THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

The role of activation functions 1 and 2 of estrogen receptor- for the effects of estradiol and selective estrogen receptor modulators in male mice

Citation for published version:

Borjesson, A, Farman, HH, Engdahl, C, Koskela, A, Sjogren, K, Kindblom, JM, Stubelius, A, Islander, U, Carlsten, H, Antal, MC, Krust, A, Chambon, P, Tuukkanen, J, Lagerquist, MK, Windahl, SH & Ohlsson, C 2013, 'The role of activation functions 1 and 2 of estrogen receptor- for the effects of estradiol and selective estrogen receptor modulators in male mice', *Journal of Bone and Mineral Research*, vol. 28, no. 5, pp. 1117-1126. <https://doi.org/10.1002/jbmr.1842>

Digital Object Identifier (DOI):

[10.1002/jbmr.1842](https://doi.org/10.1002/jbmr.1842)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Bone and Mineral Research

Publisher Rights Statement:

Copyright © 2013 American Society for Bone and Mineral Research

Available under Open Access

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

The Role of Activation Functions 1 and 2 of Estrogen Receptor- α for the Effects of Estradiol and Selective Estrogen Receptor Modulators in Male Mice

Anna E Börjesson,¹ Helen H Farman,¹ Cecilia Engdahl,¹ Antti Koskela,² Klara Sjögren,¹ Jenny M Kindblom,¹ Alexandra Stubelius,¹ Ulrika Islander,¹ Hans Carlsten,¹ Maria Cristina Antal,³ Andrée Krust,³ Pierre Chambon,³ Juha Tuukkanen,² Marie K Lagerquist,¹ Sara H Windahl,¹ and Claes Ohlsson¹

¹Centre for Bone and Arthritis Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

²Department of Anatomy and Cell Biology, University of Oulu, Oulu, Finland

³Department of Functional Genomics, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Université de Strasbourg (UdS), Collège de France, Illkirch, France

ABSTRACT

Estradiol (E2) is important for male skeletal health and the effect of E2 is mediated via estrogen receptor (ER)- α . This was demonstrated by the findings that men with an inactivating mutation in aromatase or a nonfunctional ER α had osteopenia and continued longitudinal growth after sexual maturation. The aim of the present study was to evaluate the role of different domains of ER α for the effects of E2 and selective estrogen receptor modulators (SERMs) on bone mass in males. Three mouse models lacking either ER α AF-1 (ER α AF-1⁰), ER α AF-2 (ER α AF-2⁰), or the total ER α (ER α ^{-/-}) were orchidectomized (orx) and treated with E2 or placebo. E2 treatment increased the trabecular and cortical bone mass and bone strength, whereas it reduced the thymus weight and bone marrow cellularity in orx wild type (WT) mice. These parameters did not respond to E2 treatment in orx ER α ^{-/-} or ER α AF-2⁰ mice. ER α AF-1⁰ mice were tissue-dependent, with a clear response in cortical bone parameters and bone marrow cellularity, but no response in trabecular bone. To determine the role of ER α AF-1 for the effects of SERMs, we treated orx WT and ER α AF-1⁰ mice with raloxifene (Ral), lasofoxifene (Las), bazedoxifene (Bza), or vehicle. These SERMs increased total body areal bone mineral density (BMD) and trabecular volumetric BMD to a similar extent in orx WT mice. Furthermore, only Las increased cortical thickness significantly and only Bza increased bone strength significantly. However, all SERMs showed a tendency toward increased cortical bone parameters. Importantly, all SERM effects were absent in the orx ER α AF-1⁰ mice. In conclusion, ER α AF-2 is required for the estrogenic effects on all evaluated parameters, whereas the role of ER α AF-1 is tissue-specific. All evaluated effects of Ral, Las and Bza are dependent on a functional ER α AF-1. Our findings might contribute to the development of bone-specific SERMs in males. © 2013 American Society for Bone and Mineral Research.

KEY WORDS: ESTROGEN RECEPTOR; BONE; TRABECULAR; CORTICAL; SERM

Introduction

Estrogen has previously been considered the female sex steroid and testosterone the male sex steroid, but now there are several studies, in both man and mouse, showing that estradiol (E2) is of importance for male skeletal health.^(1–4) The importance of E2 in males was clearly demonstrated by the finding that men with an inactivating mutation in the aromatase gene had osteopenia and a continued linear growth after sexual maturation.^(5,6) A similar skeletal phenotype was found in a man with a nonfunctional estrogen receptor (ER)- α . This indicates that

ER α is the main mediator of the skeletal effects of estrogen in men.⁽⁷⁾ In addition, it has been shown that ER α , but not ER β , is required for the skeletal effects of estrogen in male mice.^(8–12) G protein-coupled ER-1 (GPER-1 or GPR30) has also been suggested to be an ER, but we recently demonstrated that the E2 response on bone mass is independent of the GPER-1.⁽¹³⁾ These results, together with the fact that men have higher E2 levels than postmenopausal women, suggest that E2 is a hormone of importance for male bone health.^(14,15)

ER α interacts with several classes of coactivators/corepressors and the balance between coactivators and corepressors is

Received in original form September 10, 2012; revised form November 12, 2012; accepted November 26, 2012. Accepted manuscript online December 7, 2012.

Address correspondence to: Claes Ohlsson, MD, PhD, Claes Ohlsson, Centre for Bone and Arthritis Research, Klin Farm Lab, Vita Stråket 11, Department of Internal Medicine and Clinical Nutrition, Sahlgrenska University Hospital, SE-41345 Göteborg, Sweden. E-mail: Claes.Ohlsson@medic.gu.se

Journal of Bone and Mineral Research, Vol. 28, No. 5, May 2013, pp 1117–1126

DOI: 10.1002/jbmr.1842

© 2013 American Society for Bone and Mineral Research

dependent on the cell type.^(4,16) The balance of cofactors is a critical determinant of the ability of ER α to regulate gene transcription. Therefore, estrogen can exert vastly diverse effects in different tissues. In vitro studies have shown that the estrogen-induced transactivation of ER α is mediated by the ligand-independent activation function (AF)-1 and/or the ligand-dependent AF-2 in ER α . This is dependent on the cell type and promoter context,^(17–19) and could depend on the cofactors and/or balance of cofactors in the cell type evaluated. Several cofactors bind to ER α AF-1 and ER α AF-2; some are specific for either AF-1 or AF-2, whereas some cofactors bind to both.⁽²⁰⁾ It has also been shown that the full ligand-dependent transcriptional activity of ER α is reached through a synergism between AF-1 and AF-2.^(17–19,21–23) Variation in the expression of cofactors and the recruitment of cofactors to the ER α in different cell types also appear to have an important role for the tissue-specific effects of the selective ER modulators (SERMs).^(17,24) A SERM acts as an ER agonist or antagonist in a tissue-specific manner. Compared to E2, the SERMs have a bulky side chain, which upon binding to ER α protrudes from the ligand-binding pocket. This hinders the optimal conformational change of the ligand-binding domain of ER α by preventing the folding of helix 12 in the agonistic orientation. Consequently, ER α AF-2 is not formed correctly, which prevents ER α from interacting with certain cofactors.^(17,24–26) From these in vitro studies it has, therefore, been suggested that the effects of a specific SERM are mainly mediated by the ER α AF-1 and other regions of ER α than ER α AF-2, and that the importance of the different regions of ER α is decided by the particular conformational change of ER α induced by the SERM.^(17,24–26)

Raloxifene (Ral), which was the first SERM approved for the prevention and treatment of postmenopausal osteoporosis, was shown to be an ER agonist in bone but an ER antagonist in breast.^(27–29) Today, two more SERMs; lasofoxifene (Las) and bazedoxifene (Bza), are approved in Europe for treatment of postmenopausal osteoporosis. These SERMs and Ral have similar structures and they all reduce the risk for vertebral fractures. In addition, Las and Bza have also been suggested to be more effective in reducing the risk for nonvertebral fractures than Ral.^(27,29,30) Unfortunately, these three SERMs are all associated with side effects; eg, an increased risk for thromboembolism.^(27,29–31) Ral treatment has been shown to increase the bone mass in men with serum E2 levels below a certain threshold, without having feminizing effects, but there is not yet any approved SERM treatment available for male osteoporosis.^(32,33) Therefore, it is possible that bone-specific SERMs may be useful in the treatment of male osteoporosis. Thus, it is of importance to further characterize the signaling pathways of estrogen and SERMs in the male bone.

In this study, we have evaluated the roles of ER α AF-1 and ER α AF-2 in male mice for the effects of E2 in bone, and some other major estrogen-responsive tissues by analyzing mice with inactivation of the entire ER α protein (ER $\alpha^{-/-}$), ER α AF-1 (ER α AF-1⁰), or ER α AF-2 (ER α AF-2⁰). In addition, because in vitro experiments have suggested that the ER α AF-1 is the main region for mediating the tissue-specific effects of SERMs, we treated orx wild-type (WT) and orx ER α AF-1⁰ mice with Ral, Las, and Bza to clarify the role of ER α AF-1 for the effects of these SERMs in vivo.

The results obtained will clarify the relevance of different regions of the ER α for the effect of E2 and SERMs in males, thus facilitating the design of novel bone-specific SERMs.

Materials and Methods

Generation of mice

All experimental procedures involving animals were approved by the Ethics Committee of the University of Gothenburg. The generation of ER α -deficient mice (ER $\alpha^{-/-}$),⁽³⁴⁾ ER α AF-1⁰ mice,^(35,36) and ER α AF-2⁰ mice⁽³⁶⁾ have been described. Briefly, the ER $\alpha^{-/-}$ mice have a deletion in exon 3 of the ER α gene and they do not express any of the isoforms of the ER α protein.⁽³⁴⁾ The ER α AF-1⁰ mice have a specific deletion of AF-1 and do not express any full-length 66-kDa protein. Instead, they express a truncated 49-kDa ER α protein that lacks AF-1 and also the physiologically occurring but less abundantly expressed 46-kDa ER α isoform. The ER α AF-1⁰ protein has been shown, in vivo, to be expressed in similar amounts as the WT ER α protein.⁽³⁵⁾ The ER α AF-2⁰ mice have a deletion of the AF-2 core that resides within exon 9 and corresponds to amino acids 543 to 549. The sizes of the ER α proteins in ER α AF-2⁰ mice are slightly smaller than the WT ER α proteins of 66-kDa and 46-kDa, respectively, and they have been shown, in vivo, to be expressed in similar amounts as the WT ER α proteins.^(36,37) All three mouse models and their WT littermate controls were inbred C57BL/6 mice and generated by breeding heterozygous females and males.

In the experiment where the importance of ER α AF-1 and ER α AF-2 for an effect of estrogen was evaluated, orchidectomy (orx) or sham operation was performed on 12-week-old ER $\alpha^{-/-}$, ER α AF-1⁰, ER α AF-2⁰ and WT male mice. The orx mice were treated with either placebo or E2 (167 ng/mouse/d) and the sham-operated mice were treated with placebo for 4 weeks, using slow-release pellets inserted subcutaneous (s.c.) at the time of surgery (Innovative Research of America, Sarasota, FL, USA; $n = 8–11$ per group).

In the experiment where the different SERMs were evaluated, orx was performed on 20-week-old ER α AF-1⁰ and WT male mice. The orx mice rested for 6 days before they received s.c. injections 5 days/week during 3 weeks with either vehicle (veh; Miglyol 812; OmyaPeralta GmbH, Hamburg, Germany), raloxifene (120 μ g/mouse/d; Sigma Aldrich, St. Louis, MO, USA), lasofoxifene (8 μ g/mouse/d; Pfizer Inc., Groton, CT, USA), or bazedoxifene (48 μ g/mouse/d; Pfizer) ($n = 6–10$ per group).

Measurement of serum hormone levels

Commercially available radioimmunoassay (RIA) kits were used to assess serum concentrations of testosterone (ICN Biomedicals, Costa Mesa, CA, USA), according to the manufacturer's instructions.

Dual-energy X-ray absorptiometry

Analyses of total body areal bone mineral density (aBMD) were performed by dual-energy X-ray absorptiometry (DXA) using the Lunar PIXImus mouse densitometer (Wipro GE Healthcare, Madison, WI, USA).

Peripheral quantitative computer tomography

Computer tomography scans were performed with the peripheral quantitative computer tomography (pQCT) XCT RESEARCH M (version 4.5B; Norland, Fort Atkinson, WI, USA), operating at a resolution of 70 μm as described.⁽³⁸⁾ The scans were positioned in the metaphysis, at a distance proximal from the distal growth plate of the femur corresponding to 3.4% of the total length of the femur, and at a distance distal from the proximal growth plate of the tibia corresponding to 2.6% of the total length of the tibia. The trabecular bone region was defined as the inner 45% of the total cross-sectional area. Cortical bone parameters were analyzed in the mid-diaphyseal region of the femur and tibia.⁽¹²⁾

Micro-computed tomography

Micro-computed tomography (μCT) analyses were performed on the lumbar vertebra 5 (L_5) by using Skyscan 1072 scanner (Skyscan N.V., Aartselaar, Belgium), imaged with an X-ray tube voltage of 100 kV and current 98 μA , with a 1-mm aluminum filter.⁽⁹⁾ The scanning angular rotation was 180 degrees and the angular increment 0.90 degrees. The voxel size was 6.51 μm isotropically. Datasets were reconstructed using a modified Feldkamp algorithm and segmented into binary images using adaptive local thresholding.⁽³⁹⁾ The trabecular bone in the vertebral body caudal of the pedicles was selected for analyses as described.⁽³⁶⁾

Three-point bending

Immediately after the dissection, the femurs were fixed in Bürkhardt's formaldehyde for 2 days and after that stored in 70% ethanol. Just before the mechanical testing the bones were rinsed in PBS for 24 hours. The three-point bending test (span length 5.5 mm, loading speed 0.155 mm/sec) at the mid femur was made by the Instron universal testing machine (Instron 3366; Instron Corp., Norwood, MA, USA). Based on the recorded load deformation curves, the biomechanical parameters were acquired from raw files produced by Bluehill 2 software version 2.6 (Instron) with custom-made Excel macros.

Bone marrow cellularity and cells

Bone marrow cells were harvested by flushing 5 mL PBS through the bone cavities of one femur and one humerus, from each mouse, using a syringe. After centrifugation at 515 g for 5 minutes, pelleted cells were resuspended in Tris-buffered 0.83% NH_4Cl solution (pH 7.29) for 5 minutes to lyse erythrocytes and then washed in PBS. Bone marrow cells were resuspended in RPMI culture medium (PAA Laboratories, Pasching, Austria) before use. The total number of leucocytes in bone marrow was calculated using an automated cell counter (Sysmex, Hamburg, Germany). For flow cytometry analyses, cells were stained with phycoerythrin (PE)-conjugated antibodies to CD19 (Beckton-Dickinson, Biosciences, Pharmingen, San Diego, CA, USA) for detection of B-lymphocytes. The cells were then subjected to fluorescence activated cell sorter analysis (FACS) on a FACSCalibur (Beckton-Dickinson, Biosciences, Pharmingen) and analyzed using FlowJo software. Results are expressed as cell frequency (%).

Statistical analysis

For statistical evaluation, Student's t test was used when comparing the estrogen-treated mice with the placebo-treated mice, p values less than 0.05 were considered statistically significant. When comparing the Ral-, Las-, and Bza-treated groups with the vehicle-treated group (three comparisons), Student's t test with Bonferroni correction was used.

Results

The E2 response in trabecular bone requires $\text{ER}\alpha\text{AF-1}$ and AF-2 whereas the E2 response in cortical bone requires $\text{ER}\alpha\text{AF-2}$ but not AF-1

As expected, the serum testosterone levels in gonadal-intact (sham) male $\text{ER}\alpha^{-/-}$ mice were elevated compared to sham WT controls (WT: 0.68 ± 0.27 ng/mL; $\text{ER}\alpha^{-/-}$: 5.26 ± 0.88 ng/mL; $p < 0.01$; Fig. 1A). These sham $\text{ER}\alpha^{-/-}$ mice showed a normal trabecular bone volume/tissue volume (BV/TV; WT: $27.3\% \pm 2.1\%$; $\text{ER}\alpha^{-/-}$: $26.9\% \pm 1.4\%$; Fig. 1B), but a significantly decreased cortical thickness (WT: 0.228 ± 0.006 mm; $\text{ER}\alpha^{-/-}$:

Fig. 1. Elevated serum testosterone levels in male $\text{ER}\alpha^{-/-}$ mice lead to compensatory effects in trabecular bone but not in cortical bone. Gonadal-intact (sham) male $\text{ER}\alpha^{-/-}$ mice and their corresponding sham wild-type (WT) mice were evaluated. (A) Serum testosterone levels were measured. (B) Trabecular bone (ie, bone volume/tissue volume, BV/TV) in L_5 vertebra was analyzed by μCT . (C) Cortical thickness (Crt. Thk) was analyzed by pQCT.

0.204 ± 0.003 mm; $p < 0.01$; Fig. 1C) compared to sham WT controls. This is consistent with previous results showing that compensatory mechanisms via the androgen receptor (AR) can maintain the trabecular bone mass, but not the cortical bone mass in male ER $\alpha^{-/-}$ mice.⁽⁹⁾ To avoid compensatory effects from elevated serum testosterone acting via the AR when evaluating the effect of E2, ER $\alpha^{-/-}$, ER α AF-2⁰, and ER α AF-1⁰ mice were orx and treated with either placebo or E2.

DXA measurements showed that E2-treated orx WT mice had a normal estrogenic response on the total body aBMD (Fig. 2). E2 treatment did not increase total body aBMD in the orx ER $\alpha^{-/-}$ or in the orx ER α AF-2⁰ mice. However, E2 treatment led to a significantly increased total body aBMD in the orx ER α AF-1⁰ mice, although the E2 response was of less magnitude compared to the E2 response in orx WT mice (Fig. 2).

Trabecular bone analyses of L₅ vertebrae, using μ CT, demonstrated a clear estrogenic response in trabecular bone in orx WT mice (Fig. 3). In contrast, there was no estrogenic response in orx ER $\alpha^{-/-}$, orx ER α AF-2⁰, or orx ER α AF-1⁰ mice. Cortical bone analyses of femur showed that E2 treatment increased the cortical thickness and vBMD in orx WT mice (Fig. 4A, B). The orx ER $\alpha^{-/-}$ and orx ER α AF-2⁰ mice showed no increase in cortical thickness or vBMD when treated with E2. In contrast, the orx ER α AF-1⁰ mice demonstrated a marked estrogenic response on both the cortical thickness and vBMD (Fig. 4A, B). This suggests that the E2 response in trabecular bone requires both ER α AF-1 and AF-2, whereas the E2 response in cortical bone requires ER α AF-2 but not AF-1.

Three-point bending tests demonstrated that the stiffness and the maximal load at failure increased by E2 treatment in the orx WT mice, but not in orx ER $\alpha^{-/-}$ or in orx ER α AF-2⁰ mice (Fig. 4C, D). Interestingly, the E2-treated orx ER α AF-1⁰ mice showed an increase in both stiffness and maximal load at failure, although the increase was not as pronounced as in the orx WT controls (Fig. 4C, D). Thus, the effect of E2 on bone strength requires ER α AF-2 but not AF-1.

Fig. 2. Role of ER α AF-1 and AF-2 for the effect of E2 on total body areal bone mineral density (aBMD) in orchidectomized (orx) male mice. Total body aBMD analyzed by DXA in orx ER $\alpha^{-/-}$, ER α AF-1⁰, and ER α AF-2⁰ mice and their corresponding orx wild-type (WT) mice after placebo or estradiol (E2) treatment for 4 weeks. KO = knockout. * $p < 0.05$ versus placebo-treated orx mice, Student's t test. Values are given as means $\pm$ SEM ($n = 8-11$).

Fig. 3. Role of ER α AF-1 and AF-2 for the effect of E2 on trabecular bone volume in orchidectomized (orx) male mice. Trabecular bone (ie, bone volume/tissue volume [BV/TV]) in L₅ vertebra was analyzed by μ CT in orx ER $\alpha^{-/-}$, ER α AF-1⁰, and ER α AF-2⁰ mice and their corresponding orx wild-type (WT) mice after placebo or estradiol (E2) treatment for 4 weeks. KO = knockout. * $p < 0.05$ versus placebo-treated orx mice, Student's t test. Values are given as means $\pm$ SEM ($n = 8-11$).

Role of ER α AF-1 is tissue-dependent

The immune system is known to be involved in the regulation of bone metabolism; therefore the role of ER α AF-1 and AF-2 for the E2 response in thymus and bone marrow was investigated. As expected, the thymus weight, bone marrow cellularity, and the frequency of B-lymphocytes in the bone marrow were decreased in E2-treated orx WT mice. No E2 response was seen for thymus weight or bone marrow parameters in the orx ER $\alpha^{-/-}$ or orx ER α AF-2⁰ mice (Table 1), showing that an intact ER α AF-2 is required for the effects of E2 on these parameters. Interestingly, the E2 response in the ER α AF-1⁰ mice varied between the evaluated parameters. Similarly, as seen for trabecular bone parameters (BV/TV: 8.1% $\pm$ 7.5% and trabecular number: 4.7% $\pm$ 7.3% of E2 response in WT mice; Fig. 5, Table 1), no significant E2 response was seen on thymus weight/body weight (5.7% $\pm$ 9.1% of E2 response in WT mice; Fig. 5, Table 1), and similar to the E2 response in cortical thickness and vBMD (32% $\pm$ 5.9% and 45% $\pm$ 6.3% of E2 response in WT mice; Fig. 5) a clear E2 response was seen for the bone marrow cellularity (33% $\pm$ 6.3% of E2 response in WT mice; Fig. 5, Table 1) and frequency of B-lymphocytes (54% $\pm$ 9.6% of E2 response in WT mice; Fig. 5, Table 1). This suggests that ER α AF-2 is required for all E2 effects evaluated while the role of ER α AF-1 is tissue-dependent (Fig. 5).

The effects of SERMs are dependent on ER α AF-1

Orx WT mice were treated with three different SERMs (Ral, Las, or Bza) or veh to evaluate their effects on bone and other estrogenic target tissues. These SERMs increased the total body aBMD and the trabecular vBMD to a similar extent compared to veh (Fig. 6A, B). All three SERMs appeared to increase cortical bone parameters compared to vehicle. However, after the conservative Bonferroni correction (three comparisons), only Las increased cortical thickness significantly (Ral: +5.6%, $p = 0.047$; Las: +9.9%, $p = 0.011$; and Bza: +8.1%, $p = 0.039$; Fig. 6C,

Fig. 4. Role of ERαAF-1 and AF-2 for the effect of E2 on cortical bone parameters and bone strength in orchidectomized (orx) male mice. orx ERα^{-/-}, ERαAF-1⁰, and ERαAF-2⁰ mice and their corresponding orx wild-type (WT) mice were treated with placebo or estradiol (E2) for 4 weeks. (A) Cortical thickness (Crt. Thk.) and (B) cortical volumetric bone mineral density (Crt. vBMD) were analyzed by pQCT. (C) Stiffness and (D) maximal load at failure (Max. Load) were analyzed by three-point bending. KO = knockout. **p* < 0.05 versus placebo-treated orx mice, Student's *t* test. Values are given as means ± SEM (*n* = 8–11).

Bonferroni correction requires *p* < 0.017 for significance) and only Bza increased bone strength significantly (maximal load at failure; Ral: +7.8%, *p* = 0.285; Las: +11.3%, *p* = 0.092; and Bza: +16.2%, *p* = 0.006; Fig. 6D). None of the SERMs reduced the thymus weight/body weight (veh: 2.6 ± 0.5 mg/g; Ral: 2.5 ± 0.1 mg/g; Las: 2.5 ± 0.1 mg/g; Bza: 2.6 ± 0.1 mg/g).

Our finding, that the role of ERαAF-1 for the effect of E2 is tissue-dependent in male mice, together with the fact that the effects of SERMs in vitro have been suggested to be mediated mainly via the ERαAF-1, led us to evaluate if the ERαAF-1 is required for different SERMs to exert their effects in vivo. Therefore, orx ERαAF-1⁰ mice were treated with Ral, Las, Bza, or veh. None of the treatments; Ral, Las, or Bza, increased total body aBMD, trabecular bone, cortical bone, or bone strength in the orx ERαAF-1⁰ mice (Fig. 6A–D). Therefore, the effects of these three SERMs are dependent on a functional ERαAF-1 in male mice.

Discussion

Estrogens are crucial for male bone health. When serum E2 levels are below a certain threshold in males, there are indications that SERM treatment could have positive effects on bone without

having feminizing effects.^(1–4,32,33) Hence, it is of importance to further characterize the signaling pathways of estrogen and SERMs in male bone, and other estrogenic target tissues, in order to facilitate the development of new bone-specific treatment strategies for male osteoporosis. Because the bone-sparing effects of estrogen in both men and male mice are primarily mediated via ERα,^(4,7) we have evaluated the roles of ERαAF-1 and ERαAF-2 in male mice by using mouse models with specific deletions of AF-1 or AF-2 in ERα. These specific deletions leave all other domains of ERα intact, which ensures that ligand can bind to the receptor and that the receptor can bind to the DNA.^(19,36,40–42) Therefore, the phenotypes of the ERαAF-1⁰ and ERαAF-2⁰ mice are due to the lack of the specific AF and not due to inability of the ERαAF-1⁰ and ERαAF-2⁰ proteins to bind the DNA or the ligand. Our main findings in this study are that the estrogenic effects on all evaluated parameters are dependent on ERαAF-2, whereas the role of ERαAF-1 for the estrogenic effects is tissue-specific, where the trabecular bone is dependent on ERαAF-1 but the cortical bone and bone strength do not require ERαAF-1. In contrast, all effects of the three evaluated SERMs require an intact ERαAF-1.

Sex steroids are important for skeletal growth and maintenance in both the female and male skeleton. The effects of

Table 1. The Effect of Estradiol on Thymus Weight, Trabecular Number, and Bone Marrow in Orx ER $\alpha^{-/-}$, ER α AF-2 0 , and ER α AF-1 0 Male Mice

	ER $\alpha^{-/-}$						ER α AF-2 0						ER α AF-1 0					
	WT			KO			WT			KO			WT			KO		
	Placebo	E2		Placebo	E2		Placebo	E2		Placebo	E2		Placebo	E2		Placebo	E2	
Thymus weight/BW (mg/g)	2.9 $\pm$ 0.1	2.2 $\pm$ 0.3*		3.6 $\pm$ 0.1	3.3 $\pm$ 0.1		3.5 $\pm$ 0.2	2.4 $\pm$ 0.4*		3.7 $\pm$ 0.2	3.8 $\pm$ 0.1**		3.0 $\pm$ 0.2	1.0 $\pm$ 0.1*		3.2 $\pm$ 0.2	3.1 $\pm$ 0.2**	
Trabecular number (1/mm)	4.2 $\pm$ 0.2	5.3 $\pm$ 0.4*		3.9 $\pm$ 0.3	4.1 $\pm$ 0.3		4.2 $\pm$ 0.2	5.3 $\pm$ 0.4*		4.5 $\pm$ 0.2	4.1 $\pm$ 0.2**		4.5 $\pm$ 0.1	8.3 $\pm$ 0.6*		4.0 $\pm$ 0.2	4.2 $\pm$ 0.2**	
Bone marrow (BM)																		
BM cellularity, 1 $\times$ 10 ⁶	22.9 $\pm$ 1.1	16.0 $\pm$ 2.7*		21.4 $\pm$ 1.6	20.4 $\pm$ 1.5**		23.8 $\pm$ 1.4	14.4 $\pm$ 2.3*		20.0 $\pm$ 1.0	19.8 $\pm$ 1.0**		21.4 $\pm$ 1.5	2.7 $\pm$ 1.4*		18.0 $\pm$ 2.3	12.8 $\pm$ 1.0**	
Frequency CD19+ in BM (%)	30.7 $\pm$ 3.7	18.3 $\pm$ 3.3*		21.7 $\pm$ 3.3	24.0 $\pm$ 2.3**		22.0 $\pm$ 2.9	17.6 $\pm$ 2.7		31.3 $\pm$ 3.4	32.7 $\pm$ 3.2		35.2 $\pm$ 3.2	15.6 $\pm$ 3.6*		37.9 $\pm$ 2.9	26.4 $\pm$ 2.0**	

Values are given as means $\pm$ SEM ($n = 8-11$).

WT = wild type; KO = knockout; Placebo = orchidectomized (orx) mice treated with placebo; E2 = orx mice treated with estradiol; BW = body weight; BM = bone marrow.

* $p < 0.05$ versus Placebo; ** $p < 0.05$ E2 effect in KO versus E2 effect in WT.

testosterone can be exerted directly through the AR or indirectly via aromatization to E2 and activation of ER α , but not ER β , in male mice.^(43,44) When deleting the ER α in male mice, the serum testosterone levels are elevated due to a disturbed negative feedback mechanism. These high testosterone levels have compensatory effects on the skeleton via activation of the AR.^(9,45) When evaluating the results for the intact, sham-operated, male mice it was clear that ER α was necessary to maintain cortical thickness, whereas the elevated testosterone levels could prevent the trabecular bone loss (BV/TV) in mice devoid of ER α .

To analyze the importance of different domains of ER α for the estrogenic effects in male mice without having confounding compensatory effects of elevated serum testosterone, ER $\alpha^{-/-}$, ER α AF-2 0 , ER α AF-1 0 , and control WT mice were orx and treated with either placebo or E2. As expected, E2 increased the total body aBMD in all orx WT control mice and this was due to an increase in both trabecular and cortical bone mass. In contrast, no effect on any of these bone parameters was seen in the orx ER $\alpha^{-/-}$ or in the orx ER α AF-2 0 mice. This demonstrates that ER α , is of importance in the male skeleton and that ER α AF-2 is crucial for the estrogenic effects in both trabecular and cortical bone. Interestingly, there was a significant effect of E2 on total body aBMD in the orx ER α AF-1 0 mice. Further analyses of the trabecular and cortical bone of these mice showed that the orx ER α AF-1 0 mice had no E2 response in the trabecular bone whereas they had a clear response to E2 in the cortical bone. These results are consistent with a previous study in female mice.⁽³⁶⁾ Because cortical bone comprises more than 80% of the skeleton and is increasingly recognized as a major structural determinant of bone strength,⁽¹⁾ we continued to analyze the effect of E2 on the biomechanical properties of the three male knockout mouse models. We concluded that all E2-treated orx WT mice showed an increase in bone strength. The increase in cortical bone mass in the E2-treated orx ER α AF-1 0 mice also led to an increased bone strength, whereas there was no E2 effect on these parameters in the orx ER $\alpha^{-/-}$ or orx ER α AF-2 0 mice. Therefore, ER α AF-1 is not required for an estrogenic effect on bone strength.

The immune system is known to be involved in the regulation of bone metabolism; therefore the role of ER α AF-2 and ER α AF-1 for the E2 response in bone marrow and thymus was investigated. The orx WT mice had a normal estrogenic response on bone marrow cellularity and thymus weight, whereas there was no E2 response on these parameters in the orx ER $\alpha^{-/-}$ or in the orx ER α AF-2 0 mice. The orx ER α AF-1 0 mice displayed an estrogenic response on the bone marrow cellularity and on the frequency of B-lymphocytes in the bone marrow, whereas they had no response on the thymus weight. This demonstrates that the E2 response on bone marrow cellularity and on the percentage of B-lymphocytes in the bone marrow in male mice is dependent on ER α AF-2 but not on AF-1, whereas the thymus weight is dependent on both ER α AF-2 and AF-1.

SERMs have been shown to increase BMD in men with low BMD^(32,33) and there are studies in orx mice and orx rats that have shown that both Ral and Las have an effect on male bone mass.^(46,47) Here, we have for the first time in orx male mice, evaluated the effects of the three SERMs; Ral, Las, and Bza in the

Fig. 5. The role of ER α AF-1 is tissue-dependent. Orchidectomized (orx) ER α AF-1⁰ mice and their corresponding orx wild-type (WT) mice were treated with placebo or estradiol (E2) for 4 weeks. As expected, E2 treatment resulted in a significant effect on several estrogen-responsive bone parameters (increased total body areal bone mineral density [aBMD], cortical thickness, cortical volumetric BMD [vBMD], trabecular bone volume/tissue volume [BV/TV], trabecular number [N]), bone marrow parameters (reduced bone marrow [BM] cellularity and frequency of B-lymphocytes), and non-bone parameters (reduced thymus weight) in orx WT mice. To illustrate the role of ER α AF-1 for the effect of E2 on these parameters, the estrogenic response in E2-treated orx WT mice, for each parameter, is set to 100%. The bars represent the estrogenic response in percent for the E2-treated orx ER α AF-1⁰ mice compared with the E2 response in orx WT mice. Thus, 0% means no E2 response whereas 100% is a normal WT E2 response. The results show that whereas some parameters are dependent on a functional ER α AF-1, many do not require ER α AF-1 for an estrogenic response. Values are means $\pm$ SEM ($n = 8-11$).

same study and evaluated their effects on several bone parameters and thymus weight. These three SERMs increased total body aBMD and trabecular bone mass in orx WT mice to a similar extent. Las-treated orx WT mice showed a significant increase in cortical thickness, whereas Ral- and Bza-treated mice had a tendency toward an increase. Bza-treated orx WT mice displayed significantly increased bone strength as analyzed by three-point bending, and there was a tendency toward an increase in the Ral- and Las-treated mice. Our results indicate that Las and Bza treatment increase cortical bone parameters more than Ral treatment. This is consistent with the fact that Las and Bza, but not Ral, have been shown to have significant effects on nonvertebral fractures.^(27,29-31)

Our study demonstrates that the estrogenic effects of ER α AF-1 are tissue-dependent in male mice, whereas the estrogenic effects of ER α AF-2 are crucial for all evaluated parameters. In addition, the three SERMs; Ral, Las, and Bza exert tissue-specific effects in orx WT mice. Taken together, these results led us to further evaluate the importance of ER α AF-1 for different SERMs to exert their effects in vivo, by evaluating the effects of Ral, Las, and Bza in orx ER α AF-1⁰ mice. Our results show that there are no agonistic effects of the SERMs when ER α AF-1 is deleted. This finding further strengthens the theory that it is the ER α AF-1 that mainly mediates the tissue-specific effects of the SERMs, and that the SERM interactions with ER β cannot replace ER α in the evaluated tissues in these male mice. When E2 binds to the ligand binding domain of ER α , helix 12 is folded in the agonistic orientation. This enables helices 3, 4, 5, and 12 to form the ER α AF-2 hydrophobic patch to which coactivators can bind, where the most important interaction site is found in helix 12.

Fig. 6. Tissue-specific effects of selective estrogen receptor modulators (SERMs) in wild-type (WT) mice are dependent on ER α AF-1. Orchidectomized (orx) WT and ER α AF-1⁰ mice were treated with vehicle (Veh), Raloxifene (Ral), Lasofoxifene (Las), or Bazedoxifene (Bza) for 3 weeks. (A) Total body areal bone mineral density (aBMD) was analyzed by DXA. Trabecular volumetric BMD (Trab. vBMD) (B) and cortical thickness (Crt. Thk.) (C) were analyzed by pQCT. (D) Maximal load at failure (Max. Load) was analyzed with a three-point bending test. * $p < 0.05$ versus corresponding Veh-treated orx mice, Student's t test Bonferroni corrected. Values are means $\pm$ SEM ($n = 6-10$).

However, when a SERM binds to the ER α , helix 12 in the ligand binding domain is prevented from folding in the agonistic orientation and the ER α AF-2 hydrophobic patch is not formed correctly. Instead, helix 12 is able to bind to the static region of AF-2; formed by residues from helices 3, 4, and 5. This leads to a limitation and/or loss of interaction between ER α AF-2 and its corepressors and coactivators.^(48–50) Consequently, when ER α AF-1 is deleted, the interaction of coactivators with any of the two AFs in ER α , after SERM activation, is greatly affected and little or no regulation of ER α -dependent gene transcription will occur in the evaluated tissues. Because there was no response in ER α AF-1⁰ mice, on bone parameters or thymus weight, after SERM treatment, we conclude that the ER α AF-1 probably is the most important mediator for the effects of the presently evaluated SERMs, but it is possible that some other region/regions of ER α are also involved. The lack of E2 response in the ER α AF-2⁰ mice suggests that ER α AF-1 is not able to activate the ER α AF-2⁰ protein. The ER α AF-2⁰ protein lacks the most important coactivator interaction site in the ER α AF-2 hydrophobic patch, found in helix 12. This suggests that it is not possible for coactivators to bind to the ER α AF-2⁰ protein, whereas corepressors are still able to interact with the static region of AF-2. Therefore, corepressor binding to the ER α AF-2⁰ protein could prevent the ER α AF-1 from activating ER α , thereby inhibiting estrogenic responses in the E2 treated ER α AF-2⁰ mice. In contrast, when a SERM binds to the full length ER α the binding of helix 12 to the static region of AF-2 prevents corepressor interaction.^(48–52)

In conclusion, ER α AF-2 is required for all the evaluated estrogenic effects in orx male mice, whereas the role of AF-1 is tissue-specific, where the trabecular bone is dependent on ER α AF-1 but the cortical bone and bone strength do not require ER α AF-1. In addition, the tissue-specific effects of Ral, Las, and Bza are dependent on ER α AF-1. These results further clarify the signaling pathways of E2 and SERMs via different domains of ER α in male mice, which suggest that it would be beneficial to develop a new class of SERMs that, in contrast to the SERMs presently available, would not activate the ER α AF-1. This SERM would have positive effects on the cortical bone and bone strength, while minimizing the adverse effects in other tissues. Importantly, cortical bone comprises more than 80% of the skeleton and is likely the major contributor to overall fracture risk. To screen for such new SERMs, the ER α AF-1⁰ mice will be a valuable model for evaluating the effects of the SERMs in vivo. The results from this study could facilitate the design of novel, bone-specific SERMs for male osteoporosis.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

This study was supported by the Swedish Research Council, COMBINE, an ALF/LUA research grant in Göteborg, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, the Novo Nordisk Foundation, the King Gustav V's 80 Years'

Foundation, the association against Rheumatism, the Åke Wiberg Foundation and the NIH/NIDDKD Grant DK071122.

Authors' roles: Study design: AEB, SHW, and CO. Data collection: AEB, HHF, CE, AKo, KS, JMK, AS, UI, HC, MCA, AKr, JT, MKL, and SHW. Data analysis: AEB, HHF, CE, AKo, PC, JT, and CO. Data interpretation: AEB, SHW, and CO. Drafting manuscript: AEB and CO. Approving final version of manuscript: All authors. AEB and CO take responsibility for the integrity of the data analysis.

References

- Khosla S, Melton LJ 3rd, Riggs BL. The unitary model for estrogen deficiency and the pathogenesis of osteoporosis: Is a revision needed? *J Bone Miner Res*. 2011;26:441–51.
- LeBlanc ES, Nielson CM, Marshall LM, Lapidus JA, Barrett-Connor E, Ensrud KE, Hoffman AR, Laughlin G, Ohlsson C, Orwoll ES. The effects of serum testosterone, estradiol, and sex hormone binding globulin levels on fracture risk in older men. *J Clin Endocrinol Metab*. 2009;94:3337–46.
- Mellström D, Vandenput L, Mallmin H, Holmberg AH, Lorentzon M, Oden A, Johansson H, Orwoll ES, Labrie F, Karlsson MK, Ljunggren O, Ohlsson C. Older men with low serum estradiol and high serum SHBG have an increased risk of fractures. *J Bone Miner Res*. 2008;23:1552–60.
- Vandenput L, Ohlsson C. Estrogens as regulators of bone health in men. *Nat Rev Endocrinol*. 2009;5:437–43.
- Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER. Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl J Med*. 1997;337:91–5.
- Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J Clin Endocrinol Metab*. 1995;80:3689–98.
- Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med*. 1994;331:1056–61.
- Lindberg MK, Moverare S, Skrtic S, Alatalo S, Halleen J, Mohan S, Gustafsson JA, Ohlsson C. Two different pathways for the maintenance of trabecular bone in adult male mice. *J Bone Miner Res*. 2002;17:555–62.
- Moverare S, Venken K, Eriksson AL, Andersson N, Skrtic S, Wergedal J, Mohan S, Salmon P, Bouillon R, Gustafsson JA, Vanderschueren D, Ohlsson C. Differential effects on bone of estrogen receptor alpha and androgen receptor activation in orchidectomized adult male mice. *Proc Natl Acad Sci U S A*. 2003;100:13573–8.
- Vandenput L, Ederveen AG, Erben RG, Stahr K, Swinnen JV, Van Herck E, Verstuyf A, Boonen S, Bouillon R, Vanderschueren D. Testosterone prevents orchidectomy-induced bone loss in estrogen receptor-alpha knockout mice. *Biochem Biophys Res Commun*. 2001;285:70–6.
- Venken K, De Gendt K, Boonen S, Ophoff J, Bouillon R, Swinnen JV, Verhoeven G, Vanderschueren D. Relative impact of androgen and estrogen receptor activation in the effects of androgens on trabecular and cortical bone in growing male mice: a study in the androgen receptor knockout mouse model. *J Bone Miner Res*. 2006;21:576–85.
- Vidal O, Lindberg MK, Hollberg K, Baylink DJ, Andersson G, Lubahn DB, Mohan S, Gustafsson JA, Ohlsson C. Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. *Proc Natl Acad Sci U S A*. 2000;97:5474–9.
- Windahl SH, Andersson N, Chagin AS, Mårtensson UE, Carlsten H, Olde B, Swanson C, Moverare-Skrtic S, Savendahl L, Lagerquist MK, Leeb-Lundberg LM, Ohlsson C. The role of the G protein-coupled

- receptor GPR30 in the effects of estrogen in ovariectomized mice. *Am J Physiol Endocrinol Metab.* 2009;296:E490–6.
14. Khosla S, Melton LJ 3rd, Atkinson EJ, O'Fallon WM. Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *J Clin Endocrinol Metab.* 2001;86:3555–61.
 15. Labrie F, Cusan L, Gomez JL, Martel C, Berube R, Belanger P, Belanger A, Vandenput L, Mellstrom D, Ohlsson C. Comparable amounts of sex steroids are made outside the gonads in men and women: strong lesson for hormone therapy of prostate and breast cancer. *J Steroid Biochem Mol Biol.* 2009;113:52–6.
 16. Mödder UI, Sanyal A, Xu J, O'Malley BW, Spelsberg TC, Khosla S. The skeletal response to estrogen is impaired in female but not in male steroid receptor coactivator (SRC)-1 knock out mice. *Bone.* 2008;42:414–21.
 17. Berry M, Metzger D, Chambon P. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO J.* 1990;9:2811–8.
 18. Metzger D, Losson R, Bornert JM, Lemoine Y, Chambon P. Promoter specificity of the two transcriptional activation functions of the human oestrogen receptor in yeast. *Nucleic Acids Res.* 1992;20:2813–7.
 19. Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P. The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell.* 1989;59:477–87.
 20. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev.* 1999;20:321–44.
 21. Kumar R, Thompson EB. The structure of the nuclear hormone receptors. *Steroids.* 1999;64:310–9.
 22. Metzger D, Ali S, Bornert JM, Chambon P. Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. *J Biol Chem.* 1995;270:9535–42.
 23. Norris JD, Fan D, Kerner SA, McDonnell DP. Identification of a third autonomous activation domain within the human estrogen receptor. *Mol Endocrinol.* 1997;11:747–54.
 24. Smith CL, Nawaz Z, O'Malley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol Endocrinol.* 1997;11:657–66.
 25. Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C, Brown M. Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science.* 1994;264:1455–8.
 26. Pike AC. Lessons learnt from structural studies of the oestrogen receptor. *Best Pract Res Clin Endocrinol Metab.* 2006;20:1–14.
 27. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA.* 1999;281:2189–97.
 28. Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, Draper M, Christiansen C. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. *N Engl J Med.* 1997;337:1641–7.
 29. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, Christiansen C, Delmas PD, Zanchetta JR, Stakkestad J, Gluer CC, Krueger K, Cohen FJ, Eckert S, Ensrud KE, Avioli LV, Lips P, Cummings SR. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *JAMA.* 1999;282:637–45.
 30. Silverman SL, Chines AA, Kendler DL, Kung AW, Teglbjaerg CS, Felsenberg D, Mairon N, Constantine GD, Adachi JD. Sustained efficacy and safety of bazedoxifene in preventing fractures in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled study. *Osteoporos Int.* 2012;23:351–63.
 31. Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, Goldstein S, Sriram U, Lee A, Thompson J, Armstrong RA, Thompson DD, Powles T, Zanchetta J, Kendler D, Neven P, Eastell R. Lasofoxifene in postmenopausal women with osteoporosis. *N Engl J Med.* 2010;362:686–96.
 32. Doran PM, Riggs BL, Atkinson EJ, Khosla S. Effects of raloxifene, a selective estrogen receptor modulator, on bone turnover markers and serum sex steroid and lipid levels in elderly men. *J Bone Miner Res.* 2001;16:2118–25.
 33. Smith MR, Fallon MA, Lee H, Finkelstein JS. Raloxifene to prevent gonadotropin-releasing hormone agonist-induced bone loss in men with prostate cancer: a randomized controlled trial. *J Clin Endocrinol Metab.* 2004;89:3841–6.
 34. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development.* 2000;127:4277–91.
 35. Billon-Gales A, Fontaine C, Filipe C, Douin-Echinard V, Fouque MJ, Flouriot G, Gourdy P, Lenfant F, Laurell H, Krust A, Chambon P, Arnal JF. The transactivating function 1 of estrogen receptor alpha is dispensable for the vasculoprotective actions of 17beta-estradiol. *Proc Natl Acad Sci U S A.* 2009;106:2053–8.
 36. Börjesson AE, Windahl SH, Lagerquist MK, Engdahl C, Frenkel B, Möverare-Skrtic S, Sjögren K, Kindblom JM, Stubelius A, Islander U, Antal MC, Krust A, Chambon P, Ohlsson C. Roles of transactivating functions 1 and 2 of estrogen receptor-alpha in bone. *Proc Natl Acad Sci U S A.* 2011;108:6288–93.
 37. Billon-Gales A, Krust A, Fontaine C, Abot A, Flouriot G, Toutain C, Berges H, Gadeau AP, Lenfant F, Gourdy P, Chambon P, Arnal JF. Activation function 2 (AF2) of estrogen receptor-alpha is required for the atheroprotective action of estradiol but not to accelerate endothelial healing. *Proc Natl Acad Sci U S A.* 2011;108:13311–6.
 38. Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C. Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta(–/–) mice. *J Clin Invest.* 1999;104:895–01.
 39. Waarsing JH, Day JS, Weinans H. An improved segmentation method for in vivo microCT imaging. *J Bone Miner Res.* 2004;19:1640–50.
 40. Kumar V, Chambon P. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell.* 1988;55:145–56.
 41. Lees JA, Fawell SE, Parker MG. Identification of two transactivation domains in the mouse oestrogen receptor. *Nucleic Acids Res.* 1989;17:5477–88.
 42. Tasset D, Tora L, Fromental C, Scheer E, Chambon P. Distinct classes of transcriptional activating domains function by different mechanisms. *Cell.* 1990;62:1177–87.
 43. Ohlsson C, Vandenput L. The role of estrogens for male bone health. *Eur J Endocrinol.* 2009;160:883–9.
 44. Riggs BL, Khosla S, Melton LJ, 3rd, Sex steroids and the construction and conservation of the adult skeleton. *Endocr Rev.* 2002;23:279–302.
 45. Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, Gaillard-Kelly M, Baron R. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. *Bone.* 2002;30:18–25.
 46. Broulik PD, Broulikova K. Raloxifene prevents bone loss in castrated male mice. *Physiol Res.* 2007;56:443–7.
 47. Ke HZ, Qi H, Crawford DT, Chidsey-Frink KL, Simmons HA, Thompson DD. Lasofoxifene (CP-336,156), a selective estrogen receptor modulator, prevents bone loss induced by aging and orchidectomy in the adult rat. *Endocrinology.* 2000;141:1338–44.

48. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*. 1997; 389:753–8.
49. Nettles KW, Greene GL. Ligand control of coregulator recruitment to nuclear receptors. *Annu Rev Physiol*. 2005;67:309–33.
50. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*. 1998; 95:927–37.
51. Danielian PS, White R, Lees JA, Parker MG. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J*. 1992;11:1025–33.
52. Webb P, Nguyen P, Kushner PJ. Differential SERM effects on corepressor binding dictate ERalpha activity in vivo. *J Biol Chem*. 2003;278:6912–20.