Estrogen receptor specificity in the regulation of the skeleton in female mice

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Abstract

There are two known estrogen receptors, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), which may mediate the actions of estrogen. The aim of the present study was to compare fat content, skeletal growth and adult bone metabolism in female mice lacking ERα (ERKO), ERβ (BERKO) or both ERs (DERKO). We demonstrate that endogenous estrogens decrease the fat content in female mice via ERα and not ERβ. Interestingly, the longitudinal bone growth was decreased in ERKO, increased in BERKO, but was intermediate in DERKO females, demonstrating that ERα and ERβ exert opposing effects in the regulation of longitudinal bone growth. The effects on longitudinal bone growth were correlated with similar effects on serum levels of IGF-I. A complex regulation of the trabecular bone mineral density (BMD), probably caused by a disturbed feedback regulation of estrogen and testosterone, was observed in female ER-inactivated mice. Nevertheless, a partial functional redundancy for ERα and ERβ in the maintenance of the trabecular BMD was observed in the female mice at 60 days of age. Thus, ERα and ERβ may have separate effects (regulation of fat), opposing effects (longitudinal bone growth) or partial redundant effects (trabecular BMD at 60 days of age), depending on which parameter is studied.

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Introduction

Estrogen is of importance for the regulation of pubertal growth and adult bone metabolism in females. The importance of estrogen in maintaining the trabecular bone mineral density (BMD) in the adult skeletal tissue is clearly illustrated by the pronounced trabecular bone loss observed in estrogen-deficient states, such as in postmenopausal osteoporosis and after ovariectomy. The exact mechanism whereby estrogen exerts its effect in the skeleton remains poorly understood.

There are two known estrogen receptor (ER) subtypes, denoted ERα and ERβ (Green *et al.* 1986, Kuiper *et al.* 1996). The role of these two receptors in mediating the effect of estrogen on bone is not fully understood. However, it has been demonstrated that both ERα and ERβ are expressed in the growth plate as well as in the bone, suggesting a role for both ER subtypes in the regulation of skeletal homeostasis (Arts *et al.* 1997, Onoe *et al.* 1997, Kusec *et al.* 1998, Lim *et al.* 1999, Nilsson *et al.* 1999, Vidal *et al.* 1999*a*, Windahl *et al.* 2000).

We have previously shown that female mice lacking ERα (ERKO) display decreased appendicular growth, while female mice lacking ERβ (BERKO) display an increased appendicular growth (Vidal *et al.* 1999*b*, Windahl *et al.* 1999), demonstrating an important role for estrogen in the regulation of longitudinal bone growth. The fact that some estrogenic effects are preserved in ERKO mice (Das *et al.* 1997, Iafrati *et al.* 1997, Shughrue *et al.* 1997) and BERKO mice (Karas *et al.* 1999, Windahl *et al.* 1999) indicates that alternative pathways may be involved or that possible compensatory activity of the remaining ER subtype may exist, i.e. functional redundancy.

The recent development of Double-Estrogen Receptor Knockout (DERKO) mice makes it possible to functionally distinguish between ERα and ERβ as well as other estrogen dependent pathways *in vivo* (Couse *et al.* 1999). In the present study, we investigated the role of ER subtypes in the regulation of skeletal growth and adult bone metabolism in female mice.

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Materials and Methods

Animals

Male double heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were mated with female double heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice, resulting in $ER\alpha^{+/+}ER\beta^{+/+}$ (wild type, WT), $ER\alpha^{-/-}ER\beta^{+/+}$ (ERKO), $ER\alpha^{+/+}ER\beta^{-/-}$ (BERKO) and $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO) offspring with a mixed C57BL/6J/129 background (Lubahn *et al.* 1993, Krege *et al.* 1998). Disruption of the mouse ERα gene was obtained by insertion of a neomycin-resistance gene into exon 2, resulting in a disruption of the reading frame, as previously described (Lubahn *et al.* 1993). Disruption of the DNA-binding domain of the ERβ gene was obtained by an insertion of a neomycin-resistance gene into exon 3 of the ERβ gene as previously described (Krege *et al.* 1998). The numbers of animals in the first experiment (60 days of age) were WT $n=14$, ERKO $n=8$, BERKO $n=19$ and DERKO $n=15$. In the second experiment (140 days of age) the numbers of animals were WT $n=10$, ERKO $n=9$, BERKO $n=10$ and DERKO $n=10$. Genotyping of tail DNA was performed at 3 weeks of age as previously described (Vidal *et al.* 2000). Animals had free access to fresh water and food pellets (B&K Universal AB, Sollentuna, Sweden) consisting of cereal products (76·9% barley, wheat feed, wheat and maize germ), vegetable proteins (14·0% hipro soya) and vegetable oil (0·8% soya oil).

Dual-energy X-ray absorptiometry (DXA)

Measurement of bone mineral content (BMC) and areal bone mineral density (aBMD) was performed with the Norland pDEXA Sabre (Norland, Fort Atkinson, WI, USA) and the Sabre Research software (v3·6) as previously described (Windahl *et al.* 1999). Briefly, the machine was calibrated daily with a phantom provided by the manufacturer. Medium resolution scans, with line spacing set at 0·05 cm, were used. Three mice were analyzed at a time, including one mouse that was sacrificed at the beginning of the experiment and included in all the scans to avoid inter-scan variation. The inter-assay coefficient of variation was less than 5% (Andersson *et al.* 2001). An *in vivo* prediction of fat content was performed as described previously (Ohlsson *et al.* 2000).

Peripheral quantitative computerized tomography (pQCT)

Computerized tomography was performed with the Stratec pQCT XCT Research M (Norland; v5·4B) operating at a resolution of 70 μm, as previously described (Windahl *et al.* 1999).

Cortical parameters were determined with a mid-diaphyseal pQCT scan of the tibiae. Trabecular BMD was determined with a metaphyseal pQCT scan of the proximal tibiae positioned at a distance from the growth plate corresponding to 1·3% of the total length of the tibiae. The trabecular bone compartment was defined as the inner 45% of the total area. The inter-assay coefficients of variation (CV) for the pQCT measurements were less than 2%. We have previously shown that this measurement of trabecular BMD is very well correlated to measurement of the ratio of trabecular bone volume (BV) to total volume (TV) (BV/TV) by histomorphometry (Windahl *et al.* 2001).

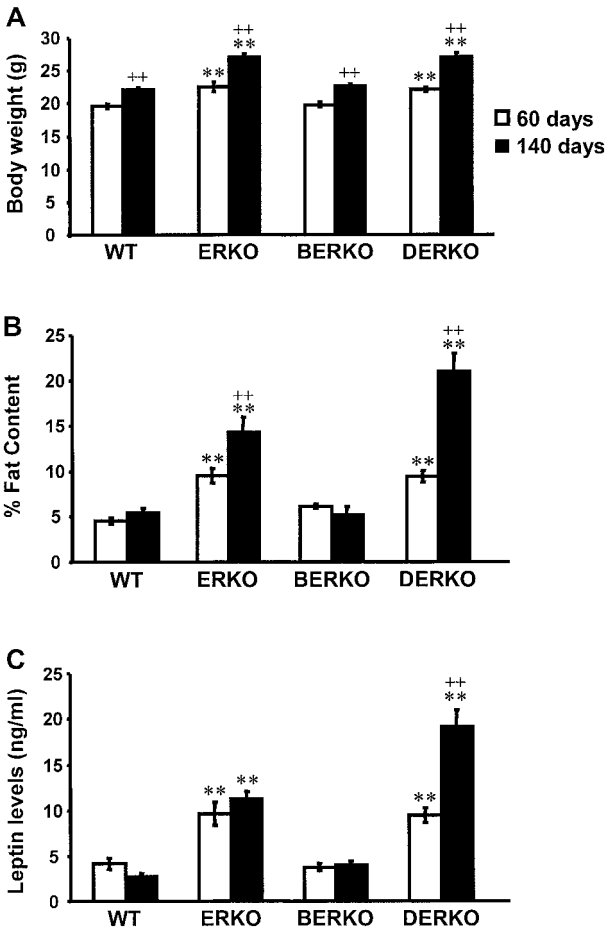

Figure 1 Body weight (A), fat content, as determined using DXA (B), and serum leptin levels (C) in WT, ERKO, BERKO and DERKO females at 60 days of age (open bars) and at 140 days of age (filled bars). Values are given as means ± S.E.M.S (60 days old: WT $n=14$, ERKO $n=8$, BERKO $n=19$, DERKO $n=15$; 140 days old: WT $n=10$, ERKO $n=9$, BERKO $n=10$, DERKO $n=10$). Values were first analyzed with one-way ANOVA followed by Student–Newman–Keuls multiple range test. $^{**}P<0.01$ versus WT, $^{++}P<0.01$ versus 60 days old.

RIA

Serum insulin-like growth factor-I (IGF-I) levels were measured by double antibody IGF binding protein-blocked RIA (Blum & Breier 1994). Serum osteocalcin

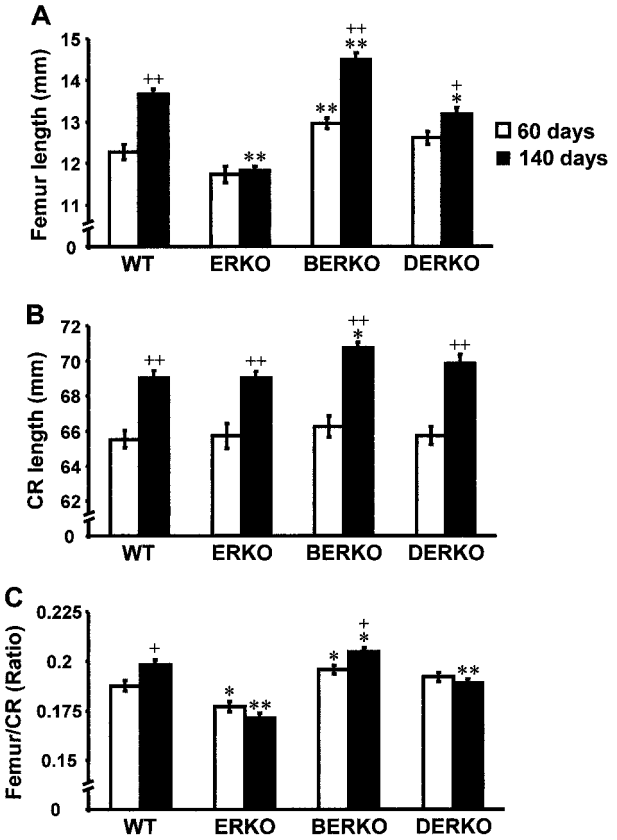

Figure 2 Femur length (A), crown–rump length (CR) (B), and the femur/CR ratio (C) in WT, ERKO, BERKO and DERKO females at 60 days of age (open bars) and at 140 days of age (filled bars). Values are given as means ± S.E.M.S (60 days old: WT $n=14$, ERKO $n=8$, BERKO $n=19$, DERKO $n=15$; 140 days old: WT $n=10$, ERKO $n=9$, BERKO $n=10$, DERKO $n=10$). Values were first analyzed with one-way ANOVA followed by Student–Newman–Keuls multiple range test. $^{*}P<0.05$, $^{**}P<0.01$ versus WT, $^{+}P<0.05$, $^{++}P<0.01$ versus 60 days old.

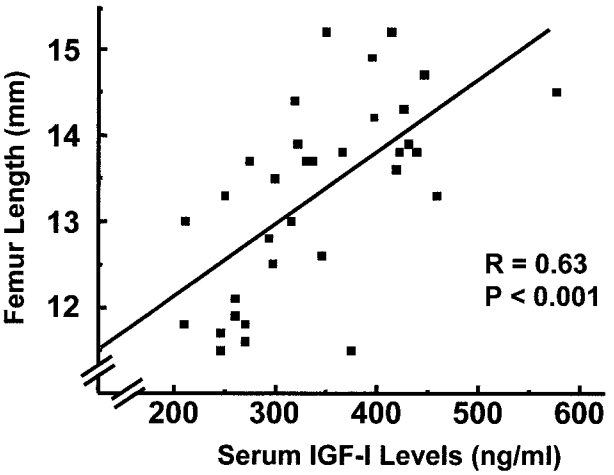

Figure 3 Linear regression analysis of femur length as independent variable and serum IGF-I levels as dependent variable. Femur length and serum IGF-I levels were measured in 140-day-old female WT ($n=10$), ERKO ($n=9$), BERKO ($n=10$) and DERKO ($n=7$) mice. Pearson's correlation coefficient (r) was calculated. t-tests were used and a P value<0·05 was considered significant.

levels were measured by RIA using rabbit anti-mouse osteocalcin antiserum and purified mouse osteocalcin as standard and tracer (Richman *et al.* 1999). The sensitivity of the mouse osteocalcin assay was 0·5 ng/ml and intra- and inter-assay CVs were less than 8%. 17β-estradiol was measured using a RIA detecting estradiol (DiaSorin, Saluggia, Italy), with a sensitivity below 5 pg/ml at 95% confidence limit. Testosterone was measured using Immuchem Double Antibody Testosterone RIA, with a sensitivity of 0·1 ng/ml (ICN Biomedicals Inc., Costa Mesa, CA, USA). Levels of c-telopeptide were measured in serum by an ELISA which measures degradation products of type I collagen that is generated by osteoclastic bone resorption (Srivastava *et al.* 2000). The sensitivity of the ELISA was less than 0·1 ng/ml. The average intra- and inter-assay CVs were less than 12%. Serum leptin levels were measured by an ELISA (Chrystal Chem Inc., Chicago, IL, USA) with an intra-assay and inter-assay CVs of 5·4 and 6·9% respectively.

Tartrate-resistant acid phosphate (TRAP) 5b immunoassay

TRAP 5b, purified from human osteoclasts as described (Halleen *et al.* 1996), was used as antigen to develop a polyclonal antiserum in rabbits (Alatalo *et al.* 2000). The antiserum was incubated on antirabbit IgG-coated microtiter plates (EG & G Wallac) for 1 h. Diluted mouse serum samples were incubated in the wells for 1 h, and bound enzyme activity was detected using 8 mmol/l 4-nitrophenyl phosphate (4-NPP) as substrate in 0·1 mol/l sodium acetate buffer, pH 6·1, for 2 h at 37 °C. The enzyme reactions were terminated by adding 25 µl of 0·32 mol/l NaOH, and A_{405} was measured using model 2 Victor equipment (EG & G Wallac).

Results

Body growth

At 60 days of age, shortly after sexual maturation in WT mice, the body weight was increased in female ERKO and DERKO but not in BERKO mice compared with WT mice (Fig. 1A). These effects on body weight were more pronounced at 140 days of age (Fig. 1A). Because of the increase in body weight in ERα inactivated mice, body fat content and serum leptin levels were analyzed. The body fat content, as measured with DXA, was increased in ERKO and DERKO but not in BERKO females

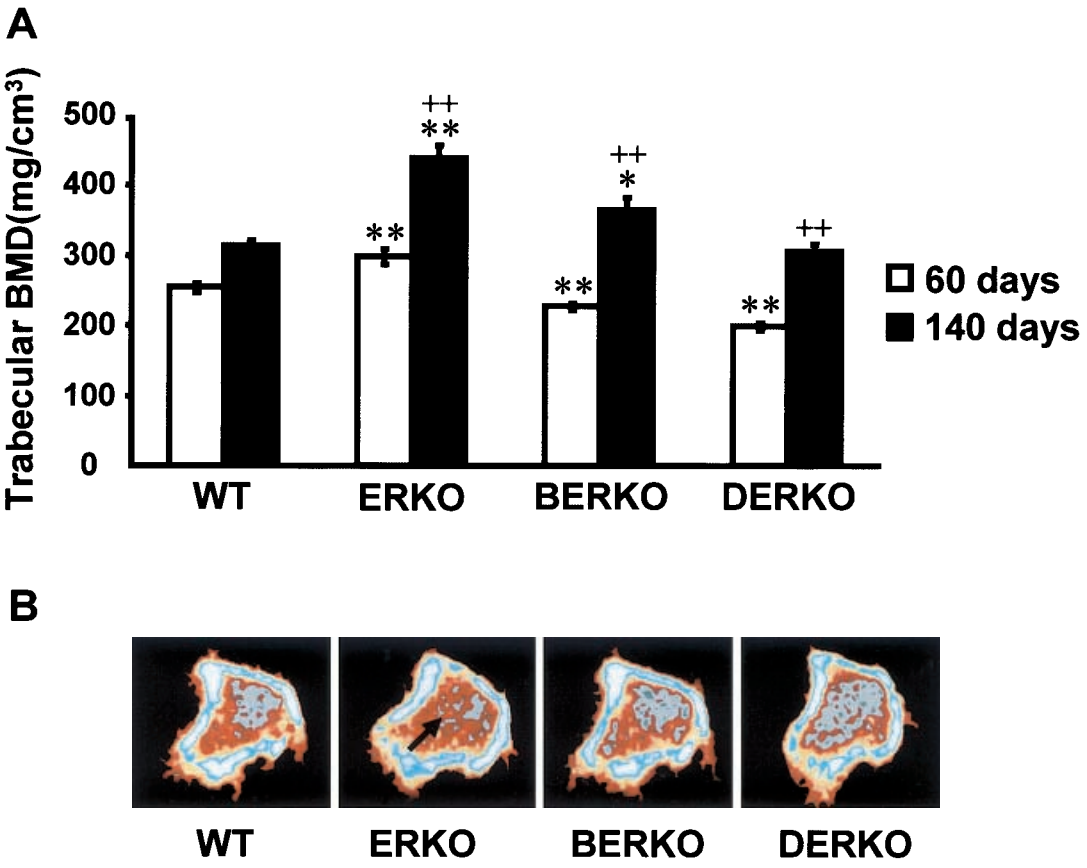

Figure 4 Trabecular BMD in WT, ERKO, BERKO and DERKO females at 60 days of age (open bars) and at 140 days of age (filled bars), as measured in a metaphyseal pQCT scan of the proximal tibiae (A). Values are given as means ± S.E.M.S (60 days old: WT $n=14$, ERKO $n=8$, BERKO $n=19$, DERKO $n=15$; 140 days old: WT $n=10$, ERKO $n=9$, BERKO $n=10$, DERKO $n=10$). Values were first analyzed with one-way ANOVA followed by Student–Newman–Keuls multiple range test. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ versus WT, $^{++}P<0{\cdot}01$ versus 60 days old. Representative metaphyseal pQCT scan of the proximal tibiae in female WT, ERKO, BERKO and DERKO mice at 60 days of age (B). The arrow indicates a central area consisting of trabecular bone.

(Fig. 1B). The effects on fat content were associated with similar effects on serum levels of leptin (Fig. 1C).

The femur length was used as a measurement of appendicular growth. At 60 days of age there was an increase in the BERKO (6%), while no change was seen in the DERKO as compared with WT mice. At 140 days of age the femur length was decreased in ERKO (–14%) while it was increased in BERKO mice (6%). A small decrease was seen in 140 days old DERKO (–4%) mice as compared with WT mice. However, the femur length in female DERKO mice, at 140 days of age, was intermediate between the lengths of ERKO and BERKO mice (Fig. 2A). The crown–rump (CR) length was used as a measurement of the axial skeletal growth. It was unchanged between all genotypes except for a small increase (3%) in BERKO mice compared with WT at 140 days of age (Fig. 2B). The differential effects on the appendicular and the axial skeletal growth were illustrated by the fact that ERKO mice displayed a decreased femur/CR ratio, while BERKO mice displayed an increased ratio (Fig. 2C). DERKO mice had a femur/CR ratio that was intermediate between ERKO and BERKO mice. An altered length of femur is often associated with a disturbed growth hormone–IGF-I axis. A two-way ANOVA, in which ER$\alpha^{-/-}$ and ER$\beta^{-/-}$ were regarded as separate treatments, demonstrated that serum levels of IGF-I were decreased in ER$\alpha^{-/-}$ while they were increased in ER$\beta^{-/-}$ mice at 140 days of age (WT, 354 ± 20 ng/ml; ERKO, 277 ± 15 ng/ml; BERKO, 412 ± 23 ng/ml; DERKO, 319 ± 22 ng/ml, $P<0{\cdot}05$, two-way ANOVA followed by Student–Newman–Keuls multiple range test). Furthermore, the length of femur was correlated with serum IGF-I levels in ER inactivated mice (Fig. 3).

Table 1 Cortical bone parameters measured by pQCT. Values are given as means ± S.E.M.s

	WT		ERKO		BERKO		DERKO	
	60 days (n=14)	140 days (n=10)	60 days (n=8)	140 days (n=9)	60 days (n=19)	140 days (n=10)	60 days (n=15)	140 days (n=10)
BMC (mg/mm)	0·58 ± 0·02	0·74 ± 0·02^{++}	0·63 ± 0·02	0·82 ± 0·01^{*++}	0·54 ± 0·02	0·81 ± 0·01^{**++}	0·54 ± 0·02	0·80 ± 0·02^{++}
Area (mm^2)	0·57 ± 0·02	0·67 ± 0·02^{++}	0·61 ± 0·01	0·71 ± 0·02^{++}	0·53 ± 0·01	0·72 ± 0·01^{++}	0·54 ± 0·02	0·71 ± 0·02^{++}
Thickness (mm)	0·15 ± 0·003	0·18 ± 0·004^{++}	0·17 ± 0·003*	0·20 ± 0·002^{**++}	0·15 ± 0·003	0·19 ± 0·002^{**++}	0·14 ± 0·004	0·19 ± 0·003^{**+}
Periosteal circumference (mm)	4·23 ± 0·06	4·26 ± 0·05	4·20 ± 0·03	4·17 ± 0·06	4·04 ± 0·06	4·35 ± 0·05^{++}	4·22 ± 0·05	4·31 ± 0·06
Endosteal circumference (mm)	3·27 ± 0·05	3·12 ± 0·04^{+}	3·15 ± 0·03	2·91 ± 0·06^{*+}	3·12 ± 0·05	3·13 ± 0·04	3·31 ± 0·05	3·11 ± 0·06^{+}
BMD (mg/mm^3)	1·003 ± 0·009	1·099 ± 0·007^{++}	1·033 ± 0·007*	1·164 ± 0·010^{**++}	1·030 ± 0·004**	1·127 ± 0·006^{**++}	1·000 ± 0·006	1·127 ± 0·007^{**+}

*P<0·05, **P<0·01 versus WT, one-way ANOVA followed by Student–Newman–Keuls multiple range test. ^{+}P<0·05, ^{++}P<0·01 versus 60 days, Student's t-test.

Cancellous BMD

The trabecular BMD (tBMD) was measured *in vivo* using pQCT in the metaphyseal region of the proximal tibiae. In 60-day-old mice the tBMD was increased in ERKO (18%), slightly decreased in BERKO (–11%) and clearly decreased in DERKO (–22%) mice (Fig. 4A, B). At 140 days of age the increase in ERKO was more pronounced (40%), and an increase was also seen in BERKO mice (16%). The decrease in tBMD seen in 60-day-old DERKO mice disappeared in the 140-day-old DERKO mice (Fig. 4A). When comparing 140- with 60-day-old mice an increase in tBMD was seen in ERKO, BERKO and DERKO but not in WT mice (Fig. 4A).

Cortical bone parameters

Cortical bone parameters were analyzed with a mid-diaphyseal pQCT scan of the tibiae. The mid-diaphysis is a region containing mainly cortical bone in mice. The cortical bone mineral content (BMC) was increased in 140-day-old ERKO (11%), BERKO (10%) and DERKO (8%) mice as compared with WT mice (Table 1). This increase was associated with an increased cortical thickness. When comparing 140- with 60-day-old mice an increase was seen in cortical content, area, thickness and BMD in all genotypes. The endosteal circumference was decreased (–8%) by age in ERKO mice, while the periosteal circumference was increased (+8%) by age in BERKO mice (Table 1).

Bone mineral status as determined by DXA

BMC and aBMD of total body, cranium, spine and femur were measured with DXA. The results obtained from these measurements are a combination of effects on size, trabecular BMD and cortical bone parameters. BMC and aBMD were unchanged in ERKO mice while they were increased at all sites in 140-day-old BERKO mice as compared with WT (Table 2). The spine and femur BMC as well as aBMD were decreased in 60-day-old DERKO mice. DERKO mice aged 140 days had decreased BMC in the total body and in the spine (Table 2).

Sex steroids and biochemical bone parameters

The serum levels of 17β-estradiol were strongly increased in ERKO (550%) and DERKO (251%) mice, while the levels in BERKO mice were unchanged as compared with WT mice (Table 3). Serum levels of testosterone were not detectable in female WT and BERKO mice, while they were increased in female ERKO and DERKO mice (Table 3). The serum levels of osteocalcin were decreased in ERKO (–34%), BERKO (–48%) and DERKO (–44%) mice as compared with WT (Table 3). TRAP 5b activity

Table 2 DXA measurements of aBMD and BMC. Values are given as % WT ± S.E.M.

	ERKO		BERKO		DERKO	
	60 days (n=8)	140 days (n=9)	60 days (n=19)	140 days (n=10)	60 days (n=15)	140 days (n=10)
aBMD Total	2·6 ± 0·8	1·6 ± 0·6	2·7 ± 0·2	4·9 ± 0·7**	2·1 ± 1·0	1·5 ± 0·7
Cranial	3·2 ± 2·1	3·9 ± 0·9	2·7 ± 1·1	7·0 ± 1·1**	− 1·5 ± 1·0	1·6 ± 1·2
Spine	− 1·7 ± 1·4	− 3·7 ± 0·1	− 0·4 ± 1·1	8·2 ± 0·1**	− 5·0 ± 0·8**	− 5·1 ± 0·1
Femur	0·3 ± 1·5	4·6 ± 1·7	0·5 ± 1·0	8·6 ± 2·1**	− 5·7 ± 1·6**	0·2 ± 1·3
BMC Total	− 3·1 ± 3·6	3·2 ± 2·2	− 3·4 ± 3·7	14·4 ± 3·8**	− 16·5 ± 2·0**	− 12·1 ± 2·8*
Cranial	3·0 ± 3·1	1·4 ± 0·9	0·4 ± 2·4	8·4 ± 1·9**	− 2·9 ± 1·6	− 5·2 ± 1·7
Spine	− 21·2 ± 6·6	− 8·7 ± 4·9	− 3·6 ± 6·1	16·8 ± 4·8**	− 28·1 ± 3·5**	− 17·8 ± 3·7*
Femur	1·6 ± 6·7	− 2·6 ± 4·6	0·8 ± 3·4	18·0 ± 3·6**	− 24·9 ± 4·9**	− 10·5 ± 2·9

*P<0·05, **P<0·01 versus WT, one-way ANOVA followed by Student–Newman–Keuls multiple range test.

was unchanged in all genotypes, while serum levels of c-telopeptide were increased in ERKO mice (54%) compared with WT mice (Table 3).

Discussion

Estrogen exerts a variety of important physiological effects including regulation of longitudinal bone growth, body composition and trabecular bone mineral density. The ER specificity for these effects was in the present study characterized in female mice.

Estrogen is of importance for the regulation of fat mass in mice. Ovariectomy increases while substitution with estrogen inhibits weight gain in mice (Ornoy *et al.* 1994). Furthermore, aromatase deficiency results in obesity (Fisher *et al.* 1998). Increased adipose tissue has recently been described in ERα-inactivated female mice (Heine *et al.* 2000). We have in the present study demonstrated that female ERKO and DERKO, but not BERKO, mice have increased fat content associated with increased serum levels of leptin. We cannot say if the increased body weight in ERKO and DERKO only is dependent on this increase in fat mass, as the muscle mass was not measured. However, our DXA data clearly demonstrates that the proportion of body fat is increased in female mice lacking ERα. Thus, endogenous estrogens decrease the fat content in female mice via ERα and not ERβ. Similarly, we have recently described that ERα but not ERβ is of importance for the regulation of fat mass in male mice as well (Ohlsson *et al.* 2000).

We have previously shown that female ERKO mice have decreased femur length, and that this decrease is associated with a decrease in serum levels of IGF-I (Vidal *et al.* 1999*b*). These results in female ERKO mice were confirmed in the present study. Furthermore, ERβ inactivated mice displayed, both in the present study and in a previous study, an increased length of femur, which in the present study was shown to be associated with an increase of serum levels of IGF-I (Windahl *et al.* 1999). Interestingly, the length of femur and serum levels of IGF-I were, in female DERKO mice, intermediate between ERKO and BERKO mice. A fact supporting the notion that the GH–IGF-I axis might be involved in the estrogenic regulation of appendicular bone growth is that the serum IGF-I levels were strongly correlated to the femur length in the mice with ER inactivation. These data clearly demonstrate that ERα and ERβ have opposing effects regarding the effect on appendicular growth in female mice. We propose that ERα promotes, while ERβ represses, longitudinal bone growth in female mice. These findings are in line with the notion that ERβ sometimes

Table 3 Serum parameters in 140-day-old female mice. Values are given as means ± S.E.M.s (WT n=4–16, ERKO n=3–14, BERKO n=4–17, DERKO n=4–16)

	WT	ERKO	BERKO	DERKO
17β-Estradiol (pmol/l)	32·9 ± 1·1	213·7 ± 12·8**	36·1 ± 0·7	115·5 ± 5·5**
Testosterone (ng/ml)	ND	1·73 ± 0·12**	ND	0·19 ± 0·05*
Osteocalcin (ng/ml)	261 ± 23	178 ± 27*	141 ± 21**	151 ± 22**
TRAP 5b (U/l)	24·4 ± 1·2	20·7 ± 1·9	23·2 ± 2·1	23·1 ± 2·0
c-telopeptide (ng/ml)	10·8 ± 1·1	16·6 ± 1·3**	9·0 ± 0·6	9·7 ± 1·6

*P<0·05, **P<0·01 versus WT, ND=not detectable. One-way ANOVA followed by Student–Newman–Keuls multiple range test.

acts as a repressor of ERα-mediated effects, and is also supported by our recent study, demonstrating that ERβ is a negative modulator of ERα activity in the immature uterus (Weihua *et al.* 2000). Furthermore, ERα and ERβ have previously been shown to exert opposing effects *in vitro* where ERα promotes, while ERβ inhibits transcription when complexed with 17β-estradiol at an AP-1 element (Paech *et al.* 1997). The estrogenic regulation of appendicular growth is somewhat different in males compared with females. In ERKO males the growth is repressed, resulting in shorter long bones, comparable with females. However, no effect is seen in BERKO males. Male DERKO mice follow the same growth pattern as male ERKO mice, in contrast to the intermediate growth seen in female DERKO mice. Thus, ERβ represses longitudinal bone growth in female mice, while it has no function in the regulation of longitudinal bone growth in male mice.

The trabecular BMD was increased in female ERKO mice. The mechanism behind this increase is unclear but one might speculate that the strongly elevated levels of testosterone in female ERKO mice might result in an increased activation of the androgen receptor. An alternative explanation is that the dramatically increased levels of 17β-estradiol seen in ERKO mice may result in an elevated activation of ERβ- and/or of ERα/β-independent pathways and thereby result in increased trabecular BMD. The increased trabecular BMD in ERKO mice was unexpectedly associated with decreased levels of osteocalcin and increased levels of c-telopeptide. In the present study the trabecular BMD was measured *in vivo* in anesthetized mice using pQCT. We have previously shown that this sort of measurement of trabecular BMD is very well correlated to measurement of the ratio of trabecular bone volume (BV) to total volume (TV) (BV/TV) by histomorphometry (Windahl *et al.* 2001). The mice included in the present study were later used for other purposes, making it technically impossible to perform histomorphometric analysis on excised bones. BERKO mice aged 140 days had increased trabecular BMD and this finding is in accordance with what we have recently reported in 1-year-old female BERKO mice (Windahl *et al.* 1999). This increased trabecular BMD in 140-day-old BERKO mice might be due to increased ERα expression in bone tissue (Windahl *et al.* 2001). Both the aBMD in femur and spine as well as the trabecular BMD in the proximal tibia were clearly decreased in 60-day-old female DERKO mice. The observed decrease in trabecular BMD is similar to the effect of ovariectomy and is in accordance with the recent description of trabecular osteopenia in female aromatase-deficient (ArKO) mice, which lack endogenous production of estrogen (Oz *et al.* 2000). These data indicate that a partial functional redundancy exists between ERα and ERβ regarding the protective effects of estrogen on trabecular BMD in female mice at 60 days of age, representing a young adult stage. However, in females at 140 days of age, the trabecular BMD is no longer decreased in DERKO mice. One explanation may be compensatory mechanisms that do not begin to act until after 60 days of age. Levels of estrogen as well as testosterone are elevated in female DERKO mice at 140 days of age, and one may speculate that these elevated levels of sex steroids result in a normalization of the trabecular BMD in the female DERKO via an ERα/β-independent mechanism including, for instance, activation of the androgen receptor. Thus, a complex regulation of the trabecular BMD, probably caused by a disturbed feedback regulation of estrogen and testosterone, was observed in adult female ER-inactivated mice.

In the present study at least three different mechanisms of action of estrogen regarding ER specificity were observed. The first was effects mediated by only one receptor, as seen regarding regulation of fat content, which was an ERα-dependent effect. The second type was the interesting opposing effect between ERα and ERβ seen in the regulation of longitudinal bone growth. A partial functional redundancy was the third type of effect, seen in 60-day-old mice regarding trabecular BMD, where ERα and ERβ could partly replace each other in the maintenance of a normal trabecular BMD. Thus, ERα and ERβ may have separate effects, opposing effects or redundant effects, depending on which parameter is studied.

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