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Characterization of the NF-κB Activation Induced by TR8, an Osteoclastogenic Tumor Necrosis Factor Receptor Family Member

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TR8 is a recently identified member of the tumor necrosis factor (TNF) receptor superfamily. TR8 seems to play important roles in bone metabolism as stimulation of this receptor with its ligand, TL8 or osteoclast differentiation factor (ODF), induced the differentiation and activation of osteoclasts. Despite its important biological functions, the biochemical events ensuing from TR8 activation have not been revealed in detail. Most of TNF receptor family proteins provoke the activation of the NF-κB transcription factor. In the present study, we examined the signaling potential of TR8 to induce NF-B activation. When overexpressed in a human embryonic kidney cell line by transient transfection, TR8 caused a strong activation of NF-κB, which was further increased upon stimulation with TL8. The TR8-induced NF-B activation was abrogated by the co-expression of the TRAF6 mutant lacking the Ring and zinc finger domains and that of the kinase-inactive mutant NIK. Taken together, our study suggests that the presence of intact TRAF6 and the kinase activity of NIK may be essential for TR8 to induce NF-κB activation.

Key words: TR8, NF-κB, Osteoclastogenesis, TRAF, NIK

INTRODUCTION

TR8 was identified by search of an expressed sequence tag (EST) cDNA database for genes with sequence homology to TNFR. This gene was also cloned by direct sequencing of a dendritic cell cDNA library and named RANK (receptor activator of NF-κB) (Anderson *et al.*, 1997). The human TR8 protein is a type I transmembrane molecule and consisted of 616 amino acids with a relatively long cytoplasmic domain of 383 amino acids. The extracellular domain contains 184 amino acids and shows the highest homology to CD40. The ligand for TR8, TL8 or RANKL, is a TNF family type II transmembrane protein of 317 amino acids (Anderson *et al.*, 1997). TL8 is also identical to TRANCE (TNF-related activation-induced cytokine) induced by T cell receptor stimulation (Wong *et al.*, 1997) and ODF (osteoclast differentiation factor) identified as a ligand for OPG (osteoprotegerin)/OCIF (osteoclastogenesis inhibitory factor) (Lacey *et al.*, 1998; Yasuda *et al.*, 1998). The interaction between TR8 and TL8 has been reported to play important roles in the differentiation and activation of osteoclasts (Fuller *et al.*, 1998; Lacey *et al.*, 1998) and in the regulation of dendritic and T cell functions (Anderson *et al.*, 1997).

Stimulation of many of the TNFR family proteins has been shown to result in the activation of a transcription factor NF-κB and a serine/threonine kinase JNK and the induction of apoptosis (Aizawa *et al.*, 1997; Cao *et al.*, 1996; Natoli *et al.*, 1997; Rothe *et al.*, 1995). The activation of NF-κB and JNK by TNFR family proteins is regulated by cytoplasmic TRAF (TNF receptor-associated factor) proteins (Baker and Reddy, 1996; Rothe *et al.*, 1994). TRAF 2, 5, and 6 can interact with NIK (NF-κB-inducting kinase) (Malinin *et al.*, 1997; Song *et al.*, 1997) that phosphorylates and activates IKK (IκB-kinase), which in turn phosphorylates and induces degradation of IκB, resulting in the translocation of NF-κB into the nucleus (Regnier *et al.*, 1997). TNF and IL-1 receptors were shown to employ this TRAF-NIK-IKK-IκB degradation pathway (Malinin *et al.*, 1997; Regnier *et al.*, 1997). Activation of NF-κB induced by TR8/RANK has been shown by electrophoretic mobility shift assays in transfected 293 cells and pre-activated peripheral blood T cells (Anderson *et al.*, 1997). In the present study, we explored the mechanism by which TR8/RANK activates NF-κB using a

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NF-κB responsive luciferase reporter system.

MATERIALS AND METHODS

Cells and transfection

293-EBNA cells (Invitrogen) were cultured in DMEM containing 10% FCS and 250 μg/ml G418. For transfection, 1.5×10^5 cells per well were plated onto 24-well plates. The next day, transfection was carried out with a mixture of the indicated amount of TR8 or TRAF DNA and 3 μl SuperFect reagent (Qiagen) or 4 μl Tfx reagent (Promega) according to the manufacturers' instructions. Each transfection contained 100 ng of the NFκB-responsive reporter construct $(κB)_4$-IL-2-Luc plasmid and the total amount of DNA was kept constant by supplementation with vector control DNAs.

For generation of stable TL8-expressing cells, 293-EBNA cells (4×10^5 cells/35 mm dish) were transfected with 2 μg of pCEP4-TL8 (see below) and SuperFect reagent. The next day selection was started with DMEM medium containing 250 μg/ml hygromycin as well as 250 μg/ml G418. The control stable cell line was established n the same manner after transfecting pCEP4 vector DNA (Invitrogen).

Reporter assays

16-24 h after transfection, cells were washed with phosphate buffered saline (PBS) and lysed in Cell Culture Lysis Reagent (Promega). Luciferase activity was measured with 4-20 μl of cell lysates after mixing with 100 μl of Luciferase Assay Substrate (Promega) using a luminometer (EG&G Berthold).

Crude membrane preparation

Cells were washed with PBS and lysed in a hypotonic lysis buffer (10 mM Tris ·HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT). After 15 strokes of homogenation, whole cell lysates were centrifuged for 5 min at 1000 g to remove unbroken cells and nuclei. The supernatants were collected and centrifuged for 10 min at 10,000 g. The pellet fractions were used as crude membrane preparations.

Plasmid constructions

The cDNA encoding a full-length human TR8 was obtained from Human Genome Sciences, Inc (Rockville, MD) and subcloned into a mammalian expression vector (pSRa-TR8-WT).

Eukaryotic expression vectors of human full-length TRAF5 and TRAF6, the TRAF2 mutant lacking the N-terminal Ring domain, and the kinase-inactive form of NIK (NIK-KKAA) were generously provided by Dr. H.Y. Song (Lilly Co. Center, Indianapolis, IN). Deletion mutants of TRAF5 and TRAF6 that are lacking the N-terminal Ring and zinc finger domains were generated by PCR.

The full-length TL8 cDNA was subcloned into pCEP4 eukaryotic expression vector by PCR.

RESULTS

Activation of NF-κB by TR8

Activation of the NF-κB transcription factor is one of the consequences of signaling by TNF receptor family proteins. In order to assess the ability of TR8 to signal NF-κB activation, a plasmid of the luciferase gene placed under the control of NF-κB transcription factor was transfected into 293-EBNA cells and a reporter assay was performed. Stimulation of these cells with TNF caused an increase in the luciferase activity (3.9 fold) (Fig. 1A), indicating the presence of some TNF receptors in these cells. When the cells were transfected with TR8, greater activation of NF-κB was observed (58.7 fold) (Fig. 1A).

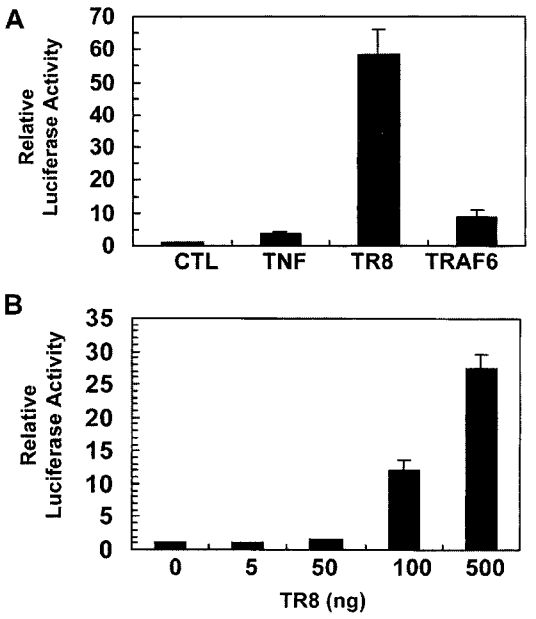

Fig. 1. A: Reporter assay for NF-κB activity induced by TNF, TR8, and TRAF6. 293-EBNA cells (1×10^5) were transfected with 100 ng of the $(kB)_4$-IL-2-Luc reporter construct and 500 ng of TR8, 300 ng of TRAF6, or control vector plasmid as described in "Materials and Methods". Cells were stimulated with 50 ng/ml TNF-a (TNF lane) or control vehicle (other lanes) for 5 h. Luciferase activity was measured with cell lysates as described in "Materials and Methods". Error bars indicate standard deviations between samples in triplicate. The representative data of three independent experiments are presented. B: Dose response of TR8 in the NF-κB activation. Cells were transfected with indicated amounts of TR8 plasmid and 100 ng of the reporter construct. 20 h after transfection, cells were lysed in 150 μl of lysis buffer and luciferase activity was measured with 20 μl of the lysate. Each bar represents the mean±S.D. from one experiment in triplicate.

As reported by other researchers (Song *et al.*, 1997), overexpression of TRAF6 also led to NF-κB activation (Fig. 1A).

When various amounts of TR8 cDNA was transfected into 293-EBNA cells, the extent of increase in the luciferase activity was dose-dependent between 50 and 500 ng of DNA per well of 24-well plates (Fig. 1B). Fold increase in the TR8-induced luciferase activity varied between experiments depending on the transfection reagents and probably on the cellular state at the time of transfection even when the same amounts of DNA were used.

Stimulation of TR8 activation of NF-κB by TL8

We next determined whether the stimulation of TR8 with its cognate ligand TL8 can enhance the TR8-induced NF-κB activation in this transfection system. Cells were transfected with TR8 and stimulated with crude membrane preparations from the TL8- or the control vector-stable transfectant cells. Stimulation with TL8 increased the TR8-induced NF-κB activation, whereas the control treatment had no effect (Fig. 2).

Involvement of TRAF proteins in the TR8 activation of NF-κB

TNF receptor associated factor (TRAF) 2, -5, and -6 have been implicated in the NF-κB activation induced by many TNFR family proteins. To this end we sought to determine whether TRAF family proteins are involved in the TR8 signaling to the NF-κB activation. The effects on TR8-induced NF-κB activation of TRAF2, -5, and -6 mutants that lack the N-terminal zinc finger and/or Ring finger domains were assessed. As shown in Fig. 3A, the mutants of TRAF2, -5, and -6 had dominant negative effects on TNF-stimulation of NF-κB activity. The TRAF6 mutant was most effective showing 92% inhibition and the TRAF2 and TRAF5 mutants resulted in 80% and 67% inhibition, respectively. Co-transfection of these mutants with TR8 also resulted in an inhibitory effect on NF-κB activity (Fig. 3B). Again the TRAF6 mutant was most efficient and the TRAF2 and 5 mutants inhibited the TR8-induced NF-κB activation by more than 90%. Therefore, the TRAF2, -5, and -6 proteins seem to be involved in the NF-κB activation caused by TR8 overexpression and perhaps TRAF6 plays most potent role among these TRAF members.

NIK mediated NF-κB activation by TR8

TRAF6 has been reported to mediate the NF-κB activation induced by IL-1 receptor through a serine/

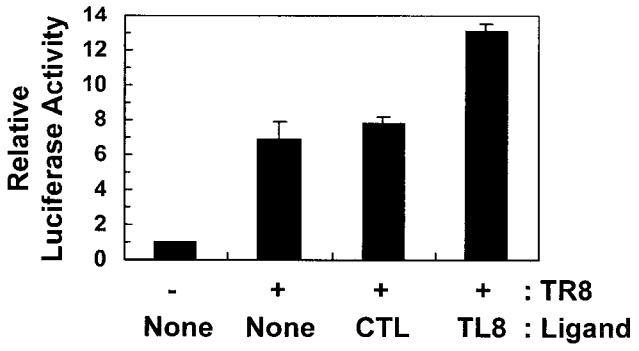

Fig. 2. Effect of ligand stimulation on the TR8-induced NF-κB activation. Cells were transfected with 200 ng of TR8 and 100 ng of the reporter DNA. 16 h after transfection, cells were stimulated with either the crude membrane preparation of TL8-expressing cells (TL8) or that of the control cells (CTL) for 5 h. Luciferase activity was determined with cell lysates as described in "Materials and Method". The data are presented as the mean±S.D. of triplicate samples from one experiment. Similar results were obtained in two other experiments.

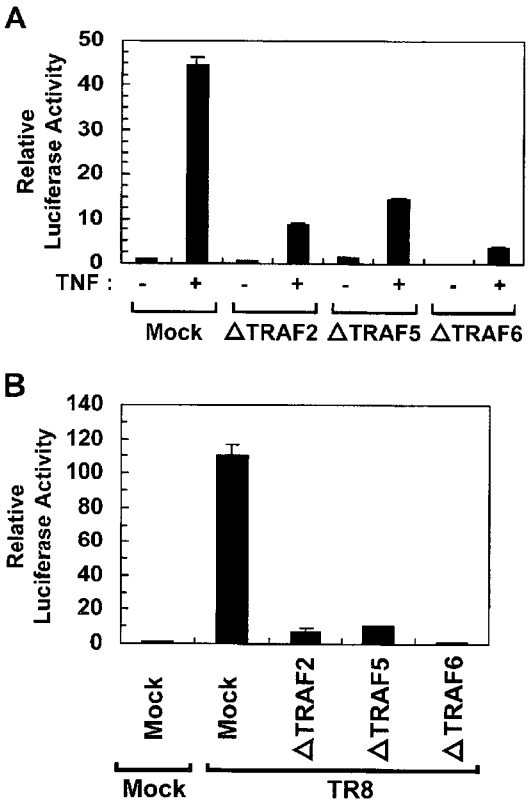

Fig. 3. A. Involvement of TRAF molecules in the NF-κB-inducing activity of TNF-α. 300 ng of the N-terminal deletion mutant of TRAF2, -5, or -6 or the control vector were cotransfected with 100 ng of the reporter plasmids. The next day cells were stimulated with 50 ng/ml TNF-α for 4 h and luciferase assay was performed. **B.** Effect of TRAF mutants on TR8-induced NF-κB activity. 50 ng of TR8 or the control vector were co-transfected with 300 ng of the TRAF2, -5, or -6 mutant and 100 ng of the reporter plasmids. Effect of the TRAF mutant was examined by the reporter assay. Error bars denote standard deviations between three samples. These results are representative of three independent experiments.

threonine kinase NIK (Cao *et al.*, 1996; Malinin *et al.*, 1997). In order to determine whether this kinase mediates the TR8-induced NF-κB activation, a kinase-inactive NIK that contains two lysine-to-alanine mutations in its kinase domain (NIK-KKAA) was co-transfected and the effect on luciferase activity was examined. Blocking the NIK kinase activity abrogated the NF-κB activation of TR8 (Fig. 4). The same NIK mutant also significantly reduced TNF-stimulation of NF-κB activity (Fig. 4).

DISCUSSION

In this study, the ability of TR8, a TNF receptor family member essential for osteoclast differentiation, to activate the NF-κB transcription factor and the signal transduction pathways involved in the TR8-induced NF-κB activation were investigated. Transient overexpression of TR8 in human embryonic kidney cell line 293-EBNA induced a strong activation of NF-κB (Fig. 1) and this activation was further increased by stimulation with TL8, the ligand for TR8 (Fig. 2). Overexpression of cell surface receptors has been thought to lead to receptor aggregation that mimics the ligand-stimulated state (Brandt - Rauf *et al.*,1990) and thus has been employed in biochemical studies on intracellular events initiated by surface receptor activation.

The cytoplasmic domain of TR8 contains sequence motifs similar to those reported for TRAF binding to other TNFR family members: the PXQX and EE(D)E(D) motifs (Boucher *et al.*, 1997). The PXQX motifs are present at residues 302, 353, 568, and 607 and the EE(D)E(D) motifs at residues 336, 346, and 480. Deletion of the C-terminal part of the cytoplasmic domain of TR8 at the 353 amino acid residue caused a complete abrogation of the NF-κB activating potential (data not shown), indicating that this C-terminal portion is required for NF-κB activation. The TR8-induced activation of NF-κB appeared to be mediated by the TRAF2, -5, and -6 proteins (Fig. 3). TRAF6 has been reported to be involved in the activation of NF-κB in CD40 and IL-1 receptor signaling (Cao *et al.*, 1996; Ishida *et al.*, 1996). One of the common downstream targets of NF-κB-activating TRAFs (TRAF 2, -5, and -6) is NIK. The mutant form of NIK that lost either the TRAF-interacting capability by N-terminal deletion or the kinase activity by the substitution of two lysine residues for alanines in the kinase domain was shown to inhibit these TRAF-induced NF-κB activation (Song *et al.*, 1997). The kinase-inactive NIK abolished TR8-induced NF-κB activation (Fig. 4). Therefore, I conclude that the NF-κB activation induced by TR8 is mediated through TRAF6 and NIK.

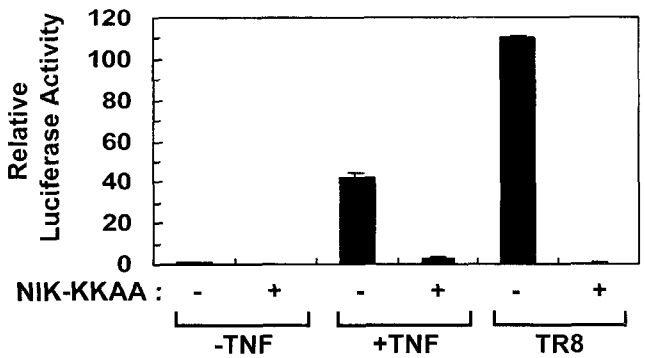

Fig. 4. Inhibition of TR8-induced NF-κB activation by the kinase-inactive NIK. Cells were transfected with 50 ng of TR8 DNA alone or together with 300 ng of the kinase-inactive NIK (NIK-KKAA) plasmid. 24 h after transfection, cells were lysed and the luciferase activity was measured. The averages and standard deviations of triplicate samples are shown. Data from one representative experiment are presented. Similar results were observed in two other experiments.

Cellular responses to the activation of most TNFR superfamily receptors include NF-κB activation, elevated JNK (c-Jun N-terminal kinase) activity, and apoptosis. We have shown the JNK activation upon overexpression of TR8 (Kim *et al.*, 1999). The activation of NF-κB and JNK induced by TR8 may explain in part the physiological roles of TR8 in the differentiation and activation of osteoclasts and the survival and function of dendritic cells (Anderson *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998). Mice lacking NF-κB-1 and -2 were found to develope osteopetrosis due to a defect in osteoclast differentiation (Iotsova *et al.*, 1997), which suggests that NF-κB plays a critical role in this process. In this regard, an intriguing question is whether inhibition of the TR8-induced NF-κB activation would block osteoclast differen-tiation. NF-κB also seems to have anti-apoptotic effects (Liu *et al.*, 1996), which supports the role of TR8 in dendritic cell survival. c-Jun, the physiological substrate of JNK, heterodimerizes with Fos to form AP-1 transcription factors, which have been shown to be involved in cell proliferation, survival, and differentiation. In addition, knock-out and anti-sense studies have implicated c-Fos in osteoclast formation, especially in the proliferation of hematopoietic osteoclast progenitor cells (Grigoriades *et al.*, 1994; Udagawa *et al.*, 1996). The TR8 protein may participate in several stages of osteoclast differentiation and differential signaling pathways of TR8 may be activated depending on the cellular context at each stage.

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REFERENCES CITED

Aizawa, S., Nakano, H., Ishida, T., Horie, R., Nagai, M.,

Ito, K., Yagita, H., Okmura, K., Inoue, J. and Watanabe, T., Tumor necrosis factor receptor-associate factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NFkappaB activation. *J. Biol. Chem.*, 272, 2042-2045 (1997).

Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tomesko, M. E., Roux, E. R., Teepe, M. C., DuBose, R.F., Cosman, D. and Galibert, L., A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature*, 390, 175-179 (1997).

Baker, S. J. and Reddy, E. P., Modulation of life and death by the TNF receptor superfamily. *Oncogene*, 12, 1-9 (1996).

Boucher, L. -M., Marengere, L. E. M., Lu, Y., Thukral, S. and Mak, T. W., Binding sites of cytoplasmic effectors TRAF1, 2, and 3 on CD30 and other members of the TNF receptor superfamily. *Biochem. Biophy. Res. Comm.*, 233, 592-600 (1997).

Brandt-Rauf, P.W., Rackovsky, S. and Pincus, M. R., Correlation of the structure of the transmembrane domain of the neu oncogene-encoded p185 protein with its function. *Proc. Natl. Acad. Sci. USA*, 87, 8660-8664 (1990).

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. and Goeddel, D. V., TRAF6 is a signal transducer for interleukin-1. *Nature*, 383, 443-446 (1996).

Fuller, K., Wong, B., Fox, S., Choi, Y. and Chambers, T. J., TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J. Exp. Med.*, 188, 997-1001 (1998).

Grigoriades, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A. and Wagner, E. F., c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science*, 266, 443-448 (1994).

Iotsova, V., Caamano, J., Loy, J., Yang, Y., Lewin, A. and Bravo, R., Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nature Med.*, 3, 1285-1289 (1997).

Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T. and Inoue, J., Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. *J. Biol. Chem.*, 271, 28745-28748 (1996).

Kim, H. -H., Lee, D. E., Shin, J. N., Lee, Y. S., Jeon, Y. M., Chung, C. -H., Kwon, B. S. and Lee, Z. H., Receptor activator of NF-κB recruits multiple TRAF family adaptors and activates c-Jun N-terminal kinase. *FEBS Lett.*, 443, 297-302 (1999).

Lacey, D. L., Timms, E., Tan, H. -L. *et al.*, Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, 93, 165-176 (1998).

Liu, Z. G., Hsu, H., Goeddel, D. V. and Karin, M., Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell*, 87, 565-576 (1996).

Malinin, N. L., Boldin, M. P., Kovalenko, A. V. and Wallach, D., MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature*, 385, 540-544 (1997).

Natoli, G., Antonio, C., Ianni, A., Templeton, D. J., Woodgett, J.R., Balsano, C. and Levrero, M., Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. *Science* 275, 200-203 (1997).

Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z. and Rothe, M., Identification and characterization of an IκB kinase. *Cell*, 90, 373-383 (1997).

Rothe, M., Sarma, V., Dixit, V. M. and Goeddel, D. V., TRAF2-mediated activation of NF-κB by TNF receptor 2 and CD40. *Science*, 269, 1424-1427 (1995).

Rothe, M., Wong, S. C., Henzel, W. J. and Goeddel, D. V., A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*, 78, 681-692 (1994).

Song H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V. and Rothe, M., Tumor necrosis factor (TNF)-mediated kinase cascades: Bifurcation of nuclear factor-kB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc. Natl. Acad. Sci. USA*, 94, 9792-9796 (1997).

Udagawa, N., Chan, J., Wada, S., Findlay, D. M., Hamilton, J. A. and Martin, T. J., c-fos antisense DNA inhibits proliferation of osteoclast progenitors in osteoclast development but not macrophage differentiation in vitro. *Bone*, 18, 511-516 (1996).

Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett III, F. S., Frankel, W. N., Lee, S. Y. and Choi, Y., 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 (1997).

Yasuda, H., Shima, N., Nakagawa, N. *et al.*, 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 (1998).