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# Dimer formation of receptor activator of nuclear factor κB induces incomplete osteoclast formation☆

Katsuya Iwamoto[a,b,1], Takeshi Miyamoto[a,*,1], Yumi Sawatani[a], Naobumi Hosogane[a], Isao Hamaguchi[a], Masamichi Takami[c], Kana Nomiyama[a], Katsumasa Takagi[b], Toshio Suda[a]

[a] *Department of Cell Differentiation, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[b] *Department of Orthopedic Surgery, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan*
[c] *Laboratory of Biochemistry, Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa, Tokyo 142-8555, Japan*

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**Abstract**

Receptor activator of nuclear factor κB-ligand (RANKL) transduces a differentiation signal appropriate to osteoclasts likely through induction a receptor homotrimer; however, biological importance of RANK-trimerizarion is unknown. To address the signaling mechanism of the RANK receptor, we analyzed the effect of two different types of homodimer inducers RANK-TM-FKBP36v and hEpoR-RANK-TM on osteoclastogenesis. Dimerizing component FKBP36v or extracellular portion of human erythropoietin receptor (hEpoR) was fused to RANK lacking the extracellular domain, and the dimerization of this fusion protein was induced by addition of the chemical inducer of dimerization AP20187 or erythropoietin, respectively. Such treatment resulted in induction of TRAP-activity, a marker of osteoclast in a dose dependent manner, with an efficiency equivalent to that of induction by RANKL. However, dimerized-RANK-induced osteoclasts showed relatively low levels of multinucleation, pit forming activity, and expression of calcitonin receptor and cathepsin K, compared with osteoclasts which were induced in the presence of RANKL. As expression of nuclear factor of activated T cells 1 (NFATc1) was also reduced in dimerized-RANK-induced osteoclasts, RANK oligomerization by RANKL is a critical event to generate fully matured osteoclasts through upregulation of NFATc1.


*Keywords:* Osteoclast; Differentiation; RANK; FKBP36v; NFATc1

Osteoclasts are bone resorbing multinuclear cells derived from hematopoietic stem cells. Two cytokines, macrophage colony stimulating factor (M-CSF) and RANKL, are essential to promote osteoclast differentiation [1,2]. M-CSF induces expression of RANK [3], and subsequent RANKL binding to RANK transduces signals required for differentiation, cell survival, and bone resorption via c-Fos and TRAF6 [4–6]. Recently, RANKL has been shown to selectively induce NFATc1 expression via c-Fos and TRAF6 pathways [7]. Since NFATc1 induces osteoclastogenesis without RANKL, NFATc1 is considered a key regulator of osteoclast differentiation.

Proteins of the TNF ligand superfamily, including RANKL, TNFα, and CD40 ligand, form homotrimers that induce transmembrane signaling through receptor trimerization [8–11]. On the other hand, cytokine binding to receptors such as M-CSF, SCF, and Epo induces receptor dimerization via ligand dimerization [12–14]. Recently, a dimerizing component, FKBP36v, has been

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* Corresponding author. Fax: +81 3 5363 3475.
*E-mail address:* miyamoto@sc.itc.keio.ac.jp (T. Miyamoto).
[1] These authors contributed equally to this work.

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genetically engineered. When FKBP36v is fused to a receptor, the CID AP20187 induces receptor dimerization through FKBP36v, and biological signals are transduced. Chimeras of FKBP36v and receptors such as c-Kit, erythropoietin, and Mpl are activated by CID to promote cell growth in the absence of ligands [15–17]. However, whether CID-induced receptor dimerization can mediate differentiation through trimer forming receptors is not known.

Here, we report that a RANK-dimerizer utilizing FKBP36v and hEpoR induces formation of multinuclear TRAP-positive cells in the presence of CID or Epo; however, the potency of that activation is not sufficient to induce pit forming activity and expression of osteoclast markers induced by RANKL. Our data demonstrate the requirement for RANK trimerization to generate fully functional osteoclasts via upregulation of NFATc1.

## Materials and methods

*Cell culture.* Osteoclastogenesis in RAW264.7 cells (a gift of K. Matsuo, Keio University, Tokyo, Japan) was induced in the presence of 25 ng/ml RANKL. Cultured cells were subjected to tartrate resistant acid phosphatase (TRAP) staining or TRAP-solution assays (Sigma Chemical, St. Louis, MO) as described previously [3,18,19]. Bone resorbing activity was analyzed in dentine slices (Wako Cemicals, Osaka, Japan); resorbing pits were visualized by toluidine blue O staining and the number of pits was counted.

*Construction of RANK-FKBP36v expressing retroviral vector.* To construct the RANK-TM-FKBP36v vector, the Ig κ chain leader sequence (pSecTag2 vector, Invitrogen), which was used as a signal sequence, and the murine RANK transmembrane/cytoplasmic domain ($Ser_{214}$–$Glu_{625}$) were ligated. Subsequently, the FKBP36v fragment (Ariad Pharmaceuticals, Cambridge, MA, www.ariad.com/regulationkits) was PCR amplified and fused to the C-terminus of RANK-TM ($Ser_{214}$–$Glu_{625}$) to yield RANK-TM-FKBP36v. hEpoR (a gift from T. Naruse, Kaketsuken, Kumamoto, Japan) was fused to the N-terminus of RANK-TM to yield hEpoR-RANK-TM. RANK-TM-FKBP36v and hEpoR-RANK-TM were subcloned into the pMXs-IRES-puro vector (a gift of T. Kitamura, University of Tokyo, Tokyo, Japan). Lyophilized AP20187 was dissolved in absolute ethanol as a 1 mM stock solution. RANK-TM-FKBP36v and hEpoR-RANK-TM clustering was induced by addition of 0.05–500 nM AP20187 or 0.12–120 U/ml Epo to the culture, respectively.

*Retrovirus infection.* Retroviral plasmids were introduced into amphotrophic Phoenix cells (ATCC, Manassas, VA) by lipofection, and the supernatant was collected on days 3–5 as a virus solution [19]. RAW264.7 cells were transduced with retrovirus-containing supernatant in the presence of 5 μg/ml polybrene (Sigma Chemical, St. Louis, MO). Culture media were changed to fresh medium on day 1 and to fresh medium containing 5 μg/ml puromycin (Sigma) on day 2. Puromycin resistant stably transfected cells were harvested on day 6 and used for the osteoclast formation assay.

*RT-PCR analysis.* Expression of osteoclastic markers and transcription factors was examined by RT-PCR in Mock, RANK-FKBP36v or hEpoR-RANK-TM transfected RAW264.7 cells cultured in the presence or absence of RANKL (25 ng/ml), AP20187 (50 nM), and Epo (12 U/ml) for 4 days. The primer sets were as follows: CTR (calcitonin receptor)-5′, 5′-TGGTTGAGGTTGTG CCCAATGGAGA-3′; CTR-3′, 5′-CTCGTGGGTTTGCCTCATC TTGGTC-3′; cathepsin K-5′, 5′-ACGGAGGCATTGACTCTGAAG ATG-3′, cathepsin K-3′, 5′-GGAAGCACCAACGAGAGGAGA AAT-3′; c-Fos-5′, 5′-ATGATGTTCTCGGGTTTCAACG-3′; c-Fos-3′, 5′-CAGTCTGCTGCATAGAAGGAACCG-3′; TRAF6-5′, 5′-AGTGAAAGATGACAGCGTGAGTGGCTCT-3′; TRAF6-3′, 5′-G CCATGGACACAGCACAGTTTACACAAG-3′; NFATc1-5′, 5′-CC CAGTATAC CAGCTCTGCCATTG-3′; and NFATc1-3′, 5′-GGAG CCTTCTCCACGAAAATGACT-3′.

*Luciferase assay.* RANK-TM-FABP36v and pNF-κB-luc vector (Becton–Dickinson, San Jose, CA) were stably transduced in RAW264.7 cells. Transduced cells were treated with either RANKL (25 ng/ml) or AP20187 (50 nM) for 24 h and luciferase activities were examined (Promega, Madison, WI).

## Results and discussion

### *RANK dimerization induces TRAP expression*

In order to analyze the effect of RANK dimerization on osteoclastogenesis, two RANK-dimerizer constructs (RANK-TM-FKBP36v and hEpoR-RANK-TM) were engineered. The dimerizing component FKBP36v or hEpoR was fused to the transmembrane and cytoplasmic portion of RANK, which lacked the extracellular domain in order to prevent binding by RANK-ligand (Fig. 1A). RANK-TM-FKBP36v, hEpoR-RANK-TM or Mock-retrovirus carrying the IRES-puromycin resistance sequence was transduced to RAW264.7 cells, and stably transduced cells were selected. Puromycin resistant cells were cultured in the presence or absence of RANKL, AP20187, and Epo. TRAP-positive cells were induced by AP20187 or Epo in a dose dependent manner in RANK-TM-FKBP36v (Fig. 1B) or hEpoR-RANK-TM (Fig. 1C) transduced cells, respectively, while TRAP-positive cells were not observed in Mock transduced cells (Figs. 1B and C). Such a dose-dependent curve is similar to that observed with CID-FKBP36v in other systems [20]. Interestingly, TRAP-activity induced by RANK-dimers was comparable to that induced by RANKL, suggesting that dimerization of RANK is sufficient to promote TRAP expression. Accordingly, multinuclear TRAP-positive, osteoclast-like cells were formed following AP20187 (Fig. 2A) and Epo (data not shown) treatment. It has been observed that overexpression of receptors can induce signal transduction in the absence of ligands. In our experiments, simple overexpression of RANK-TM-FKBP36v or hEpoR-RANK-TM in RAW264.7 cells showed increased TRAP activity compared to unstimulated Mock transduced cells (Figs. 1B and C). Moreover, multinuclear TRAP-positive cells were formed in the absence of AP20187, Epo or RANKL (Fig. 2A and data not shown), suggesting that RANK overexpression may transduce a differentiation signal. However, our previous findings demonstrated that osteoclastogenesis is not induced in the presence of M-CSF alone, and that cells differentiate into macrophages, despite high RANK expression in osteoclast

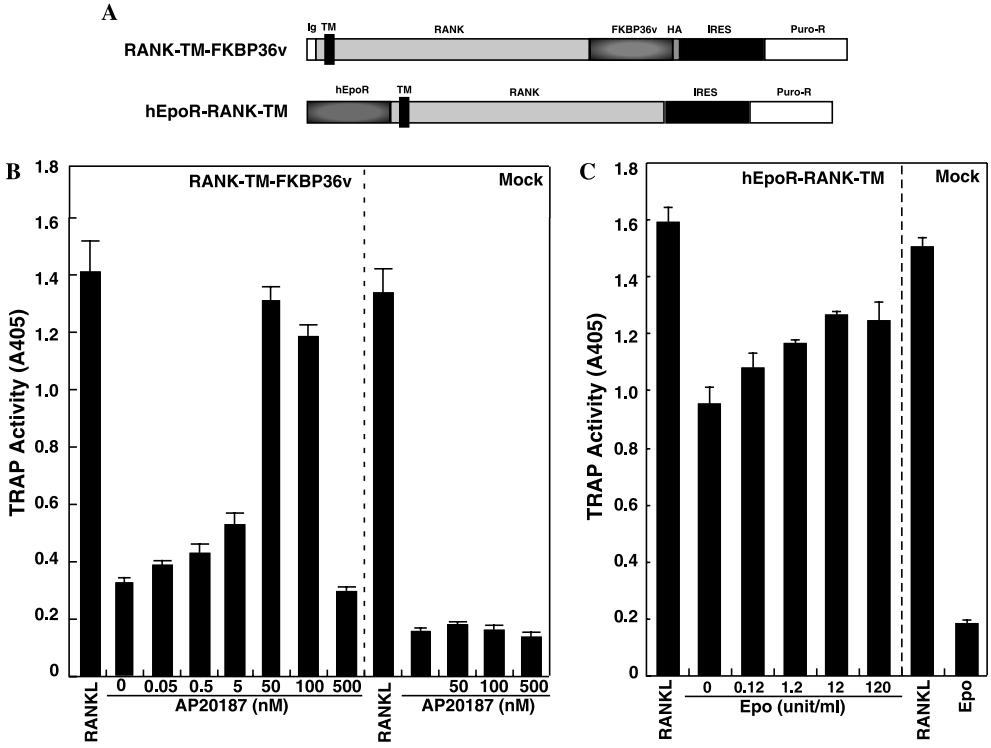


Fig. 1. Dimerized-RANK induces comparable TRAP-activity with RANKL-induced trimerized-RANK. (A) Schematic design of the RANK-TM-FKBP36v and hEpoR-RANK-TM constructs. RANK-TM-FKBP36v; the immunoglobulin (Ig) κ-chain leader sequence followed by the transmembrane (TM) and the cytoplasmic domain of murine RANK was fused to FKBP36v containing a hemagglutinin (HA) epitope tag. hEpoR-RANK-TM; The extracellular portion of human erythropoietin receptor (hEpoR) was fused to N-terminus of RANK. The expression of the transgene was driven by the pMX-IRES-Puro retroviral vector. Puro-R represents the puromycin resistance sequence. (B,C) Dose-dependency of AP20187 and Epo. RANK-TM-FKBP36v, hEpoR-RANK-TM or Mock stably transduced RAW264.7 cells were treated with various concentrations of AP20187 or Epo for 4 days and examined for osteoclastogenesis by a TRAP-solution assay. The TRAP-activity reached a plateau at 50 nM AP20187 in RANK-TM-FKBP36v transduced cells (B) or 12 U/ml Epo in hEpoR-RANK-TM transduced cells (C).

precursor cells without RANKL [3]. Since RANK expression is observed even in unstimulated RAW264.7 cells, RANK clustering by ligand is absolutely required for osteoclast differentiation. Overexpression of hEpoR-RANK-TM showed higher TRAP-activity than that of RANK-TM-FKBP36v, suggesting that intact cytoplasmic domain might be important to transduce a differentiation signal or FKBP36v may inhibit the spontaneous signal in the absence of AP20187.

*RANK-dimerization induces incomplete osteoclasts*

Next, to determine whether RANK-dimerization is sufficient to induce fully differentiated osteoclasts, the number of multinuclear TRAP-positive cells was scored. As shown in Figs. 2B and C, AP20187 and Epo induced multinuclear osteoclasts; however, that number was significantly lower than that seen in RANKL induced osteoclasts (Figs. 2B and C). Furthermore, induction of bone resorbing activity was significantly lower in RANK-dimer induced cells compared with RANKL induced osteoclasts (Figs. 3A and B). Thus, components of the signaling pathway downstream of RANK and responsible for osteoclastogenesis were not fully activated by RANK dimerization. To clarify the mechanisms underlying these differences between RANK-dimer and -trimer, signaling pathway downstream of RANK was examined. RANKL–RANK interaction activates NF-κB, and AP20187 induced NF-κB activation in a manner similar to RANKL (Fig. 4A). RANK signaling also induces expression of the transcription factor NFATc1 through c-Fos and the TRAF6 pathway, and NFATc1 triggers terminal differentiation of osteoclasts [7]. As observed in Fig. 4B, expression of c-Fos and TRAF6 does not differ between RANK-dimer and RANKL induced osteoclasts, while NFATc1 expression is relatively low in RANK-dimer-induced osteoclasts. Since the NFATc1 expression is closely related to the expression of osteoclast differentiation markers such as the calcitonin receptor and cathepsin K (Figs. 4B and C), incomplete osteoclastogenesis is likely due to low induction of NFATc1 by RANK-dimer.

Here, we have shown that the RANK dimerization induces the apparent differentiation of multinuclear TRAP-positive cells, but that the bone resorbing activity and expression of factors required for differentiation of

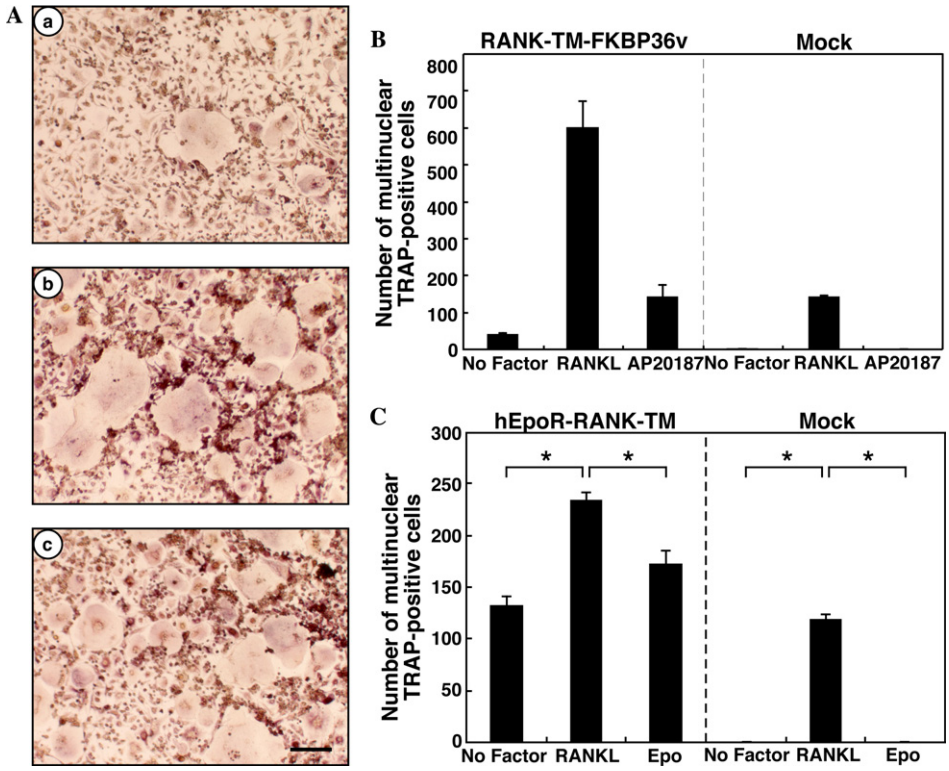


Fig. 2. Multinucleation is low in RANK-dimer induced cells. (A) Multinuclear TRAP-positive cells were formed by both RANKL (25 ng/ml) and AP20187 (50 nM). a, no factor; b, RANKL (25 ng/ml); and c, AP20187 (50 nM). Bar, 100 μm. (B,C) Number of multinuclear TRAP-positive cells was determined. Cells containing more than three nuclei were counted as multinuclear cells. Note that the number of multinuclear TRAP-positive cells is low in RANK-dimerized cells compared with RANK-trimerized cells. $*P < 0.01$.

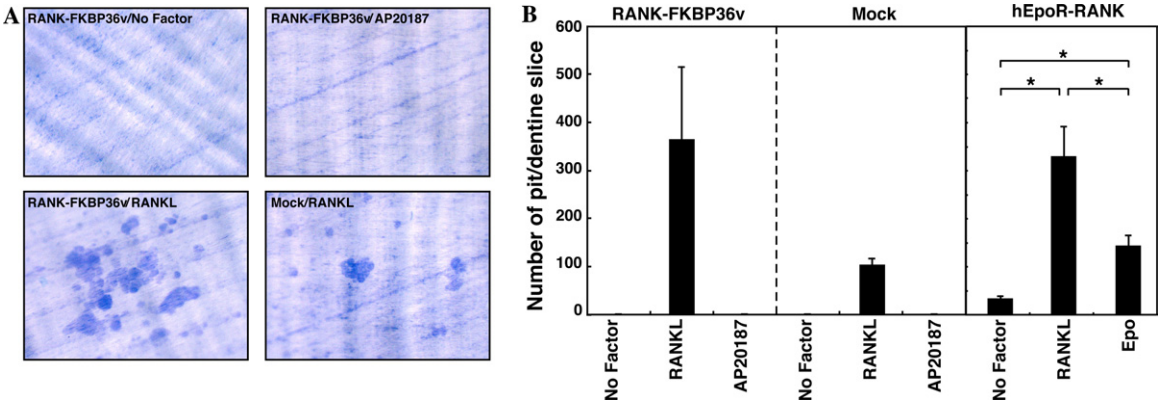


Fig. 3. Pit forming activity is reduced in RANK-dimer induced cells. (A) RANK-TM-FKBP36v stably transfected RAW264.7 cells were treated with either RANKL (25 ng/ml) or AP20187 (50 nM) on dentine slices. After 4 days of culture, resorption lacunae were visualized by toluidine blue O staining. (B) The number of resorption pits was counted. Note that the number of pits induced by RANK dimerization was significantly lower than that induced by RANKL. A representative result from three repeated experiments is shown. $*P < 0.05$.

osteoclasts are lower than those seen in RANKL stimulated cells. We postulate a possible mechanism underlying these observations: dimerization may not be sufficient for signal transduction for receptors requiring trimerization. Fas, a member of the TNF receptor superfamily, forms receptor homotrimers following ligand binding and transduces an apoptotic signal. However, since dimerization of Fas by CID transduces a signal sufficient for cell death [21], dimerization of the TNF receptor superfamily transduces a signal that in some cases is equally effective as stimulation with natural ligands, like the TRAP-expression in our system. TRAP activity is also observed in cells other than osteoclasts; however, bone resorbing activity is limited to osteoclasts, suggesting that the requirement for strong signals by RANK oligomerization is a critical event for osteoclast function. Our system using RANK-TM-FKBP36v and hEpoR-RANK-TM reveals the

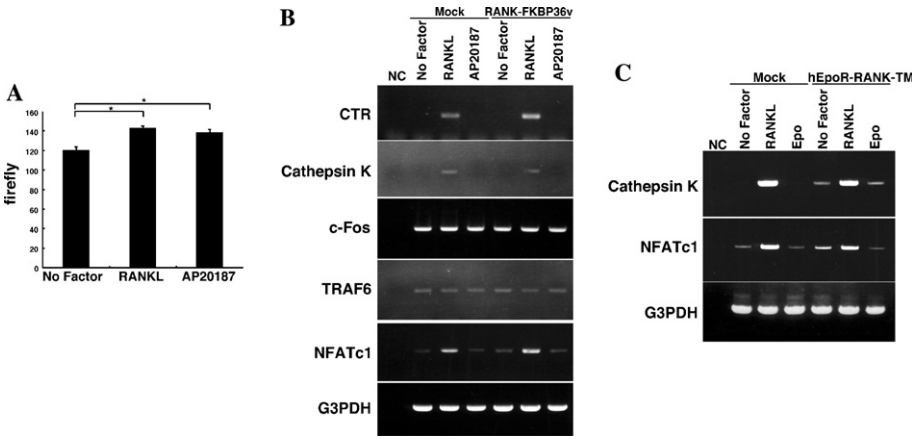


Fig. 4. NFATc1 expression is reduced in RANK-dimer induced cells. (A) Activation of NF-κB was observed in both RANKL and AP20187-induced osteoclasts. $*P < 0.01$. (B,C) Expression of osteoclastic markers and transcription factors was examined in Mock, RANK-TM-FKBP36v or hEpoR-RANK-TM transfected RAW264.7 cells cultured in the presence or absence of RANKL (25 ng/ml), AP20187 (50 nM), and Epo (12 U/ml) for 4 days by RT-PCR. CTR, calcitonin receptor; NC, no template control. Note that the expression of NFATc1, CTR, and cathepsin K is low in RANK-dimer induced cells compared with RANKL induced cells.

biological importance of RANK oligomerization to generate differentiation signals required for fully maturated osteoclasts, and clarifies the relevance of osteoclastic characteristics by the requirement of trimerized-RANK transduced signals during osteoclastogenesis.

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