SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

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Osteoclast differentiation

Bone marrow cells were isolated from the tibiae and femurs of 6-week-old ICR mice by flushing the bone marrow with α-MEM. Bone marrow cells were cultured in α-MEM containing 10 % fetal bovine serum (FBS) in the presence of macrophage colony-stimulating factor (M-CSF; 30 ng/ml) for 3 days. The adherent bone marrow-derived macrophages (BMMs) used as osteoclast precursors were cultured with M-CSF (30 ng/ml) and RANKL (20-100 ng/ml) for 3 days. Cultured cells were fixed with 10 % formalin and stained for TRAP solution. TRAP-positive cells with more than three nuclei were counted as osteoclasts.

Osteoblast differentiation

Primary osteoblast precursor cells were isolated from the calvarial bone of neonatal mice by successive enzymatic digestion with α-MEM containing 0.1 % collagenase (Thermo Fisher Scientific, Waltham, MA, USA) and 0.2 % dispase II (Roche Diagnostics, Rotkreuz, Switzerland). After removing the enzymes, the collected cells were cultured in osteogenic medium containing BMP2 (100 ng/ml), ascorbic acid (50 ng/ml), and β-glycerophosphate (100 mM). To assess osteoblast differentiation, osteoblast precursor cells cultured for 3 days were subjected to an ALP assay. Briefly, cells were lysed with osteoblast lysis buffer (50 mM Tris-HCl [pH 7.4], 1 % Triton X-100, 150 mM NaCl, and 1 mM EDTA) and the lysates were incubated with p-nitrophenyl phosphate substrate (MilliporeSigma, Burlington, MA, USA). ALP activity was measured as the change in absorbance at 405 nm using a spectrophotometer.

To assess their function, osteoblasts cultured for 9 days were fixed with 70 % ethanol and stained with 40 mM alizarin-red (pH 4.2). Nonspecific staining was removed with PBS, stained alizarin-red was dissolved with 10 % cetylpyridinium chloride (MilliporeSigma), and the absorbance of the extracted solution was measured at 562 nm.

Retroviral gene transduction

Retroviral vectors were transfected into the packaging cell line (Plat E) using FuGENE 6 (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Viral supernatants were collected from the culture medium 48 h after transfection. BMMs or osteoblasts were incubated with the viral supernatants for 6 h in the presence of polybrene (10 μg/ml) (MilliporeSigma).

Small interfering RNA transfection

The small interfering RNA (siRNA) sequences used to knock down KLF2: a nonspecific control siRNA (5'-CCU GGC GCC UUC GGU CUU UUU-3') and mouse KLF2-specific siRNA (5'-GCA CGG AUG AGG ACC UAA A-3'). siRNAs were transfected into BMMs and osteoblasts using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol.

Quantitative real-time PCR analysis

Total RNA was extracted from the cultured cells using Qiazol lysis reagent (Qiagen, Venlo, Germany) according to the manufacturer's instructions, and 2 μg RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Thermo Fisher Scientific). Quantitative real-time PCR analysis was performed in triplicate using a Rotor-Gene Q with SYBR Green (Qiagen). The following sequences were used to assess mRNA expression: c-Fos, 5′-ATG GGC

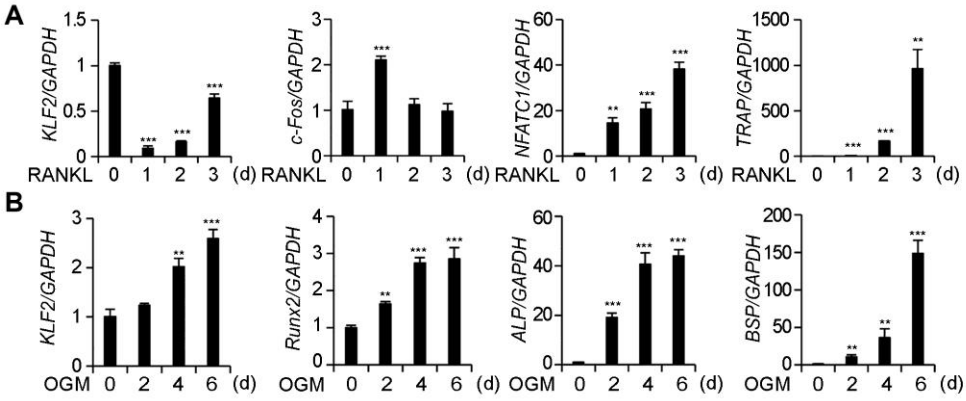
TCT CCT GTC AAC ACA-3′ and 5′-TGG CAA TCT CAG TCT GCA ACG CAG-3′; NFATc1, 5′-CTC GAA AGA CAG CAC TGG AGC AT-3′ and 5′-CGG CTG CCT TCC GTC TC ATAG-3′; TRAP, 5′-CTG GAG TGC ACG ATG CCA GCG ACA-3′ and 5′-TCC GTG CTC GGC GAT GGA CCA GA-3′; GAPDH, 5′-TGA CCA CAG TCC ATG CCATCA CTG-3′ and 5′-CAG GAG ACA ACC TGG TCC TCA GTG-3′; Runx2, 5′-CCC AGC CAC CTT TAC CTA CA-3′ and 5′-CAG CGT CAA CAC CAT CAT TC-3′; ALP, 5′-CAA GGA TAT CGA CGT GAT CAT G-3′ and 5′-GTC AGT CAG GTT GTT CCG ATT C-3′; BSP, 5′-GGA AGA GGA GAC TTC AAA CGA AG-3′ and 5′-CAT CCA CTT CTG CTT CTT CGT TC-3′; KLF2, 5′-CTG GCG CCT TCG GTC TTT TC-3′ and 5′-CGC ATC CTT CCC AGT TGC AA -3′.

Western blotting analysis

Cultured cells were lysed in extraction buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40, 1 mM PMSF, and a protease inhibitor mixture). Equal amounts of protein were subjected to SDS-PAGE and transferred electrophoretically onto a polyvinylidene difluoride membrane (MilliporeSigma). Membranes were incubated with antibodies against actin (MilliporeSigma), c-Fos, and NFATc1 (Santa Cruz Biotechnology, Dallas, TX, USA). Immune complexes were detected using ECL (MilliporeSigma) and analyzed with a LAS3000 luminescent image analyzer (GE Healthcare, Chicago, IL, USA).

Statistical analysis

Statistical analyses were performed using an unpaired Student's *t* test. All data are presented as the mean ± SD. *P* values < 0.05 were considered statistically significant.



Supplementary Figure 1

Supplementaet Figure 1. Expression profile of KLF2 in ostoeclastogenesis and Osteoblastogenesis. (A) Bone marrow-derived macrophage cells (BMMs) were cultured with M-CSF and RANKL for the indicated length of time. The mRNA levels of KLF2, c-Fos, NFATc1, and TRAP were assessed by real-time PCR. (B) Osteoblasts were cultured with osteogenic medium (OGM) containing BMP2 (100 ng/ml), ascorbic acid (50 μg/ml), and β-glycerophosphate (100 mM) for the indicated length of time. The mRNA expression of KLF2, Runx2, ALP, and BSP was assessed by real-time PCR. (B-D) Osteoblasts were transduced with either pMX-IRES-EGFP (control) or KLF2 retroviruses and cultured in an osteogenic medium.