

# Supporting Information

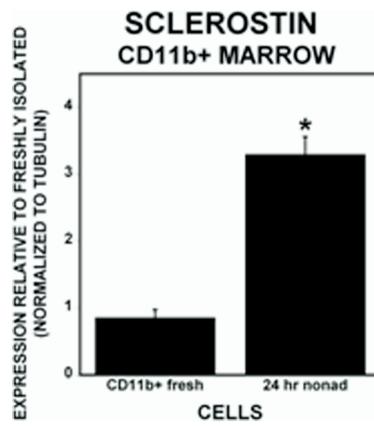
Pederson *et al.* 10.1073/pnas.0805133106

## SI Methods

**Western Blotting.** At the end of treatments, cells were placed on ice and rinsed 3 times with cold PBS. Laemmli sample buffer lacking bromophenol blue and 2-mercaptoethanol was added, and cell extracts were collected by scraping. Protein concentrations were determined by using the Bio-Rad Protein Quantitation In Detergent Analysis Kit. Forty micrograms of protein per lane was used for analysis. Before boiling and Western blotting, 2-mercaptoethanol and bromophenol blue were added to the samples. Western blotting was carried out as previously outlined (1). The following antibodies were used: SPHK1 (CalbioChem

catalog no. PC727); sclerostin (R & D System catalog no. AF1589); Wnt10b (R & D Systems catalog no. AF2110); and BMP6 (Santa Cruz Biotechnology catalog no. SC-27409). Antibodies were used at a 1:1,000 dilution for Western blotting as we have reported (1). Anti-tubulin hybridoma supernatant (E7) from the Developmental Studies Hybridoma Bank at the University of Iowa was also used at a 1:1,000 dilution. Peroxidase-conjugated secondary antibodies (Cell Signaling) were used at a 1:5,000 dilution with chemiluminescent detection using ECL Plus according to the product directions (GE Healthcare).

1. Gingery A, Bradley E, Shaw A, Oursler MJ (2003) Phosphatidylinositol 3-kinase coordinately activates the MEK/ERK and AKT/NFkappaB pathways to maintain osteoclast survival. *J Cell Biochem* 89:165–179.



**Fig. S1.** CD11b<sup>+</sup> marrow cells were purified and either collected for RNA (fresh) or cultured overnight with M-CSF as above, and nonadherent cells were harvested (24 h nonad.). RNA was harvested for real-time PCR analyses ( $n = 3$  replicates). \*,  $P < 0.05$  compared with time 0. These results are representative of 2 experiments.

**Table S1. Affymetrix array results of secreted and extracellular matrix-associated osteoclast genes**

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a disintegrin and metalloprotease domains 2, 18, 21, 24, 25, 26, 28, 3, 33, 11
a disintegrin and metalloproteinase domains 12, 15, 19, 9
a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 and 8
Activin A receptor, type 1B and type II-like 1
activin receptor IIB
ADAM-like, decysin 1
ADAMTS-like 1
adipocyte complement related protein
adipsin
allergen dl chain C2C
amelogenin X chromosome
androgen binding protein, alpha
androgen-induced 1
aquaporin 1, 2,5
biglycan
BMP-binding endothelial regulator
bone morphogenetic proteins 4, 5, 6, 7, 8b, 10, 15
bone morphogenetic protein receptor, type II (serine/threonine kinase)
C1q and tumor necrosis factor related protein 1
cadherin 1, 15, 16, 17, 2, 20, 22,23, 3, 4, 5, 6, 8,
cadherin EGF LAG seven-pass G-type receptor 1, 2, 3
calcitonin receptor
calcitonin receptor-like
calcitonin/calcitonin-related polypeptide, alpha
calcitonin-related polypeptide, beta
cathepsin 6, 7, 8, B, C, E, F, G, H, J, K, L, M, R, S, W
CDK5 regulatory subunit associated protein 1
chemokine (C motif) ligand 1
chemokine (C-C motif) ligand 1, 12, 17, 19, 2, 20, 21a, 22, 24, 25, 27, 3, 4, 5, 6, 7, 8, 9,
chemokine (C-C motif) ligand 5
chemokine (C-X3-C motif) ligand 1
chemokine (C-X-C motif) ligand 1, 10, 11, 12, 13, 14, 15, 2, 4, 5, 7, 9
colony stimulating factor 1 (macrophage)
colony stimulating factor 1 receptor
colony stimulating factor 2 (granulocyte-macrophage)
colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)
colony stimulating factor 3 (granulocyte)
colony stimulating factor 3 receptor (granulocyte)
connective tissue growth factor
decidual/trophoblast prolactin-related protein
decorin
dickkopf homolog 1, 2, 3, 4
Eph receptor A3
epidermal growth factor
epidermal growth factor receptor
fibroblast growth factor 10, 12, 15, 16, 17, 2, 20, 21, 22, 23, 3, 4, 5, 6, 7, 8,
fibroblast growth factor binding protein 1
fibroblast growth factor receptor 1, 2, 3, 4
fibroblast growth factor receptor-like 1
fibromodulin
fibronectin 1
forkhead box L1
frizzled homolog 5, 6, 7
frizzled-related protein
glucagon
glucagon receptor
glucagon-like peptide 1 receptor
granzyme A, B, C, D, E, F, G, K, M
growth arrest specific 6
growth differentiation factor 10, 11, 15, 2, 3, 5, 9
growth hormone
growth hormone receptor
growth hormone releasing hormone
Hedgehog-interacting protein
Indian hedgehog
insulin I & II
insulin receptor-related receptor
insulin-like 3, 5, 6
insulin-like growth factor 1 & 2
insulin-like growth factor binding protein 1, 2, 4, 5
insulin-like growth factor binding protein, acid labile subunit
integrin alpha 2, 2b, 7, 9, L
integrin beta 1 (fibronectin receptor beta), 2, 2-like, 3, 5, 7,

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integrin binding sialoprotein  
integrin, alpha E, epithelial-associated  
intercellular adhesion molecule 2  
intercellular adhesion molecule 4, Landsteiner-Wiener blood group  
intercellular adhesion molecule 5, telencephalin  
interferon (alpha and beta) receptor 1 & 2  
interferon alpha family, gene 1  
interferon beta, fibroblast  
interferon gamma  
interferon gamma inducible protein 30  
interferon gamma receptor  
IL 1 alpha  
IL 1 beta  
IL 1 family, member 5, 6, 7, 8, 9  
IL 1 receptor accessory protein-like 2  
IL 1 receptor antagonist  
IL 1 receptor, type I and II  
IL 1 receptor-like 1  
IL 1 receptor-like 1 ligand  
IL 10 receptor, alpha  
IL 10, 11, 12a, 12b, 13, 15, 17, 17B, 17E, 18, 2, 20, 21, 24, 25, 3, 4, 5, 6, 7, 9  
IL 10-related T cell-derived inducible factor beta  
IL 12 receptor, beta 1 and 2  
IL 13 receptor, alpha 1 and 2  
IL 15 receptor, alpha chain  
IL 17 receptor & B  
IL 18 binding protein  
IL 18 receptor 1  
IL 18 receptor accessory protein  
IL 2 receptor, alpha and beta chains  
IL 2 receptor, gamma chain  
Interleukin 21 (IL21), mRNA  
IL 21 receptor  
IL 23, alpha subunit p19  
IL 27 receptor, alpha  
IL 3 receptor, alpha chain  
IL 4 receptor, alpha  
IL 5 receptor, alpha  
IL 6 receptor, alpha  
IL 6 signal transducer  
killer cell lectin-like receptor, subfamily D, member 1  
kit ligand  
kit oncogene  
klotho  
latent transforming growth factor beta binding proteins 1 & 3  
lectin, galactose binding, soluble 3  
leptin  
leptin receptor  
leukemia inhibitory factor  
leukemia inhibitory factor receptor  
lumican  
macrophage scavenger receptor 2  
macrophage stimulating 1 (hepatocyte growth factor-like)  
macrophage stimulating 1 receptor (c-met-related tyrosine kinase)  
matrix metalloproteinase 10, 11, 12, 13, 14, 15, 16, 17, 19, 2, 20, 23, 24, 3, 7, 8, 9  
mel transforming oncogene-like 1  
meprin 1 alpha & beta  
met proto-oncogene  
midkine  
nerve growth factor, beta  
neural cell adhesion molecule 1  
neural cell adhesion molecule 2  
neural proliferation, differentiation and control gene 1  
noggin  
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta  
oncostatin M receptor  
oocyte secreted protein 1  
osteoclast inhibitory lectin related protein  
osteomodulin  
parathyroid hormone  
parathyroid hormone-like peptide  
phospholipase A2, group X, XIII  
platelet derived growth factor receptor, beta polypeptide  
platelet derived growth factor, B polypeptide

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platelet/endothelial cell adhesion molecule  
platelet-derived growth factor, C polypeptide, D polypeptide  
programmed cell death 1  
programmed cell death 1 ligand 1  
prolactin-like protein A  
protocadherin beta 1, 10, 11, 12, 13, 15, 16, 17, 18, 19, 2, 21, 22, 3, 4, 6, 7, 8, 9  
sclerostin  
sclerostin domain containing 1  
secreted acidic cysteine rich glycoprotein  
secreted and transmembrane 1  
secreted BMP-binding protein; Mus musculus twisted gastrulation protein (Tsg).  
secreted frizzled-related sequence protein 1, 2, 5  
secreted phosphoprotein 1  
secreted protein SST3  
secretin  
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A  
sonic hedgehog  
SPARC related modular calcium binding 1  
stem cell growth factor  
Sphingosine kinase 1, 2  
stromal cell derived factor 1, 2, 2-like, 4  
stromal cell derived factor receptor 1  
stromal interaction molecule 1  
superoxide dismutase 3, extracellular  
thyroid stimulating hormone receptor  
thyroid stimulating hormone, beta subunit  
tissue inhibitor of metalloproteinase 1, 2  
toll-like receptor 2, 3, 4, 5, 7, 8, 9  
transforming growth factor alpha  
transforming growth factor, beta 1, beta 3  
transforming growth factor, beta induced  
transforming growth factor, beta receptor III  
triggering receptor expressed on myeloid cells 1, 3  
tumor necrosis factor (ligand) superfamily, member 10, 6, 11a, 18, 19, 1a, 1b, 25, 4, 5, 7, 8, 9  
tumor necrosis factor alpha induced protein 6  
tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)  
twisted gastrulation homolog 1 (Drosophila)  
vascular cell adhesion molecule 1  
vascular endothelial growth factor A, B, C  
vitronectin  
Wnt 10a, 10b, 2b, 1, 11, 16, 2, 3, 3A, 4, 5A, 6, 7A, 8A, 9A, 9B  
Wnt inhibitory factor 1  
WNT1 inducible signaling pathway protein 1, 2

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Microarray analysis and validation. Total RNA was isolated from 3 independent osteoclast cultures followed by reverse transcription. Purified double-stranded cDNA was in vitro transcribed in the presence of biotinylated dUTP and dCTP, and fragmented cRNA was hybridized to the Mouse Expression Array 430A (Affymetrix), followed by staining with streptavidin-phycoerythrin. Arrays were scanned and analyzed with GeneChip3.1 software. To eliminate nonexpressed and highly variable genes, the gene's expression level had to be scored "Present" by the GeneChip software algorithm in all 3 samples. Two selection criteria were used to generate the candidate gene list: (i) extracellular matrix-associated genes and (ii) secreted protein genes. The list was further narrowed by a survey of the relevant literature to generate the list of genes that were examined for real-time PCR analysis of expression changes comparing marrow-derived preosteoclasts (day 0 or day 3 of culture) to mature osteoclasts (Table S2, left data column). Genes whose expression was significantly altered during differentiation ( $P < 0.05$ ) were further examined for expression changes during differentiation of RAW 264.7 cells into osteoclasts by real-time PCR (Table S2, right data column). The final selection of the candidates pursued involved demonstrating concordant changes by real-time PCR in both the marrow-derived and RAW 264.7 osteoclasts. Candidate coupling factors selected for study are indicated in bold. ND, not done.

**Table S2. Marrow cells were harvested and cultured as described in *Methods***

Gene	Osteoclast source	
	Marrow-derived	RAW-derived
BMP2	76.6 ± 24.7*	0.8 ± 0.04
BMP4	3.7 ± 0.2*	0.6 ± 0.5
BMP6	3.7 ± 0.6*	54.6 ± 11.3*
BMP7	3.2 ± 1.7*	0.5 ± 1.2
Cardiotrophin-1	1.7 ± 0.6	ND
CTGF	469.7 ± 367.9*	No signal
DKK1	10.2 ± 0.1*	1.1 ± 0.8
DKK2	50.2 ± 20.4*	0.15 ± 0.1*
DKK3	53.6 ± 23.1*	1.8 ± 0.3
EphrinB2	4.7 ± 0.2*	2.3 ± 0.3*
Growth hormone	0.98 ± 0.2	ND
IGF-I	1.2 ± 0.5	ND
IGF-II	1.1 ± 0.2	ND
Klotho	6.2 ± 1.3*	1.1 ± 0.2
Osteopontin	0.9 ± 0.4	ND
Sclerostin	0.06 ± 0.16*	0.001 ± 0.003*
Sclerostin domain-containing 1	0.4 ± 0.2*	ND
Semaphorin 7a	216.2 ± 88.9*	2.9 ± 1.9*
SPHK1	3.2 ± 0.2*	4.1 ± 0.2*
SPHK2	2.9 ± 0.3*	1.7 ± 0.9*
Wnt3a	2.0 ± 0.1*	1.7 ± 0.4
Wnt10b	3.8 ± 0.2*	49.2 ± 12.3*

Cells were harvested prior to fusion (precursor) or once fused (osteoclast). RAW 264.7 cells were cultured without RANKL (precursor) or after culture for 5 days with RANKL (osteoclasts). RNA was harvested and analyzed by real-time PCR as detailed in *Methods*. Values are normalized to tubulin. Below are the ratios of mature osteoclast to precursor expression levels. \*,  $P < 0.05$  comparing undifferentiated to differentiated osteoclasts. In bold are the selected candidate factors further examined in these studies. ND, not done.

