Supporting Information

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SI Materials and Methods

Generation of Mice. The generation of ERα-deficient mice ($ERα^{-/-}$) has previously been described (1). These mice have a deletion in exon 3 of the ERα gene and they do not express any of the isoforms of the ERα protein (1). The $ERα^{-/-}$ mice and WT control ($ERα^{+/+}$) littermates were inbred C57BL/6 mice and generated by breeding male $ERα^{+/-}$ with female $ERα^{+/-}$ mice. The generation of ERα AF-1–deficient ($ERαAF\text{-}1^0$) mice has previously been described (2). These mice have a deletion of 441 bp of exon 1, corresponding to aa 2 to 148, with a preserved translational initiation codon in exon 1 (ATG1; Fig. 1*A*). The $ERαAF\text{-}1^0$ mice do not express any full-length 66-kDa protein (2). Instead they express a truncated 49-kDa ERα protein that lacks AF-1 and also the physiologically occurring but less abundantly expressed 46-kDa ERα isoform initiated by a second translational initiation codon in exon 2 (ATG2; Fig. 1*A*). The $ERαAF\text{-}1^0$ mice and WT control ($ERαAF\text{-}1^{+/+}$) littermates were inbred C57BL/6 mice and generated by breeding male $ERαAF\text{-}1^{+/-}$ with female $ERαAF\text{-}1^{+/-}$ mice. The ERαAF-2–deficient ($ERαAF\text{-}2^0$) mice were generated through the strategy outlined in Fig. 1*A*. Briefly, $ERαAF\text{-}2^0$ mice have a deletion of the AF-2 core that resides within exon 8 and corresponds to aa 543 to 549 (Fig. 1*A*). Western blot analysis demonstrated that $ERαAF\text{-}2^0$ but not $ERα^{-/-}$ mice express proteins initiated from the initiation codon in exon 1 (ATG1) and the initiation codon in exon 2 (ATG2; Fig. 1*B*). The sizes of these proteins in $ERαAF\text{-}2^0$ mice are slightly smaller (corresponding to the 7-aa truncation located in the AF-2 region) than the WT ERα proteins of 66 kDa and 46 kDa, respectively. The $ERαAF\text{-}2^0$ mice and WT control ($ERαAF\text{-}2^{+/+}$) littermates were inbred C57BL/6 mice and generated by breeding male $ERαAF\text{-}2^{+/-}$ with female $ERαAF\text{-}2^{+/-}$ mice.

The following primer pairs were used for genotyping the mice. For genotyping the $ERαAF\text{-}1^0$ mice, primer pair 1 was used: P1:1 (5′-TGAAAGAACATTGAACCCGACACAAT-3′) and P1:2 (5′-GCCTTCTACAGGTACCCGCGCCACAT-3′). For genotyping the $ERαAF\text{-}2^0$ mice, primer pair 2 was used: P2:1 (5′-ATGAATTCTTAATAGGTTTAAAAAATGACT-3′) and P2:2 (5′-TGTGCTGAAGTGGAGCTGGT-3′). Primer pairs 3 and 4 were used to genotype the $ERα^{-/-}$ mice: P3:1 (5′-TTGCCCGATAACAATAACAT-3′) and P3:2 (5′-ATTGTCTCTTTCTGACAC-3′); as well as P4:1 (5′-GGCATTACCATTCTCCTGGGAGTCT-3′) and P4:2 (5′-TCGCTTTCCTGAAGACCTTTCATAT-3′) (1).

Western Blot. Western Blot and protein preparation from uteri from $ERα^{-/-}$ and $ERαAF\text{-}2^0$ mice were essentially performed as described previously (3). The uteri were homogenized in lysis buffer and a mixture of protease inhibitors (complete Mini EDTA-free; Roche Diagnostics). The rabbit polyclonal ERα antibody (MC-20) and mouse monoclonal actin antibody (C-2; Santa Cruz Biotechnology), both diluted 1:500, were used. HRP-linked secondary antibodies, anti-rabbit and anti-mouse, respectively (GE Healthcare), were diluted 1:30,000 and used to visualize the bands.

pQCT. CT scans were performed with the pQCT XCT RESEARCH M (version 4.5B; Norland), operating at a resolution of 70 as described previously (4). The scan was positioned in the metaphysis of the femur at a distance proximal from the distal growth plate corresponding to 3.4% of the total length of the femur, and the trabecular bone region was defined as the inner 45% of the total cross-sectional area. Cortical bone parameters were analyzed in the middiaphyseal region of the femur (5).

Micro-CT. Micro-CT analyses were performed on the distal femur and lumbar vertebra (L5) by using a model 1072 scanner (Skyscan), imaged with an X-ray tube voltage of 100 kV and current of 98 μA, with a 1-mm aluminum filter (6). The scanning angular rotation was 180° and the angular increment 0.90°. The voxel size was 6.51 μm isotropically. Datasets were reconstructed by using a modified Feldkamp algorithm and segmented into binary images by using adaptive local thresholding (7). In the femur, the trabecular bone proximal to the distal growth plate was selected for analyses within a conforming volume of interest (cortical bone excluded) commencing at a distance of 338.5 μm from the growth plate, and extending a further longitudinal distance of 488 μm in the proximal direction. Cortical measurements were performed in the diaphyseal region of femur starting at a distance of 5.2 mm from the growth plate and extending a further longitudinal distance of 163 μm in the proximal direction. In the vertebra, the trabecular bone in the vertebral body caudal of the pedicles was selected for analyses within a conforming volume of interest (cortical bone excluded) commencing at a distance of 6.5 μm caudal of the lower end of the pedicles, and extending a further longitudinal distance of 325.5 μm in the caudal direction. Trabecular thickness and separation were calculated by the sphere-fitting local thickness method (8).

Histomorphometric Analyses. ***Trabecular bone.*** L4 vertebrae were fixed in 4% paraformaldehyde, dehydrated in 70% EtOH, and embedded in plastic (L R White Resin; Agar Scientific). The trabecular bone was analyzed by using longitudinal coronary sections of the vertebrae. Sections 4 μm thick were stained with Masson–Goldner trichrome (9, 10). The parameters were measured by using the OsteoMeasure histomorphometry analysis system with software version 2.2 (OsteoMetrics) and following the guidelines of the American Society for Bone and Mineral Research (11).

Cortical bone. Femurs were fixed in Bürckhardt fixative, dehydrated in 70% EtOH, and embedded in plastic (L R White Resin; Agar Scientific). For the measurement of dynamic parameters, the mice were double-labeled with calcein, which was injected (i.p.) into the mice 1 and 8 d before termination. Histomorphometric analyses of cortical bone were done using 20-μm-thick transverse cross-sectional sections in the middiaphyseal region of femur.

Quantitative Real-Time PCR Analysis. Total RNA from whole humerus was prepared using TriZol Reagent (Life Technologies). The RNA was reverse-transcribed into cDNA, and real-time PCR analysis was performed using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems). We used predesigned real-time PCR assays from Applied Biosystems for analysis of OPG (Mm00435452_m1), LIFR (Mm00442940_m1), and IL-1ra (probe, CAG CGC TGT GTC AAT GCG GAG G; forward primer, GAA GCT CAG TGC CGC CA; reverse primer, TTC ATG TGG TGG TCC AGC TTT). The mRNA abundance of each gene was calculated using the standard curve method (User Bulletin 2; PE Applied Biosystems) and adjusted for the expression of 18S.

Bone Marrow and Thymus Cellularity and Cell Distribution. Bone marrow cells were harvested by flushing 5 mL PBS solution through the bone cavity of one femur and one humerus by using a syringe. After centrifugation at 515 × *g* for 5 min, pelleted cells

were resuspended in Tris-buffered 0.83% NH_4Cl solution (pH 7.29) for 5 min to lyse erythrocytes and then washed in PBS solution. Bone marrow cells were resuspended in RPMI culture medium (PAA Laboratories) before use. The total number of leukocytes in bone marrow was calculated using an automated cell counter (Sysmex). For flow cytometry analyses, cells were stained with phycoerythrin-conjugated antibodies to CD19 for detection of B lymphocytes. The cells were then subjected to FACS analysis on a FACSCalibur device (BD Pharmingen) and analyzed by using FlowJo software. Results are expressed as cell frequency (in percentages).

Enzyme-Linked Immunosorbent Spot Assay. Enumeration of IgM-, IgG-, and IgA-secreting bone marrow cells was performed using the enzyme-linked immunosorbent spot technique (12). Briefly, 96-well nitrocellulose plates (Millipore) were coated with affinity-purified $F(ab')_2$ fragments of goat anti-mouse IgM, IgG, and IgA (5 μg/mL diluted in PBS solution; Cappel; Oragon Teknika). After incubation overnight at 4 °C and blocking with 5% FCS, 50,000 freshly isolated bone marrow cells, diluted in Iscove culture medium, were added in triplicate. The plates were incubated for 3.5 h at 37 °C in 5% CO_2 and 85% humidity and thereafter rinsed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgM, IgG, and IgA antibodies (diluted 1:750 in PBS solution) at 4 °C overnight. After rinsing, the wells were incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium for 1 h for visualization of the spots. The number of Ig-secreting cells was expressed as the frequency of spot-forming cells per 10^3 $CD19^+$ cells.

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Table S1. Effect of estradiol on trabecular and cortical bone parameters in OVX ERα$^{-/-}$, ERαAF-2^0 and ERαAF-1^0 mice

	ERα$^{-/-}$				ERαAF-2^0				ERαAF-1^0			
	WT		KO		WT		KO		WT		KO	
Parameter	OVX	E2	OVX	E2	OVX	E2	OVX	E2	OVX	E2	OVX	E2
Trabecular bone parameters (μCT)												
Trabecular number (1/mm)	4.78 ± 0.15	7.25 ± 0.37*	4.88 ± 0.26	4.61 ± 0.29†	3.59 ± 0.16	6.56 ± 0.22*	3.74 ± 0.40	3.57 ± 0.15†	3.05 ± 0.19	6.01 ± 0.22*	3.42 ± 0.22	3.22 ± 0.31†
Trabecular thickness (μm)	51 ± 1	59 ± 2*	50 ± 1	48 ± 1†	46 ± 1	50 ± 1*	44 ± 1	43 ± 1†	45 ± 1	48 ± 1	46 ± 2	45 ± 1
Cortical bone parameters (pQCT)												
Cortical content (mg/mm)	0.91 ± 0.02	1.21 ± 0.03*	0.87 ± 0.03	0.86 ± 0.04†	0.85 ± 0.01	1.10 ± 0.03*	0.86 ± 0.03	0.82 ± 0.02†	0.88 ± 0.02	1.17 ± 0.04*	0.90 ± 0.03	1.11 ± 0.04*
Cortical area (mm^2)	0.78 ± 0.02	0.97 ± 0.02*	0.75 ± 0.02	0.75 ± 0.03†	0.75 ± 0.01	0.92 ± 0.02*	0.76 ± 0.02	0.73 ± 0.02†	0.75 ± 0.01	0.92 ± 0.03*	0.77 ± 0.02	0.89 ± 0.03*
Cortical vBMD (mg/cm^3)	1,167 ± 6	1,249 ± 8*	1,159 ± 8	1,153 ± 11†	1,145 ± 8	1,199 ± 8*	1,126 ± 10	1,115 ± 13†	1,179 ± 7	1,265 ± 5*	1,174 ± 8	1,248 ± 6*
Endosteal circumference (mm)	3.72 ± 0.05	3.45 ± 0.05*	3.57 ± 0.05	3.57 ± 0.05†	3.78 ± 0.06	3.61 ± 0.06*	3.91 ± 0.05	3.84 ± 0.04	3.50 ± 0.05	3.20 ± 0.07*	3.49 ± 0.04	3.23 ± 0.04*
Periosteal circumference (mm)	4.87 ± 0.05	4.91 ± 0.05	4.70 ± 0.06	4.70 ± 0.07	4.87 ± 0.05	4.95 ± 0.05	4.98 ± 0.05	4.90 ± 0.04	4.65 ± 0.04	4.67 ± 0.06	4.67 ± 0.05	4.65 ± 0.06

E2, estradiol treatment of OVX. Values are given as means ± SEM.

*$P < 0.05$ vs. OVX and $^\dagger P < 0.05$, E2 effect in KO vs. E2 effect in WT (n = 8–12).

Table S2. Histomorphometry in ERαAF-1^0 mice

	WT		KO	
Parameter	OVX	E2	OVX	E2
Trabecular bone (L4 vertebrae)				
BV/TV (%)	13.0 ± 1.3	25.3 ± 1.6*	12.4 ± 1.0	11.2 ± 1.0†
Trabecular number (1/mm)	3.5 ± 0.2	6.7 ± 0.4*	3.5 ± 0.2	3.3 ± 0.2†
Trabecular thickness (μm)	36.0 ± 1.6	37.1 ± 1.1	34.2 ± 1.3	32.3 ± 1.4
N.Oc/B.Pm (1/mm)	2.13 ± 0.19	1.84 ± 0.12	2.18 ± 0.10	2.44 ± 0.16‡
Oc.S/BS (%)	5.64 ± 0.52	5.41 ± 0.40	5.90 ± 0.33	6.72 ± 0.38‡
Cortical bone (femur diaphysis)				
Periosteal circumference (mm)	4.36 ± 0.03	4.36 ± 0.06	4.33 ± 0.06	4.33 ± 0.06
Endosteal circumference (mm)	3.22 ± 0.03	2.85 ± 0.06*	3.17 ± 0.04	2.95 ± 0.04*
MS/BS endosteal (%)	56.6 ± 2.0	93.9 ± 2.1*	59.1 ± 1.8	85.7 ± 4.3*
BFR/BS endosteal ($\mu m^2/\mu m/d$)	0.66 ± 0.06	1.75 ± 0.14*	0.67 ± 0.04	1.19 ± 0.19*
MAR endosteal (μm/d)	1.16 ± 0.10	1.85 ± 0.12*	1.13 ± 0.06	1.36 ± 0.17

BFR/BS, bone formation rate/bone surface; E2, estradiol treatment of OVX; MAR, mineral apposition rate; MS/BS, mineralized surface/BS; N.Oc/B.Pm, number of osteoclasts/bone perimeter; Oc.S/BS, osteoclast surface/BS. Values are given as means ± SEM.

*$P < 0.05$ vs. OVX and $^{\dagger}P < 0.05$, E2 effect in KO vs. E2 effect in WT.

‡Significant vs. WT E2 (n = 9–11).

Table S3. Effect of estradiol on thymus, liver, and bone marrow in OVX ERα$^{-/-}$, ERαAF-2^0, and ERαAF-1^0 mice

	ERα$^{-/-}$				ERαAF-2^0				ERαAF-1^0			
	WT		KO		WT		KO		WT		KO	
Parameter	OVX	E2	OVX	E2	OVX	E2	OVX	E2	OVX	E2	OVX	E2
Thymus weight/BW, mg/g	3.05 ± 0.13	1.29 ± 0.23*	2.89 ± 0.18	2.98 ± 0.12†	2.89 ± 0.08	0.87 ± 0.12*	3.02 ± 0.26	2.90 ± 0.17†	2.99 ± 0.20	0.81 ± 0.18*	3.00 ± 0.15	2.72 ± 0.16†
Liver weight/BW, mg/g	42.4 ± 0.7	49.4 ± 0.6*	41.6 ± 1.4	41.5 ± 2.0	44.0 ± 1.0	50.3 ± 1.1*	39.7 ± 0.8	39.1 ± 1.4†	39.6 ± 1.2	45.8 ± 0.4*	36.0 ± 1.1	42.1 ± 1.6*
Bone marrow												
Cellularity, 10^6	19.8 ± 1.7	10.0 ± 1.6*	19.1 ± 1.9	19.2 ± 2.2†	22.3 ± 1.8	8.2 ± 1.9*	21.9 ± 1.9	20.8 ± 1.9†	29.4 ± 1.2	6.8 ± 1.5*	29.5 ± 1.9	22.7 ± 2.1*,†
B-lymphocytes, %	33.0 ± 2.5	22.1 ± 1.2*	34.2 ± 2.5	35.3 ± 2.2†	27.1 ± 3.2	12.9 ± 2.2*	29.3 ± 3.4	28.2 ± 3.2†	38.9 ± 1.2	16.1 ± 1.7*	38.3 ± 0.9	25.6 ± 1.6*,†
Ig secretion												
IgG, per 10^3 CD19$^+$ cells	NA	NA	NA	NA	1.76 ± 0.99	18.43 ± 1.12*	1.76 ± 0.64	1.97 ± 0.82†	0.91 ± 0.11	3.68 ± 0.90*	0.78 ± 0.15	1.80 ± 0.29*
IgM, per 10^3 CD19$^+$ cells	NA	NA	NA	NA	1.23 ± 0.22	15.05 ± 5.52*	1.42 ± 0.48	1.66 ± 0.75†	1.52 ± 0.29	5.14 ± 1.98*	1.17 ± 0.19	2.40 ± 0.36*
IgA, per 10^3 CD19$^+$ cells	NA	NA	NA	NA	2.10 ± 0.45	12.88 ± 5.17*	2.27 ± 0.46	1.99 ± 0.30†	1.84 ± 0.23	7.43 ± 0.72*	1.68 ± 0.12	3.73 ± 0.63*,†

BW, body weight; E2, estradiol treatment of OVX; NA, not available. Ig secretion from plasma B-cells from bone marrow is given per 10^3 CD19$^+$ cells. Values are given as means ± SEM.
*$P < 0.05$ vs. OVX, $^{\dagger}P < 0.05$, E2 effect in KO vs. E2 effect in WT (n = 8–12).