Evidence for osteocyte regulation of bone homeostasis through RANKL expression

Tomoki Nakashima[1–3], Mikihito Hayashi[1–3], Takanobu Fukunaga[1,3], Kosaku Kurata[4], Masatsugu Oh-hora[1,3], Jian Q Feng[5], Lynda F Bonewald[6], Tatsuhiko Kodama[7], Anton Wutz[8], Erwin F Wagner[9], Josef M Penninger[10] & Hiroshi Takayanagi[1–3,11]

Osteocytes embedded in bone have been postulated to orchestrate bone homeostasis by regulating both bone-forming osteoblasts and bone-resorbing osteoclasts. We find here that purified osteocytes express a much higher amount of receptor activator of nuclear factor-κB ligand (RANKL) and have a greater capacity to support osteoclastogenesis *in vitro* than osteoblasts and bone marrow stromal cells. Furthermore, the severe osteopetrotic phenotype that we observe in mice lacking RANKL specifically in osteocytes indicates that osteocytes are the major source of RANKL in bone remodeling *in vivo*.

© 2011 Nature America, Inc. All rights reserved.

In vitro osteoclast differentiation is induced by the cell-cell contact between osteoclast precursor cells of the monocyte/macrophage lineage and anchorage-dependent mesenchymal cells in bone, which include osteoblasts and bone marrow stromal cells (BMSCs)[1–3]. RANKL has been identified as the membrane-bound factor representing the osteoclast differentiation factor or the stromal osteoclast-forming activity expressed by osteoclastogenesis-supporting cells[4,5]. Osteoblastic cells have been thought to be the major cell type that expresses RANKL to support osteoclastogenesis[2,5]. Germ-line targeted disruption of RANKL results in severe osteopetrosis due to a complete lack of osteoclasts, demonstrating the essential role of this cytokine in osteoclastogenesis[6,7]. However, the major source of RANKL *in vivo* remains unclear, as RANKL is expressed by several cell types in bone and bone marrow, including osteoblasts, osteocytes, BMSCs and lymphocytes[5,8,9]. Despite T cell expression of RANKL, the osteoclastogenic T cell subset is limited to autoimmune T cells[1], and RANKL expression in bone marrow cells, including T cells, is much lower than that in bone cells (including osteoblasts and osteocytes) in normal mice (**Fig. 1a**). In addition, osteopetrosis in RANKL-deficient humans does not recover upon bone marrow transfer, suggesting that the RANKL expressed by the hematopoietic lineage does not have a physiological role in osteoclastogenesis[10].

To identify the most physiologically relevant osteoclastogenesis-supporting cells among the mesenchymal lineage cells in bone, we determined the mRNA expression of *Tnfsf11* (encoding RANKL) in osteoblasts and osteocytes. We first followed the conventional method to obtain an osteocyte-rich fraction[11,12], in which cells expressed a high level of osteocyte marker genes such as *Dmp1* (encoding dentin matrix acidic phosphoprotein 1) and *Sost* (encoding sclerostin)[8], but not the osteoblast marker *Kera* (encoding keratocan)[13] (**Supplementary Fig. 1**). Notably, *Tnfsf11* was more highly expressed in the osteocyte-rich fraction than the osteoblast-rich fraction by approximately twofold, but the purity of osteocytes is reported to be around 60% using this isolation method[11].

To isolate osteocytes of higher purity, we generated mice with osteocyte-specific expression of EGFP by crossing CAG-CAT-EGFP reporter mice[14] with a transgenic line expressing *Cre* recombinase under the control of the *Dmp1* promoter (*Dmp1-Cre* mice)[15]. We sorted $EGFP^+$ and $EGFP^-$ cell populations from cells obtained by enzymatic digestion of the neonatal calvaria of these mice (**Fig. 1b**). The 99% EGFP-positive fraction (**Fig. 1b**) had a dendritic morphology characteristic of osteocytes (**Fig. 1c**). $EGFP^+$ cells abundantly expressed osteocyte-specific genes such as *Dmp1*, *Sost*, *Reln* (encoding reelin) and *Npy* (encoding neuropeptide Y), whereas the expression of these genes in the $EGFP^-$ cells was negligible (**Fig. 1d** and **Supplementary Fig. 2**). On the basis of their mutually exclusive expression of *Dmp1*, *Sost*, *Reln*, *Npy*, *Kera* and *Fmod* (encoding fibromodulin), the $EGFP^+$ and $EGFP^-$ cell populations could be considered to contain osteocytes and osteoblasts of high purity, respectively.

Using this high-purity isolation method, we confirmed that *Tnfsf11* mRNA expression is more than ten times higher in osteocytes than in osteoblasts (**Fig. 1d**). Flow cytometric (**Fig. 1e** and **Supplementary Fig. 3**) and immunohistochemical (**Fig. 1f**) analyses confirmed that the expression of RANKL in osteocytes is much higher than in osteoblasts and other mesenchymal cells. We cocultured osteoclast precursor cells with osteoblasts or osteocytes in the presence of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and prostaglandin E_2 (PGE_2), which revealed that osteocytes have a more potent ability to support osteoclastogenesis than osteoblasts (**Fig. 1g**). Despite their high level of RANKL expression, osteocytes did not induce osteoclastogenesis without $1,25(OH)_2D_3$ and PGE_2 (data not shown), suggesting that

[1]Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan. [2]Japan Science and Technology Agency, Exploratory Research for Advanced Technology Program, Takayanagi Osteonetwork Project, Yushima, Bunkyo-ku, Tokyo, Japan. [3]Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Yushima, Bunkyo-ku, Tokyo, Japan. [4]Department of Mechanical Engineering, Faculty of Engineering, Kyushu University, Motooka, Nishi-ku, Fukuoka, Japan. [5]Department of Biomedical Sciences, Baylor College of Dentistry, Texas A&M Health Science Center, Dallas, Texas, USA. [6]Department of Oral Biology, University of Missouri at Kansas City, Kansas City, Missouri, USA. [7]Research Center for Advanced Science and Technology, Department of Molecular Biology and Medicine, University of Tokyo, Tokyo, Japan. [8]Department of Biochemistry, The Wellcome Trust Centre for Stem Cell Research, University of Cambridge, Cambridge, UK. [9]Genes, Development and Disease Group, Banco Bilbao Vizcaya Argentaria Foundation, Cancer Cell Biology Program, Spanish National Cancer Center, Madrid, Spain. [10]Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria. [11]Center for Orthopaedic Research, School of Surgery, The University of Western Australia, Perth, Australia. Correspondence and requests for materials should be addressed to H.T. (taka.csi@tmd.ac.jp).

Received 25 May; accepted 25 July; published online 11 September 2011; doi:10.1038/nm.2452

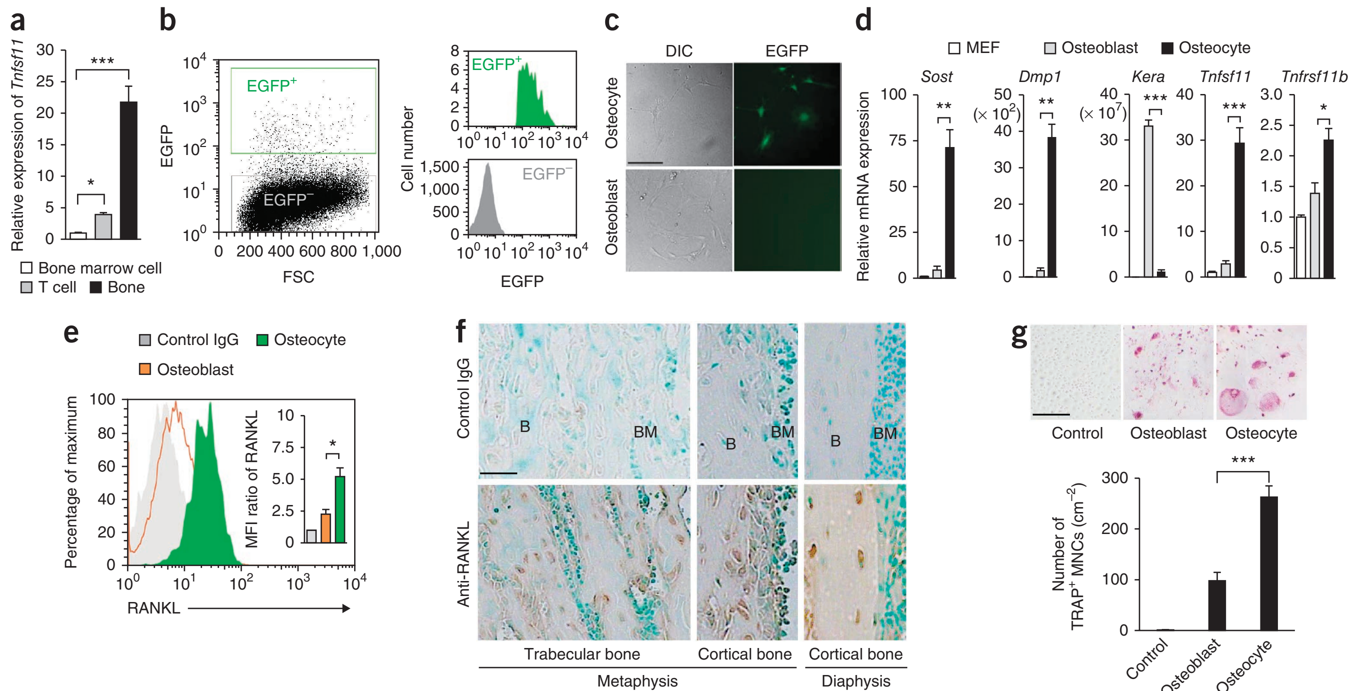

Figure 1 New method of isolating osteocytes and RANKL expression in osteocytes. (**a**) Quantitative RT-PCR analysis of *Tnfsf11* mRNA in bone tissue from which the bone marrow cells has been removed as compared to isolated bone marrow cells and T cells. (**b**,**c**) Separation of the EGFP-positive cell population by flow cytometry (**b**) and the morphology of the isolated cells (**c**). DIC, differential interference contrast microscopy. (**d**) Profiling of gene expression in mouse embryonic fibroblasts, osteoblasts and osteocytes (quantitative RT-PCR analysis). Osteoblasts and osteocytes were separated by the method described above. (**e**) Cell surface expression of RANKL in isolated osteocytes. MFI, mean fluorescence intensity. (**f**) Immunohistochemical analysis of RANKL expression in femur. Bone tissue was stained with control IgG (top) or RANKL-specific (anti-RANKL) antibodies (bottom). B, bone; BM, bone marrow. (**g**) Osteoclastogenesis in coculture with osteoblasts or osteocytes. Top, TRAP staining. Scale bars: **c**,**g**, 100 μm, **f**, 40 μm. Error bars, means ± s.e.m.; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. Mouse experiments were done with the approval of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

the downregulation of osteoprotegerin (encoded by the *Tnfrsf11b* gene) is also required for efficient osteoclastogenesis[12].

We applied the high-purity isolation method to the cells in the long bone and isolated BMSCs according to an established method (**Supplementary Methods** and **Supplementary Fig. 4a**). The osteocytes derived from long bone (**Supplementary Fig. 4b**,**c**) also expressed much more RANKL (**Supplementary Fig. 4a**,**c**,**d**) and had a more potent ability to support osteoclastogenesis (**Supplementary Fig. 4e**) than did osteoblasts or BMSCs.

To prove the importance of osteocytes as a source of RANKL *in vivo*, we generated mice carrying a $Tnfsf11^{flox}$ allele, in which exons 3 and 4 of the *Tnfsf11* gene were flanked by two *loxP* sequences (**Supplementary Fig. 5a**,**b**). Mice bearing a $Tnfsf11^{flox}$ allele were bred with transgenic mouse carrying the *Cre* transgene under the control of the β-actin promoter to generate the $Tnfsf11^{\Delta}$ allele (**Supplementary Fig. 5c**,**d**). These mice were then intercrossed with a $Tnfsf11^{flox/+}$ line to generate $Tnfsf11^{flox/\Delta}$ and $Tnfsf11^{+/\Delta}$ mice.

Global *Tnfsf11*-deficient mice ($Tnfsf11^{\Delta/\Delta}$), born at the expected Mendelian frequency (data not shown), showed growth retardation and a severe osteopetrotic phenotype identical to the phenotype observed in *Tnfsf11*-null mice[6] generated previously using a targeting vector in which the exon containing the tumor necrosis factor homology domain was disrupted (**Supplementary Fig. 6a**–**j**). We crossed $Tnfsf11^{flox/\Delta}$ mice with *Lck-Cre* mice to generate T cell–specific *Tnfsf11*-deficient mice, which did not show any discernible osteopetrotic phenotype (**Supplementary Fig. 7a**–**c**), indicating that the RANKL expressed on T cells does not substantially contribute to the physiological regulation of osteoclastogenesis.

To delete the *Tnfsf11* gene specifically in osteocytes, we crossed $Tnfsf11^{flox/\Delta}$ mice with *Dmp1-Cre* mice ($Tnfsf11^{flox/\Delta}$; *Dmp1-Cre* mice). To confirm the selective deletion of RANKL in osteocytes, we crossed $Tnfsf11^{flox/\Delta}$; *Dmp1-Cre* mice with CAG-CAT-EGFP reporter mice. We confirmed successful *Cre*-mediated recombination in osteocytes, but not in osteoblasts, at the genomic DNA and mRNA levels (**Supplementary Fig. 8a**,**b**). Flow cytometric (**Fig. 2a**) and immunohistochemical (**Supplementary Fig. 8c**) analyses indicated that RANKL was deleted in osteocytes, but not in osteoblasts. Notably, osteoctyte-specific RANKL deficiency diminished the osteoclastogenesis-supporting ability of osteocytes, but not that of osteoblasts (**Fig. 2b**). Mice deficient in *Tnfsf11* specifically in osteocytes did not show any gross abnormalities, such as defects in tooth eruption (**Supplementary Fig. 8d**), nor any growth retardation (**Supplementary Fig. 8e**). However, the bone volume was greatly increased, and the bone marrow cavity was abnormally filled with trabecular bone at the age of 12 weeks (**Fig. 2c**,**d**). The number of osteoclasts and the parameters of osteoclastic bone resorption were markedly reduced, and we observed cartilage remnants (**Fig. 2e**,**f**). These results indicate that mice deficient in *Tnfsf11* specifically in osteocytes show a severe osteopetrotic phenotype owing to a lack of osteoclasts. The bone formation rate (**Supplementary Fig. 9**) decreased, possibly through a coupling mechanism, but there was no abnormality in osteocyte density (**Fig. 2f**), morphology (data not shown) or marker gene expression, including that of *Sost* (**Supplementary Figs. 8b** and **10**), between the wild-type and osteocyte-specific *Tnfsf11*-deficient mice. Thus, osteocyte-derived RANKL has a crucial role in the regulation of osteoclastogenesis *in vivo*.

© 2011 Nature America, Inc. All rights reserved.

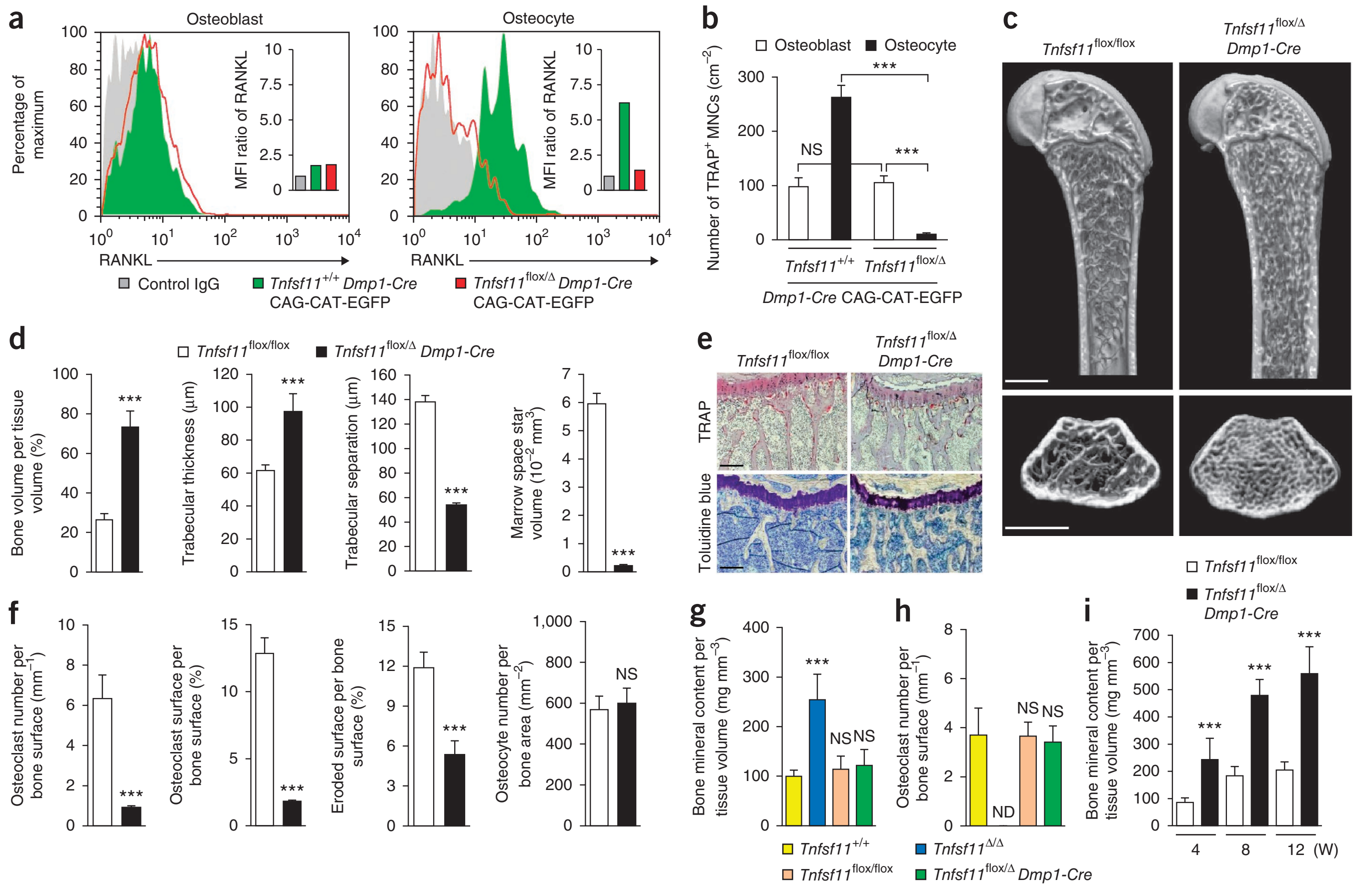

Figure 2 Osteopetrotic phenotype in osteocyte-specific *Tnfsf11*-deficient mice. (**a**) Selective deletion of RANKL in osteocytes, but not in osteoblasts, in *Tnfsf11*$^{flox/\Delta}$; *Dmp1-Cre* mice with the CAG-CAT-EGFP reporter. RANKL expression was analyzed by flow cytometry in $EGFP^+$ (osteocytes) and $EGFP^-$ (osteoblasts) populations. (**b**) Osteoclastogenesis-supporting ability of osteocytes and osteoblasts in *Tnfsf11*$^{flox/\Delta}$; *Dmp1-Cre* mice. (**c**) Microcomputed tomography (μCT) analysis of the femurs of *Tnfsf11*$^{flox/flox}$ and *Tnfsf11*$^{flox/\Delta}$; *Dmp1-Cre* littermates at 12 weeks of age (male, n = 6 or 7). Top, longitudinal view; bottom, axial view of the metaphyseal region. (**d**) Bone volume and parameters of trabecular bone in μCT analysis. (**e**) Histological analysis of the proximal tibia of *Tnfsf11*$^{flox/flox}$ and *Tnfsf11*$^{flox/\Delta}$; *Dmp1-Cre* littermates. (**f**) Bone morphometric analysis of *Tnfsf11*$^{flox/flox}$ and *Tnfsf11*$^{flox/\Delta}$; *Dmp1-Cre* littermates. (**g**) Trabecular bone mineral content per tissue volume in newborn mice in μCT analysis (postnatal day 1, male, n = 6 or 7). (**h**) Osteoclast number per bone surface (postnatal day 1). (**i**) Bone mineral content in *Tnfsf11*$^{flox/flox}$ and *Tnfsf11*$^{flox/\Delta}$; *Dmp1-Cre* littermates during growth (male, n = 5–7). Scale bars: **c**, 1 mm; **e**, 100 μm. Error bars, mean ± s.e.m.; $***P < 0.005$; NS, not significant; ND, not detected. Mouse experiments were done with the approval of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

© 2011 Nature America, Inc. All rights reserved.

Of note, no phenotype was evident in the osteocyte-specific *Tnfsf11*-deficient mice at birth (**Fig. 2g**,**h** and **Supplementary Fig. 11a**–**c**). However, a postnatal phenotype became increasingly obvious as the bone mass increased with age (**Fig. 2i**), in contrast to the *Tnfsf11*$^{\Delta/\Delta}$ mice, which had a markedly high bone mass at birth (**Fig. 2g**,**h** and **Supplementary Fig. 11a**). Thus, the RANKL expressed by osteocytes contributes more substantially to bone remodeling after birth than to the skeletal development in the embryo.

Osteocytes are mechanosensory cells, suggesting that RANKL on osteocytes may play a part in the response to mechanical stress, the importance of which increases after birth. Consistent with this notion, *Tnfsf11* expression was induced by mechanical stress inflicted on osteocyte-like cells (**Supplementary Fig. 12**). It is unclear how osteocytes express RANKL to such a greater degree than osteoblasts; both cell types equally express receptors for parathyroid hormone and $1,25(OH)_2D_3$ (refs. 16,17). The distinct bone- and age-dependent phenotypes in *Tnfsf11*$^{\Delta/\Delta}$ and osteocyte-specific *Tnfsf11*-deficient mice suggest that RANKL expressed by cells other than osteocytes have a bigger role in the regulation of osteoclastic bone resorption in the embryo, as well as tooth eruption and growth of long bones.

Osteocytes may play a key part in bone and mineral metabolism[8,18], but only a few molecules (for example, sclerostin and fibroblast growth factor-23) have been identified to be involved in this regulation[8]. Here we have provided genetic evidence showing that osteocytes regulate osteoclast differentiation through RANKL expression in bone remodeling. We cannot rule out the possibility that other cell types, including osteoblasts, are involved at distinct developmental stages and under certain pathological conditions[19,20]. Although the relative contribution of the membrane-bound and soluble forms of RANKL expressed by osteocytes remains to be elucidated, cell-cell contact was required for osteoclast formation in the coculture of osteocytes and osteoclast precursor cells (**Supplementary Fig. 13a**). Osteocyte-conditioned medium alone did not potently induce osteoclastogenesis (**Supplementary Fig. 13b**,**c**). These results suggest that membrane-bound RANKL has a more potent physiological role in osteocytes.

Anatomically, osteocytes can contact osteoclast precursor cells and mature osteoclasts through their long processes, which reach the bone surface and vascular space, thus directly communicating through membrane-bound factors[12,18]. As osteocytes originate from osteoblasts, it is technically difficult to compare osteoblast-specific deletion with osteocyte-specific deletion, which should be pursued in the future, but our study has clearly demonstrated that osteocytes are a crucial, if not exclusive, *in vivo* source of RANKL required for osteoclastogenesis. RANKL is a multifunctional cytokine that is involved in bone, the immune system, mammary gland development and in brain[1,9]. The source, function and relative contribution of RANKL in each system should be explored in the future, and mice bearing a *Tnfsf11*^{flox} allele will be a useful tool for such analyses. The key role of osteocyte expression of RANKL has shed light on an unexpected network among bone cells and may provide a molecular basis for future therapeutic approach to bone diseases.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We are grateful to J. Miyazaki for providing CAG-CAT-EGFP transgenic mice and U. Möhle-Steinlein for assisting the generation of A9 embryonic stem cells. We thank S. Fukuse, T. Suda-Kayamori, T. Nishioka-Honda, T. Ando, Y. Kunisawa, E. Idrus, K. Nishikawa, H. Inoue, K. Okamoto, T. Negishi-Koga, M. Shinohara, L. Bakiri, Ö. Uluçkan, N. Amizuka, K. Kayamori, A. Yamaguchi and S. Iseki for discussion and assistance. We also thank T. Wada, H. Hara, Y. Nakashima, R. Hanada, T. Hanada, A. Leibbrant, S.J. Cronin, G.G. Neely, N. Joza, J.A. Pospisilik and A. Meixner for technical assistance. This work was supported in part by a grant for the Exploratory Research for Advanced Technology Program, the Takayanagi Osteonetwork Project from the Japan Science and Technology Agency; Grant-in-Aids for Young Scientist A from the Japan Society for the Promotion of Science (JSPS); a Grant-in-Aid for Challenging Exploratory Research from the JSPS; grants for the Global Center of Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and grants from the Tokyo Biochemical Research Foundation, the Life Science Foundation of Japan, Takeda Science Foundation, Uehara Memorial Foundation, Nakatomi Foundation, Nagao Memorial Foundation, Kowa Life Science Foundation, Naito Foundation, Ichiro Kanehara Foundation, Senri Life Science Foundation and Astellas Foundation for Research on Metabolic Disorders.

© 2011 Nature America, Inc. All rights reserved.

AUTHOR CONTRIBUTIONS

T.N. generated conditional knockout mice, performed most of the experiments, interpreted the results and prepared the manuscript. M.H. participated in the *in vivo* analyses of the mice and prepared the manuscript. T.F. and K.K. performed experiments using the three-dimensional gel-embedded cell culture system and contributed to the osteocyte isolation experiments. M.O. assisted the *in vivo* analyses of the mice. J.Q.F. and L.F.B. provided *Dmp1-Cre* deleter mice and advice on project planning and data interpretation. L.F.B. also provided the osteocyte-like cell line MLO-Y4. T.K. conducted the GeneChip analysis. A.W. and E.F.W. generated embryonic stem cells and provided technical help. J.M.P. provided advice on project planning. H.T. directed, supervised the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemedicine/.
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Takayanagi, H. *Nat. Rev. Immunol.* **7**, 292–304 (2007).
2. Takahashi, N. *et al. Endocrinology* **123**, 2600–2602 (1988).
3. Chambers, T.J., Owens, J.M., Hattersley, G., Jat, P.S. & Noble, M.D. *Proc. Natl. Acad. Sci. USA* **90**, 5578–5582 (1993).
4. Rodan, G.A. & Martin, T.J. *Calcif. Tissue Int.* **33**, 349–351 (1981).
5. Suda, T. *et al. Endocr. Rev.* **20**, 345–357 (1999).
6. Kong, Y.Y. *et al. Nature* **397**, 315–323 (1999).
7. Kim, N., Odgren, P.R., Kim, D.K., Marks, S.C. Jr. & Choi, Y. *Proc. Natl. Acad. Sci. USA* **97**, 10905–10910 (2000).
8. Bonewald, L.F. *J. Bone Miner. Res.* **26**, 229–238 (2011).
9. Hanada, R., Hanada, T., Sigl, V., Schramek, D. & Penninger, J.M. *J. Mol. Med.* **89**, 647–656 (2011).
10. Sobacchi, C. *et al. Nat. Genet.* **39**, 960–962 (2007).
11. Gu, G., Nars, M., Hentunen, T.A., Metsikko, K. & Vaananen, H.K. *Cell Tissue Res.* **323**, 263–271 (2006).
12. Kramer, I. *et al. Mol. Cell. Biol.* **30**, 3071–3085 (2010).
13. Paic, F. *et al. Bone* **45**, 682–692 (2009).
14. Kawamoto, S. *et al. FEBS Lett.* **470**, 263–268 (2000).
15. Lu, Y. *et al. J. Dent. Res.* **86**, 320–325 (2007).
16. Franz-Odendaal, T.A., Hall, B.K. & Witten, P.E. *Dev. Dyn.* **235**, 176–190 (2006).
17. O'Brien, C.A. *et al. PLoS ONE* **3**, e2942 (2008).
18. Seeman, E. & Delmas, P.D. *N. Engl. J. Med.* **354**, 2250–2261 (2006).
19. Martin, T.J. *J. Musculoskelet. Neuronal Interact.* **4**, 243–253 (2004).
20. Pivonka, P. *et al. Bone* **43**, 249–263 (2008).