Supporting Information

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SI Materials and Methods

Isoproterenol (ISO) Treatment. A total of 31 male (9-wk-old) mice were used in the experiments. For ISO treatment, 15 WT and 16 homozygous osteopontin (OPN)-KO mice were divided into two groups. The mice in one group (ISO) were subjected to i.p. injections of ISO (10 μg/g of body weight per d) for 21 d, and the mice in the control group were subjected to i.p. injection of physiological saline (1, 2). For antibody treatment 100 μg of 2C5 anti-OPN antibody was injected each time before ISO injection.

3D X-Ray Microcomputed Tomography (μCT) Analysis. The 3D-μCT analysis was conducted with Scan-Xmate-E090 (Comscan Techno Co.) and computer software, Tri/3D-Bon (Ratoc System Engineering Co.). In some specimens, 2D-μCT pictures were also examined. Bone volume/tissue volume (BV/TV), trabecular number, and other microarchitectural parameters were analyzed in the secondary trabecular regions from 200 μm to 600 μm away from the growth plate.

Bone Histomorphometric Analysis. Calcein labeling was conducted to estimate the levels of newly formed bone within a given time period according to methods previously described (3, 4). Briefly, calcein (10 mg/kg of body weight) was given i.p. at 2 d and 6 d before sacrifice. Femora were fixed in 4% paraformaldehyde and embedded in glycomethacrylate. Sagittal histological sections were prepared, and the calcein bands were visualized based on confocal laser microscopy with an excitation wavelength at 488 nm using a 550-nm band-pass filter. Single-labeled bone surface (sLS), double-labeled bone surface (dLS), and total bone surface (BS) were separately measured. Mineralizing surface per bone surface (MS/BS) was calculated as (dLS + sLS/2)/BS. The distance between the parallel calcein lines was measured to yield the mineral apposition rate (MAR) (μm/d). The bone formation rate was obtained by multiplying MAR and MS/BS. Histomorphometric analysis was performed by focusing on the secondary trabecular regions from 200 μm to 600 μm away from the growth plate in the distal end of the femora. In the decalcified section, the number of osteoclasts per bone surface (*n* per mm) and osteoclast surface per bone surface (%) were analyzed based on tartrate-resistant acid phosphatase (TRAP) staining in sagittal sections of the tibiae. Staining was performed as described previously (5, 6). $TRAP^+$ cells that contain one or more nuclei and are located on the surface of the trabeculae were regarded as osteoclasts. One section per animal was analyzed for these parameters.

Urinary Deoxypyridinoline Measurement. Urine samples from each mouse were housed individually, and urine was collected in metabolic cages (Natsume) during the last 24 h before the mice were killed. Urinary deoxypyridinoline levels were measured by AutoAnalyzer (Mitsubishi Medicine).

Osteoclast Development in Vitro. Bone marrow cells were cultured in the presence of vitamin D_3 (10 nM) and dexamethasone (100 nM) for 5 d. Cells were then fixed and stained for TRAP. For mineralized nodule-formation assay, bone marrow cells were cultured in the presence of ascorbate and β-glycerophosphate for 3 wk and then stained for alizarin red.

RT-PCR Analysis. Bone marrow of femora including cancellous bone was homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted according to the manufacture's protocol. Reverse transcription (RT) was carried out with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad). The quantification was carried out as described previously (4). Expression values were normalized against the mRNA levels of GAPDH. Sequences of the primers are as follows. OPN: forward, 5′-TCC CTC GAT GTC ATC CCT GTT G-3′ and reverse, 5′-GCA CTC TCC TGG CTC TCT TTG G-3′; Runx2: forward, 5′-TGG CTT GGG TTTCAG GTT AGG G-3′ and reverse, 5′-TCG GTT TCT TAG GGT CTTGGA GTG-3′; TRAP: forward, 5′-GAC CAC CTT GGC AAT GTC TCT G-3′ and reverse, 5′-TGG CTG AGG AAG TCA TCT GAG TTG-3′; GAPDH: forward, 5′-AGA AGGTGG TGA AGC AGG CAT C-3′ and reverse, 5′-CGA AGG TGG AAGAGT GGG AGT TG-3′.

ELISA. The plasma samples were collected via BD Microtainer (ref. 365973; BD Biosciences). Levels of OPN in plasma samples from 129sv and C57BL/6 mice were measured based on an ELISA using a kit (no. 27351; Immuno-Biological Laboratories). Plasma and standard OPN samples were diluted in PBS containing 1% BSA and 0.05% Tween-20 in PBS and incubated for 1 h at 37 °C. After rinsing several times with 0.05% Tween-20 in PBS, horseradish peroxidase-labeled conjugated anti-mouse OPN (O-17) monoclonal antibody (100 μL of 2 ng/mL) was added and incubated for 30 min at 4 °C. After rinsing, 100 μL of tetramethyl benzidine buffer was added, and the signal was allowed to develop for 30 min at room temperature. The reaction was stopped by adding 100 μL of sulfuric acid (1 N). The absorbance at 450 nm was measured by an automatic ELISA reader (SpectraMax; Molecular Devices).

Transfection and Luciferase Assay. MC3T3-E1 osteoblastic cells and C2C12 mesenchymal precursor cells were plated on 24-well plates at a density of 104 cells per well. Transfection of siRNA and plasmid DNA was performed on day 1 with Lipofectamine 2000 (Invitrogen). The construct of siRNA for OPN was purchased from Ambion (Silencer Predesigned siRNA, no. 150969 for mouse Spp1). Silencer Negative Control siRNA (Ambion) was used as a control. cAMP response element luciferase (CRE-Luc) reporter plasmid (PathDetect; Stratagene) was used to estimate cAMP-induced transcriptional activity. *Renilla* luciferase reporter vector pGL4 (Promega) was used as an internal control. Empty vector (pcDNA3.1; Invitrogen) was used as a control for reporter plasmids. For transfection, each well received 0.2 μg of CRE-Luc plasmid DNA or control, 20 pmol of siRNA for OPN or control, and 40 ng of *Renilla* luciferase plasmid combined with 2 μl of Lipofectamine 2000. ISO (10 μM) (Sigma-Aldrich), isobutylmethylxanthine (IBMX; 100 μM; Sigma-Aldrich), or forskolin (10 μM) (Sigma-Aldrich) were added at 24 h after transfection. The luciferase assay was performed with the dual luciferase reporter assay system (Promega) at 6 h and 24 h after ISO treatment. Luciferase activity was measured with a luminometer (Lumat LB 9507; Berthold Technologies).

Western Blot Analysis. MC3T3-E1 cells were lysed in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris·HCl, pH 8.0) supplemented with cOmplete, Mini (Roche Applied Science) as a protein inhibitor, and protein extracts were cleared by centrifugation. The proteins were eluted in SDS sample buffer after heating at 95 °C for 5 min. The proteins were electrophoresed in SDS/PAGE. OPN

was detected by monoclonal anti-mouse OPN antibody (no. 18621; Immuno-Biological Laboratories). CRE binding (CREB) and phosphorylated CREB were detected by CREB antibody and rabbit polyclonal anti-rabbit phospho-CREB (Cell Signaling Technologies), respectively, by using the ECL Plus Western Blotting Detection System (GE Healthcare).

Immunoprecipitation. Cells on 10-cm dishes were transfected as described and treated with 10 μM ISO or vehicle (500 mL of PBS) for 1 min. Reversible cross-linking of proteins was then performed by incubating cells in 2.5 mM dithiobis(succinimidyl) propionate (Sigma) for 30 min at room temperature. Cells were then washed in cold PBS and incubated for 20 min in 1 mL of cold RIPA buffer. Cells were scraped, transferred to microcentrifuge tubes, and spun for 20 min at 10,000 × *g*. Postnuclear supernatant was then transferred to a new tube, and 1:10 vol Protein A/G UltraLink beads (Pierce) conjugated with polyclonal anti-GαS (Millipore) was added after collecting 50 μL of postnuclear supernatant as a loading control. Lysate/bead slurries were rotated overnight at 4 °C then washed four times in cold RIPA buffer, and beads were resuspended in 1:1 (vol/vol) Laemmli buffer (Bio-Rad) supplemented with 1:20 (vol/vol) β-mercaptoethanol (Sigma). Cross-linking was reversed by incubating beads and loading control samples for 1.5 h at 37 °C. Proteins were then analyzed by SDS/PAGE and immunoblotting.

1. Kondo H, et al. (2005) Unloading induces osteoblastic cell suppression and osteoclastic cell activation to lead to bone loss via sympathetic nervous system. *J Biol Chem* 280: 30192–30200.
2. Chruscinski AJ, et al. (1999) Targeted disruption of the β2 adrenergic receptor gene. *J Biol Chem* 274:16694–16700.
3. 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.
4. Saita Y, et al. (2007) Lack of Schnurri-2 expression associates with reduced bone remodeling and osteopenia. *J Biol Chem* 282:12907–12915.
5. Mizoguchi F, et al. (2008) Transient receptor potential vanilloid 4 deficiency suppresses unloading-induced bone loss. *J Cell Physiol* 216:47–53.
6. Ono N, et al. (2007) Constitutively active parathyroid hormone receptor signaling in cells in osteoblastic lineage suppresses mechanical unloading-induced bone resorption. *J Biol Chem* 282:25509–25516.

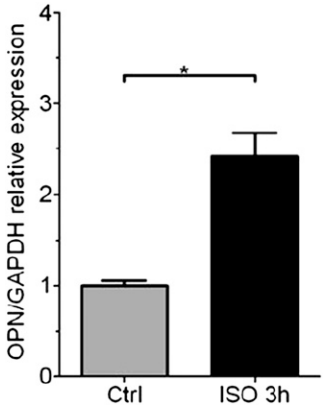

Fig. S1. OPN expression in calvariae from WT mice in organ culture. Expression of OPN mRNA normalized to GAPDH mRNA expression in calvariae from WT mice is shown. Results are expressed in arbitrary units as mean ± SEM of $n = 5$ (*$P < 0.05$).

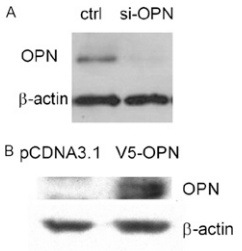

Fig. S2. Depletion (*A*) and overexpression (*B*) of OPN from MC3T3 by using siRNA (*A*) and a pCDNA3.1 plasmid encoding OPN (V5-OPN; *B*) and measured by Western blotting.

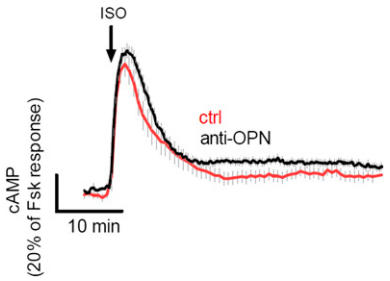

Fig. S3. Effect of anti-OPN on β2-adrenergic receptor (β2AR) signaling. Averaged cAMP responses mediated by ISO (10 μM). cAMP induction over a 30-min time course was monitored by FRET changes from MC3T3 osteoblastic cells transiently expressing epac-CFP/YFP alone (control, red), or in the presence of a neutralizing OPN antibody (anti-OPN, black). Data represent the mean ± SEM of n = 10 (ctrl) and n = 20 (anti-OPN).

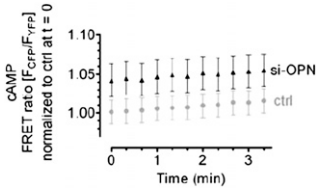

Fig. S4. Basal cAMP levels. Basal cAMP over a 3.5-min time course was monitored by FRET changes from MC3T3 live cells transiently expressing epac-CFP/YFP alone (ctrl) or in combination with OPN-specific siRNA transfected cells (si-OPN). Data represent the mean ± SEM of n = 25 (ctrl) and n = 25 (si-OPN). Statistical analysis was performed with a t test (**$P < 0.01$).

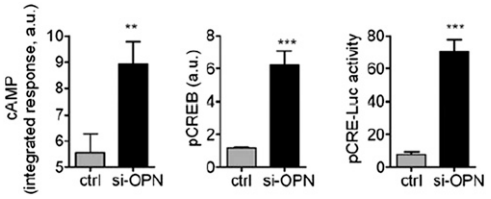

Fig. S5. Effect of OPN on the β2AR signaling cascade. Bars represent the average cAMP responses, CREB phosphorylation, and pCRE-Luc activity from MC3T3 cells transfected with si-OPN or scrambled siRNA (control). Data represent the mean ± SEM of n = 15 (cAMP), n = 3 (pCREB), and n = 4 (pCRE-Luc). Statistical analysis was performed with a t test (**$P < 0.05$, *$P < 0.01$).

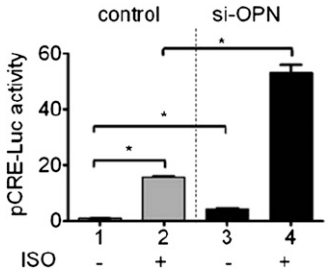

Fig. S6. Transcriptional modulation in C2C12 cells. Effect of OPN on the β2AR signaling cascade. Bars represent the average pCRE-Luc activity from C2C12 cells transfected with either si-OPN or scrambled siRNA (control). Data represent the mean ± SEM of n = 4. Statistical analysis was performed with a t test (*$P < 0.01$).