Supplementary Materials and methods

Antibodies and reagents

Rabbit anti-fibronectin antibody and human plasma fibronectin were purchased from EMD Millipore (Billerica, MA, USA). Rabbit anti-biotin antibody was purchased from Rockland (Gilbertsville, PA, USA), Mouse monoclonal anti-FN (IST-9) antibody (EDA-FN antibody), rabbit polyclonal anti-Ki67 antibody, goat anti-mouse serum albumin antibody were from Abcam (Cambridge, MA, USA). Anti-mouse osteopontin (OPN) antibody was from R&D Systems. Polyclonal goat anti-rabbit biotinylated IgG was from DAKO (Carpinteria, CA, USA). Anti-actin antibody, bovine plasma FN, Thiazolyl Blue Tetrazolium Bromide (MTT), and EZlink®- ExtrAvidin-Alkaline phosphates conjugate were obtained from Sigma Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP) linked anti-rabbit IgG was from Cell Signaling (Boston, MA, USA) and HRP-linked anti-mouse IgG was from GE Healthcare (Quebec, QC, Canada), respectively. Alexa Fluor®568 and DAPI (4', 6-diamidino-2-phenylindole) were purchased from Life Technologies (Grand Island, NY, USA). Dylight -488 conjugated NeutrAvidin protein, EZ-link sulfo-NHS-biotin and 5-(biotinamido) pentylamine (BPA) were from Pierce (Rockford, IL, USA). ECL kit was purchased from Zmtech Scientifique (Montreal, QC, Canada). All other reagents were purchased from Sigma or Fisher Scientific unless specified below.

Bone mineral density

Total body bone mineral density was measured in 1, 3, 6 and 12-month old live mice (n=11) using GE Lunar PIXImus bone densitometer.

Biomechanical testing

Biomechanical properties of femurs from 1 and 3-month-old male mice (n=4-6) were assessed by the 3-point bending test using a MACH-1™ micro-mechanical system (70). Prior to biomechanical testing, dissected femurs were stored frozen in PBS until all samples were collected.

Histology and histomorphometric analysis

Femurs from 3-month-old mouse femurs (n=6) were fixed in 3.7% formaldehyde, decalcified with 8%

EDTA, pH 7.4 and embedded in paraffin for histology. Sections were cut (5-µm thickness), de-paraffinized and antigen-retrieval was facilitated by treatment with 1 mg/ml trypsin for 20 minutes at 37°C. For 3, 3′-diaminobenzidine (DAB) staining, endogenous peroxidase activity was blocked with 3% H_2O_2. For alkaline phosphatase staining, endogenous biotin was blocked with endogenous biotin-blocking kit from Molecular Probes (Eugene, OR, USA). For antibody staining, sections were blocked for 1 h at room temperature with 1% BSA, 2% goat serum, and 0.1% Triton X-100 in PBS. Sections were then incubated with primary antibodies overnight at 4°C followed by detection with horseradish peroxidase-conjugated secondary antibody and visualization with DAB or ALP (Vector Laboratories, Burlingame, CA, US) as the substrate. Sections were counterstained with hematoxylin or methyl green (Vector, Burlingame, CA, USA).

For the staining of alkaline phosphatase enzyme activity, mouse femurs were fixed in 70% ethanol and embedded in polymethyl methacrylate. Sections were cut (5-µm thickness), de-plasticized and incubated with alkaline phosphatase substrate solution (Vector, Burlingame, CA, USA) at room temperature until the staining was visible. Counterstaining was done with methyl green (Vector, Burlingame, CA, USA).

For the assessment of mineral apposition rate (MAR), 3-month-old mice were injected intraperitoneally with 10 µl/g body weight of calcein solution (0.25% calcein and 2% $NaHCO_3$ dissolved in 0.15 M NaCl) twice at a 3-day interval. Mice were euthanized on day 5 of the experiment, and bones were processed for histomorphometric analyses. For plastic sectioning, lumbar vertebrae were fixed overnight in 4% PFA/PBS, embedded in polymethyl methacrylate, and sectioned (5-µm thickness). Additional staining was performed including von Kossa (for mineral), Toluidine Blue (TB, for histology) and tartrate-resistant acid phosphatase (TRAP, osteoclast marker). Osteoblast and osteoclast counts were done on stained bone sections, and MAR was calculated from immunofluorescence images of calcein-labelled specimens using Osteomeasure software (OsteoMetrics, Inc. Decatur, GA). Images were taken at room temperature using a light microscope (DM200; Leica, Solms, Germany) with a 2.5× (numerical aperture of 0.07), 20× (numerical aperture of 0.40) or 40× (numerical aperture of 0.65) objective. All histological images were captured using a digital camera (DP72; Olympus, Tokyo, Japan), acquired with DP2-BSW software (XV3.0; Olympus), and processed using Adobe Photoshop.

Sequential bone protein extraction

Bone protein extracts were prepared from femurs as previously described (39, 40). Briefly, 3-month-old male mice were sacrificed and the long bones were collected, the growth plates/metaphyses were removed from each end of the bone and the bone marrow was flushed out with PBS, and the bones were then flash-frozen in liquid nitrogen. Frozen bones were ground into powder with a biopulverizer (Biospec Products, Bartlesville, OK, USA) under liquid nitrogen, and the bone powder was subjected to two consecutive 24 hour extractions at 4°C using 4 M of Guanidium-HCl in 50 mM of Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mg/ml of benzamidine, and 1 mg/ml of leupeptin (Sigma). After centrifugation at 1000 *g* for 15 minutes, supernatant was collected and the two supernatants were combined together as the G1-extract. The pellet was washed twice with 50 mM of Tris-HCl, pH 7.4, and extracted twice for 24 h with 0.5 M of EDTA in 50 mM of Tris-HCl, pH 7.4 at 4°C. This extraction is named E-extract. Two additional extractions with 4 M Guanidium-HCl as described previously were processed and this extract was named the G2-extract. The final pellet was extracted with 50 mM of Tris-HCl, pH 7.4 containing 1% deoxycholic acid (DOC) and 1% sodium dodecyl sulphate (SDS) and thus termed the DOC/SDS extract. All extracts were concentrated with Centricon Plus-20 PL-10 concentrators (Millipore, Bedford, MA, USA) and in the case of the G1- and G2-extracts, buffer was changed to 8 M urea to facilitate SDS-PAGE chromatography.

Immunofluorescence microscopy

BmMSCs were cultured on Nunc® Lab-Tek® chamber slides (Fisher) for immunofluorescence staining and microscopy. Biotinylation of pFN (bpFN) was done as previously described (Cui and Kaartinen 2014, Cui, Wang et al. 2014). At day 3, 10 µg/ml bpFN was added to the culture media, and after 24 hours, cells were fixed in 3.7% for 10 minutes at room temperature, permeabilized with 0.25% Triton, and blocked with 2% bovine serum albumin (BSA) in PBS. Ki67 was detected by anti-Ki67 antibody and corresponding Alexa Fluor® 568 secondary antibody. Biotin from biotin-labeled plasma FN was visualized by Dylight-488 NeutrAvidin. Nuclei were stained with DAPI. Slides were mounted with Prolong Gold Anti-Fade Medium (Invitrogen). An Axioskop-2 upright fluorescence microscope equipped with AxioVision 4.8 imaging software (Zeiss) was applied for image acquisition.

Flow cytometry

Bone marrow cells (BM) were collected by centrifugation from mice long bones and resuspended in flow cytometry staining buffer (FACS) buffer (eBioscience) and passed through 70µm cell strainer to remove any debris. Cells were centrifuged at 500 x g for 5 minutes and resuspended in Red Blood Cell lysis buffer (Sigma) for 10 minutes on ice to lyse erythrocytes. After centrifugation the cell pellet was washed once with FACS buffer and then resuspended in the same buffer. Cells were counted and 1 million cells were used for each flow cytometry reactions. Cells were stained with Fluorescein isothiocyanate (FITC)-conjugated antibodies to mouse Gr-1/Ly-6G or Allophycocyanin (APC)-conjugated antibodies to mouse CD11b or Phycoerythrin (PE)-conjugated antibodies to mouse F4/80 (eBioscience). 7-Amino-actinomycin D viability staining solution (eBioscience) was used for the exclusion of nonviable cells in flow cytometric analysis. Flow cytometry analysis was done using LSR-II flow cytometry machine (BD Biosciences) and analyzed using FACSDIVA software. Results are expressed as cell frequency (%).

RT-PCR

Messenger RNA was extracted from the cultured cells using Trizol reagent (Gibco). PCR was performed with SuperScript™ Ⅲ One-Step RT-PCR system with Platinum® Taq DNA polymerase (Invitrogen). Primers (Invitrogen) for genes detected were as described previously: *Pparg2* (Myneni, Hitomi et al. 2014), *Csf* (Wittrant, Gorin et al. 2009), *Rankl* (Wittrant, Gorin et al. 2009), *Opg* (Wittrant, Gorin et al. 2009) and *Gapdh* (Al-Jallad, Nakano et al. 2006). PCR products were analyzed by 2% agarose gel electrophoresis.

qRT-PCR

RNA was isolated from passage two non-differentiated bone marrow mesenchymal stem cells using RNeasey Mini Kit (Qiagen). High Capacity cDNA RT kit (Life Technologies, Burlington, ON, Canada) was used to for RNA reverse transcription. Real-time PCR was performed on ABI One StepPlus real-time PCR system (Applied Biosysystems, Thermo Fisher) using the comparative CT method. Taqman Fast Advanced Master Mix was used. Expression levels of *Tnfsf11* (RANKL, Mm00441906_m1), *Tnfrsf11b* (OPG, Mm00435454_m1), *Tgm1* (Mm00498375_m1) and normalized to Gapdh (Mm99999915_g1)

Western blotting

Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). For Western blotting, proteins (10 µg/lane) were resolved by 8.5% SDS-PAGE gel electrophoresis, and transferred to PVDF membranes (Bio Rad, Mississauga, ON, Canada). Membranes were blocked with 5% non-fat milk powder in Tris-buffered saline-Tween (TBS-T) buffer, and each protein was detected with specific antibody followed by corresponding HRP-conjugated secondary antibodies. The detection was visualized with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada).