Supporting Information

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SI Materials and Methods

Animals. Genotyping of Nestin-ERα$^{-/-}$ mice on tail-biopsy DNA was performed by PCR using Cre primers P1 (5′-CCAGGCTAAGTGCCTTCTCTACA-3′) and P2 (5′-AATGCTTCTGTCCGTTTGCCGGT-3′), and estrogen receptor (ER)-αflox primers P3 (5′-TTGCCCGATAACAATAAC AT-3′) and P4 (5′-ATTGTCTCTTTCTGACAC-3′).

To exclude the possibility of ERα-independent nestin-Cre phenotypic effects, Cre-positive mice (Cre$^{+/-}$ERα$^{flox/+}$) were compared with Cre-negative control mice (ERα$^{flox/flox}$). Deletion of one ERα allele has previously been shown to result in ERα protein expression corresponding to levels in WT mice (1) and the bone phenotype in mice with one ERα allele is not different from that of WT mice (2). The results reported in this study were Cre-independent because no difference was detected between Cre$^{+/-}$ERα$^{flox/+}$ mice and control mice.

In the experiment terminated at 9 wk of age, one individual in the nestin-ERα$^{-/-}$ group was excluded because of unchanged hypothalamic ERα expression, implicating impaired Cre function or failure in genotyping.

Measurement of Central Serotonin Levels. Frozen brain tissue (−80 °C) from the cortex and hippocampus was homogenized with ultrasound (Sonifier Cell Disruptor B-30; Branson Sonic Power Co.) in 0.1 M perchloricacid with 10.75 mM EDTA·2Na·2H_2O and 0.65 mM glutathione, kept on ice and subsequently centrifuged at 14,000 × *g* for 15 min at 4 °C. The supernatant was diluted 1:50 in homogenization buffer and analyzed on the same day on a HPLC with electrochemical detection (HPLC-ED). Running buffer consisted of 20% methanol, 69.2 mM citric acid, 146 mM NaOH, 0.1 mM EDTA·2Na·2H_2O. Twenty-five microliters of the sample was injected (CMA/200 sample injector; CMA Microdialysis) and separated with a ion-exchange column (Nucleosil SA 5μ 100A 150 × 2 mm; Phenomenex) followed by amperometric detection (Waters 460; Millipore Waters) of serotonin content. Chromatograms were analyzed with Dionex Chromeleon software (Dionex).

Gene Expression Analyses. Total RNA from tibia, femur, hypothalamus, white adipose tissue, brown adipose tissue, spleen, uterus, liver, and striated muscle was prepared using TriZol Reagent (Invitrogen). The RNA was reverse transcribed into cDNA and real time (RT)-PCR analysis was performed using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems). We used predesigned RT-PCR assays from Applied Biosystems for analysis of ERα (Mm00433147_m1), ERβ (Mm00599819_m1), Leptin (Mm00434759_m1), Dentin matrix acidic phosphoprotein 1 (Mm01208363_m1), Bone sialoprotein (Mm00492555_m1), cathepsin K (Mm00484036_m1), receptor activator of NFκ-B ligand (RANKL, Mm00441908_m1), tartrate-resistant acid phosphatase 5b (Mm00475698_m1), osteoprotegerin (Mm00435452_m1), serotonin receptor 1b (Htr1b, Mm00439377_s1), uncoupling protein 1 (Mm00494069_m1), IL-6 (Mm00446190_m1), leptin receptor (ObRb, Mm00440181_m1), protein tyrosine phosphatase 1b (Mm00448427_m1), suppressor of cytokine signaling-3 (Mm00545913_s1), and class II MHC transactivator (Mm01342721_m1). 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. Cre expression was analyzed using SYBRgreen reagents (Applied Biosystems) and the following primer pair: P1 (5′-GTG TAG AGA AGG CAC TTA GC-3′) and P2 (5′-CTG ACC AGA GTC ATC CTT AG-3′). Simultaneous analysis of endogenous 18S expression was used for adjustment.

MicroCT. μCT analyses were performed on the distal femur, proximal tibia, and lumbar vertebra (L_5) by using Skyscan 1072 scanner (Skyscan), imaged with an X-ray tube voltage of 100 kV and current 98 μA, with a 1-mm aluminum filter (3). The scanning angular rotation was 180° and the angular increment 0.90°. 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 (4). In long bones, the trabecular bone proximal of the distal growth plate (femur) or distal of the proximal growth plate (tibia) 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. 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 (5).

Histomorphometry. For static histomorphometric analyses, femurs were fixed in 4% formaldehyde, dehydrated, and embedded in methylmethacrylate (Technovit 9100 New; Heraeus Kulzer). Four-micrometer sections were stained with Ladewig staining. Images were captured using a Nikon Eclipse 80i light microscope connected to a Sony DXC-S500 video camera using the Osteomeasure software (OsteoMetrics).

For dynamic histomorphometric analyses, the mice were injected (intraperitoneally) with calcein (30 mg/kg), 8 and 1 d before termination of the experiment. Tibias were fixed in 4% paraformaldehyde, dehydrated in 70% EtOH, and embedded in plastic. The trabecular bone was analyzed using longitudinal plastic sections obtained from three standardized sites of marrow cavity with the site distance of 100 μm. These three sites were named as site A, site B, and site C. In each site, the trabecular bone was analyzed in one field. Eight-micrometer sections were stained with Masson-Goldner Trichrome staining (6). The parameters were measured using OsteoMeasure histomorphometry analysis system with software version 2.2 (Osteometrics) and following the guidelines of the American Society for Bone and Mineral Research (7).

Flow Cytometry. Bone-marrow cells were harvested by flushing 5 mL PBS through the bone cavity of one femur 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. 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 FITC-conjugated antibodies to CD3 for detection of T lymphocytes. The cells were then subjected to FACS on a FACSCalibur (BD Pharmingen) and analyzed using FlowJo software. Results are expressed as cell frequency (%).

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7. Parfitt AM, et al.; Report of the ASBMR Histomorphometry Nomenclature Committee (1987) Bone histomorphometry: Standardization of nomenclature, symbols, and units. *J Bone Miner Res* 2:595–610.

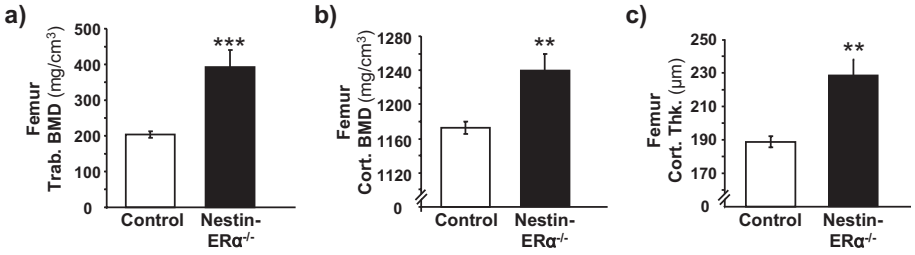

Fig. S1. Peripheral quantitative computerized tomography (pQCT) analysis of femur in nestin-ERα$^{-/-}$ and control female mice. Volumetric bone mineral density (BMD) in the (*A*) distal metaphyseal trabecular (Trab.) and (*B*) diaphyseal cortical (Cort.) bone compartments of femur. (*C*) Cortical thickness (Cort. Thk.) in the diaphyseal region of femur. Values are given as mean ± SEM, (n = 5–8). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control, Student's t test.

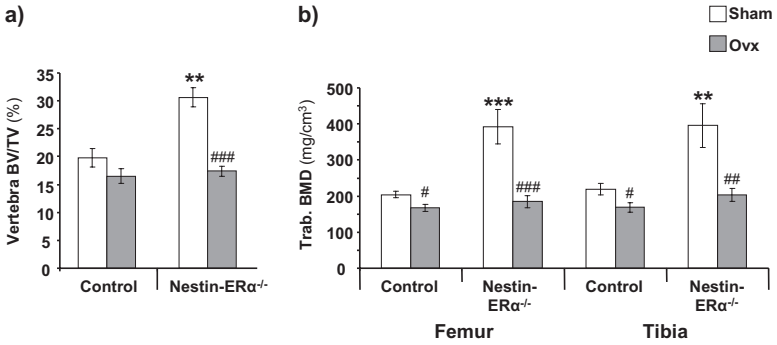

Fig. S2. Effect of ovariectomy on trabecular bone in nestin-ERα$^{-/-}$ females and control mice. Three-month-old female nestin-ERα$^{-/-}$ females and control mice were ovariectomized (Ovx) or sham-operated (Sham) and terminated after 4 wk. (*A*) μCT analysis of vertebrae, bone volume per total volume (BV/TV), and (*B*) pQCT analysis of trabecular BMD in distal femur and proximal tibia. Values are given as mean ± SEM, (n = 6). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. sham, Student's t test.

Table S1. Expression of ERβ in relevant tissues

Tissue	Control	Nestin-ERα$^{-/-}$
Bone (femur)	2.19 ± 0.67	1.36 ± 0.16
Brain	5.64 ± 0.67	3.64 ± 1.43
Spleen	1.28 ± 0.63	1.48 ± 0.35
Uterus	8.15 ± 1.64	7.78 ± 0.59
Liver	0.74 ± 0.20	1.08 ± 0.13
Muscle	1.97 ± 0.90	0.57 ± 0.13

RNA was prepared from various tissues and expression of ERβ was determined using real-time PCR. Gene expression was adjusted for the expression of 18S. Values are given as arbitrary units (mean ± SEM) (n = 3–10), Student's t test.

Table S2. Body and organ weights

Body and organ	Age (wk)	Control	Nestin-ERα$^{-/-}$
Body weight (g)	3	12.6 ± 0.5	10.9 ± 0.7
	6	19.3 ± 0.7	19.4 ± 0.4
	9	21.0 ± 0.7	21.2 ± 0.5
	12	22.3 ± 0.6	22.5 ± 0.5
Gonadal fat (mg)	9	236 ± 25	301 ± 31
Retroperitoneal fat (mg)	9	32.7 ± 4.6	51.0 ± 3.5*
Thymus (mg)	12	51.4 ± 1.8	56.9 ± 1.9

Body weights were estimated at 3, 6, 9, and 12 wk of age. Fat content (gonadal and retroperitoneal fat) and thymus weight were determined at termination at 9 or 12 wk of age. Values are given as mean ± SEM (n = 5–7), *P < 0.05, Student's t test vs. control.

Table S3. Analysis of serum FSH levels, serum markers of bone remodeling, and osteoblast/osteoclast-associated transcripts

Analyses	Serum/marker	Control	Nestin-ERα$^{-/-}$
Serum analyses	FSH (ng/mL)	6.8 ± 3.2	4.0 ± 0.3
	RatLaps (ng/mL)	21.2 ± 1.9	17.1 ± 1.5
	Osteocalcin (ng/mL)	277 ± 21	257 ± 20
Real-time PC (arbitrary units)	Cathepsin K	0.58 ± 0.08	0.88 ± 0.14
	RANKL	0.96 ± 0.26	1.05 ± 0.23
	TRAP5b	0.72 ± 0.09	1.07 ± 0.12
	OPG	0.67 ± 0.13	0.80 ± 0.10
	BSP	1.06 ± 0.12	2.03 ± 0.54*
	DMP-1	0.45 ± 0.07	0.84 ± 0.24**
	Serotonin receptor 1b	0.91 ± 0.3	0.99 ± 0.3
	LepR	0.29 ± 0.08	0.40 ± 0.06

Serum was collected from 3- to 4-mo-old nestin-ERα$^{-/-}$ and control female mice. The serum levels of C-terminal telopeptides of collagen type 1 were determined using RatLaps assay to assess bone resorption. Serum levels of osteocalcin were determined to assess bone formation. RNA was prepared from bone and expression of bone-related genes was determined using real-time PCR. Gene expression for each gene was adjusted for the expression of 18S. Values are given as mean ± SEM, (n = 4–10), *P < 0.05, **P = 0.06, Student's t test vs. control. BSP, bone sialoprotein; DMP-1, dentin matrix acidic phosphoprotein 1; FSH, follicle-stimulating hormone; LepR, Leptin receptor (ObRb); OPG, osteoprotegerin; RANKL, receptor activator of NFκB; TRAP5b, Tartrate resistant acid phosphatase isoform 5b.

Table S4. Static and dynamic histomorphometric analysis of long bones

Analyses	Control	Nestin-ERα$^{-/-}$
BV/TV (%)	16.1 ± 1.3	25.0 ± 3.2*
Trabecular thickness (μm)	42.3 ± 2.8	56.0 ± 4.8*
Trabecular number (1/mm)	3.7 ± 0.3	4.5 ± 0.5
BFR/BS ($\mu m^3 \cdot \mu m^2 \cdot y$)	140 ± 5	174 ± 27**
N.Oc/BPm (number/mm)	5.2 ± 0.8	6.4 ± 0.5

Static (performed on femur) and dynamic (performed on tibia) histomorphometric analyses on 3-mo-old nestin-ERα$^{-/-}$ and control females. For dynamic analyses, mice were injected with calcein 8 and 1 d before termination. Values are given as mean ± SEM, (n = 4–9), *P < 0.05, **P = 0.07, Student's t test vs. control. BFR/BV, bone formation rate/bone volume; BFR/BS; BFR/bone surface; BV/TV, bone volume/total volume; N.Oc/BPm, number of osteoclasts/bone perimeter.