

Supporting Information

Allan et al. 10.1073/pnas.10121411108

SI Materials and Methods

Transgenic Follicle-Stimulating Hormone (FSH) Mice and Experimental Groups. Two lines expressing human FSH (TgFSH) under the control of rat insulin II gene promoter were previously described (1, 2). The $\alpha\beta.6$ line (designated TgFSH^m) has serum TgFSH levels up to ~10 IU/L and normal serum luteinizing hormone (LH) and estradiol levels at diestrus versus age-matched non-Tg females (3). The 113 line (designated TgFSH^H) has higher serum TgFSH rising to ~41 IU/L (2) (Fig. S1). Serum levels of TgFSH progressively rise with age in female mice (3). Both TgFSH lines were maintained on the mouse strain (originally C3H/101He) carrying the hypogonadal (*hpg*) mutation (2), housed at the ANZAC Research Institute under controlled conditions (12-h light/dark cycle, 19–22 °C) with ad libitum access to food and water. Groups comprised mature TgFSH^m and TgFSH^H females having either non-*hpg* or *hpg* backgrounds and non-*hpg* or *hpg* littermate and age-matched non-Tg female controls (Table S1). Non-Tg strain-matched females from the TgFSH^m or TgFSH^H lines exhibited equivalent phenotypes so were pooled to represent one non-Tg/non-*hpg* (WT) group and one non-Tg/*hpg* control group. For dynamic histomorphometry, females in non-*hpg* groups were collected at 8.2–17.4 mo old and in the *hpg* groups were collected at 7.8–18.7 mo old.

Serum and Tissue Collection and Preparation. Females were weighed and anesthetized, and blood was collected by cardiac puncture for serum (stored at –20 °C), then uterus and bone samples were dissected. The ovary and humerus were snap-frozen in liquid nitrogen for RNA isolation. Total RNA was prepared from ovary or long bone with TRI reagent (Sigma) or TRIzol (Invitrogen), respectively. From each mouse, both tibiae and the lumbar vertebra (L2, L3, and L4) were fixed in 4% paraformaldehyde buffered with 0.1 mol/L phosphate buffer (pH 7.4) for 48 h at 4 °C. Tibial length was measured by caliper. After fixation, one tibia was decalcified with 10% EDTA, embedded in paraffin, and sectioned (5 μ m) for static histomorphometry. The contralateral tibia was stored in 70% ethanol, then the proximal end of the tibiae was embedded in methyl methacrylate and sagittal 7- μ m sections were cut from close to the midline of the proximal metaphysis. These sections were stained for 3 min in 1% xylene orange in 0.5 M Tris buffer (pH 9) to fluorescently label bone.

Bone Histomorphometry. Paraffin sections of the proximal tibial metaphysis were stained with hematoxylin/eosin or Safranin-O for general histological evaluation. To identify osteoclasts, sections were stained for tartrate-resistant acid phosphatase (TRAP) using naphthol AS-BI phosphate (Sigma) as a substrate and fast red violet Luria–Bertani salt (Sigma) as stain for the reaction product. Measurements of static indices were performed on stained 5- μ m longitudinal sections using the Bio-Quant Osteo II System (Bio-Quant). The regions of interest were a 1.5 \times 1-mm area located 0.3 mm below the growth plate of the tibia. The area of the metaphyseal bone defined for dynamic histomorphometry comprised a rectangle positioned 20 μ m below the cartilage of the proximal tibial growth plate and extending 800 μ m distally, fitting within the medullary cavity. Calcein fluorescence indicated active mineralization, with single and double

calcein labels analyzed with Bio-Quant Osteo bone histomorphometry software (Bio-Quant).

Microcomputed Tomography. Tibiae and lumbar vertebrae (L3) were analyzed by μ CT using a Skyscan 1172 scanner (Skyscan) at 60 kV and 167 μ A, with no filter, and exposure was set to 1,180 ms. In total, 1,028 projections were collected at a resolution of 7.7 μ m/pixel. Reconstruction of sections was carried out with scanner software (Nrecon), and beam hardening correction was set to 50%. VGStudio MAX 1.2 software (Volume Graphics) was used to obtain 3D visualization from reconstructed sections. CTAnalyser software (version 1.8, Skyscan) was used for morphometric quantification of trabecular and cortical bone indices. Transverse sections of tibial cortical bone were analyzed at a level 20% by length below the proximal end of the tibia, corresponding to ~3 mm down from the distal surface of proximal growth plate. Parameters of tibial trabecular bone were measured in a defined interendosteal area located 0.5–1.5 mm below the growth plate. The entire volume of trabecular bone within the vertebral body was selected for analysis of vertebral trabecular bone indices.

Serum Hormones and Bone Markers. Serum hormone levels were measured in duplicate in a single assay for each analyte. Serum levels of transgenic TgFSH were determined using a species-specific, two-site immunofluorometric assay (2, 4) that has no significant cross-reactivity to mouse FSH. Serum inhibin A levels were determined by a two-site human inhibin A ELISA modified for assaying mouse serum (5), except inhibin levels were interpolated from serially diluted mouse ovarian extract as standard using arbitrary units (AU/mL) defined as 100 AU/mL set to 100 pg/mL human standard and an assay detection limit of ~8 AU/mL. Serum testosterone levels quantified by liquid chromatography/tandem mass spectrometry (LC/MS/MS) (6) had a quantitation limit of 10 pg/mL, with samples falling below this limit assigned a nominal value of zero. Serum N-terminal propeptide of type I procollagen (PINP) was measured by immunoassay (Rat/Mouse PINP EIA, Immunodiagnostic Systems). Serum tartrate-resistant acid phosphatase form 5b (TRACP5b) was measured by immunoassay (MouseTRAP Assay, Immunodiagnostic Systems).

Osteoblast and Osteoclast Cultures. Primary osteoblastic cells were prepared from calvaria of neonatal C57BL/6J mice by digestion with 0.1% collagenase (Worthington Biomedical Co.) and 0.2% dispase (Invitrogen), then cultured in ascorbic acid (50 μ g/mL; Sigma) and β -glycerophosphate (10 mM; Sigma) to induce osteoblast differentiation as described (7). Osteoclast cultures were prepared from (i) spleen cells from adult C57BL/6J mice cultured for 7 d in medium containing 10% FCS, 25 ng/mL M-CSF (R&D Systems), and 50 ng/mL RANKL (R&D Systems) as described (8) and (ii) the murine RAW 264.7 cell line cultured for 7 d in medium containing 10% FCS and 50 ng/mL RANKL. Multinucleated osteoclasts were formed in both spleen and RAW 264.7 cell-derived cultures. Total RNA was prepared from osteoblast or osteoclast cultures using NucleoSpin as recommended (Machery-Nagel).

1. Allan CM, et al. (2004) Complete Sertoli cell proliferation induced by follicle-stimulating hormone (FSH) independently of luteinizing hormone activity: Evidence from genetic models of isolated FSH action. *Endocrinology* 145:1587–1593.

2. Allan CM, et al. (2001) A novel transgenic model to characterize the specific effects of follicle-stimulating hormone on gonadal physiology in the absence of luteinizing hormone actions. *Endocrinology* 142:2213–2220.

- McTavish KJ, et al. (2007) Rising follicle-stimulating hormone levels with age accelerate female reproductive failure. *Endocrinology* 148:4432–4439.
- Jimenez M, et al. (2005) Validation of an ultrasensitive and specific immunofluorometric assay for mouse follicle-stimulating hormone. *Biol Reprod* 72:78–85.
- Wang Y, et al. (2005) Gonadotropin control of inhibin secretion and the relationship to follicle type and number in the hpg mouse. *Biol Reprod* 73:610–618.
- Harwood DT, Handelsman DJ (2009) Development and validation of a sensitive liquid chromatography-tandem mass spectrometry assay to simultaneously measure androgens and estrogens in serum without derivatization. *Clin Chim Acta* 409:78–84.
- Zhou H, Mak W, Zheng Y, Dunstan CR, Seibel MJ (2008) Osteoblasts directly control lineage commitment of mesenchymal progenitor cells through Wnt signaling. *J Biol Chem* 283:1936–1945.
- Zhou H, et al. (2001) A novel osteoblast-derived C-type lectin that inhibits osteoclast formation. *J Biol Chem* 276:14916–14923.

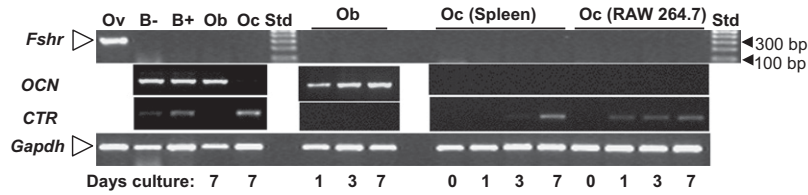


Fig. S1. Expression of the known mouse *Fshr* mRNA (RT-PCR) product (329 bp) was detected in adult ovary (Ov) as expected. In contrast, the predicted *Fshr* cDNA fragment was not detected in long bone from adult TgFSH^H (B+) and non-Tg (B-) females, isolated osteoblast (Ob), or isolated osteoclast (Oc, derived from spleen or RAW 264.7 cells) preparations after 0, 1, 3, or 7 d of culture conditions to induce Ob or Oc differentiation (*SI Materials and Methods*). A secondary round of PCR amplification using 40 cycles and the primary reaction samples as template also failed to detect the expected *Fshr* DNA product. Expression of calcitonin receptor (CTR, 245-bp product) or osteocalcin (OCN, 300-bp product) mRNA was detected in the Oc or Ob preparations by RT-PCR using primer pairs 5'-CCACTgAgCCTTCATTTCT-3'/5'-TgCCTgCTTTCCTACgAAC-3' and 5'-CCTTCATgTCCAAGCaggA-3'/5'-TgCCTgCTTTCCTACgAAC-3', respectively, both with 60 °C annealing and 35 cycles. *Gapdh* mRNA was amplified as internal cDNA control (primers 5'-AACTTggCATTgTggAAgg-3' and 5'-CTgCTTACC-ACCTTCTgAT-3'), and amplified DNA fragments were visualized by ethidium bromide staining after agarose gel electrophoresis.

