Effects of Sex Steroid Receptor Specificity in the Regulation of Skeletal Metabolism

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Abstract. The interaction between estrogens and androgens, with their protective effects in bone, and parathyroid hormone (PTH), a calcitropic peptide hormone, is complex but may be better understood with murine models. The purpose of this study was to characterize skeletal phenotypes of mice deficient in estrogen receptor alpha (ERα), androgen receptor (AR, mutant *tfm*), or both, and determine if ERα and AR alter osteoblast differentiation and/or PTH response *in vitro*. Loss of ERα resulted in increased long bone length in females, but reduced length in males, suggesting loss of ERα reversed sex steroid-dependent skeletal dimorphism. The AR deficient *tfm* mice (genetically male but phenotypically female) had the longest bones and, similar to males, lengths were reduced with loss of ERα. Loss of AR and/or ERα resulted in a reduction in femoral bone mineral density (BMD) compared to male wildtype (WT) mice, suggesting *tfm* mice follow the female sex for BMD. In males or *tfm* mice, but not females, loss of AR and/or ERα caused a reduction in cortical width of the tibia compared to male WT mice. Reduced trabecular bone was found in tibiae of female and *tfm* mice versus male littermates, suggesting that *tfm* mice follow the female sex for trabecular bone but loss of ERα did not alter trabecular bone levels. Primary calvarial osteoblasts of male WT mice were less responsive to PTH stimulation of cAMP than all other genotypes, suggesting the female chromosomal sex and/or loss of ERα or AR results in increased sensitivity to PTH. In conclusion, *tfm* mice follow the male pattern of long bone development, but imitate females in bone density and trabecular bone. Loss of ERα and/or AR results in increased osteoblast sensitivity to PTH and may explain actions of PTH noted in hypogonadal humans.

Key words: Bone — Osteoblast — Estrogen — Estrogen receptor — Androgen — Androgen receptor — Parathyroid hormone

It is well accepted that estrogen deficiency is a major risk factor for osteoporosis but the role of estrogen and androgen receptors in skeletal metabolism is not as clear. Hypogonadism and delayed puberty contribute to osteopenia in men and women [1–4]. Osteoporosis in hypogonadal males is associated with low plasma testosterone, estradiol and androstenedione concentrations which correlate with reduced bone density [5]. One case of a man with a mutant estrogen receptor and several cases of humans with aromatase deficiency confirm that estrogen and the estrogen receptor are critical for skeletal homeostasis; however, the mechanisms involved are poorly understood [6–8]. Recent evidence suggests that actions of sex steroids in bone may be via different mechanisms than those that impact reproductive organs [9].

Parathyroid hormone (PTH), unlike sex steroids, is a calcitropic peptide hormone that acts directly on the skeleton to promote calcium release and on the kidney to enhance calcium reabsorption via the PTH-1 receptor (PTH-1R). PTH has both catabolic and anabolic actions in bone [10]. Recent interest has been focused on its anabolic actions due to its therapeutic potential. Intermittent PTH causes an increase in vertebral, femoral and total body mineral density in postmenopausal osteoporosis [11]. On the other hand, infusion of PTH *in vivo* is catabolic via increased osteoclast numbers [12]. Interestingly, suppression of sex steroids increases the skeletal responsiveness to the bone-resorbing activities of PTH in elderly men [13].

The PTH-1R was the first PTH receptor isolated, cloned and sequenced, and is present in high numbers on osteoblasts [14]. The estrogen receptor α (ERα) and androgen receptor (AR) are also expressed in osteoblasts [15, 16]. Functional effects of androgens on osteoblastic cell proliferation and alkaline phosphatase have been described [17]. Furthermore, androgens, as well as estrogens, act directly on human bone cells to selectively modulate early effects of PTH [18, 19]. Still,

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relationships between PTH and sex steroids are unclear.

To better understand the effects of estrogens, androgens, and their receptors in the skeleton, animal models of receptor deficiency are valuable tools. The testicular feminized male (*tfm*) mouse has a single point mutation in the AR that inactivates the receptor resulting in a genetically male mouse with a female phenotype [20, 21]. The AR is considered necessary for full skeletal growth and it has been suggested that a modest increase in estrogens in androgen-resistant animals may prevent cancellous bone loss [22]. Genetic models of estrogen receptor dysfunction have been generated and loss of the ERα appears to impact the skeleton more dramatically than the ERβ [23, 24].

The purpose of this study was to characterize the skeletal phenotypes of ERα, AR and combined ERα/AR knockout mice and to determine whether ERα and AR regulate osteoblast differentiation *in vitro*. Further, whether these receptors are necessary for actions of PTH on osteoblasts *in vitro* was determined.

Materials and Methods

Generation of ERα and AR Mutant Mice

The generation of the ERα mutant mice has been described previously [25]. Heterozygous ERα +/− male and female mice were crossed to yield a Mendelian distribution of ERα +/+, +/− and −/− mice. Genotyping of tail-biopsy DNA was performed by PCR using standard protocols as described [25]. *Tfm* carrier female mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). *Tfm* carrier females were crossed with tabby males (The Jackson Laboratory) to continue producing *tfm* carriers used to obtain *tfm* mice. In order to generate *tfm* ERα-/-mice, male ERα+/− mice were crossed with *tfm* carrier females. The resulting ERα+/− *tfm* carrier females were crossed with male ERα+/− mice to get mice with the desired genotypes. Male and female littermates were used as controls. All studies were approved by the Institutional Animal Care and Use Committee at the University of Michigan.

Gross Evaluation and Microradiographic Analysis

The length and width of femurs and tibiae were measured using an electronic digital caliper (Max-Cal, MFG. Co., Ltd, Japan) with length reported as the longest measurement from proximal to distal and the width as the narrowest measurement at the mid-diaphyseal region. Femurs were evaluated using a microradiography system (Faxitron X-ray Corporation, IL, USA).

Dual Energy X-ray Absorptiometry (DEXA)

Mice were sacrificed at 4 and 12 weeks of age, and femurs and tibiae were dissected and placed in 70% ethanol for DEXA and 10% formalin for histomorphometry. Areal bone mineral density (BMD) and bone mineral content (BMC) were measured with the Norland pDEXA Sabre (Fort Atkinson, WI, USA) using the Sabre Small Animal Research software (version 2.2.4) as previously described [26].

Bone Histomorphometry

Tibias and lumbar vertebrae from mice were trimmed of musculature, fixed in 10% formalin at 4°C, decalcified in 10% EDTA (pH 7.4) for 7 days and embedded in paraffin. Longitudinal sections of tibiae and vertebrae were cut at 5 μm and stained with hematoxylin and eosin for histological evaluation. Histomorphometric analysis of the lumbar vertebrae and tibiae were performed using a computer-assisted bone histomorphometric analyzing system (Image-Pro Plus version 4.0, Media Cybernetics, MD, USA) as described [26]. Lumbar vertebrae measurements included total area (Tt.Ar.), and percent of trabecular bone area (Tb.Ar./Tt.Ar.). Measurements of the proximal tibia were performed as described for vertebrae and also included cortical bone width (Ct.Wi.) (10 measures of cortical width taken at the mid-diaphysis were averaged for each tibia), osteocyte number (N.Ot./mm^2) and extension of trabeculae from the growth plate as percent of trabecular length (Tb.Le./Tt.Le) [27].

Bone Ashing and Calcium Assay

Femurs were isolated, dried at 110°C overnight and weighed. Bones were then ashed at 800°C for 4 hours, weighed again, dissolved in 500 μl 6 N HCl, and amount of calcium was determined by colorimetric assay with cresolphthalein complexone (Sigma, St. Louis, MO, USA). Total calcium was expressed as percent of ash weight.

Isolation of Primary Calvarial Osteoblasts and Cell Culture

Primary calvarial cells were isolated as previously described [28]. Briefly, calvaria of 12 week mice were dissected, isolated from periosteum and subjected to sequential digestion of 20, 40 and 90 minutes in collagenase A (2 mg/ml, Boehringer-Mannheim, Indianapolis, IN, USA) with 0.25% trypsin (Invitrogen, Grand Island, NY, USA). Cells from the third digestion were washed, counted, and plated in phenol red-free α-MEM (Invitrogen) with 10% charcoal/dextran treated fetal bovine serum (HyClone, Logan, Utah, USA) containing 100 units/ml of penicillin and 100 μg/ml streptomycin. Medium was changed every other day. Primary cultures without passage were used for adenylyl cyclase stimulation and osteoblast differentiation assays.

Adenylyl Cyclase Stimulation Assay

The adenylyl cyclase stimulation and cAMP-binding protein assay were performed as previously described with minor modifications [29]. Cells were plated in triplicate at 40,000 cells/cm^2 into 24-well plates and adenylyl cyclase stimulation assay was performed on day 7 to optimize PTH-1R expression [28]. Cells were then stimulated with 10^{-8} M human PTH (hPTH) (1-34) (Bachem, Inc., Torrance, CA, USA), 10^{-7} M 17-β estradiol (Sigma, St. Louis, MO, USA), or vehicle, in calcium and magnesium-free Hanks' Balanced Salt Solution (Life Technologies, Carlsbad, CA, USA) containing 0.1% bovine serum albumin and 1 mM isobutylmethylxanthine at 37°C for 15 minutes. After aspirating the medium, the cAMP was extracted by adding 250 μl/well ice-cold 5% perchloric acid and incubating overnight at −20°C. After thawing, the pH was adjusted to 7.5 with 4 N KOH, and the neutralized extract was then assayed for cAMP using a cAMP-binding protein assay [29].

The cAMP binding assay was performed by incubating [^{3}H]-cAMP (ICN, Irvine, CA, USA) with standards or unknowns and a cAMP-binding protein sufficient to bind 40–60% of radioactivity for 90 minutes on ice. Dextran-coated charcoal was added for 20 minutes and centrifuged to remove unbound from bound cAMP-binding protein-[^{3}H]-cAMP complexes. The radioactivity of the supernatants was determined with a liquid scintillation counter and cAMP levels calculated by the log-logit method using the Graph Pad Prism program. The cAMP

Table 1. Gross evaluation of femur and tibia: ERα and AR on 12-week-old mutant mice [mean (mm) ± SEM (n)]

Genotype	Femur length	Tibia length	Femur width
Female ERα+ (12)	15.27 ± 0.14	17.92 ± 0.18	1.21 ± 0.01
Female ERα− (16)	15.60 ± 0.17∫	18.56 ± 0.15 §	1.21 ± 0.01
Male ERα+ (17)	15.56 ± 0.13	18.47 ± 0.08 §	1.25 ± 0.02
Male ERα− (11)	15.08 ± 0.12*	18.21 ± 0.08	1.33 ± 0.04*
tfm ERα+ (11)	16.04 ± 0.09*†	18.83 ± 0.09*†	1.17 ± 0.03
tfm ERα− (10)	15.19 ± 0.26†	18.40 ± 0.15†	1.18 ± 0.03

*p < 0.05 vs male ERα+, †p < 0.05 vs *tfm* ERα+, †p < 0.001 vs female ERα+, ∫p < 0.05 vs male ERα−, §p < 0.05 vs female ERα+

levels were standardized to cell numbers obtained by trypan blue enumeration of parallel triplicate wells.

Mineralization Assays

Primary osteoblasts from mice of each genotype were plated in 6o mm plates and medium was changed every other day. At day 7, plates were treated with mineralization media [ascorbic acid (50 μg/ml) + β-glycerophosphate (10 mM)] for 21 days. Cells were then fixed with 95% ethanol and stained with silver nitrate by the Von Kossa method to detect phosphate deposits in bone nodules [30], Bone nodules were analyzed morphometrically by scanning five random sites from each plate using Image-Pro Plus version 4.0. Values of mineralized nodule number per area and area of mineralization per area were calculated for primary cultures from each mouse and averaged with values from mice with the same genotype.

Statistical Analysis

The results of multiple experiments were analyzed using ANOVA followed by a Tukey-Kramer multiple comparison test and Student's *t* test with the Instat 2.1 biostatistics program (GraphPad Software).

Results

Gross Evaluation and Microradiographic Analysis

Mutations in ER have been speculated to be responsible for delay in epiphyseal closure, which increases long bone length [6]. To understand the impact of ERα and/or AR in long bone development, 4- and 12-week-old *tfm* mice with their female and male littermates were evaluated. Gross evaluation of femurs revealed that in 12-week-old mice, loss of ERα resulted in reduced femur length in males. The *tfm* ERα+/+ mice had the greatest femur length, and similar to males, lengths were reduced with loss of ERα (Table 1). In contrast, loss of ERα caused an increase in tibia length in females.

There was a 23% increase in femur length of female and male mice during the period of growth from 4 to 12 weeks. The *tfm* mice had a similar but slightly lower 22% increase in femur length during this time period. In contrast, the tibiae lengths increased only 14 and 16% in female and males, respectively, and 15% in *tfm* mice between 4 and 12 weeks (data not shown). These data suggest that sex steroids that are typically produced increasingly from the age of 4 wks in mice may impact femur growth more than tibia growth. This appears to

Table 2. Femur and tibia BMD: ERα and AR mutant mice, 12-week-old, mean (g/cm^2) ± SEM (n)

Genotype	Femur BMD	Tibia BMD
Female ERα+ (12)	0.029 ± 0.0004*	0.026 ± 0.0003
Female ERα− (16)	0.028 ± 0.0006	0.026 ± 0.0011
Male ERα+ (17)	0.032 ± 0.0010	0.026 ± 0.0006
Male ERα− (11)	0.030 ± 0.0004*	0.025 ± 0.0002*
tfm ERα+ (11)	0.029 ± 0.0005*	0.025 ± 0.0004
tfm ERα− (10)	0.028 ± 0.0005*	0.025 ± 0.0006

* p < 0.05 vs. male ERα+

be true for male and *tfm* mice, both of which demonstrated significant reductions in femur length with loss of the ERα but nonsignificant or only mildly significant reductions in tibia length. In contrast, the trend towards increased long bone length with loss of ERα in females was significant in tibia but not femurs.

Dual X-ray Absorptiometry

Developmental studies have tried to explain the role of sex steroid receptors in the skeleton. The impact of ERα was reported in orchidectomized male mice where it was proposed that AR had a significant role as well as ERα [31]. To clarify the impact of these receptors on bone content, BMD and BMC were measured on whole femurs and tibias for all mice. Younger mice (4 weeks) demonstrated a significant increase in BMD in *tfm* ERα+/+ compared to ERα+/+ males and females (data not shown). However, at 12 weeks of age the female gender and loss of ERα and/or AR resulted in a reduction in BMD for femurs compared to male WT mice. Further, absence of ERα in tibiae resulted in a statistical reduction of BMD in males (Table 2). When comparing between 4 and 12 weeks of age, femur BMD increased 13% in males but only 3% in females, suggesting that males may be more susceptible to hormonal changes during this period of growth.
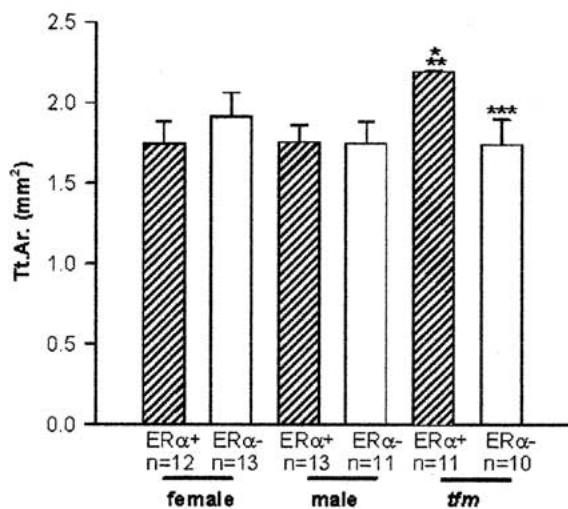
Bone Histomorphometry

Previous reports of bone histomorphometry in *tfm* rats indicated no differences in cancellous bone volume of
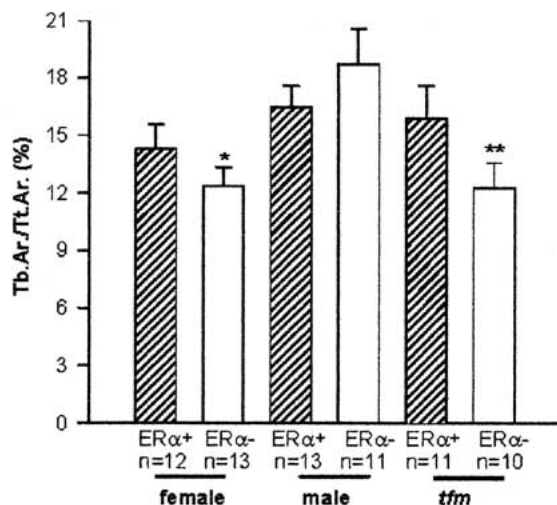
A
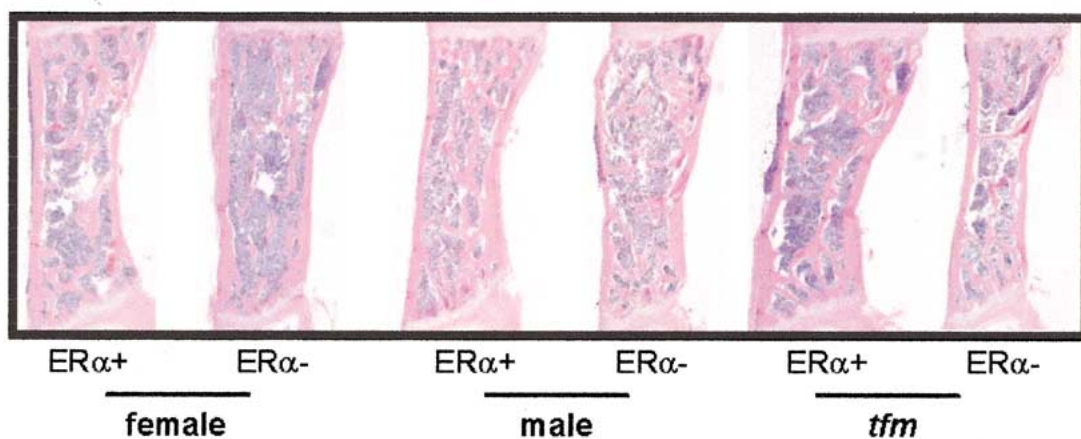
B

*p<0.02 vs female ERα+, **p<0.01 vs male ERα+

***p<0.03 vs tfm ERα+

C

*p<0.05 vs male ERα-, **p<0.01 vs male ERα-

Fig. 1. Bone histomorphometry of vertebrae. (**A**) Representative lumbar vertebra from each of the six genotypes, (**B**) total bone area and (**C**) trabecular bone area expressed as percent of total bone. Data are expressed as means ± SEM. The *tfm* mice had the greatest overall vertebral size, and loss of ERα in the *tfm* background resulted in a reduction in vertebral size. Trabecular bone area was reduced in the absence of ERα in female and *tfm* mice compared to males.

the proximal tibia compared to normal female and male rats, suggesting that the AR is not critical for bone volume [22]. In contrast, ERα was shown to protect trabecular bone development in gonadectomized male mice suggesting the ERα has protective effects in the skeleton [31]. Accordingly then, the ERα and AR would appear to have different effects on the skeleton. Static histomorphometric analysis of lumbar vertebrae indicated that *tfm* mice had the greatest overall vertebral area and loss of ERα in the *tfm* background, resulting in a reduction in total vertebral area (Fig. 1A, B). A significant reduction in trabecular area of the vertebrae was noted in the absence of ERα in female and *tfm* mice compared to male ERα mice but not when compared to same gender wildtype mice (Fig. 1C).

Histomorphometry of the proximal tibias showed reduced trabecular bone for female mice compared to males. *Tfm* ERα +/+ mice had similar trabecular bone as females and also had reduced trabecular bone compared to male ERα+/+ mice. Loss of ERα in the *tfm* mice did not alter bone area versus the *tfm* ER α+/+ but bone area was reduced compared to ER α−/− males (Fig. 2). When evaluating the extension of trabecular bone into the diaphysis, a similar pattern emerged. Male
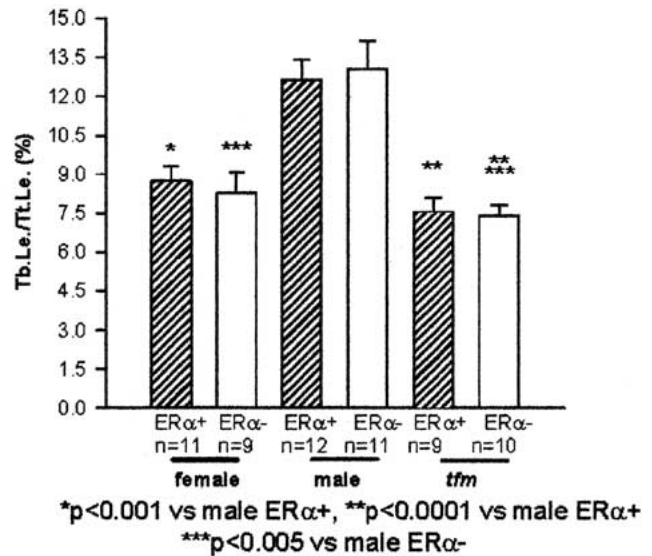
A
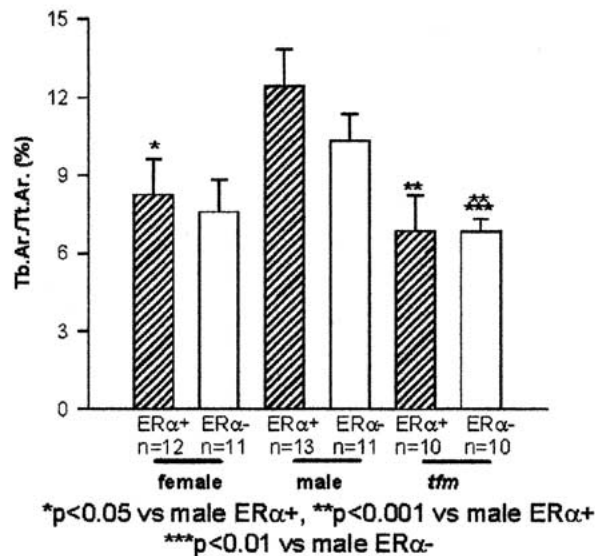
Fig. 2. Bone histomorphometry of proximal tibia. (**A**) Representative sections of proximal tibia from each of the six genotypes, (**B**) trabecular bone area expressed as percent of total bone and (**C**) trabecular bone length (measure of the distance trabeculae extend from the growth plate into the diaphysis) presented as a percent of the total length of tibia. Values are given as means ± SEM. Trabecular bone area and trabecular bone lengths were decreased for female and *tfm* tibiae compared to male mice, suggesting the *tfm* mice follow the female gender for tibial trabecular bone.

ERα+/+ or −/− mice had increased extension of trabeculae as compared to its *tfm* or female counterpart.

Male mice had the greatest tibial cortical width of all genotypes (Fig. 3). Loss of ERα and/or AR caused significant reduction in the cortical width of males, but compared to females or *tfm* mice, the male ER α−/− cortical width was still higher. Osteocyte density followed a similar trend, with the lowest density found in the tibial cortical bone of female WT mice, whereas *tfm* ERα+/+ mice had the highest number of osteocytes.

Bone Ashing and Calcium Assay

The femoral ashweight generally paralleled the BMD levels found, with the exception that no significant dif-
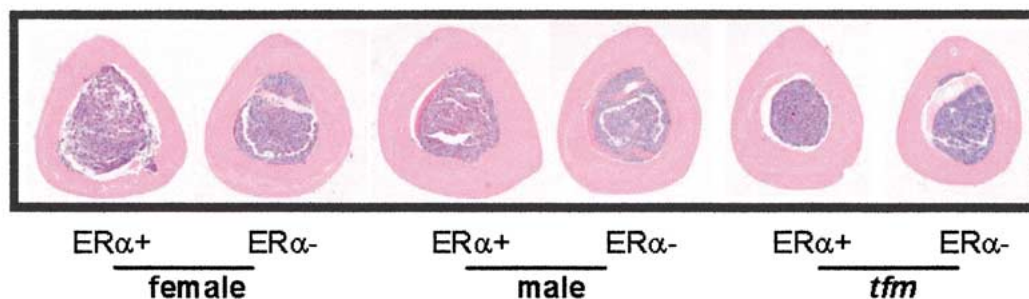
ference was noted in the *tfm* ER α+/+ versus the male ER α+/+ (Fig. 4). Moreover, there was no significant difference in total calcium amount and total calcium expressed as percent of ashweight for femurs amongst any genotypes (data not shown) suggesting that there were no alterations in the calcium incorporation in bone.
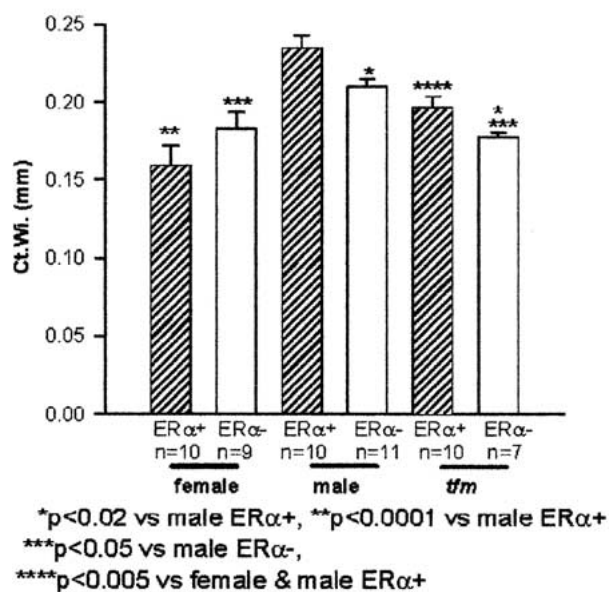
Mineralization Assay

Many studies have reported that estrogens and/or androgens alter osteoblast differentiation [17, 32]. In the present study, we found no significant alterations in the mineralized nodule number or area for any osteoblastic cultures from the various genotypes after 21 days of *in*
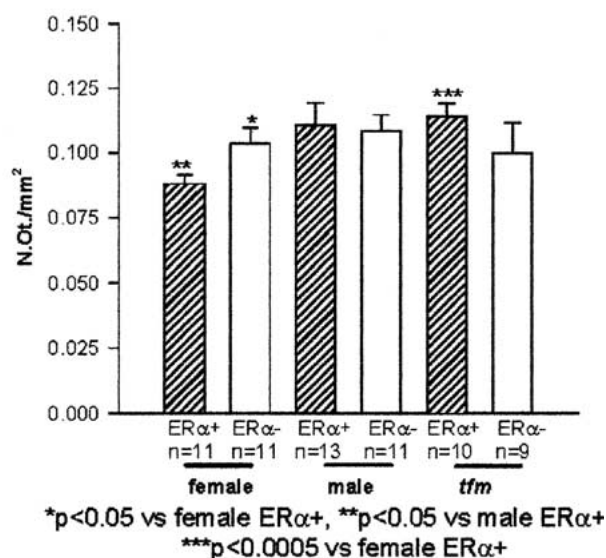
A

ERα+ ERα- ERα+ ERα- ERα+ ERα-
female male tfm

B

Ct.Wi. (mm)

ERα+ ERα- ERα+ ERα- ERα+ ERα-
n=10 n=9 n=10 n=11 n=10 n=7
female male tfm

*p<0.02 vs male ERα+, **p<0.0001 vs male ERα+
***p<0.05 vs male ERα-,
****p<0.005 vs female & male ERα+

C

N.Ot./mm^2

ERα+ ERα- ERα+ ERα- ERα+ ERα-
n=11 n=11 n=13 n=11 n=10 n=9
female male tfm

*p<0.05 vs female ERα+, **p<0.05 vs male ERα+
***p<0.0005 vs female ERα+

Fig. 3. Cortical bone of the tibia. (**A**) Representative photos of cortical bone from mid-diaphysis of tibiae from each of the six genotypes, (**B**) cortical width of tibiae and (**C**) number of osteocytes/mm^2 cortical bone. Data are expressed as means ± SEM. Loss of ERα and/or AR caused a reduction in cor-

tical width of tibia compared to male WT where female mice had also reduced cortical width but loss of the ERα did not alter cortical width in female tibia. Further, loss of ERα or AR increased the numbers of osteocytes per area of cortical bone in female tibiae.

vitro differentiation (data not shown). These data suggest that neither ERα nor AR is necessary for normal differentiation and mineralization *in vitro*.

Adenylyl Cyclase Stimulation Assay

PTH binds to the PTH-1R and activates adenylyl cyclase, which leads to increased cAMP production [14]. Recently it has been demonstrated that estrogens stimulate surface receptors, activate adenylyl cyclase, and stimulate cAMP production [33, 34]. To verify this effect in primary osteoblasts, 17-β estradiol was tested for its ability to stimulate adenylyl cyclase in a manner similar to PTH. The hPTH (1–34) and 17-β estradiol-treated cAMP levels were calculated relative to cell numbers and basal cAMP levels.

Results indicated a 1.5 to 2.5-fold increase in cAMP levels with 17-β estradiol but no significant difference among genotypes (data not shown). All groups demonstrated dramatic (40-200-fold) cAMP elevation following hPTH (1–34) stimulation that reflected the biological activity of PTH-1R in primary osteoblasts (Fig. 5). Cells from female WT mice had increased PTH-stimulated cAMP levels compared to male WT where the absence of ERα and/or AR resulted in increased ability for PTH to elevate cAMP levels.
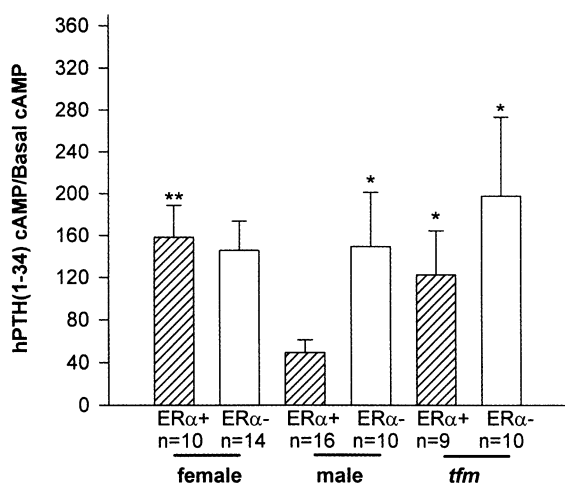
Discussion

Sex steroids exert several physiological effects on skeletal metabolism, including effects on longitudinal bone
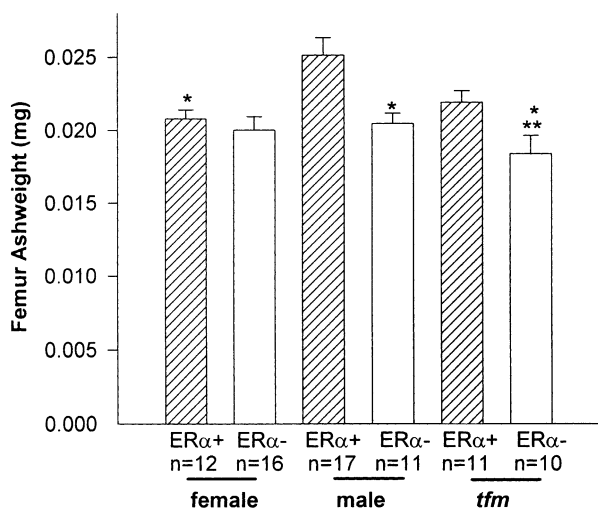
Fig. 4. Ash weight of femurs from 12-old wk mice. Values are given as means ± SEM. Loss of ERα in males or AR (*tfm*) but not in female mice resulted in a reduction in ash weight.

Fig. 5. PTH-stimulated cAMP levels in primary calvarial osteoblastic cells from the six genotypes of mice. Data are expressed as means ± SEM for cultures from indicated numbers of mice/genotype. Female gender and/or loss of ERα or AR result in increased sensitivity to PTH-stimulated increases in cAMP.

growth, bone mineral density, and composition of bone [35]. These effects of estrogens and androgens may be through direct stimulation of ERs and the AR, respectively. Further, androgens may express their actions indirectly by aromatization of androgens into estrogen, followed by the stimulation of ERs. Extensive work has been performed with animals to explain these effects of androgens and estrogens on skeletal metabolism [23, 36, 37].

In the present study, *tfm* ERα mutant mice displayed altered phenotypes in bone. Previous reports demon-

strated a decrease or no change in long bone length for male and female ERα knockout mice [26, 38, 39]. We found no statistical difference in femur length in the absence of ERα in female mice. Our results for male femur length were consistent with other studies, which show a reduction in the absence of ERα [40, 41] or aromatase [42]. Moreover, *tfm* mice had the longest femurs compared to both male and female WT and ERα knockout mice, which was different from a previous study [22], where *tfm* rats had intermediate femur length between male and female littermates. An interesting finding in our study was an increase in tibia length of female ERα knockout, but not male ERα knockout mice. It has been speculated that increased femur and tibia size in females is due to delayed epiphyseal fusion since estrogen induces epiphyseal maturation [2]. In human cases, estrogen receptor mutations lead to a delay in epiphyseal closure with a resultant increase in the length of bone [6]. Hence, estrogen may be significant in limiting long bone development. In the rat model, the tibia increases in length at a more rapid rate in males than in females and gonadectomy reverses this sexual dimorphism, an effect that can be blocked by the administration of exogenous sex steroids [43]. Zhang et al. [44] reported that ovariectomy allowed appendicular growth to continue and orchidectomy resulted in a reduction in both appendicular and axial skeletal development [44].

Our data suggest that lack of ERα in females is not critical before 12 weeks. Since sex hormones start to increase at puberty they may not show their effects until this later age where estrogen inhibits long bone length. Similarly, aromatase-deficient male mice, but not females, show an absence of accelerated growth during puberty [42]. Taken together, we propose that loss of ERα may reverse sex steroid-dependent skeletal dimorphism for adult females. ERα is necessary for appendicular development in males and is the mediator of estrogenic effects on growth and maturation of the skeleton for males [40]. Loss of ERα causes a significant reduction in longitudinal growth for *tfm*, hence *tfm* mice appear to follow the male phenotype in the appendicular skeleton. The contrast in findings of ERα loss of function in mice with the human condition are still an enigma but may be attributable to differences in timing of growth plate closure which occurs relatively later in life in mice.

It is clear that orchidectomized rodents and hypogonadal humans develop osteopenia [2, 4]. After androgen replacement therapy, bone mass is restored in gonadectomized male rats, and estrogen has reversed bone loss in adult orchidectomized rats [45, 46]. Recently, Lindberg et al. [31] showed that daily 17-β estradiol injections increased trabecular bone values in orchidectomized adult male WT and ERβ knockout mice, which suggests that estrogen shows its protective

Table 3. Relative summary of genotype changes in males[1]

	Loss of AR	Loss of ERα	Loss of AR & ERα
Femur length	↑	↓	↓(Slight)
Femur BMD	↓	↓	↓
Cortical thickness	↓	↓	↓
Trabecular bone (tibia)	↓	NC	↓
Trabecular bone (vertebra)	NC	NC	NC

↑ increase; ↓ decrease; NC, no change
[1] Relative to wildtype mice

Table 4. Relative summary of genotype changes in females[1]

	Loss of AR	Loss of ERα	Loss of AR & ERα
Femur length	↑	↑(Slight)	NC
Femur BMD	NC	NC	NC
Cortical thickness	↑	NC	NC
Trabecular bone (tibia)	NC	NC	NC
Trabecular bone (vertebra)	NC	NC	NC

↑ increase; ↓ decrease; NC, no change
[1] Relative to wildtype mice

effects on trabecular bone via ERα. They also proposed that in addition to ERα, the AR has equal importance in the regulation of trabecular bone. In our study, we also speculate that both ERα and AR have importance in skeletal metabolism. The DEXA data support our previous results of femur BMD and are in agreement with Vidal et al. [39, 40] that male WT mice have the highest femur BMD and BMC. After orchidectomy, adult male mice given estrogen have increased BMD and BMC values in male WT and ERβ knockout mice [31].

In our study, when ERα and/or AR were deleted, femur BMD decreased significantly which supports the notion that sex steroids exert important effects on the skeleton via ERα as well as AR in males. For adult female and *tfm* mice, loss of ERα does not significantly have an impact on femur and tibia BMD compared to their WTs. The *tfm* mice seem to imitate the female gender for BMD. Further, at 4 wks of age, femurs from *tfm* mice had higher BMD compared to males and females (data not shown). However, by 12 weeks, femur BMD of males started to increase compared to *tfm* and females. This increase between the age of 4 and 12 weeks was much greater for males than females. This finding is in agreement with Vidal et al. [40] who stated that ERαwas an important mediator of estrogenic effects on the maturation of the skeleton in male mice.

Previously, Vanderschueren et al. [22] reported that *tfm* mice had lower vertebral ashweight than male littermates. In the present study, there was a significant reduction in femur ashweight with the loss of ERα but not AR compared to male WT. Interestingly, in *tfm* ERα−/− mice, femur ashweight decreased significantly compared to male WT and *tfm* ERα+/+, which may indicate that loss of ERα causes a reduction in the mineral content of long bones. According to these changes, *tfm* mice follow a similar pattern as males for skeletal mineral content.

Recent interest has focused on receptor knockout models as well as gonadectomy models, in which the effects of sex steroids on trabecular bone have been reported. Treatment with estrogen was shown to prevent the reduction in trabecular BMD and histomorphometric parameters of bone in adult orchidectomized WT compared to ERα knockout males [31]. The ERα has also been reported to be critical for the stimulatory action of high dose estrogen on cancellous bone formation [47].

Turner et al. [48] reported that orchidectomy did not alter static bone histomorphometry measurements whereas ovariectomy increased cross-sectional areas of tibiae, and either orchidectomy or ovariectomy reduced trabecular area and trabecular bone length of tibiae in 4-week-old rodents. Lindberg et al. [31] also concluded that ERα protects trabecular bone loss in male mice and that there is redundancy with AR in the regulation of trabecular bone. In contrast, our ERα knockout mice did not show any significant alterations in amount of trabecular bone of the tibiae when compared to their wildtype counterparts. Further, Vanderschueren et al. demonstrated that *tfm* female rats had no difference in cancellous bone volumes for tibial histomorphometric analysis than male rats [22]. However, we found significantly decreased tibial trabecular bone area and trabecular bone length compared to male littermates. These results may indicate that *tfm* mice follow the female gender for trabecular bone.

The impact of ERα and/or AR on skeletal development as compared to wildtype mice are summarized for male/*tfm* and female/*tfm* mice in Tables 3 and 4, respectively. In general, changes are more dramatic for male mice than for female mice. In female mice, loss of the AR has more impact than loss of ERα or the combination of AR and ERα. In male mice, loss of AR or ERα impacts the skeleton with opposing effects on femur length but similar detrimental effects on bone mineral density and cortical bone. In the present study, numbers of osteocytes were elevated in female ERα−/− versus wildtype mice and also in *tfm* mice. Recently, it has been proposed that osteocytes play a role in inhibiting bone remodeling [49]. Since previous reports have indicated that loss of ERα results in decreased bone remodeling [38], our data are in concert with this and suggest that loss of the AR may also have the same impact on bone remodeling, however, an in depth analysis of dynamic parameters is needed to verify this. Loss of ERα did not have an impact on trabecular bone whereas loss of AR resulted in reduced trabecular bone in the tibia but not in the vertebrae. According to these results it appears that the impact of ERα and AR are more detrimental for males than females.

In order to better understand the cellular and molecular actions of ERα and AR in bone, an *in vitro* evaluation of osteoblastic differentiation and receptor function was made. It is established that steroid hormones exert their effects by binding to their nuclear receptors in osteoblasts. This classic estrogen receptor-signaling pathway occurs through the entry of estrogen into the cell, interaction with ER, and transcriptional activation of estrogen-responsive genes. Recently, it has been speculated that estrogen and growth factors might share the same pathway in their signaling via plasma membrane receptors [34, 50, 51]. One of these studies reported that estrogen was found to increase cAMP in breast cancer and uterine cells as detected by enhanced membrane adenylate cyclase activity. Others have demonstrated activation of the MAPK pathway in bone or osteoblastic cells by estrogen and that this action is likely mediated through a plasma membrane receptor [51, 52]. Our results showed that 17-β estradiol induced cAMP 1.5–2.5-fold in primary osteoblasts, a result similar among all six genotypes, suggesting that the ER or AR had no impact on this cell surface effect.

It has been suggested that sex steroids induce osteoblastic differentiation via their receptors, ERα and AR, in bone cells *in vitro* [17, 32, 53]. In our study, primary osteoblasts demonstrated mineralized nodule formation; however, there was no significant difference in nodule formation from cells of the different genotypes, indicating that loss of ERα and/or AR does not modify osteoblastic nodule formation in the absence of sex steroid hormone.

It is also clear that both *in vitro* and *in vivo* PTH stimulates cAMP and *c-fos*, which are early events in the PTH signaling cascade [54]. In human trials, intermittent administration of PTH (1–34) is anabolic and increases bone density in both men and women with osteoporosis [11, 55]. However, in hypogonadal men, infusion of PTH (1–34) caused a significant increase in bone-resorbing markers but did not affect bone formation markers [13]. In our study, primary osteoblasts with ablation of ERα and/or AR and from female WT mice had higher PTH (1–34) cAMP levels than male WT mice. We can speculate that the female chromosomal sex and loss of ERα and/or AR resulted in an increased sensitivity to PTH.

In summary, these data suggest that *tfm* mice follow the male pattern for appendicular skeletal development, but imitate the female sex in bone density and trabecular bone. Further, loss of ERα and/or AR result(s) in increased osteoblastic sensitivity to PTH and may explain the actions of PTH noted in hypogonadal humans. These models will help to understand the altered mechanisms of skeletal metabolism, and may facilitate improved therapeutic approaches for the treatment of hypogonadal-associated osteoporosis.

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