is a unique finding from this study. Between 6 and 13 months of age, WT mice lose trabecular bone density, trabecular bone volume and increase marrow cavity area and endocortical circumference, while bone formation and bone resorption parameters remain similar. However, these age-related decreases in cancellous and endocortical bone did not occur in mice lacking ERβ (BERKO). In fact, BERKO mice had significantly higher trabecular bone volume and trabecular number at 13 months compared with 6 months. However, trabecular content and density remained similar between 6 to 13 months of age in BERKO mice. The reason for the discrepancy between pQCT and histomorphometric measurements is not known. It may be due to a misassignment of part of trabecular bone to endocortical bone or even cortical bone by pQCT, since we observed a large amount of trabecular bone near the endocortical surface in the marrow cavity in 13-month-old BERKO mice. Dynamic histomorphometric analysis of cancellous bone indicated that BERKO had much higher cancellous bone mass and much lower cancellous bone resorption and turnover than WT did at 13 months of age. These results may indicate that ERβ plays a role in stimulating cancellous bone resorption and bone turnover that leads to age-related cancellous bone loss during the aging process. These results are in partial agreement with the most recent report of Windahl and colleagues[28], which indicates that ERβ knockout partially protected against age-related trabecular bone loss. However, these results differ from those reported by Sims et al.[16], which showed that BERKO female mice had higher trabecular bone volume at 10 weeks and 16 weeks of age, but decreased to the level of WT controls by 12 months of age. We have no explanation for the difference between our results and those of Sims et al.[16] with response of trabecular bone to ERβ knockout in female mice. In their study, Windahl and colleagues[28] found that serum bone resorption markers and osteoclast number did not differ, but messenger RNA expression levels of osteoblast marker core-bind factor a1 (Cbfa1) were increased in 1-year-old BERKO compared with WT mice. Therefore, they concluded that ERβ knockout leads to an increase in bone formation, thus protecting against age-related trabecular bone loss. However, the results from histomorphometric analysis of cancellous bone of the DFM in the current study clearly show that bone resorption indices such as osteoclast number and osteoclast surface were significantly decreased (more than 50%) in BERKO versus WT at 13 months of age. In addition, bone formation and bone

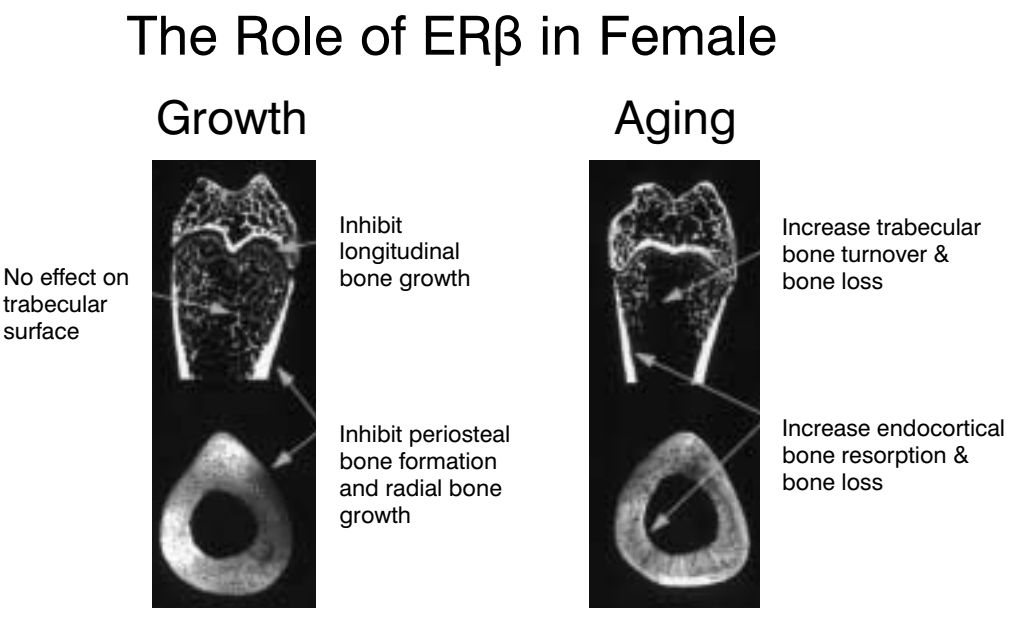

Figure 5. Illustration showing the physiological roles of estrogen receptor-β (ERβ) on different bone surfaces during growth and aging.

turnover indices such as mineralizing surface, mineral apposition rate and bone formation rate/bone surface referent were significantly decreased (more than 60%) in BERKO versus WT at 13 months of age. The most recent report by Sims and colleagues[16] showed that osteoclast surface decreased in 10- and 16-week-old but increased in 12-month-old BERKO female mice as compared with their WT controls. The reason for the difference of bone resorption in response to ERβ knockout in aged female mice between the current study and the study by Windahl et al.[28] and Sims et al.[16] is not clear. It may be due to the different measurement techniques.

As reported by Windahl et al.[28], mice lacking ERβ did not have altered serum estradiol levels. However, BERKO mice had increased messenger RNA expression levels of ERα. Upregulation of ERα in these BERKO mice may lead to the increased sensitivity of the skeletal system to estrogen which may contribute to the protection of age-related bone loss. We can't completely exclude this possibility from the available study results.

In summary, as illustrated in Figure 4, the knockout of the ERβ gene does not affect cancellous bone up to 6 months old in female mice. Thereafter, the ERβ knockout protects against age-related cancellous bone loss and in fact slightly increases cancellous bone mass up to 13 months by decreasing bone resorption and bone turnover compared with WT mice. In cortical bone, ERβ knockout does not affect cortical bone surface (periosteal and endocortical surfaces) or cortical bone mass in pre-pubertal female mice (4-week-old). However, ERβ knockout mice have higher cortical bone mass in mature (6-month-old) and aged (13-month-old) female mice. These findings reveal the potential physiological roles of ERβ in female skeletal regulation as shown in Figure 5. During puberty, ERβ plays a role in inhibiting periosteal bone formation, radial and longitudinal bone growth in females. At the beginning of the aging process, ERβ plays a role in stimulating trabecular and endocortical bone resorption and bone turnover leading to bone loss. These results reveal that antagonizing ERβ in growing females may not only increase peak cortical bone mass during maturation but may also protect against age-related cancellous bone loss during the aging process.

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