Receptor activator of NF- κ B ligand induces the fusion of mononuclear preosteoclasts into multinucleated osteoclasts

Je-Tae Woo*, Masanori Kato, Masamichi Takami & Kazuo Nagai

Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

(* Author for correspondence)

Received 18 June 1999; accepted 18 September 1999

Key words: fusion, IL-1 β , NF- κ B, osteoclast, RANKL

Abstract

The osteoclasts are bone-resorbing multinucleated cells formed by the fusion of mononuclear preosteoclasts (pOCs) of hematopoietic origin. Although receptor activator of NF- κ B ligand (RANKL) has been shown to regulate osteoclast differentiation and function, its effect on the fusion of pOCs into multinucleated osteoclast-like cells (OCLs) has not been known. Using our fusion assay system, that is not contaminated with multinucleated cells (MNCs) and osteoblastic cells, we determined the effect of RANKL on the fusion of pOCs into MNCs. When pOCs were cultured on the plates, most of pOCs died and disappeared from the plates within 24 h in the absence of additives, but pOCs were fused to MNCs within 6 h in the presence of RANKL. RANKL-induced MNCs showed typical properties of OCL such as tartrate-resistant acid phosphatase (TRAP) activity, actin ring formation, and bone-resorbing activity. The fusion of pOCs into OCLs induced by osteoblastic cells or RANKL was inhibited by OPG/OCIF, but that induced by IL-1 β was not. Both RANKL- and IL-1 β -induced OCL formation from pOCs was inhibited by ZLLL-H, a peptide inhibitor of proteasome. These findings indicate that RANKL supports the survival of pOCs and induces the fusion of pOCs into OCLs and suggest that NF- κ B activation is involved in these processes induced by RANKL and IL-1 β .

Abbreviations: 1,25(OH) $_2$ D $_3$, 1 α ,25-dihydroxyvitamin D $_3$; FCS, fetal calf serum; I κ B, inhibitor of NF- κ B; IL-11, interleukin-11; IL-1 β , interleukin-1 β ; MEM, minimal essential medium; MNCs, multinucleated osteoclasts; NF- κ B, nuclear factor κ B; OCIF, osteoclastogenesis inhibitory factor; OCLs, multinucleated osteoclast like cells; ODF, osteoclast differentiation factor; OPG, osteoprotegerin; PGE $_2$, prostaglandin E $_2$; pOCs, mononuclear preosteoclasts; PTH, parathyroid hormone; RANKL, receptor activator of NF- κ B ligand; TRAF, TNF receptor-associated factor; TRANCE, TNF-related activation induced cytokine; TRAP, tartrate-resistant acid phosphatase; ZLLL-H, carbobenzoxy-leuciny-leuciny-leucinal.

Introduction

Osteoclasts are bone-resorbing multinucleated cells (having between 2 to 100 nuclei per cell) that play a critical role in bone remodeling. They are formed by the fusion of mononuclear preosteoclasts (pOCs), that are differentiated from hematopoietic precursors (Athanasou, 1996; Roodman, 1996; Suda et al., 1996). Osteoclasts are activated to have bone-resorbing activity (Jimi et al., 1999; Wesolowski et

al., 1995). It has been shown that osteoblastic/stromal cells are essential for osteoclast differentiation and activation (Suda et al., 1995, 1996). To study the mechanism of osteoclast differentiation and function, a co-culture system of mouse bone marrow cells and osteoblastic/stromal cells has been used (Roodman, 1996; Suda et al., 1996). Multinucleated osteoclast-like cells (OCLs) have been known to be formed in the cultures containing osteoclast-inducing factors such as 1 α , 25-dihydroxyvitamin D $_3$ [1,25(OH) $_2$ D $_3$], para-

thyroid hormone and interleukin 11 (IL-11) (Takahashi et al., 1988). Due to the difficulty of isolating the pOCs without contamination of MNCs and osteoblastic cells, the mechanism of the fusion of pOCs has hardly been known. We have established a procedure to isolate pOCs without contamination with MNCs and osteoblastic cells from co-cultures of mouse osteoblastic cells and bone marrow cells in the presence of 1,25(OH)₂D₃ (Takami et al., 1998). Using the isolated pOCs, we have developed a fusion assay system and reported that osteoblasts are essential for the fusion of pOCs into OCLs (Takami et al., 1998).

Receptor activator of NF- κ B ligand (RANKL, Wong et al., 1997a, b), also called TNF-related activation induced cytokine (TRANCE, Anderson et al., 1997), osteoclast differentiation factor (ODF, Yasuda et al., 1998), osteoprotegerin ligand (OPGL, Lacey et al., 1998), has been identified as a novel member of the TNF superfamily that regulates immune responses and bone resorption. RANKL, expressed in T cells, has been reported to support the survival of dendritic cells (Wong et al., 1997a, b). Furthermore, RANKL expressed in bone marrow stromal or osteoblastic cells that are stimulated with bone-resorbing factors such as PTH, 1,25(OH)₂D₃, IL-11 and PGE₂, has been shown to induce osteoclasts differentiation from their progenitors in the presence of M-CSF in the absence of stromal or osteoblastic cells and to stimulate osteoclastic bone resorption (Yasuda et al., 1998; Lacey et al., 1998). It was demonstrated that RANKL-induced osteoclast formation is inhibited by osteoprotegerin (OPG; also called OCIF), which is a soluble member of the TNFR superfamily (Yasuda et al., 1998; Lacey et al., 1998). Recently, RANK, which is a member of the TNF receptor superfamily that activates NF- κ B, has been reported to be an essential signaling receptor for osteoclast differentiation induced by RANKL (Nakagawa et al., 1998; Hsu et al., 1999). IL-1 β is known to promote the survival of mature osteoclasts through IL-1 type I receptors by stimulating NF- κ B activation (Jimi et al., 1995, 1998; Suda et al., 1997). Recently, IL-1 β has been reported to induce multinucleation of osteoclasts in the absence of osteoblastic/stromal cells (Jimi et al., 1999). The effect of RANKL on the survival of pOCs and the fusion of pOCs into OCLs has not been determined, although it was revealed that RANKL clearly induces osteoclast differentiation and stimulates the function of mature osteoclasts.

The object of this study is to examine the effect of RANKL on the survival of pOCs and the fusion of pOCs into MNCs. We show here that IL-1 β or

Figure 1. Effect of RANKL on MNC formation from pOCs. pOCs (1×10^5 cells/100 μ l/well) were cultured in 96-well plates in the presence of RANKL (100 ng ml⁻¹). After culturing for indicated periods, cells were fixed and stained for TRAP. (A) Number of MNCs formed and (B) Number of pOCs remained in the presence of RANKL (●) or in the absence of any additives (■). TRAP-positive MNCs containing more than 3 nuclei were counted. Data are expressed as the mean $\pm$ SD of 4 cultures.

RANKL supports the survival of pOCs and then induces the fusion of pOCs into OCLs, that the fusion of pOCs induced by RANKL is inhibited by OCIF/OPG, but the fusion of those induced by IL-1 β is not, and that both RANKL- and IL-1 β -induced fusion of pOCs are blocked by ZLLL-H, a proteasome inhibitor.

Figure 2. Time dependent formation of the MNCs from pOCs. pOCs (1×10^5 cells/ $100 \mu\text{l}$ /well) were cultured in 96-well plates in the presence (A–D) or absence (E–G) of RANKL (100 ng ml^{-1}). After culturing for indicated periods, cells were fixed and stained for TRAP. Arrowheads and arrows indicate pOCs and OCLs, respectively. Bar = $100 \mu\text{m}$.

Figure 3. Morphology and pit forming activity of MNCs induced by osteoblastic cells, IL-1 β and RANKL. pOCs (1×10^5 cells/100 μ l/well) were cultured in 96-well plates (A–D) or on the dentine slices (E–H) in the presence of osteoblastic cells (5×10^3 cells) (A, E), IL-1 β (50 ng ml $^{-1}$) (B, F) and RANKL (100 ng ml $^{-1}$) (C, G) for 24 h or in the absence of any additives (D, H). Cells (A–D) were then fixed and stained for TRAP. Resorption pits (E–H) were stained with hematoxylin. Arrows indicate OCLs or resorption pits. Bar = 100 μ m.

Figure 4. Dose-dependent effects of RANKL on the fusion of pOCs into MNCs. pOCs (1×10^5 cells/100 μ l/well) were cultured in 96-well plates in the presence of indicated concentrations of RANKL for 24 h. Cells were then fixed and stained for TRAP. TRAP-positive MNCs containing more than 4 nuclei were counted as OCLs. Data are expressed as the mean $\pm$ SD of 3 cultures.

Materials and methods

Animals and chemicals

Five- to eight-week-old male mice and newborn mice of ddY strain were obtained from Sankyo Labo Service Co.. Chemicals such as sodium tartrate, Fast Red Violet LB Salt, Mayer's hematoxylin and Naphthol AS-MX Phosphate were purchased from Sigma Chemical Co.. Recombinant human RANKL and recombinant murine IL-1 β were purchased from Pepro Tech EC Ltd. (London, England) and R&D Systems (Minneapolis, MN). Collagenase, dispase and collagen gel solution were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Godo Shusei and Nitta Gelatin Co., respectively. Fetal calf serum was obtained from CSL Ltd. ZLLL-H was purchased from Peptide Institute Inc (Osaka, Japan). OPG was a generous gift from Dr. Kwon (Indiana University School of Medicine).

Preparation of pOCs

Bone marrow cells were collected from femora and tibiae of five- to eight-week-old male mice as described (Takahashi et al., 1988). Primary osteoblastic cells were prepared from calvaria collected from newborn mice. A total of 40 to 50 calvaria were subjected to five sequential digestions using 0.1% collagenase

and 0.2% dispase as described. Mouse osteoblastic cells (2×10^6 cells/dish) were cocultured with bone marrow cells (2×10^7 cells/dish) in α -MEM containing 10% fetal calf serum and 10^{-8} M 1,25(OH) $_2$ D $_3$ in 100 mm culture dish. All cultures were maintained at 37 $^\circ$ C in a humidified atmosphere of 5% CO $_2$ in air. After culturing for 5 days, floating cells were removed and cells attached on osteoblastic cell layers were recovered as pOCs by pipetting with fresh α -MEM. Prepared pOCs were fixed and stained for TRAP, a marker enzyme of osteoclasts, and TRAP-positive mononuclear cells were counted as pOCs. This preparation of pOCs was not contaminated with MNCs or osteoblastic cells, and composed of more than 50–60% of TRAP-positive mononuclear cells without osteoblastic cells and MNCs.

Fusion assay system

pOCs (1×10^5 cells/well) were cultured in each well of 96 well tissue culture plates (100 μ l/well) in the presence or absence of osteoblastic cells, RANKL or IL-1 β . After culturing for 24 h, cells were fixed and stained for TRAP. The number of TRAP-positive MNCs with more than 4 nuclei was counted.

Pit formation assay

pOCs (1×10^5 cells/well) were placed on dentine slices (4 mm diameter) in the presence or absence of osteoblastic cells, RANKL and IL-1 β in each well of 96-well plates containing 0.2 ml α -MEM supplemented with 10% FCS. After incubation for 48 h, cells were removed with the treatment of 0.05 M NH $_4$ OH for 30 min from dentine slices and resorption pits were stained with Mayer's hematoxylin as described in Nakamura et al. (1995).

Results

We have previously reported that osteoblastic cells are essential for the fusion of pOCs into OCLs (Takami et al., 1998). Since RANKL induces osteoclast differentiation from their precursor cells in the presence of M-CSF without osteoblastic or stromal cells (Yasuda et al., 1998; Lacey et al., 1998), we determined whether RANKL induces the fusion of pOCs into OCLs in the absence of osteoblastic cells. When pOCs were cultured without any additives on plates, they survived for 2 h. Their survival allowed the spontaneous fusion of pOCs into MNCs having more than 3 nuclei

Figure 5. Effect of OPG on MNC formation induced by RANKL or IL-1 β . pOCs (1×10^5 cells/100 μ l/well) were cultured with OPG (1000 ng ml $^{-1}$) in 96-well plates in the presence of RANKL (100 ng ml $^{-1}$) and IL-1 β (10 ng ml $^{-1}$) for 24 h. Cells were then fixed and stained for TRAP. TRAP-positive MNCs having more than 3 nuclei were counted as OCLs. Data are expressed as the mean $\pm$ SD of 3 cultures.

(Figures 1A and 2E), but most of the TRAP-positive pOCs and MNCs died within 24 h and disappeared from the plates (Figures 1A, B and Figures 2F, G). However, when pOCs were cultured with RANKL for 24 h, TRAP-positive pOCs survived, and consequently the pOCs fused to form MNCs (Figures 2B–D). The number of MNCs was increased during the culture for 24 h (Figure 1A). After 12 h both TRAP-positive and MNCs having more than 4 nuclei were retained on the plates in the presence of RANKL. The decrease of the number of pOCs in the presence of RANKL, shown in Figure 2B, is considered to be due to multinucleation of pOCs as a consequence of fusion. To compare the effect of RANKL with that of IL-1 β , the effect of IL-1 β on the fusion of pOCs into MNCs was determined. IL-1 β , also supported the survival of pOCs and induced the fusion of pOCs into MNCs (Figure 3C). The effect of RANKL on the multinucleation of pOCs (having more than 4 nuclei) was dose-dependent in the concentration range of 10–100 ng ml $^{-1}$ (Figure 4). The potency of RANKL on the fusion was comparable to that on osteoclast differentiation from hematopoietic precursor cells without osteoblastic/stromal cells. Next, it was examined whether MNCs induced by RANKL from pOCs have osteoclastic features.

RANKL-induced OCLs showed typical properties of osteoclast in terms of TRAP activity (Figures 3A–C) and actin ring formation, as detected by staining F-actin with rhodamine-conjugated phalloidin (data not shown). pOCs were cultured on the dentine slices to determine the bone-resorbing activity of OCLs induced by RANKL. In the absence of additives no resorption pit was detected (Figure 3H). When pOCs were cultured for 48 h in the presence of RANKL, many resorption pits were formed on dentine slices similar to these cultured in the presence of IL-1 β or osteoblastic cells (Figures 3E–G). Thus, RANKL-induced MNCs are considered to be OCLs, like IL-1 β - or osteoblastic cell-induced osteoclasts.

Since RANKL-induced osteoclast differentiation is inhibited by OPG/OCIF, a decoy receptor of RANKL (Yasuda et al., 1998; Lacey et al., 1998), we examined the effect of OPG/OCIF on the fusion of pOCs induced by osteoblastic cells, IL-1 β or RANKL. Multinucleation of pOCs induced by osteoblastic cells or RANKL was inhibited by OPG, but that induced by IL-1 β was not (Figure 5). When pOCs were treated with 1000 ng ml $^{-1}$ OCIF in the presence of RANKL, neither TRAP-positive pOCs nor OCLs were retained on the plates (data not shown). RANKL is shown to

Figure 6. Effect of ZLLL-H on the fusion of pOCs induced by RANKL (100 ng ml^{-1}) or IL-1 β . pOCs (1×10^5 cells/ $100 \mu\text{l}$ /well) were cultured with ZLLL-H ($10 \mu\text{g ml}^{-1}$) in 96-well plates in the presence of RANKL (100 ng ml^{-1}) and IL-1 β (10 ng ml^{-1}) for 24 h. Cells were then fixed and stained for TRAP. TRAP-positive MNCs having more than 4 nuclei were counted as OCLs. Data are expressed as the mean $\pm$ SD of 3 cultures.

be a ligand of RANK that activates NF- κ B (Wong et al., 1998; Darnay et al., 1998; Nakagawa et al., 1998), and IL-1 β also promotes the survival of mature osteoclasts by activating NF- κ B through IL-1 β type I receptors (Jimi et al., 1998). Thus, a peptide inhibitor of proteasome ZLLL-H, which inhibits NF- κ B activation by blocking proteasome activity participating in the degradation of I κ B of NF- κ B/I κ B complex, was used to investigate whether NF- κ B-signaling pathway is involved in the process of fusion of pOCs induced by these two cytokines. When pOCs were treated with ZLLL-H in the presence of RANKL or IL-1 β the formation of OCLs having more than 4 nuclei were inhibited (Figure 6). The number of pOCs were also decreased in the presence of ZLLL-H (data not shown).

Discussion

Osteoblastic/stromal cells play a critical role in the differentiation and activation of osteoclast through a cell-to-cell interaction (Fuller et al., 1998; Suda et al., 1997, 1995). RANKL is expressed as a membrane-bound type protein in stromal/osteoblastic cells that support osteoclast differentiation and activate mature

osteoclast to resorb bone (Yasuda et al., 1998; Lacey et al., 1998). These RANKL effects on the differentiation and activation of osteoclasts are inhibited by OPG/OCIF, a decoy receptor of RANKL (Yasuda et al., 1998; Simonet et al., 1997). It has been shown that stromal cells are involved in osteoclast survival (Jimi et al., 1995). Recently, OPG/OCIF was also reported to inhibit osteoclast survival and induce apoptosis in the presence of stromal cells (Akatsu et al., 1998). It is indicated that OPG/OCIF inhibits osteoclast formation, activation and survival by disrupting the interaction between RANKL on the stromal cells and its receptor on osteoclast progenitors or mature osteoclasts (Yasuda et al., 1998; Lacey et al., 1998; Akatsu et al., 1998). We previously reported that osteoblastic cells are also essential for the survival of pOCs and the fusion of pOCs into OCLs (Takami et al., 1998). In this study we showed that RANKL promotes the survival of pOCs, and then induces the fusion of pOCs into OCLs in the absence of M-CSF without the help of osteoblastic/stromal cells. MNCs formed from pOCs in the presence of RANKL showed typical osteoclast properties with TRAP activity, actin ring formation and bone-resorbing activity. The survival and fusion of pOCs induced by RANKL as well as osteoblastic cells were inhibited by OPG/OCIF,

suggesting that OPG functions as a decoy receptor of RANKL to interfere the binding of RANKL to its receptors expressed on pOCs. These findings indicate that RANKL could substitute for the role of osteoblastic/stromal cells in the survival of pOCs and the fusion of pOCs into OCLs.

Activation of the NF- κ B family of transcriptional factors are known to regulate the expression of genes that participate in the pathways involving cell proliferation and apoptosis (Baldwin, 1996). The activation of NF- κ B is also an important process for survival of mature osteoclast (Jimi et al., 1995). Recently, RANK, which is known to have the capacity of activating NF- κ B, has been shown to be an essential signaling receptor for osteoclast differentiation from their progenitors (Nakagawa et al., 1998). RANKL-mediated osteoclastogenesis can be induced by the activation of NF- κ B through RANK in progenitors (Nakagawa et al., 1998). IL-1 β also promotes the survival of mature osteoclasts by activating NF- κ B through IL-1 β type I receptors and induces the fusion of pOCs into OCLs (Jimi et al., 1998, 1999). A common feature of NF- κ B regulation is their sequestration in the cytoplasm as an inactive complex with I κ B to keep the molecule from being translocated into the nucleus (Siebenlist et al., 1995). It is known that degradation of I κ B by proteasome is a prerequisite for NF- κ B activation (Siebenlist et al., 1995). An experiment using ZLLL-H has revealed that IL-1 β -induced survival of purified mature osteoclasts is due to a result of activation of NF- κ B through the degradation of I κ B (Jimi et al., 1998). In the present study ZLLL-H inhibited the fusion of pOCs into OCLs induced by RANKL or IL-1 β . It is thought that the effect of ZLLL-H is resulted from the decrease of the number of pOCs survived. OPG/OCIF also inhibited the fusion of pOCs induced by osteoblastic cells or RANKL in a similar manner. When pOCs were cultured without RANKL or IL-1 β , TRAP-positive pOCs also died by apoptosis (our unpublished data) and then no OCLs were formed. Thus, inhibitory effect of ZLLL-H on the fusion of pOCs, like that of OPG/OCIF, is considered to be a consequence of pOC apoptosis caused by blocking the activation of NF- κ B for survival signaling. These facts suggest that NF- κ B activation is an important process for the survival and the fusion of pOCs induced by RANKL or IL-1 β .

Although the effect of RANKL on the fusion of pOCs into OCLs and on the activation of OCLs is similar to that of IL-1 β (Suda et al., 1997; Fuller et al., 1998), IL-1 β could not substitute for the ef-

fect of RANKL on the early stage, the differentiation of progenitor cells into preosteoclasts. Several members of TNF receptor superfamily interact with signaling adapter proteins (TNF receptor-associated factors, TRAFs) to activate NF- κ B (Rothe et al., 1994). TRAF2, TRAF5 and TRAF6 of TRAF molecules have been demonstrated to mediate signaling of NF- κ B (Malinin et al., 1997; Song et al., 1997; Regnier et al., 1997; Mercurio et al., 1997; Woronicz et al., 1997). Recently, Darnay et al. (1998) and Wong et al. (1998) have independently reported that distinct intracellular region of RANK associates with TRAF2, TRAF5 and TRAF6 to initiate NF- κ B activation. RANKL may play a critical role in the polyfunctions of dendritic cells and osteoclasts through the RANK by activating NF- κ B via these three TRAFs. It should be studied which TRAFs are involved in the signaling pathway of RANKL and IL-1 β , and if the difference between RANKL and IL-1 β activity is attributed to the difference of TRAFs involved.

In summary, we found that RANKL induces the survival of pOCs and fusion of pOCs into OCLs, that osteoblastic cell- or RANKL-induced fusion of pOCs are inhibited by OPG/OCIF and that IL-1 β - or RANKL-induced fusion of pOCs are inhibited by peptide inhibitor of proteasome. These findings suggest that NF- κ B activation is an important process for the survival and fusion of pOCs induced by osteoblastic/stromal cells. It was also suggested that NF- κ B activation by RANKL is an essential signaling pathway for all steps in osteoclastogenesis, the differentiation of progenitors into pOCs, the survival of pOCs, the fusion of pOCs into OCLs, and the activation of mature osteoclasts.

References

- Akatsu T, Murakami T, Nishikawa M, Ono K, Shinomiya N, Tsuda E, Mochizuki SI, Yamaguchi K, Kinoshita M, Higashio K, Yamamoto M, Motoyoshi K and Nagata N (1998) Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclasts. *Biochem Biophys Res Commun* 250: 229–234.
- Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D and Galibert L (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 390: 175–179.
- Athanasou NA (1996) Cellular biology of bone-resorbing cells. *J Bone Joint Surg Am* 78: 1096–1112.
- Baeuerle PA and Baltimore D (1996) NF- κ B: Ten years after. *Cell* 87: 13–20.
- Baldwin AS (1996) The NF- κ B and I κ B proteins: new discoveries and insights. *Annu Rev Immunol* 14: 649–683.

- Darnay BG, Haridas V, Ni J, Moore PA and Aggarwal BB (1998) Characterization of the intracellular domain of receptor activator of NF- κ B (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF- κ B and c-Jun N-terminal kinase. *J Biol Chem* 273: 20551–20555.
- Fuller K, Wong B, Fox S, Choi Y and Chambers TJ (1998) TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med* 188: 997–1001.
- Jimi E, Shuto T and Koga T (1995) Macrophage colony-stimulating factor and interleukin-1 α maintain the survival of osteoclast-like cells. *Endocrinology* 136: 808–811.
- Jimi E, Nakamura I, Ikebe T, Akiyama S, Takahashi N and Suda T (1998) Activation of NF- κ B is involved in the survival of osteoclasts promoted by interleukin-1. *J Biol Chem* 273: 8799–8805.
- Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA and Suda T (1999) Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. *Exp Cell Res* 247: 84–93.
- Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L, Xia XZ, Elliott R, Chiu L, Black T, Scully S, Capparelli C, Morony S, Shimamoto G, Bass MB and Boyle WJ (1999) Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA* 96: 3540–3545.
- Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J and Boyle WJ (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93: 165–176.
- Malinin NL, Boldin MP, Kovalenko AV and Wallach D (1997) MAP3K-related kinase involved in NF- κ B induction by TNF, CD95 and IL-1. *Nature* 385: 540–544.
- Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A and Rao A (1997) IKK-1 and IKK-2: cytokine-activated I κ B kinases essential for NF- κ B activation. *Science* 278: 860–866.
- Nakagawa N, Kinoshita M, Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T and Higashio K (1998) RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Commun* 253: 395–400.
- Nakamura I, Takahashi N, Sasaki T, Tanaka S, Udagawa N, Murakami H, Kimura K, Kabuyama Y, Kurokawa T, Suda T and Fukui Y (1995) Wortmannin, a specific inhibitor of phosphatidylinositol-3 kinase, blocks osteoclastic bone resorption. *FEBS Lett* 361: 79–84.
- Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z and Rothe M (1997) Identification and characterization of an I κ B kinase. *Cell* 90: 373–383.
- Rothe M, Wong SC, Henzel WJ and Goeddel DV (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* 78: 681–692.
- Roodman GD (1996) Advances in bone biology: the osteoclasts. *Endocrine Rev* 17: 308–332.
- Scheven BA, Visser JW and Nijweide PJ (1986) In vitro osteoclast generation from different bone marrow fractions, including a highly enriched haematopoietic stem cell population. *Nature* 321: 79–81.
- Siebenlist U, Franzoso G and Brown K (1994) Structure, regulation and function of NF- κ B. *Annu Rev Cell Biol* 10: 405–455.
- Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P and Boyle WJ (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 89: 309–319.
- Song HY, Regnier CH, Kirschning CJ, Goeddel DV and Rothe M (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor- κ B and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc Natl Acad Sci USA* 94: 9792–9796.
- Suda T, Nakamura I, Jimi E and Takahashi N (1997) Regulation of osteoclast function. *J Bone Miner Res* 12: 869–879.
- Suda T, Udagawa N and Takahashi N (1996) Cells of bone: osteoclast generation. In: Bilezikian JP, Rase LG and Rodan GA (eds) *Principles of bone biology* (pp. 87–102). Academic Press San Diego.
- Suda T, Takahashi N and Martin TJ (1995) Modulation of osteoclast differentiation: Update. *Endocrine Rev* 4: 266–270.
- Takami M, Woo JT and Nagai K (1998) Requirement of osteoblastic cells for the fusion of preosteoclasts. *J Bone Miner Metabol* 16: 151–157.
- Takahashi N, Akatsu T, Udagawa N, Sasaki T, Yamaguchi A, Moseley JM, Martin TJ and Suda T (1988) Osteoblastic cells are involved in osteoclast formation. *Endocrinology* 123: 2600–2602.
- Wesolowski G, Duong LT, Lakkakorpi PT, Nagy RM, Tezuka K, Tanaka H, Rodan GA and Rodan SB (1995) Isolation and characterization of highly enriched, prefusion mouse osteoclastic cells. *Exp Cell Res* 219: 679–686.
- Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FS, Frankel WN, Lee SY and Choi Y (1997a) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* 272: 25190–25194.
- Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM and Choi Y (1997b) TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* 186: 2075–2080.
- Wong BR, Josien R, Lee SY, Vologodskaya M, Steinman RM and Choi Y (1998) The TRAF family of signal transducers mediates NF- κ B activation by the TRANCE receptor. *J Biol Chem* 273: 28355–28359.
- Woronicz JD, Gao X, Cao Z, Rothe M and Goeddel DV (1997) I κ B kinase-beta: NF- κ B activation and complex formation with I κ B kinase-alpha and NIK. *Science* 278: 866–869.
- Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinoshita M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N and Suda T (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 95: 3597–3602.