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Distinct effects of loss of classical estrogen receptor signaling versus complete deletion of estrogen receptor alpha on bone

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Abstract

Estrogen receptor (ER) α is a major regulator of bone metabolism which can modulate gene expression via a “classical” pathway involving direct DNA binding to estrogen-response elements (EREs) or via “non-classical” pathways involving protein-protein interactions. While the skeletal consequences of loss of ERE binding by ER α have been described, a significant unresolved question is how loss of ERE binding differs from complete loss of ER α . Thus, we compared the skeletal phenotype of wild-type (ER $\alpha^{+/+}$) and ER α knock out (ER $\alpha^{-/-}$) mice with that of mice in which the only ER α present had a knock-in mutation abolishing ERE binding (non-classical ER α knock-in [NERKI], ER $\alpha^{-/-}$ NERKI). All three groups were in the same genetic background (C57BL/6). As compared to both ER $\alpha^{+/+}$ and ER $\alpha^{-/-}$ mice, ER $\alpha^{-/-}$ NERKI mice had significantly reduced cortical volumetric bone mineral density and thickness at the tibial diaphysis; this was accompanied by significant decreases in periosteal and endocortical mineral apposition rates. Colony forming unit (CFU)-fibroblast, CFU-alkaline phosphatase, and CFU-osteoblast numbers were all increased in ER $\alpha^{-/-}$ compared to ER $\alpha^{+/+}$ mice, but reduced in ER $\alpha^{-/-}$ NERKI mice compared to the two other groups. Thus, using mice in identical genetic backgrounds, our data indicate that the presence of an ER α that cannot bind DNA but can function through protein-protein interactions may have more deleterious skeletal effects than complete loss of ER α . These findings suggest that shifting the balance of classical versus non-classical ER α signaling triggers pathways that impair bone formation. Further studies defining these pathways may lead to novel approaches to selectively modulate ER signaling for beneficial skeletal effects.

Keywords

estrogen; bone; mouse

INTRODUCTION

Estrogen plays a critical role in regulating bone metabolism in both sexes in humans and in mice [1]. The effects of estrogen in various tissues, including bone, are mediated by two

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related receptors, estrogen receptor (ER) α and β [2], and the importance of receptor-mediated estrogen signaling in bone has been well characterized using mice with deletions in ER α (ER α knock out [ER α KO]), ER β (ER β KO), or both receptors (double ER KO, DERKO). These studies have found that ER α is the major ER mediating the effects of estrogen on bone in females [3] and in males [4]; however, ER β may play a role in estrogen action in trabecular bone [3, 5] and can, under certain circumstances, substitute for [5] or enhance [3] ER α action.

Estrogen can regulate gene expression through either ER α or β using a number of signaling pathways (Figure 1). The “classical” pathway involves direct DNA binding of the liganded ER to estrogen response elements (EREs) [6], as in the case of the prolactin [7], progesterone receptor [8], and *c-fos* [9] genes. In addition, however, the ER can also regulate gene expression via a number of “non-classical” pathways which do not involve direct DNA binding by the ER but rather are due to specific protein-protein interactions. Thus, the ER can modulate the transcription of genes containing AP-1 sites, as in the case of the collagenase [10] and IGF-I [11] genes. Similarly, suppression of IL-6 gene expression by estrogen occurs via interactions of the liganded ER with the NF- κ B complex [12]. Finally, estrogen can also regulate gene expression through membrane pathways which involve alterations in MAP kinase activity [13]; these effects appear to be particularly important for the anti-apoptotic effects of estrogen on osteoblasts.

In order to better define non-classical estrogen signaling and the balance between the classical and non-classical estrogen signaling pathways of ER α *in vivo*, Jakacka and colleagues generated mice which have two substitution mutations (E207A/G208A) in the first zinc finger of the DNA binding domain in one of the ER α alleles (non-classical ER knock-in mice, NERKI) [14]. This mutant receptor fails to activate ERE reporter activity *in vitro*, but has intact transcriptional activity for AP-1 reporter constructs and can bind *c-jun* normally in a mammalian two-hybrid cell assay [15]. Heterozygote female NERKI mice are infertile and display pronounced reproductive defects [14], suggesting that the balance between classical and non-classical estrogen signaling has important biological consequences *in vivo*. We subsequently generated female ER α ^{-/-}NERKI mice which completely lack classical ER α signaling but have intact signaling via the non-classical ER α pathway and characterized the skeletal phenotype of these mice [16]. Relative to wild type (ER α ^{+/+}) mice, female ER α ^{-/-}NERKI mice had reduced cortical volumetric BMD (vBMD) and thickness at the tibial diaphysis, preserved trabecular vBMD at the tibial metaphysis, and a trend for reduced bone volume/total volume (BV/TV) in trabecular bone at the lumbar spine [16]. Similar to ER α ^{-/-} females [5, 16], female ER α ^{-/-}NERKI mice also had significant increases in circulating estradiol levels, which may account for the relative preservation of trabecular bone in these mice through actions on ER β [5, 16].

While our previous studies indicated that the NERKI receptor had negative effects on bone, we were unable to address the important question of whether the presence of the NERKI receptor was, in fact, more deleterious for bone than simply the absence of ER α . This was because our ER α ^{-/-}NERKI mice were in a 50:50 C57BL/6:129/SvJ background, whereas the ER α ^{-/-} were in a C57BL/6 background, making it impossible to directly compare the skeletal phenotypes of ER α ^{-/-} and ER α ^{-/-}NERKI mice [16]. Thus, in this study, we assessed bone parameters in ER α ^{+/+}, ER α ^{-/-}, and ER α ^{-/-}NERKI mice, all of who were in a C57BL/6 background for > 6 generations. Overall, our findings indicate that loss of classical estrogen receptor signaling may have distinct skeletal consequences as compared to complete deletion of ER α .

METHODS

Generation, breeding, and care of animals

Heterozygote $ER\alpha^{+/NERKI}$ male mice from our earlier study [16] in a 50:50 C57BL/6:129/SvJ background were backcrossed with heterozygote $ER\alpha^{+/-}$ female mice [17] in a C57BL/6 background for more than 6 generations. The female $ER\alpha^{+/+}$, $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ littermates were then studied at 3–5 months of age. To assess dynamic measurements of bone formation, the mice received two calcein injections intraperitoneally (10 mg/kg) a week apart, with the last injection given two days prior to sacrifice. The animals were housed in a temperature controlled room ($22 \pm 2^\circ\text{C}$) with a daily 12-h light/12-h dark schedule. During the experiment, animals had free access to water and were pair fed a casein based diet (AIN 93M; Dyets Inc). Pups were genotyped at 4–5 weeks of age by PCR as described previously [18]. The Institutional Animal Care and Use Committee approved all animal procedures.

Sex steroid measurements

Serum estradiol (E_2) was measured using an ultra-sensitive quantitative radioimmunoassay (Beckman Coulter, Brea, CA; inter-assay CV<12%). Testosterone (T) was also measured by radioimmunoassay (Siemens, Los Angeles, CA; inter-assay CV 11%).

Bone Phenotypic Characterization

Three-month old female $ER\alpha^{+/+}$, $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice were scanned by dual energy x-ray absorptiometry (DXA) to obtain whole body, femur and spine areal BMD (aBMD) measurements as well as whole body % fat mass. The tibial metaphysis and diaphysis were scanned by peripheral quantitative computed tomography (pQCT) to obtain total, trabecular and cortical volumetric BMD (vBMDs) and other bone structural parameters. For the *in vivo* BMD measurements, the mice were anesthetized with Avertin (2,2,2 tribromoethanol, 720mg/kg i.p.). DXA measurements were carried out using a Lunar PIXImus densitometer (software version 1.44; Lunar Corp.). Mice were placed on an animal tray in a prone position and whole body scans were carried out. After scanning, regions of interest (whole body, right femur and lumbar spine) were analyzed. The coefficients of variation (CVs) for the total body, lumbar and femoral areal BMD (aBMD) were 4.9%, 2.7%, and 4.3%, respectively. pQCT measurements were performed with the mice placed in a supine position on a gantry using the Stratec XCT Research SA Plus using software version 5.40 (Norland Medical Systems Inc.). Slice images were measured at 1.9 mm (corresponding to the proximal tibial metaphysis) and at 9 mm (corresponding to the diaphysis of the tibia) from the proximal end of the tibia. For trabecular bone, the threshold was set at 480 mg/cm^3 and for cortical bone at 710 mg/cm^3 . The CV was 4.4% for the total tibial vBMD at the metaphysis and 1.4% for the cortical vBMD at the diaphysis.

Histology and histomorphometry

The lumbar vertebrae and femurs were dehydrated in alcohol, embedded in methyl methacrylate, sectioned and stained using Goldner's stain. The bone volume/total volume (BV/TV), trabecular number (TbN, /mm), trabecular thickness (TbTh, μm) and trabecular separation (TbSp, mm) were obtained using standard methods. The measurements excluded the primary spongiosa. All measurements were done at a 10 $\times$ magnification using the OsteoMeasure analysis system (Osteometrics). The dynamic measurements were carried out as described previously [18, 19].

MicroCT Measurements

The quantitative analysis of the metaphyseal cancellous bone of the proximal tibia was done by μ CT (μ CT 35, Scanco Medical AG). Using two-dimensional data from scanned slices, three-dimensional analysis was conducted to calculate morphometric parameters defining trabecular bone mass and micro-architecture, including BV/TV, TbN, TbTh, and ThSp.

Osteogenic and adipogenic assays

Three-month old $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/NERK1}$ mice were sacrificed by CO_2 inhalation and the tibiae and femora were excised under aseptic conditions. The metaphyseal ends of the bones were removed in order to expose the marrow cavity. The marrow was flushed using a 27 gauge syringe into maintenance medium (phenol-free α MEM containing 10% heat inactivated fetal bovine serum and 1% antibiotic/antimycotic, GIBCO/BRL). The cells were then plated after counting on a hemocytometer using trypan blue exclusion of dead cells. These freshly isolated cells derived from 5–7 individual mice of each genotype were then plated at a density of 1×10^7 cells/well for the colony-forming unit-fibroblast (CFU-F), CFU-alkaline phosphatase (AP), CFU-osteoblast (OB), and adipocyte assays. Osteogenic conditions (for the CFU-AP and CFU-OB assays) included the addition of 0.01M β glycerophosphate (Sigma), 10^{-8} M dexamethasone (DAKO), and 50 μ g/ml ascorbic acid (Sigma). Adipogenic conditions included the addition of 10^{-6} M rosiglitazone. Half media changes were carried out on day 3. On day 15, the cells were stained with methylene blue for enumerating CFU-Fs, with an AP stain (Sigma) for CFU-APs, and with an Oil Red O stain for adipocytes. The CFU-OBs were enumerated on day 24 by von Kossa staining. To identify CFU-Fs, methylene blue positive colonies (>20 cells) were counted; similar criteria were used to identify CFU-AP and CFU-OB colonies. For the adipocyte assay, we counted the number of Oil Red O positive cells per plate.

In vitro effects of estrogen on osteoblast versus adipogenic differentiation

The following experimental groups used in order to assess the osteogenic potential of bone marrow stromal cells derived from the different genotypes and their modulation by estrogen : (i) osteogenic conditions without estradiol (E_2) (α MEM +10% triple charcoal stripped fetal bovine serum + 1% antibiotic/antimycotic solution [GIBCO/BRL] containing 0.01M β glycerophosphate [Sigma], 10^{-8} M dexamethasone [DAKO] and 50 μ g/ml ascorbic acid [Sigma]); and (ii) osteogenic conditions with E_2 (α MEM +10% triple charcoal stripped fetal bovine serum + 1% antibiotic/antimycotic solution [GIBCO/BRL] containing 0.01M β glycerophosphate (Sigma), 10^{-8} M dexamethasone [DAKO], 50 μ g/ml ascorbic acid [Sigma] and 10^{-8} M E_2 [Sigma]). Cells were replenished every 4 days with fresh media and on day 24 cells were washed with PBS and fixed with cold 70% ethanol and stained with 6% silver nitrate solution. The excess stain was bleached by briefly incubating with 5% sodium thiosulfate solution. The von Kossa-positive colonies were counted and expressed as a percent of the osteogenic control. To evaluate estrogen effects on adipogenesis in the various genotypes, we used the following groups: (i) adipogenic Treatment without E_2 (α MEM +10% triple charcoal stripped fetal bovine serum + 1% antibiotic/antimycotic solution [GIBCO/BRL] containing 10^{-6} M rosiglitazone) and (ii) adipogenic treatment with E_2 (α MEM +10% triple charcoal stripped fetal bovine serum + 1% antibiotic/antimycotic solution [GIBCO/BRL] containing 10^{-6} M rosiglitazone and 10^{-8} M E_2 [Sigma]). Cells were replenished every 4 days with fresh media and on day 15 cells were stained with Oil Red O. The number of Oil Red O Positive cells were counted and the results expressed as percentage of the adipogenic control.

Statistical Methods

For comparisons between groups, we used an ANOVA; where this was significant, the Fisher Protected Least Significant Difference test was used for pairwise comparisons between groups. All data are presented as mean $\pm$ SEM, and $P < 0.05$ was considered significant.

RESULTS

Body weight and composition

Figure 2 shows the body weights (Figure 2A) and percent fat mass (Figure 2B), assessed by DXA, in the $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/-NERKI}$ mice. As is evident, either complete deletion of $ER\alpha$ or loss of ERE signaling led to increased body weight and in percent body fat, consistent with a role for $ER\alpha$ and specifically, ERE signaling, in regulating body composition in mice.

Serum sex steroid levels

We previously found that $ER\alpha^{-/-NERKI}$ mice in a 50:50 C57BL/6:129/SvJ background had significantly increased serum E_2 levels as compared to $ER\alpha^{+/+}$ mice in the same background [16]. We now reassessed serum E_2 and T levels in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/-NERKI}$ mice who were all in the C57BL/6 background (Table 1). As is evident, in this genetic background, while serum E_2 and T levels were significantly higher in the $ER\alpha^{-/-}$ mice as compared to the $ER\alpha^{+/+}$ mice, serum E_2 levels in the $ER\alpha^{-/-NERKI}$ mice were similar to those in the $ER\alpha^{+/+}$ mice. However, the $ER\alpha^{-/-NERKI}$ mice did have significant increases in serum T levels as compared to the $ER\alpha^{+/+}$ mice.

Areal and volumetric BMD

Figure 3 shows total body aBMD (Figure 3A), spine aBMD (Figure 3B), and femur aBMD (Figure 3C) in the 3 groups of mice. In the $ER\alpha^{-/-}$ mice, the increases in E_2 and T levels noted above likely activate $ER\beta$ and the androgen receptor, respectively [5], leading to the observed preservation of aBMD at all 3 sites in these mice; by contrast, despite the observed increased T levels, the $ER\alpha^{-/-NERKI}$ mice had reduced total body, spine, and femur aBMD values as compared to both the $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice (Figure 3 A – C).

Total vBMD at the tibial metaphysis was reduced in the $ER\alpha^{-/-}$ and in the $ER\alpha^{-/-NERKI}$ mice (Figure 4A), with greater reductions in the $ER\alpha^{-/-NERKI}$ mice. However, trabecular vBMD was increased in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/-NERKI}$ mice (albeit to a lesser extent in the $ER\alpha^{-/-NERKI}$ as compared to the $ER\alpha^{-/-}$ mice) (Figure 4B). By contrast, the reduction in total vBMD in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/-NERKI}$ mice was largely due to reductions in cortical vBMD (Figure 4C).

Since the tibial diaphysis is a more robust site for assessment of cortical bone, we also assessed cortical parameters at this site. Cortical vBMD (Figure 5A) and thickness (Figure 5B) were slightly higher in $ER\alpha^{-/-}$ as compared to $ER\alpha^{+/+}$ mice, but both parameters were significantly reduced in the $ER\alpha^{-/-NERKI}$ as compared to both the $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice. Periosteal circumference was increased in the $ER\alpha^{-/-}$, but not the $ER\alpha^{-/-NERKI}$ mice (Figure 5C), whereas the $ER\alpha^{-/-NERKI}$ mice had significant increases in endosteal circumference at this site as compared to both the $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice (Figure 5D).

μ CT and histomorphometry

We also assessed trabecular bone parameters in more detail at the proximal tibial metaphysis using μ CT (Table 2) and at the lumbar spine using histomorphometry (Table 3). This did

reveal site-specificity in trabecular bone changes in the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice as compared to the $ER\alpha^{+/+}$ mice. Thus, BV/TV at the tibial metaphysis was increased in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice (albeit to a lesser extent in the $ER\alpha^{-/NERKI}$ mice) as compared to the $ER\alpha^{+/+}$ mice (Table 2). TbTh was reduced in the $ER\alpha^{-/NERKI}$ as compared to both the $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice. Both the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice had increases in TbN and decreases in TbSp as compared to the $ER\alpha^{+/+}$ mice (Table 2). Figure 6A shows representative μ CT images from the tibial metaphysis in the 3 groups of mice.

In contrast to the increase in BV/TV at the tibial metaphysis in the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice as compared to the $ER\alpha^{+/+}$ mice, all 3 groups of mice had similar values for BV/TV at the lumbar spine (Table 3). However, while trabecular microarchitecture was relatively well preserved in the $ER\alpha^{-/-}$ mice, the $ER\alpha^{-/NERKI}$ mice had significant reductions in TbN and increases in TbSp at the lumbar spine; this did result in a small but non-significant decrease in BV/TV in the $ER\alpha^{-/NERKI}$ mice at this site. Overall, $ER\alpha^{-/NERKI}$ mice clearly had more severe deficits in trabecular structure at the lumbar spine as compared to the $ER\alpha^{-/-}$ mice. Figure 6B shows representative histological sections from the lumbar spine in the 3 groups of mice.

Table 3 shows additional trabecular static and dynamic histomorphometric parameters at the lumbar vertebrae. Osteoid surface/bone surface (OS/BS) and osteoblast surface/bone surface (Ob.S/BS) were reduced in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ as compared to the $ER\alpha^{+/+}$ mice, with a similar trend for number of osteoblasts/bone perimeter (N.Ob/B.Pm). Eroded surface/bone surface (ES/BS) or number of osteoclasts/bone perimeter (N.Oc/B.Pm) did not differ between the groups, while osteoclast surface/bone surface (Oc.S/BS) was reduced in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ as compared to the $ER\alpha^{+/+}$ mice. Mineral apposition rate (MAR), but not the surface referent bone formation rate (BFR/BS), was also reduced in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ as compared to the $ER\alpha^{+/+}$ mice.

Table 4 shows the cortical static and dynamic histomorphometric parameters at the femur diaphysis in the 3 groups of mice. Consistent with the pQCT data at the tibial diaphysis (Figure 5B), histologically assessed cortical thickness was reduced at the femur diaphysis in the $ER\alpha^{-/NERKI}$, but not the $ER\alpha^{-/-}$, mice. Periosteal MAR and BFR were reduced in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ as compared to the $ER\alpha^{+/+}$ mice. By contrast, endocortical MAR (but not BFR) was significantly reduced in the $ER\alpha^{-/NERKI}$ mice, but not in the $ER\alpha^{-/-}$ mice, consistent with the more severe reductions in cortical thickness present in the $ER\alpha^{-/NERKI}$ mice.

Collectively, these data demonstrate that while trabecular bone volume at the tibial metaphysis is increased in both the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice as compared to the $ER\alpha^{+/+}$ mice, this is not the case for trabecular bone volume at the spine. However, at the spine, $ER\alpha^{-/NERKI}$ mice do have microstructural changes (reduced TbN and increased TbSp, with a trend for reduction in BV/TV) not present in the $ER\alpha^{-/-}$ mice. In addition, cortical vBMD and thickness are reduced in the $ER\alpha^{-/NERKI}$ mice as compared to either the $ER\alpha^{+/+}$ or $ER\alpha^{-/-}$ mice. MAR, which represents the work done by a team of osteoblasts, is reduced in trabecular and in cortical bone in the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice, with the deficit in MAR in cortical bone tending to be more severe in the $ER\alpha^{-/NERKI}$ as compared to the $ER\alpha^{-/-}$ mice.

In vitro studies

To better understand the cellular basis for the alterations in skeletal structure and dynamics in the $ER\alpha^{-/-}$ and $ER\alpha^{-/NERKI}$ mice, we performed CFU-F, CFU-AP, and CFU-OB assays in the 3 groups of mice. As shown in Figure 7, all 3 parameters were increased in the $ER\alpha^{-/-}$, but reduced in the $ER\alpha^{-/NERKI}$, as compared to the $ER\alpha^{+/+}$ mice. These data

indicate that in contrast to loss of ER α , the presence of the NERKI receptor leads to a reduction in osteoblast progenitors in the ER α ^{-/-}/NERKI mice. Figure 8 shows the number of adipocytic (Oil Red O+) cells in marrow cultures from the 3 genotypes. As is evident, these were similar in the ER α ^{+/+} and ER α ^{-/-} cultures, but reduced in the ER α ^{-/-}/NERKI cultures, indicating that the presence of the NERKI receptor is suppressive also of adipocytic precursors.

Since both osteoblasts and adipocytes share a common progenitor [20], in further studies we also examined the effects of *in vitro* estrogen treatment of bone marrow stromal cells from the 3 groups of mice on osteoblast versus adipocyte colonies. As shown in Figure 9A, while estrogen treatment significantly increased the number of osteoblastic colonies in cells from the ER α ^{+/+} mice, estrogen failed to have this effect on cells from the ER α ^{-/-} or the ER α ^{-/-}/NERKI mice. By contrast, estrogen treatment reduced the numbers of adipocytes *in vitro* in cells from ER α ^{+/+} as well as ER α ^{-/-}/NERKI mice, but failed to have this effect in cells from the ER α ^{-/-} mice (Figure 9B). These findings thus demonstrate that while stimulation of mineralization by estrogen requires both ER α and intact ERE activity, the suppression of adipogenesis by estrogen, although requiring ER α , can also occur in cells from ER α ^{-/-}/NERKI mice.

DISCUSSION

The effects of estrogen on bone are complex and mediated by multiple signaling pathways [18]. In the present study, we compared the skeletal phenotype of wild type (ER α ^{+/+}) mice with those having complete deletion of ER α (ER α ^{-/-}) versus mice in whom the only active ER α was the NERKI receptor, which is unable to bind DNA (ER α ^{-/-}/NERKI) [14]. All genotypes were in the same genetic background, allowing for a direct comparison between groups. In cortical bone at the tibial diaphysis, we found that ER α ^{-/-}/NERKI mice had significantly reduced values for cortical vBMD and thickness as compared to either the ER α ^{+/+} or ER α ^{-/-} mice. This was accompanied by significant decreases in periosteal and endocortical MARs in the ER α ^{-/-}/NERKI mice. These data thus suggest that for cortical bone, the presence of the NERKI receptor has effects that are distinct from simple loss of the receptor. Similar to our findings, Almeida et al. [21] also found reduced cortical thickness at the tibial diaphysis in ER α ^{-/-}/NERKI as compared to ER α ^{+/+} mice, but these investigators did not compare values for cortical bone parameters in the ER α ^{-/-}/NERKI mice versus the ER α ^{-/-} mice.

In our previous study [16], we found that ER α ^{-/-}/NERKI mice in a 50:50 C57BL/6:129/SvJ background had significantly increased serum E₂ levels as compared to ER α ^{+/+} mice in the same background. Interestingly, when we compared ER α ^{-/-}/NERKI mice in a complete C57BL/6 background to ER α ^{+/+} mice, serum E₂ levels did not differ between the two groups, but serum T levels were significantly higher in the ER α ^{-/-}/NERKI mice. While we did not previously assess serum T levels in the ER α ^{-/-}/NERKI mice in a 50:50 C57BL/6:129/SvJ background, these findings do indicate that there may be significant strain effects on changes in serum sex steroid levels in mice with ER α mutations.

The trabecular bone phenotype of the ER α ^{-/-}/NERKI mice was more complex than the cortical bone phenotype and differed somewhat for trabecular bone at the tibial metaphysis versus the spine. As compared to the ER α ^{+/+} mice, trabecular vBMD and BV/TV at the tibial metaphysis was increased in both the ER α ^{-/-} and ER α ^{-/-}/NERKI mice, although lower in the ER α ^{-/-}/NERKI as compared to the ER α ^{-/-} mice. Similar to our findings, Sims et al. [5] reported that ER α ^{-/-} had increased trabecular BV/TV at the femoral metaphysis; the authors postulated that this was likely secondary to the elevated E₂ levels in the female ER α ^{-/-} mice activating ER β signaling [5].

In contrast to the tibia, trabecular BV/TV was not increased in either the $ER\alpha^{-/-}$ or $ER\alpha^{-/-}NERKI$ mice at the lumbar spine; instead, as compared to both the $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice, $ER\alpha^{-/-}NERKI$ mice had significantly reduced trabecular numbers and increased trabecular separation. Trabecular BV/TV was somewhat reduced (by 9%) in the $ER\alpha^{-/-}NERKI$ as compared to the $ER\alpha^{+/+}$ mice, but this difference was not statistically significant. While Almeida et al. [21] did not compare the $ER\alpha^{-/-}NERKI$ to the $ER\alpha^{-/-}$ mice, the $ER\alpha^{-/-}NERKI$ mice in their study did have significant reductions in spine BV/TV as compared to the $ER\alpha^{+/+}$ mice. These reductions were of a greater magnitude than those observed in our study, perhaps due to the fact that the mice used by Almeida et al. [21] were in a mixed C57BL/6–129Sv/J background, rather than in a complete C57BL/6 background. This difference notwithstanding, our findings in trabecular bone at the spine do demonstrate that the $ER\alpha^{-/-}NERKI$ mice have significant structural deficits as compared to the $ER\alpha^{-/-}$ mice. It should also be noted that, as compared to the $ER\alpha^{+/+}$ mice, osteoclast numbers or eroded surfaces were not increased in either the $ER\alpha^{-/-}$ or $ER\alpha^{-/-}NERKI$ mice. Thus, while osteoclast-specific deletion of $ER\alpha$ clearly results in increased osteoclast numbers and enhanced bone resorption [22, 23], global deletion of $ER\alpha$ or global knock-in of the $NERKI$ receptor does not increase either parameter, perhaps due to the increase in sex steroid levels in these mice activating $ER\beta$ or the androgen receptor in osteoclasts. Further studies are needed, however, to address this important issue.

Our *in vitro* studies provide further support for differential effects of deletion of $ER\alpha$ versus the $NERKI$ mutation on CFU-F, -AP, and OB numbers. All three parameters were increased in bone marrow cells from the $ER\alpha^{-/-}$ mice, consistent with previous work from Di Gregorio et al. [24] showing that estrogen suppresses the proliferation of early mesenchymal progenitors. By contrast, all three parameters were reduced in bone marrow cells from $ER\alpha^{-/-}NERKI$ as compared to either the $ER\alpha^{+/+}$ or $ER\alpha^{-/-}$ mice. Finally, our studies with estrogen treatment of bone marrow stromal cells derived from mice of the three genotypes indicated that the presence of $ER\alpha$ and ERE signaling was necessary for the stimulation of mineralization by these cells. However, estrogen was able to suppress adipocyte colony formation in cells from $ER\alpha^{+/+}$ and $ER\alpha^{-/-}NERKI$, but not $ER\alpha^{-/-}$, mice, indicating that ERE signaling was not required for this effect. The molecular mechanisms mediating these differential effects of $ER\alpha$ on mineralization versus adipocyte formation warrant further study, but these data do suggest that $ER\alpha$ (and in particular, the classical function of $ER\alpha$) plays a role in osteoblastic versus adipocytic differentiation of mesenchymal progenitor cells. Additional studies are also needed to examine changes in marrow fat with aging in $ER\alpha^{-/-}$ and $ER\alpha^{-/-}NERKI$ as compared to wild type mice.

It is also important to address the apparent discrepancy between our data in Figure 7, where complete loss of $ER\alpha$ leads to an increase in CFU-F, CFU-AP, and CFU-OB (implying a suppressive effect of $ER\alpha$ on these parameters, which also is present in the $ER\alpha^{-/-}NERKI$ mice), whereas in Figure 9, estrogen treatment facilitates osteoblast differentiation in wild type mice. We believe this is due to the fact that the CFU assays measure progenitor cells for CFU-F, CFU-AP, and CFU-OB *in vivo* in the absence of estrogen treatment *in vitro* (which are increased in the absence of $ER\alpha$ and reduced in the presence of the $NERKI$ receptor), which is distinct from the effects of estrogen in enhancing differentiation (which is what is assessed in Figure 9). Thus, estrogen appears to suppress the pool of progenitor cells (as demonstrated, for example, by Di Gregorio et al. [24]), leading to the increase in CFU-F, CFU-AP, and CFU-OB populations in the $ER\alpha^{-/-}$ mice, but when progenitor cells are exposed to estrogen, they do show enhanced differentiation. In short, effects of estrogen on the progenitor pool seem to be different from the effects on estrogen on osteoblast differentiation. In this context, it is also important to note that the MAR reflects the activity of a team of osteoblasts in the basic multicellular unit, whereas the CFU assays reflect the numbers of progenitor cells *in vivo*. Thus, changes in these two parameters may differ,

depending on the effects of estrogen on the progenitor pool versus osteoblast differentiation and/or activity

Since our 3 genotypes were wild type ($ER\alpha^{+/+}$), complete $ER\alpha$ deletion ($ER\alpha^{-/-}$), or mice where the only receptor is the NERKI receptor ($ER\alpha^{-/NERKI}$), and we did not rely on or use heterozygous mice (i.e., $ER\alpha^{+/NERKI}$) mice, the issue of the NERKI receptor being a dominant negative is not a concern with any of the data in this paper. Moreover, previous work by Jackaka et al. [14] has provided evidence that the NERKI receptor does not function as a dominant negative receptor for wild type $ER\alpha$.

Previous studies by Jackaka and colleagues [15] have shown that the NERKI receptor fails to activate ERE reporter activity *in vitro*, but has intact transcriptional activity for AP-1 reporter constructs and can bind *c-jun* normally in a mammalian two-hybrid cell assay. Of note, recent studies have provided evidence for a requirement for “pioneer” factors, such as FoxA1, in mediating direct ER binding to DNA, at least in breast cancer cells [25]. Based on what is known regarding preserved protein-protein interactions of the NERKI receptor [14,15], this receptor should be able to bind factors such as FoxA1, although further studies are needed to address this possibility.

In summary, our study provides a detailed skeletal characterization of $ER\alpha^{-/NERKI}$ compared to $ER\alpha^{-/-}$ mice and indicates that for a number of bone parameters, the presence of the NERKI receptor results in greater skeletal deficits than complete loss of the receptor. We recognize that our models are limited by the fact that the deletion of $ER\alpha$ or the NERKI mutation was present in all tissues, and not specifically restricted to osteoblasts or osteoclasts. However, generating mice with replacement of the wild-type $ER\alpha$ by the NERKI receptor in specific cell types is not a simple exercise, although the construction of such mice has been initiated in our laboratory. This limitation notwithstanding, our findings point to the need for further studies aimed at defining the cellular and molecular mechanisms whereby shifting the balance of $ER\alpha$ signaling from the classical (ERE) to the non-classical (non-ERE) pathway leads to deleterious skeletal effects. In addition to better understanding the fundamental biology of ER signaling, such studies may identify novel approaches to selectively modulate ER signaling pathways for beneficial skeletal effects.

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Figure 1.

ER signaling pathways. The classical pathway of ER α action (left) requires direct binding to EREs found in the control regions of estradiol (E₂)-regulated genes. The non-classical pathway can involve either nuclear (middle) or membrane (right) signaling. In non-classical nuclear signaling, the ER interacts with other transcription factors, such as the jun/fos complex which binds to AP-1 sites, whereas in the membrane pathway, ER signaling is via alterations in kinase activity.

Figure 2. (A) Body weight (n = 48–49 per group) and (B) percent body fat by DXA in ERα^{+/+}, ERα^{-/-}, and ERα^{-/-}NERK1 mice (n = 14–25 per group). **P < 0.01 and ***P < 0.001 versus ERα^{+/+}; †††P < 0.001 versus ERα^{-/-}.

Figure 3.

(A) Total body aBMD, (B) spine aBMD, and (C) femur aBMD by DXA in ERα^{+/+}, ERα^{-/-}, and ERα^{-/-}NERKI mice (n = 14–25 per group). ***P < 0.001 versus ERα^{+/+}; †††P < 0.001 versus ERα^{-/-}.

Figure 4.

(A) Total vBMD, (B) trabecular vBMD, and (C) cortical vBMD at the tibial metaphysis by QCT in ERα^{+/+}, ERα^{-/-}, and ERα^{-/-}NERK1 mice (n = 18–31 per group). ***P < 0.001 versus ERα^{+/+}; †††P < 0.001 versus ERα^{-/-}.

Figure 5.

(A) Cortical vBMD, (B) cortical thickness, (C) periosteal circumference, and (D) endosteal circumference at the tibial diaphysis in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/NERKI}$ mice ($n = 18-31$ per group). ^a $P = 0.051$, ^{*} $P < 0.05$, ^{**} $P < 0.01$ and ^{***} $P < 0.001$ versus $ER\alpha^{+/+}$; ^{††} $P < 0.01$ and ^{†††} $P < 0.001$ versus $ER\alpha^{-/-}$.

Figure 6.
(A) Representative μ CT images of the tibial metaphysis and (B) representative histological sections of the lumbar spine in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/-}$ NERKI mice.

Figure 7. (A) CFU-F, (B) CFU-AP, and (C) CFU-OB colonies (per 10^7 cells plated) in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/-}NERKI$ mice (n = 4 per group). *P < 0.05, **P < 0.01 and ***P < 0.001 versus $ER\alpha^{+/+}$; †††P < 0.001 versus $ER\alpha^{-/-}$.

Figure 8. Oil-red O+ (adipocyte) cells in bone marrow cultures from ERα^{+/+}, ERα^{-/-}, and ERα^{-/-}NERKI mice (n = 5–10 per group). *P < 0.05 versus ERα^{+/+} mice.

Figure 9.

(A) Osteoblast colonies and (B) Oil-red O+ (adipocyte) cells (open bars, vehicle control; solid bars, 10^{-8} M E_2) in bone marrow cultures from $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/-}NERKI$ mice (n = 5–10 per group). *P < 0.05 and **P < 0.01 versus the vehicle control.

Table 1

Serum E₂ and T levels in the ER $\alpha^{+/+}$, ER $\alpha^{-/-}$, and ER $\alpha^{-/NERK1}$ mice (n = 7–11 per group).

	ER $\alpha^{+/+}$	ER $\alpha^{-/-}$	ER $\alpha^{-/NERK1}$
E ₂ , pg/mL	9 ± 2	21 ± 4*	11 ± 3
T, ng/dL	40 ± 9	111 ± 23*	72 ± 11*

* P < 0.05 versus ER $\alpha^{+/+}$

Table 2

Trabecular bone parameters at the proximal tibial metaphysis by μ CT in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/NERKI}$ mice (n = 3–5 per group). BV/TV, bone volume/tissue volume; TbTh, trabecular thickness; TbN, trabecular number; TbSp, trabecular separation.

	$ER\alpha^{+/+}$	$ER\alpha^{-/-}$	$ER\alpha^{-/NERKI}$	ANOVA
BV/TV, %	4.8 ± 0.28	$12.7 \pm 1.1^{***}$	$8.4 \pm 1.1^{*,\dagger}$	<0.001
TbTh, mm	0.036 ± 0.001	$0.041 \pm 0.001^{*}$	$0.031 \pm 0.001^{*,\dagger,\ddagger}$	<0.001
TbN, mm	3.18 ± 0.12	$4.71 \pm 0.11^{***}$	$5.00 \pm 0.14^{***}$	<0.001
TbSp, mm	0.322 ± 0.011	$0.221 \pm 0.007^{***}$	$0.202 \pm 0.006^{***}$	<0.001

* $P < 0.05$,

** $P < 0.01$ and

*** $P < 0.001$ versus $ER\alpha^{+/+}$;

$\dagger$ $P < 0.05$ and

$\ddagger$ $P < 0.001$ versus $ER\alpha^{-/-}$.

Table 3

Static and dynamic histomorphometric parameters at the lumbar spine in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/NERKI}$ mice (n = 9–12 per group). BV/TV, bone volume/tissue volume; TbTh, trabecular thickness; TbN, trabecular number; TbSp, trabecular separation; OS/BS, osteoid surface/bone surface; Ob.S/BS, osteoblast surface/bone surface; N.Ob/B.Pm, number of osteoblasts/bone perimeter; ES/BS, eroded surface/bone surface; Oc.S/BS, osteoclast surface/bone surface; N.Oc/B.Pm, number of osteoclasts/bone perimeter; MAR, mineral apposition rate; BFR/BS, bone formation rate, surface referent.

	$ER\alpha^{+/+}$	$ER\alpha^{-/-}$	$ER\alpha^{-/NERKI}$	ANOVA
Static parameters				
BV/TV, %	23.0 ± 1.1	21.9.0 ± 1.4	21.0 ± 1.1	0.492
TbTh, mm	0.050 ± 0.002	0.044 ± 0.003 ^a	0.051 ± 0.002 [†]	0.095
TbN, /mm	4.6 ± 0.2	5.0 ± 0.1	4.1 ± 0.1 ^{*,††}	0.0014
TbSp, mm	0.170 ± 0.008	0.158 ± 0.004	0.193 ± 0.008 ^{*,††}	0.007
OS/BS, %	6.25 ± 0.72	2.92 ± 0.70 ^{**}	3.92 ± 0.59 [*]	0.01
Ob.S/BS, %	17.2 ± 1.4	9.3 ± 1.3 ^{***}	13.9 ± 0.9 ^{a, †}	0.002
N.Ob/B.Pm, /mm	20.0 ± 1.7	12.4 ± 1.5 ^{**}	17.4 ± 1.3 [†]	0.009
ES/BS, %	1.27 ± 0.22	1.15 ± 0.19	0.85 ± 0.09	0.273
Oc.S/BS, %	4.43 ± 0.39	2.81 ± 0.55 [*]	3.06 ± 0.24 [*]	0.032
N.Oc/B.Pm, /mm	1.68 ± 0.14	1.37 ± 0.24	1.41 ± 0.10	0.415
Dynamic parameters				
MAR, $\mu\text{m}/\text{d}$	0.936 ± 0.059	0.805 ± 0.016 [*]	0.805 ± 0.041 [*]	0.060
BFR/BS, $\mu\text{m}^3/\mu\text{m}^2/\text{d}$	0.192 ± 0.020	0.152 ± 0.015	0.188 ± 0.026	0.308

^aP = 0.075,

^{*}P < 0.05,

^{**}P < 0.01 and

^{***}P < 0.001 versus $ER\alpha^{+/+}$;

[†]P < 0.05 versus $ER\alpha^{-/-}$.

Table 4

Static and dynamic histomorphometric parameters at the femur diaphysis in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{-/NERKI}$ mice (n = 6 per group).

	$ER\alpha^{+/+}$	$ER\alpha^{-/-}$	$ER\alpha^{-/NERKI}$	ANOVA
Static parameters				
Cortical thickness, mm	0.185 ± 0.008	0.190 ± 0.010	$0.127 \pm 0.012^{***, \dagger \dagger \dagger}$	<0.001
Dynamic parameters				
Periosteal surface				
MAR, $\mu\text{m}/\text{d}$	0.925 ± 0.038	$0.620 \pm 0.167^*$	$0.366 \pm 0.081^{***}$	0.001
BFR, $\mu\text{m}^2/\mu\text{m}/\text{d}$	0.140 ± 0.025	$0.032 \pm 0.016^{***}$	$0.026 \pm 0.012^{***}$	<0.001
Endocortical surface				
MAR, $\mu\text{m}/\text{d}$	0.796 ± 0.063	0.692 ± 0.082	$0.508 \pm 0.050^{*,a}$	0.013
BFR, $\mu\text{m}^2/\mu\text{m}/\text{d}$	0.122 ± 0.013	0.091 ± 0.046	0.073 ± 0.022	0.430

* $P < 0.05$,

** $P < 0.01$ and

*** $P < 0.001$ versus $ER\alpha^{+/+}$;

^a $P = 0.068$

$\dagger \dagger \dagger$ $P < 0.001$ versus $ER\alpha^{-/-}$.