HIF1 α is required for osteoclast activation by estrogen deficiency in postmenopausal osteoporosis

Yoshiteru Miyauchi^{a,b}, Yuiko Sato^{a,c}, Tami Kobayashi^{a,d}, Shigeyuki Yoshida^e, Tomoaki Mori^a, Hiroya Kanagawa^a, Eri Katsuyama^a, Atsuhiko Fujie^a, Wu Hao^a, Kana Miyamoto^{a,d}, Toshimi Tando^a, Hideo Morioka^a, Morio Matsumoto^a, Pierre Chambon^f, Randall S. Johnson^g, Shigeaki Kato^h, Yoshiaki Toyama^a, and Takeshi Miyamoto^{a,b,d,1}

Departments of ^aOrthopedic Surgery, ^bIntegrated Bone Metabolism and Immunology, and ^cMusculoskeletal Reconstruction and Regeneration Surgery, ^dKeio Kanrinmaru Project, and ^eDepartment of Dentistry and Oral Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan; ^fDepartment of Functional Genomics, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Collège de France, Université de Strasbourg, FR-67404 Illkirch, France; ^gMolecular Biology Section, Division of Biological Sciences, School of Medicine, University of California, San Diego, La Jolla, CA 92093; and ^hDepartment of Radiation Protection, Soma Central Hospital, Fukushima 976-0013, Japan

Edited by Sakae Tanaka, The University of Tokyo, Tokyo, Japan, and accepted by the Editorial Board August 16, 2013 (received for review May 8, 2013)

In women, estrogen deficiency after menopause frequently accelerates osteoclastic bone resorption, leading to osteoporosis, the most common skeletal disorder. However, mechanisms underlying osteoporosis resulting from estrogen deficiency remain largely unknown. Here we show that in bone-resorbing osteoclasts, estrogen-dependent destabilization of hypoxia-inducible factor 1 alpha (HIF1 α), which is unstable in the presence of oxygen, plays a pivotal role in promoting bone loss in estrogen-deficient conditions. In vitro, HIF1 α was destabilized by estrogen treatment even in hypoxic conditions, and estrogen loss in ovariectomized (Ovx) mice stabilized HIF1 α in osteoclasts and promoted their activation and subsequent bone loss in vivo. Osteoclast-specific HIF1 α inactivation antagonized bone loss in Oxv mice and osteoclast-specific estrogen receptor alpha deficient mice, both models of estrogen-deficient osteoporosis. Oral administration of a HIF1 α inhibitor protected Oxv mice from osteoclast activation and bone loss. Thus, HIF1 α represents a promising therapeutic target in osteoporosis.

Bone mass is tightly regulated by a delicate balance between osteoblastic bone formation and osteoclastic bone resorption. Estrogen loss in women after menopause frequently promotes activation of osteoclastic bone resorption, causing osteoporosis. Osteoporotic bone phenotypes are seen in ovariectomized female mice, and estrogen deficiency-induced bone loss in both mouse models and women is ameliorated by estrogen treatment (1, 2). However, estrogen administration reportedly increases risk of cardiovascular events and carcinogenesis of the mammary gland and uterus (3). Bioavailable estrogens including selective estrogen receptor modulators (SERMs) also protect bone from estrogen deficiency-induced osteoporosis (4), and estrogen and SERMs primarily act via estrogen receptors (ER), ER α and ER β (5, 6). However, how SERMs act on bone remains largely unknown. Thus, understanding of osteoclast activation following estrogen loss is crucial for development of safe therapeutic reagents.

Both the endosteal zone of bone marrow cavities and epiphyseal growth plates are hypoxic areas, and the hypoxia-inducible factor (HIF) signaling pathway governs chondrocyte and osteoblast function in these respective areas (7, 8). The HIF1 transcription factor consists of an oxygen-regulated α subunit, HIF1 α , and a constitutively expressed β subunit, HIF1 β . Under normoxia, HIF1 α is posttranslationally modified by prolyl hydroxylases, which catalyze hydroxylation of proline residues in the presence of O₂ and Fe²⁺. Recognition of hydroxylated HIF1 α by the von Hippel-Lindau tumor suppressor protein recruits an E3 ubiquitin ligase complex targeting HIF1 α for proteasomal degradation. Conversely, under hypoxia, proline hydroxylation is inhibited by substrate (O₂) deprivation, allowing HIF1 α accumulation and formation of an active transcriptional complex with HIF1 β (9). Recently, regulation of HIF1 α protein levels by factors other than O₂, including reactive oxygen species and growth factors, has been reported (10), although hormonal HIF1 α regulation has not been characterized.

HIF1 α plays specific roles in vasculogenesis and tumorigenesis (11). Stabilization and activation of HIF1 α in an avascular area due to hypoxia promotes vascularization by inducing typical HIF1 α target genes, such as vascular endothelial growth factor (VEGF) (12). Such neovascularization through HIF1 α -induced VEGF is activated in tumor growth, ischemic collateral formation, and wound healing (12–14). HIF1 α is also reportedly activated and functions in tumor development in renal cell carcinoma (15).

Here we show that HIF1 α is suppressed by estrogen in osteoclasts and that loss of estrogen stabilizes HIF1 α in those cells, which in turn activates bone resorption and promotes bone loss. Inactivating HIF1 α , either genetically or pharmacologically, protected mice from estrogen depletion and concomitant osteoclast activation and bone loss. Our observations confirm HIF1 α as a potent therapeutic target for osteoporosis.

Results

Estrogen Depletion Stabilizes HIF1 α in Osteoclasts in Vivo. HIF1 α is reportedly stabilized in hypoxic regions such as the endosteal zone of bone marrow cavities and growth plates (16). Indeed, immunofluorescence analysis indicated that HIF1 α was stabilized in chondrocytes of the mouse growth plate (Fig. S1). By contrast, we

Significance

Estrogen deficiency after menopause frequently accelerates osteoclastic bone resorption, leading to osteoporosis, the most common skeletal disorder in women. However, mechanisms underlying osteoporosis resulting from estrogen deficiency remain largely unknown. We report a unique mechanism underlying postmenopausal osteoporosis, and a therapeutic target, hypoxia-inducible factor 1 alpha (HIF1 α), to treat this condition. HIF1 α is unstable in the presence of oxygen, but is stabilized under hypoxic conditions. However, we found that HIF1 α is destabilized by estrogen even under hypoxic conditions. Following estrogen deficiency due to menopause, HIF1 α is stabilized in osteoclasts, leading to osteoclast activation. Oral administration of a HIF1 α inhibitor protected postmenopausal osteoporosis model mice from osteoclast activation and bone loss. Thus, HIF1 α represents a promising therapeutic target in osteoporosis.

Author contributions: H.M., M.M., Y.T., and T. Miyamoto designed research; Y.M. and Y.S. performed research; T.K., P.C., R.S.J., and S.K. contributed new reagents/analytic tools; S.Y., T. Mori, H.K., E.K., A.F., W.H., K.M., and T.T. analyzed data; and T. Miyamoto wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. S.T. is a guest editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

¹To whom correspondence should be addressed. E-mail: miyamoto@z5.keio.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1308755110/-DCSupplemental.

Fig. 1. Estrogen destabilizes HIF1 α in vivo and in vitro. (A) Immunofluorescence for HIF1 α (green) and Ctsk (red) in bone sections from Sham or Ovx mice. Nuclei are stained with TOTO-3 (blue). *, HIF1 α /Ctsk double-positive cells. (B) *Hif1 α* mRNA levels in C-Fms⁺Mac1^{high} osteoclasts sorted from Sham or Ovx mouse bone marrow as determined by quantitative PCR. Cells were prepared 5 wk after surgery. (C, Left) Immunofluorescent detection of hypoxic areas (Pimo, green) and Ctsk (red) on bone sections from Sham or Ovx mice. Nuclei are stained with TOTO-3 (blue). (Scale bar, 100 μ m.) (C, Right) Signal intensities of Pimo and Ctsk shown as mean values relative to TOTO-3 $\pm$ SD (n = 3). (D) Western blot analysis of HIF1 α in Raw264.7 cells cultured with or without E2. (E) *Hif1 α* mRNA levels in Raw264.7 cells as determined by quantitative PCR in the presence or absence of E2 under normoxic or hypoxic conditions. Data in B and E represent mean HIF1 α expression relative to *Actb* $\pm$ SD (n = 3).

detected lower levels of HIF1 α protein in Cathepsin K (Ctsk)-positive osteoclasts localized to the hypoxic endosteal zone of sham-operated (Sham) mice compared with growth plate chondrocytes (Fig. 1A). However, ovariectomy (Ovx), which results in estrogen depletion, induced HIF1 α stabilization in osteoclasts (Fig. 1A). Osteoclasts are reportedly c-Fms⁺Mac1^{high} (17); interestingly, *Hif1 α* expression levels in sorted c-Fms⁺Mac1^{high} osteoclasts were comparable between Sham and Ovx mice (Fig. 1B), further suggesting that estrogen depletion stabilizes HIF1 α protein rather than inducing *Hif1 α* mRNA in osteoclasts. Assessment of hypoxia using pimonidazole (Pimo) (18), which detects low oxygen tension areas, showed that the hypoxic state was comparable in the endosteal zone of Sham and Ovx mouse bones (Fig. 1C). These results suggest that estrogen deficiency stabilizes HIF1 α in osteoclasts without altering hypoxic conditions in the endosteal zone. In contrast, the uterus is an estrogen-responsive tissue; however, HIF1 α protein was not stabilized in the uterus following Ovx (Fig. S2), suggesting that HIF1 α protein stabilization following estrogen deficiency is tissue-specific.

To confirm estrogen-dependent HIF1 α destabilization in osteoclasts, we used immunoblotting to detect HIF1 α in an osteoclast progenitor cell line, Raw264.7 cell, and primary osteoclasts derived from wild-type mouse bone marrow cells treated with or without the β -estradiol (E2) in vitro (Fig. 1D and Fig. S3). Under hypoxic conditions, HIF1 α protein was stabilized in the absence of E2 but destabilized by treatment with E2 (Fig. 1D and Fig. S3). Quantitative PCR analysis showed that *Hif1 α* was induced in osteoclasts over the course of differentiation stimulated by receptor activator of nuclear factor kappa B ligand (RANKL), while the expression of *Hif2 α* , a different hypoxia-inducible transcription factor, was not detected (Fig. S4). *Hif1 α* mRNA levels were unchanged in osteoclasts treated with E2 in both hypoxia and normoxia (Fig. 1E), strongly suggesting that it is HIF1 α protein levels that are destabilized by E2, even in hypoxic conditions.

Osteoclast-Specific HIF1 α Deletion Protects Bones from Ovx-Induced Osteoporosis. To analyze the role of E2-dependent HIF1 α protein

destabilization in osteoclasts in vivo, we generated osteoclast-specific HIF1 α conditional knockout mice (HIF1 α cKO: *Ctsk*^{Cre/+}; *Hif1 α* ^{fl/fl}) by crossing Ctsk-Cre mice, in which Cre recombinase was knocked into Ctsk gene locus, with *Hif1 α* -floxed mice (Fig. 2).

HIF1 α cKO mice exhibit little change in bone mineral density (BMD), as measured by dual energy X-ray absorptiometry (DEXA) compared with control (Sham) mice (Fig. 2 *A* and *B*). Osteoclast formation detected by tartrate resistance acid phosphatase (TRAP) staining was normal in HIF1 α cKO mice (Fig. S5*A*). Bone morphometric analysis demonstrated that parameters such as bone volume per tissue volume (BV/TV), osteoblast surface per bone surface (Ob.S/BS), osteoclast surface per bone surface (Oc.S/BS), and bone formation rate per bone surface (BFR/BS) did not differ between HIF1 α cKO and control mice (Fig. S5*B*). However, although Sham HIF1 α cKO mice exhibited few bone phenotypes in vivo (Fig. 2*A* and Fig. S5 *A* and *B*), they were protected from Ovx-induced bone loss (Fig. 2 *A* and *B*). Osteoclast progenitor cells were isolated from HIF1 α cKO and

Fig. 2. HIF1 α cKO mice are resistant to ovariectomy-induced bone loss and osteoclast activation. (A and B) BMD of femurs divided equally longitudinally from Sham or Ovx control (Hif1 α^{flf}) and HIF1 α cKO (Ctsk^{Cre/+}; Hif1 α^{flf}) mice. (C) ES/BS of femurs from sham-operated or ovariectomized control and HIF1 α cKO mice. (D) Levels of serum CTx, a marker of bone resorption, in control and HIF1 α cKO mice. Analysis was undertaken 5 wk after Ovx or Sham surgery. All data are means $\pm$ SD ($n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; NS, not significant.

HIF1 α and ER α double conditional knockout mice (WcKO: *Ctsk*^{Cre/+}; *Hif1 α* ^{fl/f}; *ER α* ^{fl/f}). Significantly, decreased BMD seen in ER α cKO mice relative to controls was rescued in WcKO mice (Fig. 3B). In addition, bone morphometric analysis demonstrated that reduced BV/TV, trabecular number (Tb.N), and increased trabecular separation (Tb.Sp) seen in ER α cKO were restored to basal levels in WcKO mice (Fig. 3C). Indicators of increased osteoclastogenesis seen in ER α cKO mice, such as osteoclast number per bone perimeter (Oc.N/B.Pm), Oc.S/BS, and erosion surface per bone surface (ES/BS), were negated in WcKO mice (Fig. 3C), indicating that estrogen/ER α -dependent HIF1 α destabilization antagonizes acceleration of osteoclast activation and bone loss. In ER α cKO cells grown in vitro, osteoclastogenesis was not facilitated in normoxia but was significantly stimulated in hypoxic conditions, as evidenced by TRAP staining (Fig. 3D). Furthermore, marker analysis indicated that accelerated osteoclastogenesis seen in ER α cKO cells under hypoxia was inhibited

in WcKO cells (Fig. 3D). These results indicate that ER α is required in osteoclasts for HIF1 α stability shifts seen in the presence of estrogen. HIF1 α protein is reportedly targeted by the ubiquitin-proteasome system (9). To elucidate molecular mechanisms underlying estrogen-induced HIF1 α destabilization, we treated Raw264.7 cells with inhibitors of serine proteases, cysteine proteases, calpains, or the proteasome in the presence of E2 under hypoxic conditions. However, none of these inhibitors blocked estrogen-induced destabilization of HIF1 α (Fig. S7). These results suggest that estrogen-induced HIF1 α destabilization is mediated via a unique mechanism.

Administration of a HIF1 α -Inhibitor Protects Bone from Ovx-Induced Osteoporosis. Because HIF1 α stabilization following Ovx-induced estrogen deficiency is predicted to promote bone loss, we evaluated phenotypes seen in cultured cells and Ovx mice following treatment with 2-methoxyestradiol (2ME2), a specific inhibitor

Fig. 4. Pharmacological HIF1 α ablation antagonizes Ovx-induced bone loss and osteoclast activation. (A) Suppression of HIF1 α protein expression in Raw264.7 cells by E2 or 2ME2 in hypoxic conditions. (B) Effects of E2 or 2ME2 on *Hif1 α* mRNA levels in Raw264.7 cells. Data represent mean *Hif1 α* expression relative to that of *Actb* $\pm$ SD ($n = 5$). (C, *Top, Middle, and Bottom*) BMD of femurs from Sham or Ovx treated with vehicle or 2ME2. (D) ES/BS of femurs from Sham or Ovx treated with vehicle or 2ME2. (E) Serum CTx of Sham or Ovx mice treated with vehicle or 2ME2. Error bars indicate means $\pm$ SD ($n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; NS, not significant.

staining (17, 23). Raw264.7 cell was maintained in DMEM (Sigma-Aldrich Co.) containing 10% (vol/vol) heat-inactivated FBS (JRH Biosciences) and Gluta-Max (Invitrogen Corp.). For chemical treatment, cells were cultured in phenol red-free media containing 10% (vol/vol) charcoal-stripped FBS (Thermo Fisher Scientific K.K.), and treated by 17 β -estradiol (E2; Wako Pure Chemicals Industries, 10⁻⁶ M) or 2-methoxyestradiol (2ME2; EMD Millipore Corporation, 10⁻⁵ M). Hypoxic culture was performed at 5% O₂ and 5% CO₂ using an INVIVO2 hypoxia workstation (Ruskin Technology Ltd.) according to the manufacturer's instructions. Protease inhibitor treatment was carried out under hypoxic conditions in the presence of E2 using 4-(2-aminoethyl)-benzenesulfonyl fluoride as a serine protease (AEBSF; Wako Pure Chemicals Industries, 10⁻⁴ M), E-64d as a cysteine protease (Wako Pure Chemicals Industries, 10⁻⁵ M), ALLN as a calpain (EMD Millipore Corporation, 10⁻⁵ M) inhibitor, or MG-132 as a proteasome (EMD Millipore Corporation, 10⁻⁵ M) inhibitor.

Quantitative PCR Analysis. Total RNAs were isolated from bone marrow cultures by TRIzol reagent (Invitrogen Corp.), and cDNA synthesis was done by using oligo(dT) primer and reverse transcriptase (Wako Pure Chemicals Industries). Quantitative PCR was performed using SYBR Premix ExTaq II reagent and the DICE Thermal cycler (Takara Bio Inc.), according to the manufacturer's instructions. Samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR reactions. β -actin (*Actb*) expression served as an internal control. Primers for *Nfatc1*, *Ctsk*, *Dc-stamp*, and *Actb* were described previously (24). Other primers were purchased from Takara Bio Inc.

Immunoblotting. Whole cell lysates were prepared using radio immunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH7.5) supplemented with a protease inhibitor mixture (Sigma-Aldrich Co.) and MG-132 (EMD Millipore Corporation), and the insoluble fraction was removed by centrifugation followed with Bradford protein analysis (Bio-Rad Laboratories Inc.). Equivalent amounts of protein were separated by SDS/PAGE and transferred to a PVDF membrane (EMD Millipore Corporation). To quantify immunoblotting and control for nonequivalent protein loading, we used densitometric analysis using ImageJ software. Proteins were detected using the following antibodies: anti-HIF1 α (Novus Biologicals), anti-ER α (Santa Cruz Biotechnology, Inc), and anti-Vinculin and anti-Tubulin (Sigma-Aldrich Co.).

Immunohistochemical Analysis. Mouse femurs were dissected, fixed in 10% buffered formalin, decalcified in 8% Na₂EDTA, paraffin-embedded, and subjected to immunohistochemistry. Antigen retrieval was performed by treatment with 0.4 mg/mL Proteinase K for 8 min at room temperature. Sections were incubated in 5% BSA-PBS and then incubated with antibodies against HIF1 α (Novus Biologicals) and Ctsk (Daiichi Finechemical Co.). Nuclei

were counterstained using TOTO-3 (Invitrogen Corp.). Detection of low oxygen tension areas in tissue was performed using pimonidazole (Hypoxyprobe-1 Kit; Hypoxyprobe Inc.) according to the manufacturer's instructions. A confocal laser scanning microscope (FV1000; OLYMPUS Corp.) was used to examine immunostained sections. Quantification of signal intensity was performed by using operating software for FV1000 according to the manufacturer's manual.

ER α Knockdown. Raw264.7 transformants were generated using MISSION shRNA lentiviruses targeting ER α or nontarget control constructs (Sigma-Aldrich Co.) according to the manufacturer's instructions.

2ME2 Treatment. Wild-type C57BL/6 mice were obtained from CLEA Japan, Inc., and subjected to ovariectomy or sham operation at 12 wk old, immediately followed by 2ME2 (EMD Millipore Corporation) treatment. The 2ME2 was prepared at 15 mg/mL in a 0.5% methylcellulose solution (Wako Pure Chemicals Industries) and administered daily at 75 mg/kg via intragastric infusion for 4 wk. Vehicle groups were treated with solvent.

Analysis of Skeletal Morphology. Sham or OvX HIF1 α cKO and control littermates were necropsied, and their hind limbs were removed, fixed with 70% ethanol, and subjected to DEXA analysis to measure bone mineral density and for bone histomorphometric analysis. Bones were collected from female HIF1 α cKO, ER α cKO, and control littermates 8 wk of age. Mice treated with 2ME2 or vehicle were used for bone analysis at the end of drug administration. Mice were additionally administered 16 mg/kg calcein (Dojindo Co.) injected intraperitoneally at 6 and 1 d before sacrifice to evaluate bone formation rate.

TUNEL Staining. Paraffin-embedded sections of decalcified femurs from Sham or OvX mice were subjected to TUNEL staining. Experiments were carried out using a MEBSTAIN Apoptosis TUNEL Kit (MBL Co. Ltd.) following the manufacturer's instructions. After staining, sections were reacted with an anti-Ctsk antibody and TOTO-3.

ELISA. C-terminal telopeptide of type I collagen (CTX) serum levels were measured using a RatLaps EIA kit (Immunodiagnostic Systems Ltd.) based on the manufacturer's instructions.

Statistical Analyses. Statistical analyses were performed using the unpaired two-tailed Student's *t* test (**P* < 0.05; ***P* < 0.01; ****P* < 0.005; NS, not significant, throughout the paper). All data are expressed as the mean $\pm$ SD.

ACKNOWLEDGMENTS. We thank Prof. M. Suematsu and Dr. Y. A. Minamishima for critical reading of the manuscript. T. Miyamoto was supported by a Grant-in-Aid for Scientific Research, the Takeda Science Foundation. Y.M. was supported by a Grant-in-Aid for Scientific Research.

- Delmas PD (2002) Treatment of postmenopausal osteoporosis. *Lancet* 359(9322): 2018–2026.
- Rodan GA, Martin TJ (2000) Therapeutic approaches to bone diseases. *Science* 289(5484):1508–1514.
- Nelson ER, Wardell SE, McDonnell DP (2013) The molecular mechanisms underlying the pharmacological actions of estrogens, SERMs and oxysterols: Implications for the treatment and prevention of osteoporosis. *Bone* 53(1):42–50.
- Riggs BL, Hartmann LC (2003) Selective estrogen-receptor modulators—Mechanisms of action and application to clinical practice. *N Engl J Med* 348(7):618–629.
- Couse JF, Korach KS (1999) Estrogen receptor null mice: What have we learned and where will they lead us? *Endocr Rev* 20(3):358–417.
- Shang Y, Brown M (2002) Molecular determinants for the tissue specificity of SERMs. *Science* 295(5564):2465–2468.
- Schipani E, et al. (2001) Hypoxia in cartilage: HIF-1 α is essential for chondrocyte growth arrest and survival. *Genes Dev* 15(21):2865–2876.
- Wang Y, et al. (2007) The hypoxia-inducible factor α pathway couples angiogenesis to osteogenesis during skeletal development. *J Clin Invest* 117(6):1616–1626.
- Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. *Cell* 148(3): 399–408.
- Koh MY, Spivak-Kroizman TR, Powis G (2008) HIF-1 regulation: Not so easy come, easy go. *Trends Biochem Sci* 33(11):526–534.
- Ryan HE, et al. (2000) Hypoxia-inducible factor-1 α is a positive factor in solid tumor growth. *Cancer Res* 60(15):4010–4015.
- Ahluwalia A, Tarnawski AS (2012) Critical role of hypoxia sensor—HIF-1 α in VEGF gene activation. Implications for angiogenesis and tissue injury healing. *Curr Med Chem* 19(1):90–97.
- Liao D, Johnson RS (2007) Hypoxia: A key regulator of angiogenesis in cancer. *Cancer Metastasis Rev* 26(2):281–290.
- Andrikopoulou E, et al. (2011) Current insights into the role of HIF-1 in cutaneous wound healing. *Curr Mol Med* 11(3):218–235.
- Keith B, Johnson RS, Simon MC (2012) HIF1 α and HIF2 α : Sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer* 12(1):9–22.
- Maes C, Carmeliet G, Schipani E (2012) Hypoxia-driven pathways in bone development, regeneration and disease. *Nat Rev Rheumatol* 8(6):358–366.
- Miyamoto T, et al. (2000) An adherent condition is required for formation of multinuclear osteoclasts in the presence of macrophage colony-stimulating factor and receptor activator of nuclear factor kappa B ligand. *Blood* 96(13):4335–4343.
- Fujita N, et al. (2008) Vascular endothelial growth factor-A is a survival factor for nucleus pulposus cells in the intervertebral disc. *Biochem Biophys Res Commun* 372(2): 367–372.
- Nakamura T, et al. (2007) Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell* 130(5):811–823.
- Mabjeesh NJ, et al. (2003) 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell* 3(4):363–375.
- Arjamaa O, Nikinmaa M (2006) Oxygen-dependent diseases in the retina: Role of hypoxia-inducible factors. *Exp Eye Res* 83(3):473–483.
- Dupont S, et al. (2000) Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* 127(19):4277–4291.
- Yagi M, et al. (2005) DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *J Exp Med* 202(3):345–351.
- Miyauchi Y, et al. (2010) The Blimp1-Bcl6 axis is critical to regulate osteoclast differentiation and bone homeostasis. *J Exp Med* 207(4):751–762.