Inc. (Santa Cruz, CA), for the V5 tag from Invitrogen, for actin from Sigma-Aldrich, and for RANKL from Active Motif (Carlsbad, CA).

Division of ST2 cDNA Library and Plasmid Purification—Procedures for the construction of the ST2 cDNA library were previously described (3). Competent high DH5α (Toyobo) *Escherichia coli* cells were transformed by heat shock with DNA solution of the ST2 cDNA library, and the library was divided into 1000 subpools, each of which contained ~100 clones. After the first screening was completed, every positive pool was divided into 48 subpools each of which contained ~10 clones and was subjected to a second screening, and the resulting positive pools were divided into 48 single clones. The plasmids were purified from *E. coli* using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany).

Constructs—The expression vectors for the mutants of Ras, pcDNA3-RasV12 (a constitutively active mutant) and pcDNA3-RasN17 (a dominant negative mutant), were generous gifts from Dr. C. Kitanaka (Yamagata University). An expression vector for a constitutively active form of Mek1, pcDNA3.1(+)-Mek^{CA}, was constructed by insertion of the cDNA fragments digested from pGEM-Mek^{CA} (generous gift from K. Arai, Research Institute, National Rehabilitation Center for Persons with Disabilities) to NotI restriction site of pcDNA3.1(+) (Invitrogen). The tRANKL-SEAP expression vector was constructed as follows. The cDNA encoding for SEAP was subcloned from pSEAP2-Control (Clontech) by PCR using a set of primers, 5′-CGCTCGAGAATCATCCCAGTTGAGGAGGAGA-ACC-3′ and 5′-GCGTCTAGAGTAACCCGGGTGCGCGG-3′, and the cDNA fragment of the cytoplasmic region, transmembrane domain, and stalk region of the mouse RANKL (corresponding to amino acids 4–157) were subcloned from ST2 cDNA library by PCR using a set of the following primers: 5′-AAGCTTGCCACCATGGCCAGCCGAGACTACGGCA-A-3′ and 5′-GCGGCCGCCGCCTCGCTGGGCCACATCCA-3′. The PCR fragments were ligated into pCR-blunt II TOPO (Invitrogen) using protocols recommended by the manufacturer. The SEAP fragment and the RANKL fragment were digested from TOPO vectors with XhoI/XbaI and HindIII/NotI, respectively, and inserted into the corresponding restriction sites of pcDNA3.1-V5HisA (Invitrogen). The full-length RANKL was cloned from the cDNA of primary osteoblasts by PCR and inserted into the HindIII/NotI sites of pcDNA3.1-V5HisB (Invitrogen). The wild type CAPRI was cloned from the cDNA of primary osteoblasts, and ΔCAPRI was subcloned from pcDL-ΔCAPRI by PCR (for pcDL-SRα vector, see Ref. 18) and inserted into the EcoRI/NotI sites of pEF1-HisC (Invitrogen) and the EcoRI/XhoI sites of pcDNA3.1-V5HisA (Invitrogen). The cDNA for full-length MMP14 was digested from pcDL-MMP14 at SalI sites and inserted into XhoI sites of pcDNA3.1(+). The cDNA for MMP13 was cloned from the cDNA of primary osteoblasts by PCR, and inserted into the HindIII/XbaI sites of pcDNA3.1-V5HisA. Small interfering RNA plasmids for MMP14 were constructed using piGENE U6 vector (iGENE Therapeutics Inc., Ibaraki, Japan) according to manufacturer's protocol. The target sites were 5′-GGGCTGAGATCAAGGCCAA-TG-3′ and 5′-GCGGGTGAGGAATAACCAAGT-3′.

Cell Culture and Transfection—The human kidney cell line 293T, human osteosarcoma cell line SaOS2, and mouse fibroblast cell line NIH3T3 were cultured with Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (Sigma) at 37 °C in a humidified atmosphere containing 5% CO_2. The plasmids were transfected into the cells using the FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions.

Alkaline Phosphatase Assay of Culture Medium—293T cells were seeded in 96-well cell culture plates at a concentration of 5 × 10^5 cells/ml. 24 h later, 625 pg of pcDNA3.1-tRANKL-SEAP and 49.375 ng of a

subpool of the ST2 cDNA library or other constructs were co-transfected to 293T using FuGENE 6. 72 h after the transfection, 50 μl of the culture medium was collected from each well and subjected to an alkaline phosphatase assay. In brief, the culture medium was incubated with 125 μl of dH$_2$O, 100 mM NaHCO$_3$, 100 mM Na$_2$CO$_3$, 1 mM MgCl$_2$, and 2 mM *p*-nitrophenyl phosphate at 65 °C for 90 min, and then the absorbance of the solution at a wavelength of 405 nm was measured using the MTP-300 microplate reader (CORONA Electric, Ibaraki, Japan).

DNA Sequencing of Positive Clones—DNA sequences of the positive clones were determined by the cycle sequencing method. In brief, samples were prepared using the BigDye terminator Ver 1.0 (Applied Biosystems, Foster City, CA) and DyeEx 2.0 spin kit (Qiagen) and then analyzed using the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

Reverse Transcription-PCR—The procedures for preparation of the primary osteoblasts from the calvaria of newborn C57/BL6 mice and for formation of osteoclasts were previously described (19, 20). The total RNA was purified from primary osteoblasts and osteoclasts using ISO-GEN (Nippon Gene Co. Ltd., Toyama, Japan) according to the method recommended by the manufacturer. The cDNA was synthesized from purified RNA using SuperScript II reverse transcriptase (Invitrogen). The primers used for the PCR analysis of ΔCAPRI were 5′-GGCTGGC-CAAGGACTTTCTG-3′ and 5′-CTATGTTCTGGACCGCCTGG-3′.

Western Blotting—The procedure for Western blotting was described previously (20). To detect the soluble RANKL released into the supernatants, 3 ml of the culture medium were incubated with 6 μl of recombinant protein G-agarose (Invitrogen) and 1 μg of recombinant osteoprotegerin-Fc chimeric protein (R&D Biosystems) for 16 h at 4 °C, then recovered by brief centrifugation. The pellets were suspended in TNE buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) and subjected to SDS-PAGE. Membrane fraction was gained using Mem-PER mammalian protein extraction reagent (Pierce Chemical Co.).

Immunocytochemistry—NIH3T3 cells were transfected with pcDNA3.1-ΔCAPRI-V5HisA or -wtCAPRI and, 24 h later, were unstimulated or stimulated with 5 μg/ml ionomycin for 2 min. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X, and immunostained with anti-V5 antibody and Alexa Fluor® 488 goat anti-mouse IgG (Molecular probes, Eugene, OR).

Real Time PCR—The reaction mixture for the real time PCR was prepared using qPCR QuickGoldStar Mastermix Plus for SYBR® Green I (Nippon Gene, Tokyo, Japan) and was analyzed using the Mx3000P^{TM} QPCR System (Stratagene, La Jolla, CA). The set of primers used were 5′-GCTCACCCCAGTCACTCTCA-3′ and 5′-CTCATGGCCTTC-ATGGTGTC-3′ for MMP14 and 5′-GAAGGTGAAGGTCGGAGT-CA-3′ and 5′-GAAGATGGTGATGGGATTTC-3′ for glyceralde-hyde-3-phosphate dehydrogenase.

Statistical Analysis—A statistical analysis was performed using the Student's *t* test for alkaline phosphatase assay and the unpaired Student's *t* test for real time PCR.

RESULTS

Development of a Screening System for Molecules with RANKL Shedding Activity—To identify molecules potentially involved in the regulation of RANKL shedding, we developed a new library screening system. We constructed an expression vector encoding the fusion protein of SEAP with the C-terminally truncated form of RANKL, which contains the stalk region, transmembrane domain, and intracellular domain of RANKL (tRANKL-SEAP) (Fig. 1A). Clear up-regulation of the alkaline phosphatase activity was detected in the culture medium when this
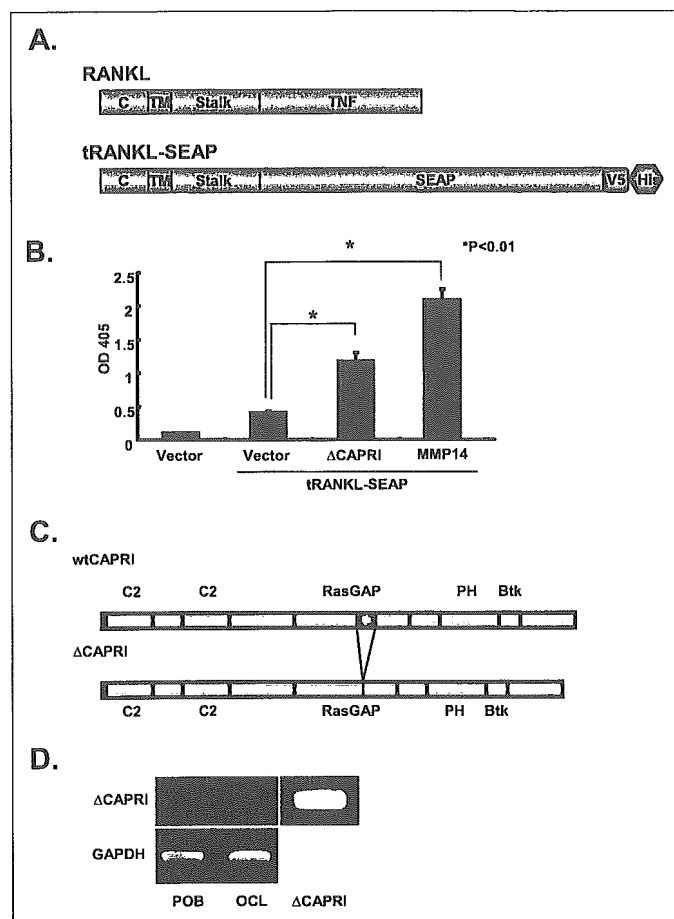
–306–

FIGURE 1. **Identification of ΔCAPRI as a possible regulator of RANKL shedding.** *A*, schematic representation of RANKL and the tRANKL-SEAP fusion protein. tRANKL-SEAP is a fusion protein of SEAP with C-terminally truncated RANKL, which contains the stalk region but lacks the TNF-like domain of RANKL, and with V5 and His$_6$ tags at the C terminus. *C*, cytoplasmic domain; *TM*, transmembrane domain; *Stalk*, stalk region; *TNF*, TNF-like domain. *B*, alkaline phosphatase activity of the culture medium of 293T cells transfected with pcDNA3.1-tRANKL-SEAP and pcDL-SRα, pcDL-ΔCAPRI or pcDL-MMP14. *, significantly different, $p < 0.01$ *C*, schematic representation of an alternative splice variant of CAPRI (ΔCAPRI), which lacks part of the RasGAP domain. *PH*, pleckstrin homology domain; *Btk*, Bruton's tyrosine kinase motif. *D*, expression of ΔCAPRI in primary osteoblasts. Reverse transcription-PCR with specific primers for ΔCAPRI showed clear expression of ΔCAPRI in primary osteoblasts (*left lane*), but low expression in osteoclasts (*middle lane*). The *right lane* shows the positive control of ΔCAPRI using pcDNA3.1-ΔCAPRI plasmids as templates. *POB*, primary osteoblast; *OCL*, osteoclast.

plasmid was co-transfected with the MMP14 expression vector into 293T cells (data not shown), indicating that this assay system is suitable for screening molecules with the RANKL shedding activity. Using this assay system, we screened the cDNA library of the mouse bone marrow-derived stromal cell line, ST2 cells for molecules with RANKL shedding activity. From 1×10^6 clones, 12 positive clones were isolated. Nucleotide sequences of these cDNA fragments revealed that one of the positive clones encoded a full-length cDNA for a novel splice variant of CAPRI, which lacks one exon (138 bp) in the RasGAP domain of the wild type CAPRI (ΔCAPRI) (Fig. 1, *B* and *C*). The other clones included full-length MMP14 (Fig. 1*B*), which was already reported to increase the RANKL shedding (16). The reverse transcription-PCR analysis showed that ΔCAPRI is expressed in mouse primary osteoblasts and osteoclasts (Fig. 1*D*).

ΔCAPRI Expression Increases RANKL Shedding—We next examined the effect of ΔCAPRI and wtCAPRI on the RANKL shedding. Either the ΔCAPRI or the wtCAPRI expression plasmid was transfected to the 293T cells with the tRANKL-SEAP construct, and the alkaline phospha-
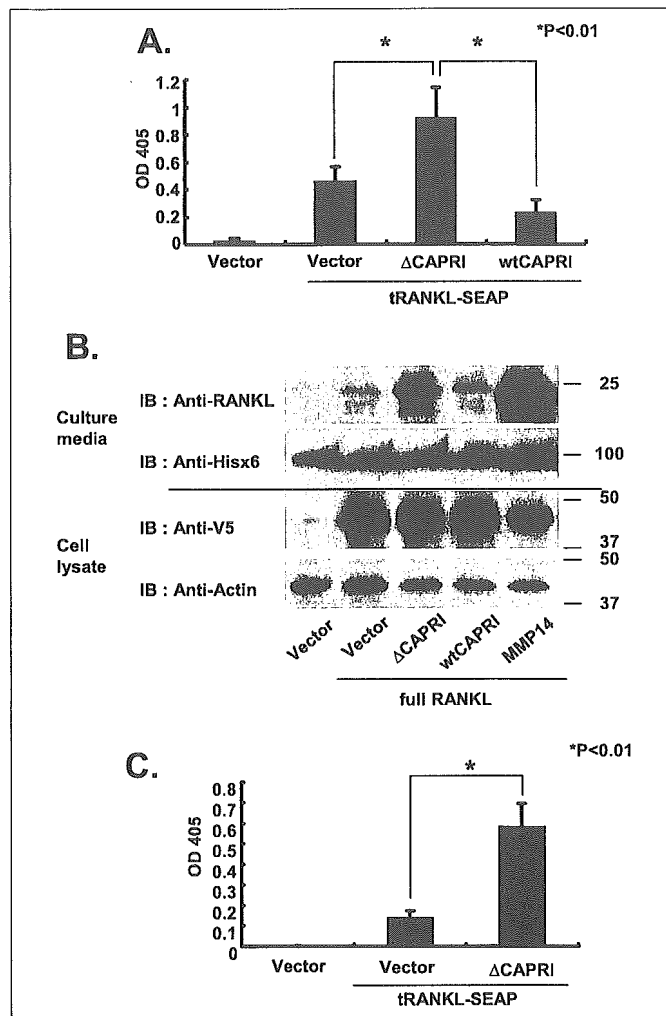

FIGURE 2. **ΔCAPRI expression promotes RANKL shedding.** *A*, alkaline phosphatase activity in the culture supernatants of 293T cells co-transfected with pcDNA3.1-tRANKL-SEAP and pEF1-His, pEF1-ΔCAPRI, or pEF1-wtCAPRI. Up-regulation of alkaline phosphatase activity was observed only in the pEF1-ΔCAPRI-transfected cell supernatants. *, significantly different, $p < 0.01$ *B*, Western blot analysis of cleaved RANKL. 293T cells were co-transfected with full-length RANKL expression vector and pEF1-His,-ΔCAPRI,-wtCAPRI, or pcDNA3.1(+)-MMP14 and incubated for 72 h. Osteoprotegerin-Fc in the culture media and RANKL in the cell lysates were probed with anti-His and anti V5 antibody, respectively. *C*, ΔCAPRI expression induces RANKL shedding in osteoblastic cells. Alkaline phosphatase activity of the culture media of SaOS2 cells transfected with pcDNA3.1-tRANKL-SEAP and pcDL-SRα or pcDL-ΔCAPRI. Up-regulation of alkaline phosphatase activity in pcDL-ΔCAPRI-transfected cells was observed. *, significantly different, $p < 0.01$.

tase activity in culture media was measured using *p*-nitrophenyl phosphate as substrate. The 293T cells co-transfected with the tRANKL-SEAP expression plasmid and empty vector exhibited a very low level of alkaline phosphatase activity in the supernatant. Similarly, a low alkaline phosphatase activity was detected in the conditioned medium of the cells expressing wtCAPRI. In contrast, the co-expression of ΔCAPRI significantly increased the amount of tRANKL-SEAP released into the supernatant, suggesting that the ΔCAPRI expression induces the cleavage of membrane-anchored RANKL in transfected 293T cells (Fig. 2*A*). In addition, the co-transfection of ΔCAPRI and the full-length RANKL expression plasmids markedly promoted the release of RANKL as detected by Western blotting with the anti-RANKL antibody (Fig. 2*B*). We next examined the effect of ΔCAPRI on the RANKL shedding in osteoblastic cells. Overexpression of ΔCAPRI also promoted the RANKL shedding in the osteoblastic cell line SaOS2 cells (Fig. 2*C*).
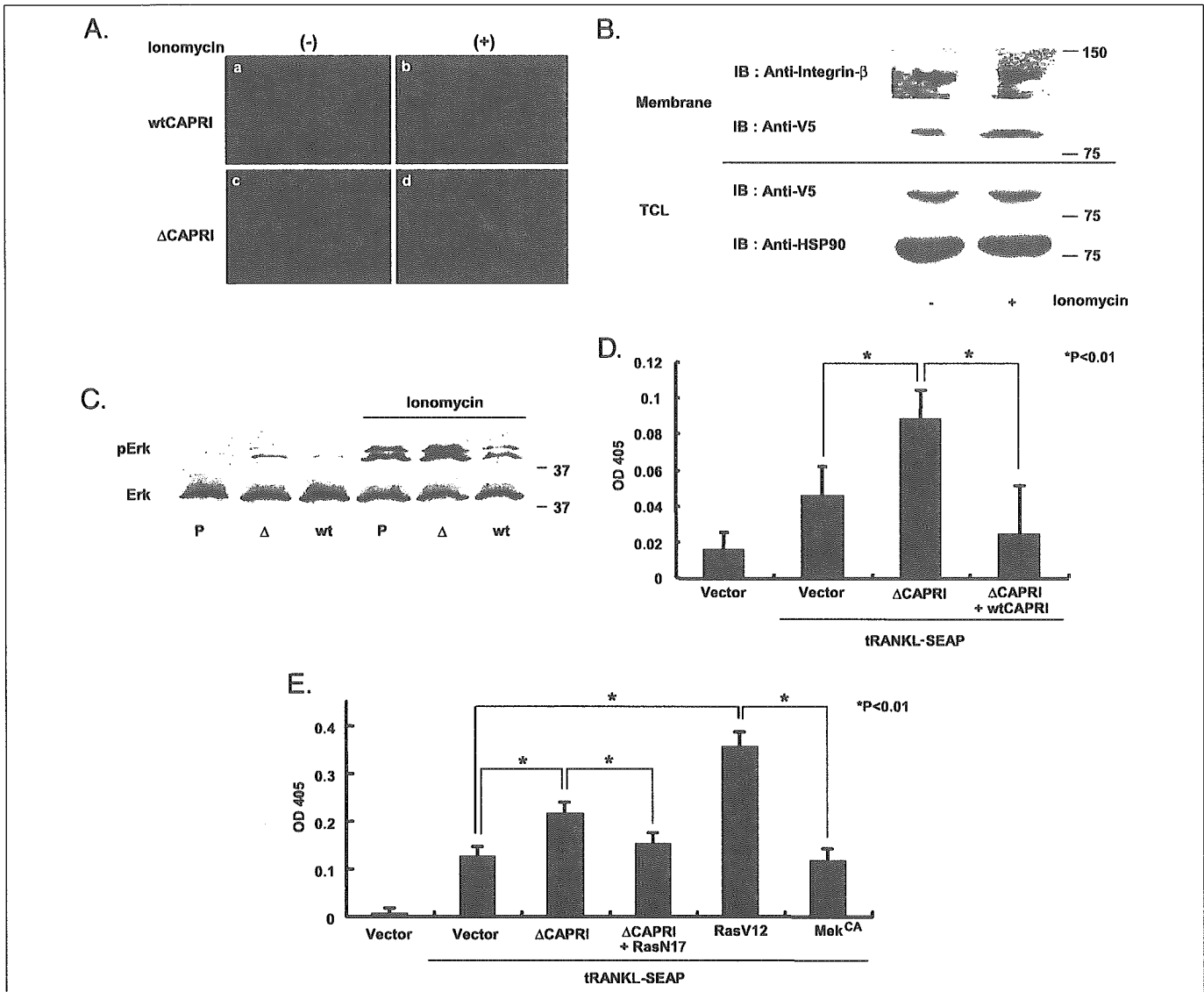
FIGURE 3. **Modulation of Ca^{2+}-induced intracellular signaling by ΔCAPRI.** *A* and *B*, translocation of wtCAPRI and ΔCAPRI to plasma membrane in response to ionomycin stimulation. *A*, NIH3T3 cells transfected with either pcDNA3.1-ΔCAPRI-V5HisA or -wtCAPRI were unstimulated (*a* and *c*) or stimulated with 5 μg/ml ionomycin for 2 min (*b* and *d*) and immunostained with anti-V5 antibody. Note the translocation of both wtCAPRI and ΔCAPRI from cytoplasm to plasma membrane upon stimulation with ionomycin. *B*, 293T cells were transfected with pcDNA3.1-ΔCAPRI-V5HisA, and 48 h later, membrane fraction and total cell lysates of cells unstimulated (*left lane*) or stimulated (*right lane*) with 5 μg/ml ionomycin for 2 min were subjected to SDS-PAGE. Integrin-β was used as a marker for the membrane fraction, and HSP90 as an internal control of total cell lysates. Ionomycin treatment up-regulated the membrane localization of ΔCAPRI as shown by the anti-V5 blotting, whereas the amount of ΔCAPRI in the total cell lysates did not appear to differ. *TCL*, total cell lysates. *C*, ΔCAPRI promotes ionomycin-induced Erk activation. 293T transfected with pcDNA3.1-V5HisA, -ΔCAPRI, or -wtCAPRI were incubated for 36 h and serum-starved for 12 h. Cells were stimulated by 5 μg/ml of ionomycin for 2 min and subjected to Western blot analysis. ΔCAPRI expression increased ionomycin-induced Erk activation, whereas wtCAPRI expression suppressed it. *P*, pcDNA3.1-V5HisA; *Δ*, pcDNA3.1-ΔCAPRI-V5HisA; *wt*, pcDNA3.1-wtCAPRI-V5HisA. *D* and *E*, alkaline phosphatase activity of the supernatants of the 293T cell cultures transfected with pcDNA3.1-tRANKL-SEAP and pcDNA3.1-V5HisA, -ΔCAPRI, wtCAPRI, pcDNA3-RasV12, -RasN17, or pcDNA3.1(+)-Mek^{CA}. *, significantly different, $p < 0.01$.

ΔCAPRI Activates Ras Signaling Pathways—Lockyer *et al.* (21) reported that the elevation of the intracellular Ca^{2+} causes a rapid C2 domain-dependent translocation of wtCAPRI to the plasma membrane, resulting in the activation of the RasGAP activity and the inactivation of the Ras/Mek/Erk pathways. Therefore, we investigated the translocation of wt and ΔCAPRI after the Ca^{2+} influx by the Ca^{2+} ionophore ionomycin treatment and found that both the wt and ΔCAPRI moved to the plasma membrane of NIH3T3 and 293T cells 2 min after the ionomycin stimulation as shown in Fig. 3*A* by immunocytochemistry and Fig. 3*B* by cell fractionation analysis. To investigate whether ΔCAPRI modulates the Ras/Erk signaling pathway, we examined the Erk activity in the ΔCAPRI- or wtCAPRI-overexpressing 293T cells. As shown in Fig. 3*C*, the overexpression of ΔCAPRI enhanced the ionomycin-induced Erk phosphorylation, whereas the wtCAPRI expression reduced the Erk activation, indicating that ΔCAPRI works in a

dominant negative fashion. In fact, wtCAPRI suppressed RANKL shedding promoted by ΔCAPRI (Fig. 3*D*).

Ras Activation, but Not Mek/Erk Pathway, Is Involved in RANKL Shedding—Because CAPRI is a Ca^{2+}-sensitive RasGAP, we next tested whether the Ras activity is involved in the RANKL shedding. As shown in Fig. 3*E*, the expression of the constitutively active Ras strongly promoted the RANKL shedding in the 293T cells. RANKL shedding induced by the ΔCAPRI overexpression was significantly suppressed by the co-expression of a dominant negative mutant of Ras (RasN17). These results indicate that the Ras activity plays an important role in the regulation of the RANKL shedding induced by ΔCAPRI. Interestingly, however, the up-regulation of Erk activity in the 293T cells with the constitutively active Mek1 expression (Mek^{CA}) did not increase the alkaline phosphatase activity released into the supernatant (Fig. 3*E*).
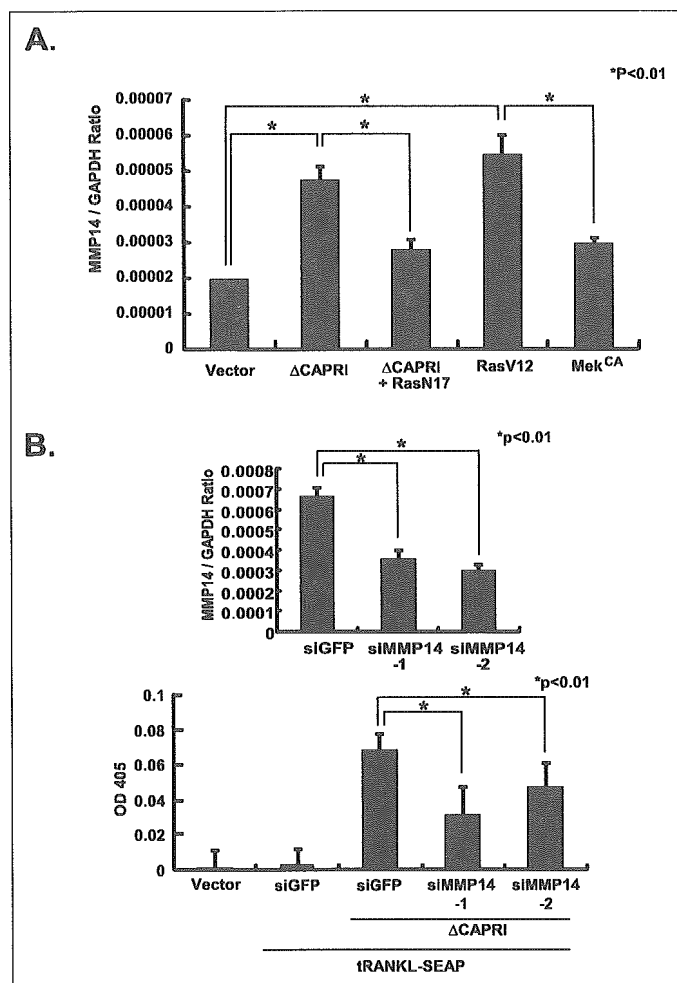
FIGURE 4. **ΔCAPRI-Ras-MMP14 axis plays an important role in the RANKL shedding.** *A,* ΔCAPRI and Ras^{CA} increases the expression of MMP14. mRNA level of MMP14 in 293T cells measured by real time PCR. 293T cells were transfected with pcDNA3.1-V5HisA, -ΔCAPRI, pcDNA3-RasV12, -RasN17, or pcDNA3.1(+)-Mek^{CA}. Forty-eight hours later, RNA was extracted and subjected to real time PCR. *, significantly different, $p < 0.01$ *B,* MMP14 knockdown suppresses RANKL shedding. SaOS2 cells were transfected with pU6-siGFP, -siMMP14-1, or -2, and MMP14 expression was analyzed by real time PCR (*upper panel*). MMP14 knockdown using these constructs suppressed ΔCAPRI-induced RANKL shedding as shown by alkaline phosphatase activity in the culture media (*lower panel*).

FIGURE 5. **MMP13 has much weaker effect on RANKL shedding compared with MMP14.** Alkaline phosphatase activity of the culture medium of 293T cells transfected with pcDNA3.1-tRANKL-SEAP and pcDNA3.1-V5HisA, -MMP13, or -MMP14. *, significantly different, $p < 0.01$.

Ras Activity Regulates the Expression Level of MMP14—ST2 cDNA library screening for the RANKL shedding confirmed that MMP14 could be a candidate for the RANKL sheddase, and in fact, the co-transfection of MMP14 expression vector with pcDNA3.1-tRANKL-SEAP significantly increased the amount of tRANKL-SEAP released into the supernatant (Fig. 1*B*). We next examined whether or not the Ras activation downstream of ΔCAPRI is involved in the expression level of MMP14. In the real time PCR analysis from mRNA of the 293T cells, ΔCAPRI up-regulated MMP14, which was significantly suppressed by the co-expression of Ras^{DN}. Ras^{CA} induced the up-regulation of MMP14, whereas Mek^{CA} did not affect the expression level of MMP14 (Fig. 4*A*). This result suggested that ΔCAPRI would promote the RANKL shedding through up-regulation of MMP14. To confirm this, we constructed siRNA vectors for MMP14. As shown in Fig. 4*B*, siMMP14-1 and -2 suppressed expression of endogenous MMP14 in SaOS2 cells (*upper panel*), and the RANKL shedding promoted by ΔCAPRI (*lower panel*).

MMP14 Has Much Stronger RANKL Shedding Activity than MMP13—Inada *et al.* (22) recently reported that MMP13-deficient mice had skeletal abnormalities such as increased trabecular bone mass.
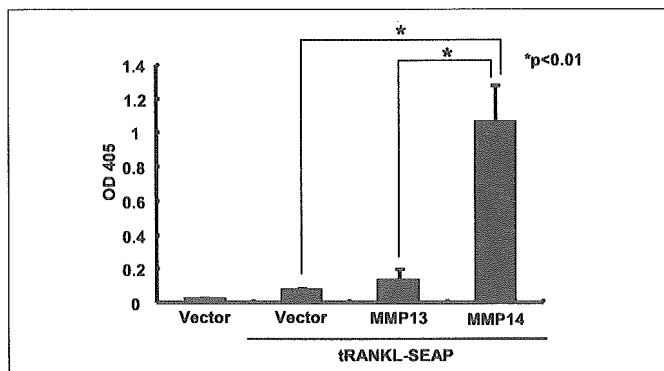
To examine the relevance of this proteinase in RANKL shedding, we transfected the MMP13 expression vector and tRANKL-SEAP to 293T cells. Overexpression of MMP13 exhibited little effect on the up-regulation of the alkaline phosphatase activity in the supernatant, indicating that MMP13 had much weaker RANKL shedding activity compared with MMP14 (Fig. 5).

DISCUSSION

Ectodomain shedding is a highly regulated process that affects a number of transmembrane proteins, and is considered to play an important role in regulating various pathophysiological events. The role of ectodomain shedding varies between substrate proteins. For example, shedding allows some local growth factors, such as TNF-α and epidermal growth factor, to be released from the local environments and participate in the paracrine and endocrine signalings (13, 14, 23). Interestingly, proteolytic processing via shedding is important even for the local effects of the growth factors such as epidermal growth factor (24).

RANKL is a key molecule for the osteoclastogenesis and bone-resorbing activity of mature osteoclasts. RANKL is produced as a membrane-bound cytokine and released into the paracrine and the endocrine milieu via ectodomain shedding, although the biological significance of RANKL shedding is still unknown. The soluble form of RANKL induces *in vitro* osteoclastogenesis from bone marrow cells, and several studies have revealed an increase in the intraarticular level of soluble RANKL under pathological conditions such as rheumatoid arthritis (25). Recently, Mizuno *et al.* (26) generated transgenic animals overexpressing soluble RANKL in the liver after birth that exhibited a marked decrease in bone mineral density with aging, indicating that the excessive production of soluble RANKL can promote *in vivo* osteoclastogenesis. On the other hand, it was reported that the membrane-bound RANKL is more potent in stimulating osteoclast differentiation than its soluble form (12). To reveal the biological and pathological relevance of RANKL shedding, elucidating the molecular mechanism underlying RANKL shedding is indispensable.

It is difficult to detect soluble RANKL in the culture medium by the usual Western blot analysis because the expression level of RANKL is relatively low. Nakashima *et al.* (12) developed a ligand-receptor precipitation Western blot analysis using osteoprotegerin, which specifically binds to RANKL, but this system is not suitable for systematic screening of a large number of molecules. Blobel and coworkers (27) reported a simple and quantitative assay for TNF-α shedding using alkaline phosphatase-tagged TNF-α. This method allows the rapid and reproducible quantitation of the TNF-α shedding. We first constructed an expression vector that encodes a fusion protein of mouse full-length RANKL and

SEAP (full RANKL-SEAP) and transfected it into 293T cells. Although we could find the expression of the full RANKL-SEAP by Western blotting, we failed to detect alkaline phosphatase activity in the supernatants even when the vector was co-transfected with a putative RANKL sheddase, MMP14, the reason of which remains unclear (data not shown). For several type II transmembrane proteins including TNF-α, the juxtamembrane sequence surrounding the cleavage site has been shown to be sufficient to target the protein for regulated shedding (16). Therefore, we next constructed an expression vector encoding a fusion protein of SEAP with the C-terminally truncated RANKL, which contained the stalk region but lacked the TNF-like domain of RANKL (pcDNA3.1-tRANKL-SEAP), and transfected it together with the ST2 cell-derived cDNA library. Using this assay system, we could isolate 12 independent positive clones, which showed alkaline phosphatase activity in the supernatant when transfected with pcDNA3.1-tRANKL-SEAP. They could also increase the amount of soluble RANKL when transfected into the 293T cells with the full-length RANKL, confirming the relevance of this screening system.

One of the positive clones was a spliced variant of CAPRI. The protease activity inducing ectodomain shedding of the transmembrane proteins has been reported to be modulated by protein kinase signaling such as the Ras/Mek/Erk pathway (28). CAPRI was originally identified as a member of the RasGAP, negative regulators of Ras signaling pathways (21, 29), and the R473S mutation decreased the RasGAP activity of CAPRI and enhanced ATP- or ionomycin-induced Erk phosphorylation (21). ΔCAPRI lacks the Arg-473-containing exon in the RasGAP domain in which the FLR motif stabilizes the catalytic arginine-finger loop (30), suggesting that it can work in a dominant negative fashion like the R473S mutant, and in fact, the ΔCAPRI expression increased the ionomycin-induced Erk activation, and co-expression of wtCAPRI diminished the RANKL shedding promoted by ΔCAPRI. The expression of a dominant negative mutant of Ras (RasN17) suppressed the RANKL shedding induced by ΔCAPRI, and a constitutively active mutant of Ras (RasV12) expression stimulated the RANKL shedding, indicating that ΔCAPRI induces RANKL shedding via activating Ras pathways.

The only protease we could isolate in this screening system was MMP14 (MT1-MMP), which was previously reported as a candidate of the RANKL sheddases (16). It should be noticed that MMP14 knock out mice showed an increase in osteoclast number and developed severe osteopenia (31). This suggests that MMP14 negatively regulates the local osteoclastogenesis by reducing membrane-bound RANKL through ectodomain shedding, although it may increase the amount of soluble RANKL. The serum concentration of soluble RANKL in the physiological condition is reportedly less than 1 ng/ml, which is not high enough to induce general osteopenia (32).

Our data do not exclude the possibility that other proteases than MMP14 are involved in this process. Recent studies have demonstrated the role of MMP13 in regulating bone integrity; however, RANKL shedding activity of MMP13 was much weaker than that of MMP14. The overexpression of ΔCAPRI or Ras^{CA} increased the expression of MMP14, and MMP14 knockdown suppressed RANKL shedding promoted by ΔCAPRI, indicating that the ΔCAPRI/Ras pathways stimulate the RANKL shedding by regulating the expression of MMP14.

Interestingly, the constitutively active Mek expression failed to up-regulate the amount of the cleaved RANKL or MMP14 expression, although the Erk activity was strongly up-regulated (data not shown). These results suggest that the downstream cascades of Ras other than the Mek/Erk pathway are implicated in the RANKL shedding. A further investigation is required to clarify the signal transduction pathway(s) downstream of Ras that regulates the RANKL shedding.

In conclusion, we established a novel library screening system for identifying the molecules involved in the RANKL shedding and identified a splice variant of CAPRI, ΔCAPRI, as a possible candidate. ΔCAPRI activates the Ras pathways, which increase the expression of MMP14 in a Mek/Erk-independent manner and lead to the RANKL shedding. These data suggest that the ΔCAPRI-Ras-MMP14 axis plays an important role in the RANKL shedding.

REFERENCES

1. Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Teepe, M. C., DuBose, R. F., Cosman, D., and Galibert, L. (1997) *Nature* **390**, 175–179
2. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F. S., III, Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) *J. Biol. Chem.* **272**, 25190–25194
3. Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, 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) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3597–3602
4. Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., and Boyle, W. J. (1998) *Cell* **93**, 165–176
5. Kong, Y. Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., Capparelli, C., Li, J., Elliott, R., McCabe, S., Wong, T., Campagnuolo, G., Moran, E., Bogoch, E. R., Van, G., Nguyen, L. T., Ohashi, P. S., Lacey, D. L., Fish, E., Boyle, W. J., and Penninger, J. M. (1999) *Nature* **402**, 304–309
6. Wong, B. R., Josien, R., Lee, S. Y., Vologodskaia, M., Steinman, R. M., and Choi, Y. (1998) *J. Biol. Chem.* **273**, 28355–28359
7. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniguchi, T. (2002) *Dev. Cell* **3**, 889–901
8. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J., and Penninger, J. M. (1999) *Nature* **397**, 315–323
9. Dougall, W. C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., Daro, E., Smith, J., Tometsko, M. E., Maliszewski, C. R., Armstrong, A., Shen, V., Bain, S., Cosman, D., Anderson, D., Morrissey, P. J., Peschon, J. J., and Schuh, J. (1999) *Genes Dev.* **13**, 2412–2424
10. Bucay, N., Sarosi, I., Dunstan, C. R., Morony, S., Tarpley, J., Capparelli, C., Scully, S., Tan, H. L., Xu, W., Lacey, D. L., Boyle, W. J., and Simonet, W. S. (1998) *Genes Dev.* **12**, 1260–1268
11. Lum, L., Wong, B. R., Josien, R., Becherer, J. D., Erdjument-Bromage, H., Schlondorff, J., Tempst, P., Choi, Y., and Blobel, C. P. (1999) *J. Biol. Chem.* **274**, 13613–13618
12. Nakashima, T., Kobayashi, Y., Yamasaki, S., Kawakami, A., Eguchi, K., Sasaki, H., and Sakai, H. (2000) *Biochem. Biophys. Res. Commun.* **275**, 768–775
13. Kriegler, M., Perez, C., DeFay, K., Albert, I., and Lu, S. D. (1988) *Cell* **53**, 45–53
14. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) *Nature* **385**, 729–733
15. Schneider, P., Holler, N., Bodmer, J. L., Hahne, M., Frei, K., Fontana, A., and Tschopp, J. (1998) *J. Exp. Med.* **187**, 1205–1213
16. Schlondorff, J., Lum, L., and Blobel, C. P. (2001) *J. Biol. Chem.* **276**, 14665–14674
17. Chesneau, V., Becherer, J. D., Zheng, Y., Erdjument-Bromage, H., Tempst, P., and Blobel, C. P. (2003) *J. Biol. Chem.* **278**, 22331–22340
18. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **8**, 466–472
19. Suda, T., Jimi, E., Nakamura, I., and Takahashi, N. (1997) *Methods Enzymol.* **282**, 223–235
20. Yamamoto, A., Miyazaki, T., Kadono, Y., Takayanagi, H., Miura, T., Nishina, H., Katada, T., Wakabayashi, K., Oda, H., Nakamura, K., and Tanaka, S. (2002) *J. Bone*

–310–

Miner. Res. **17**, 612–621

21. Lockyer, P. J., Kupzig, S., and Cullen, P. J. (2001) *Curr. Biol.* **15**, 981–986

22. Inada, M., Wang, Y., Byrne, M. H., Rahman, M. U., Miyaura, C., Lopez-Otin, C., and Krane, S. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 17192–17197

23. Harris, R. C., Chung, E., and Coffey, R. J. (2003) *Exp. Cell Res.* **284**, 2–13

24. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402**, 884–888

25. Kotake, S., Udagawa, N., Hakoda, M., Mogi, M., Yano, K., Tsuda, E., Takahashi, K., Furuya, T., Ishiyama, S., Kim, K. J., Saito, S., Nishikawa, T., Takahashi, N., Togari, A., Tomatsu, T., Suda, T., and Kamatani, N. (2001) *Arthritis Rheum.* **44**, 1003–1012

26. Mizuno, A., Kanno, T., Hoshi, M., Shibata, O., Yano, K., Fujise, N., Kinosaki, M., Yamaguchi, K., Tsuda, E., Murakami, A., Yasuda, H., and Higashio, K. (2002) *J. Bone Miner. Metab.* **20**, 337–344

27. Zheng, Y., Schlondorff, J., and Blobel, C. P. (2002) *J. Biol. Chem.* **277**, 42463–42470

28. Fan, H., and Derynck, R. (1999) *EMBO J.* **18**, 6962–6972

29. Bivona, T. G., Perez De Castro, I., Ahearn, I. M., Grana, T. M., Chiu, V. K., Lockyer, P. J., Cullen, P. J., Pellicer, A., Cox, A. D., and Philips, M. R. (2003) *Nature* **424**, 694–698

30. Ahmadian, M. R., Stege, P., Scheffzek, K., and Wittinghofer, A. (1997) *Nat. Struct. Biol.* **4**, 686–68931

31. Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S. A., Mankani, M., Robey, P. G., Poole, A. R., Pidoux, I., Ward, J. M., and Birkedal-Hansen, H. (1999) *Cell* **99**, 81–92

32. Nakamura, M., Udagawa, N., Matsuura, S., Mogi, M., Nakamura, H., Horiuchi, H., Saito, N., Hiraoka, B. Y., Kobayashi, Y., Takaoka, K., Ozawa, H., Miyazawa, H., and Takahashi, N. (2003) *Endocrinology* **144**, 5441–5449

Sakae Tanaka
Kozo Nakamura
Naoyuki Takahasi
Tatsuo Suda

Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL–RANK signaling system

Authors' addresses
Sakae Tanaka[1], Kozo Nakamura[1], Naoyuki Takahasi[2], Tatsuo Suda[3]
[1]Department of Orthopaedic Surgery, Faculty of Medicine, The University of Tokyo, Tokyo, Japan.
[2]Institute for Oral Science, Matsumoto Dental University, Nagano, Japan.
[3]Saitama Medical School Research Center for Genomic Medicine, Saitama, Japan.

Correspondence to:
Sakae Tanaka
Department of Orthopaedic Surgery
Faculty of Medicine
The University of Tokyo
7-3-1 Hongo
Bunkyo-ku
Tokyo 113-0033, Japan
Tel.: +81 3 3815 5411x33376
Fax: +81 3 3818 4082
E-mail: tanakas-ort@h.u-tokyo.ac.jp

Summary: Osteoclasts are primary cells for physiological and pathological bone resorption, and receptor activator of nuclear factor-κB ligand (RANKL) is critically involved in the differentiation, activation, and survival of these cells. Recently, therapeutics for pathological bone destruction targeting RANKL pathways has attracted a great deal of attention. Herein, we review the recent advances in the research on osteoclast biology and discuss the advantages and disadvantages of anti-RANKL therapies.

Introduction

The integrity of the skeletal tissues is maintained by a well-organized balance of old bone resorption by osteoclasts and new bone formation by osteoblasts (1, 2). Osteoblasts are bone-forming cells derived from multipotent mesenchymal stem cells, which also give rise to chondrocytes, muscle cells, and adipocytes, whereas osteoclasts are multinucleated giant cells differentiated for bone resorption and derived from the monocyte/macrophage lineage precursor cells (3–8). The bone remodeling cycle is a sequential process that is highly regulated. The 'activation' phase starts with the interaction between osteoblasts and osteoclast precursors, which differentiate into mature osteoclasts in the 'resorption' phase. In the 'reversal' phase, osteoclasts complete the resorption process and produce the signals that initiate bone formation directly or indirectly, and in the final 'formation' phase, mesenchymal cells differentiate into functional osteoblasts to make bone matrix. It has been estimated that about 10% of the total bone in adult humans is remodeled per year, and bone remodeling is important not only to maintain the skeletal structure and strength but also to regulate calcium homeostasis (9). In the remodeling cycle, the length of the resorption phase is very

30

short (2–4 weeks) compared with that of the formation phase (4–6 months), and the lifespan of the osteoclast is much shorter than that of the osteoblast (9, 10). Because the recruitment of osteoclasts on the bone surface initiates the remodeling cycle in the activation phase, the process of osteoclast differentiation and activation determines the activation frequency and regulates the bone homeostasis.

Osteoclasts are specifically differentiated for bone resorption (8, 11). Osteoclasts are highly motile cells, and once attached on the bone surface, the cells become highly polarized and form a tight ring-like zone of adhesion called the sealing zone (11). The cytoplasm of the sealing zone is devoid of cellular organelles, except for numerous actin filaments surrounded by a ring containing the adhesion-related cytoskeletal molecules, vinculin and talin, the structure called 'actin ring.' The space between the cells and the bone matrix constitutes the bone-resorbing compartment. Osteoclasts synthesize several proteolytic enzymes, such as cathepsin K and matrix metalloproteinase 9, which are transported toward the apical side of the cells and secreted into the sealed compartment and play important roles in bone mineral degradation (4, 11, 12). Simultaneously, osteoclasts acidify this compartment by extruding protons via proton pumps (vacuolar type H^+-adenosine triphosphatase) located on the apical membrane, and the low pH (pH 3–4) of the compartment contributes to demineralization of the bone matrix. The apical membrane of the cells, the so-called ruffled border membrane, develops a characteristic structure with numerous folds (11, 13). The extensive folding of this structure is probably due to the intense vesicular traffic associated with secretion as well as to the need for increasing the number of proton pumps via an amplification of the apical membrane. Osteoclasts show high levels of tartrate-resistant acid phosphatase (TRAP) activity, which is widely used as a specific histochemical marker of the cells.

Remarkable progress in research has occurred during the last decade on the molecular mechanism of osteoclast differentiation and activation, especially by the finding of the receptor activator of nuclear factor (NF)-κB ligand (RANKL). RANKL is also known as TRANCE [tumor necrosis factor (TNF)-related activation induced cytokine], osteoprotegrin (OPG) ligand, osteoclast differentiation factor (ODF), or TNF superfamily member 11 (14–17). RANKL is a type 2 (carboxy-terminus outside the cell) transmembrane protein that belongs to the TNF family cytokines, and it is produced in various tissues and abundantly expressed in bone and lymphoid tissues. Although RANKL was originally identified as a dendritic cell survival factor produced by activated T cells, subsequent studies have established a critical role of RANKL in the differentiation and activation of osteoclasts (Fig. 1). RANKL assembles into functional trimers, binds to its specific receptor RANK, and induces the differentiation of osteoclasts from their precursor cells (15, 16). RANKL also promotes the bone resorbing activity of osteoclasts and prolongs their survival (18–20). A large body of research has shown that the RANKL–RANK system is not only involved in the physiological bone development by regulating the bone remodeling cycle but also plays an important role in the pathological bone
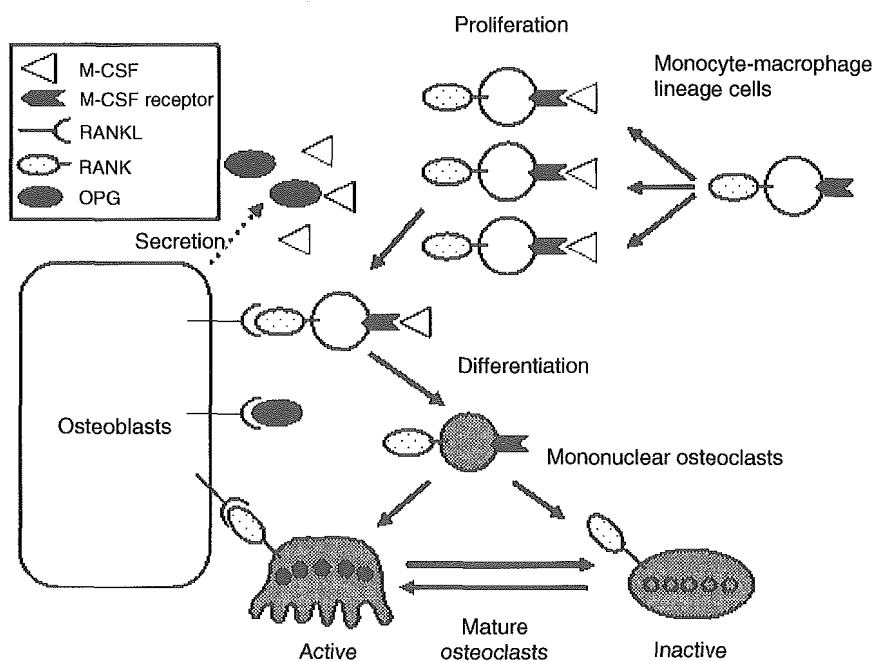

Fig. 1. Schematic representation of the cytokine milieu involved in osteoclast differentiation. Macrophage colony-stimulating factor (M-CSF)-dependent macrophages are differentiated into osteoclasts in the presence of receptor activator of nuclear factor-κB ligand (RANKL), which binds to the specific receptor RANK. Osteoprotegrin (OPG) binds to RANKL and suppresses osteoclast differentiation by competitively inhibiting RANKL–RANK interaction. M-CSF is indispensable for both proliferation of osteoclast progenitors and their differentiation into mature osteoclasts.

destruction. RANK–RANKL signaling therefore can be a potent target of the therapeutic intervention for various bone diseases.

Physiological role of RANKL–RANK pathways

In the murine coculture system of osteoblasts and spleen cells, osteoblasts stimulated by osteotropic factors such as $1\alpha,25$-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], parathyroid hormone (PTH), TNF-α, and prostaglandins express RANKL and support osteoclast differentiation from their precursors. RANKL binds to its receptor RANK, a type I transmembrane receptor belonging to the TNF receptor superfamily, which is expressed in monocyte-macrophage lineage osteoclast precursor cells as well as in mature osteoclasts and dendritic cells. In the presence of soluble RANKL (sRANKL) and macrophage colony-stimulating factor (M-CSF), spleen cells can differentiate into mature osteoclasts in the absence of osteoblasts. Binding of RANKL to RANK induces various intracellular signaling cascades including NF-κB, c-Jun N-terminus kinase (JNK), p38 mitogen-activated protein tyrosine kinase (MAPK), and NF of activated T cells c1 (NFATc1) activation (5). The adapter molecule TNF receptor-associated factor 6 (TRAF6) is critical in RANK signaling, as its specific binding sites exist in the intracellular domain of RANK (21). The other important component in this signaling system is OPG, a soluble decoy receptor of RANKL named for its effects to protect against bone loss, which also belongs to the TNF receptor superfamily (22, 23). OPG specifically binds to RANKL and inhibits RANKL activity by preventing its binding to RANK (24).

The essential role of RANKL–RANK pathways in osteoclast development was further established by a series of gene ablation experiments (25). The targeted disruption of either RANKL or RANK caused severe osteopetrosis in mice, and very few osteoclasts were observed in the skeletal tissues of these knockout mice (26–29). Conversely, the targeted disruption of OPG caused osteopenia with increased numbers of activated osteoclasts in the bone tissue (30, 31).

Subsequent research has revealed that the bone-resorbing activity and apoptosis of osteoclasts are also regulated by RANKL–RANK pathways. sRANKL treatment not only elongates the survival of mature osteoclasts but also stimulates their bone-resorbing activity both in vitro and in vivo, while administration of OPG induces apoptotic cell death of mature osteoclasts in vivo (18–20). These in vitro and in vivo observations uncovered that the RAKL/RANK/OPG axis is a critical regulator of normal osteoclast development and therefore essential for healthy bone growth.

Signal transduction pathways of the RANKL–RANK system

TRAF6

There has been remarkable progress in the study of the molecular mechanism implicated in the signal transduction pathways of RANKL/RANK system (Fig. 2). RANK is expressed on the surface of osteoclast progenitor cells and induces intracellular signals leading to osteoclastogenesis upon ligand binding or agonistic anti-RANK antibody stimulation. Like other TNF receptor superfamily members, RANK stimulation induces NF-κB, JNK, and p38 MAPK activation (Fig. 2).

TRAF family proteins are adapter molecules, which mediate intracellular signaling of various cytokine receptors including TNF receptor superfamily and Toll/interleukin-1 receptor (IL-1R) family members (21, 32). Seven TRAF family members have been identified, and several studies showed that RANK recruits TRAF2, TRAF3, TRAF5, and TRAF6 upon binding to its ligand (33–35). TRAF6 has the most divergent TRAF-C domain, and it is the only TRAF that is involved in the signal from members of the Toll/IL-1R family by interacting with the IL-1R-associated kinase (21). TRAF2, TRAF3, and TRAF5 bind to the membrane-distal domain in the cytoplasmic tail of RANK, whereas TRAF6 interacts with the membrane-proximal domain (36). The essential role of TRAF6 in normal bone development was established through experiments utilizing TRAF6-deficient mice, which displayed an osteopetrotic phenotype (37, 38). Very few osteoclasts were found in the skeletal tissues of the knockout mice, and Naito et al. (38) demonstrated that the spleen cells obtained from TRAF6$^{-/-}$ mice did not differentiate into osteoclasts, even in the presence of M-CSF and sRANKL. Interestingly, Lomaga et al. (37) found that although their knockout mice also exhibited osteopetrotic phenotypes, there were abundant dysfunctional osteoclasts. These results suggest that TRAF6 is involved not only in the differentiation of osteoclast progenitors but also in the bone-resorbing function of mature osteoclasts.

It was recently reported that there are three putative TRAF6-binding sites and two binding sites for other TRAF family members, including TRAF2, TRAF3, and TRAF5, in the cytoplasmic tail of RANK (36, 39, 40). Using retrovirus vector-mediated gene transduction system, Kadono et al. (39) and Gohda et al. (40) showed that binding sites for TRAF2, TRAF3, and TRAF5 in RANK are dispensable for osteoclast formation and that a single TRAF6-binding site is sufficient to promote osteoclastogenesis.

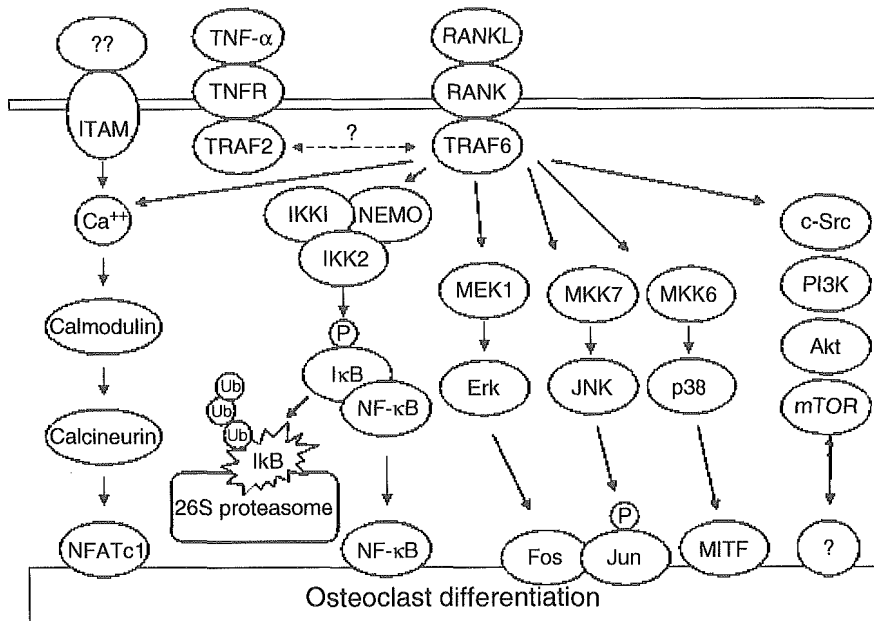When M-CSF-dependent bone marrow macrophages (M-BMMφ) were stimulated with sRANKL, rapid activation

Fig. 2. Signaling events implicated in osteoclast differentiation. When receptor activator of nuclear factor (NF)-κB ligand (RANKL) binds to RANK, multiple signaling pathways can be acftivated, including NF-κB, extracellular regulated kinase (Erk), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein tyrosine kinase, and phosphatidylinositol 3-kinase (PI3K)/Akt downstream of tumor necrosis factor (TNF) receptor-associated factor (TRAF6), and osteoclast differentiation is induced. IκB, inhibitor of nuclear factor-κB; IKK IκB kinase; mTOR, mammalian target of rapamycin; NFATc1, nuclear factor of activated T cells c1; Ub, ubiquitin.

of extracellular regulated kinase (Erk), JNK, and p38 as well as NF-κB activation was observed, while such activation was severely abrogated in M-BMMφ from TRAF6$^{-/-}$ mice. These results suggest a critical role of TRAF6 in the osteoclast differentiation signaling downstream of RANK. All TRAF proteins share a common stretch of amino acids (TRAF domain) at their carboxyl-terminus (21, 32). This domain can further be divided into two subregions, i.e. TRAF-C and TRAF-N domains. The TRAF-C domain is highly conserved among TRAF family members, and the more unique TRAF-N domain is predicted to form a coiled-coil structure. Two additional structures, the RING finger domain and zinc finger domain, are predicted to be shared by all TRAF family members except TRAF1. Retrovirus vector-mediated introduction of wildtype TRAF6 into TRAF6$^{-/-}$ M-BMMφ completely rescued the osteoclast differentiation and recovered Erk, JNK, p38, and NF-κB activation in response to the sRANKL treatment. Interestingly, the TRAF6 mutant without the RING finger domain and the first zinc finger domain could also rescue the osteoclast formation from TRAF6$^{-/-}$ cells. These cells were strongly positive for TRAP activity, and they expressed various marker molecules of osteoclasts, including matrix metalloprotease-9, cathepsin K, and calcitonin receptors. However, when cultured on dentine slices, these cells had an impaired ability for bone resorption, while full-length TRAF6 expression fully rescued the bone-resorbing activity of the cells. These results indicate that the RING finger domain of TRAF6 is not absolutely required for the differentiation of M-BMMφ to multinucleated osteoclasts, but it is essential for the full activation of the cells. The RING finger domain was also found to be

critical for the full activation of JNK and p38 (41). These results demonstrate that the JNK and p38 activation in response to RANKL plays a critical role in osteoclast development, which is mediated by the RING finger domain of TRAF6. Further study is required to define the exact mechanism of action of TRAF6 in osteoclast development.

Several studies suggest the role of TNF-α receptor/TRAF2 and TRAF5 pathways in osteoclast differentiation, and TNF-α can substitute for RANKL in pathological conditions (42–46). However, no definite in vivo evidence has been proposed to confirm the significance of TNF receptor signaling pathways on physiological and/or pathological osteoclast differentiation independently of RANKL/RANK pathways. Further studies are required to establish the involvement of other TRAFs in osteoclast development.

NF-κB and activator protein-1

RANKL stimulation induces intracellular signaling pathways including JNK, NF-κB, p38, and NFATc1 (Fig. 2). NF-κB exists in the cytoplasm of resting cells but enters the nuclei in response to various stimuli, including cytokines, pathogens, and stress (47, 48). NF-κB activation requires sequential phosphorylation, ubiquitination, and degradation of the inhibitor of NF-κB (IκB), as well as the consequent exposure of a nuclear localization signal on NF-κB. The IκB kinase (IKK) signalosome is the protein complex that contains an inducible IKK activity and consists of IKK1 (IKKα), IKK2 (IKKβ), and NF-κB essential modulator (NEMO) (IKKγ) (47–49). Among these three components of the IKK signalosome, both IKK1 and IKK2 appear to play a critical role in IκB phosphorylation.

However, the studies of IKK1 and IKK2 knockout mice indicate that IKK2 is more potent for NF-κB activation by pro-inflammatory stimuli than IKK1 (50–53). This finding suggests that IKK2 may also play a critical role in RANKL-induced NF-κB activation. Mice deficient in both p50 and p52 subunits of NF-κB are osteopetrotic, due to the failure in osteoclast differentiation, indicating a crucial role of NF-κB in osteoclastogenesis (54, 55). Recently, using a gene ablation approach, Ruocco et al. (56) demonstrated that IKK1 is required for osteoclast formation in vitro but not in vivo, while IKK2 is indispensable for both in vitro and in vivo osteoclastogenesis.

JNK activation facilitates the phosphorylation of c-Jun and increases its transcriptional activity, leading to activator protein-1 (AP-1) activation. c-Fos knockout mice exhibited an osteopetrotic phenotype, which can be rescued by not only transgenic c-Fos expression but also the expression of other Fos family members such as Fra-1 (57, 58). Ikeda et al. (59) generated a transgenic mouse in which dominant negative c-Jun (DN-c-Jun) is overexpressed under the control of TRAP promoter. The mice also showed severe osteopetrotic phenotypes, and TRAP staining showed there were very few osteoclasts in the skeletal tissues of the mice. c-Jun phosphorylation by JNK1 is necessary for the efficient osteoclastogenesis in response to RANKL using JNK1$^{-/-}$ mice, and Srivastava et al. (60) recently reported that estrogen inhibits the osteoclast differentiation by downregulating JNK1 activation in response to RANKL. These results demonstrate that both NF-κB and AP-1 activation is necessary for the normal osteoclast development.

JNK is known to activate AP-1 and to be activated by the phosphorylation of Thr and Tyr residues via MAPK kinase (MKK)-4 and/or -7. MKK-7 appears to be more specific in JNK activation than MKK-4, because MKK-4 is known to activate p38 MAPK as well (61, 62). Using adenovirus vectors carrying DN IKK2 gene or DN MKK-7 gene, we successfully segregated RANKL-induced NF-κB activation and JNK activation. We found that the activation of NF-κB and JNK is independently regulated, and both of these pathways are crucial for the osteoclast development (63).

NFAT

Ishida et al. (64) reported that NFATc1 is induced in response to sRANKL stimulation of RAW264.7 cells and that inhibiting NFAT activity by either cyclosporine A or anti-sense oligonucleotides suppressed osteoclast differentiation in vitro. Takayanagi et al. (65) also demonstrated that NFATc1 is induced in osteoclast precursors by sRANKL stimulation

using DNA microarray analysis and that embryonic stem cells deficient in NFATc1 were unable to differentiate into osteoclasts in vitro. Matsuo et al. (66) showed that NFATc1 was not induced by sRANKL in c-Fos$^{-/-}$ background, and constitutively active NFAT expression at least partially restored the osteoclast-specific gene expression in c-Fos$^{-/-}$ precursors. Ikeda et al. (59) showed that the osteoclast differentiation induced by active NFAT introduction was abrogated by DN-c-Jun overexpression. These results suggest that AP-1 and NFATc1 act coordinately to regulate osteoclast differentiation. Although it is still obscure what molecules lie upstream of NFATc1 activation, Koga et al. (67) recently showed that intracellular calcium oscillation is an important stimuli upstream of NFATc1 activation, and molecules containing immunoreceptor tyrosine-based activation motif, such as DAP12 (DNAX-activating protein 12) and Fc receptor common γ chain, facilitate the calcium-mobilizing mechanism during osteoclastogenesis (Fig. 2).

Other transcription factors

Tondravi et al. (68) demonstrated that ETS (a winged helix-turn-helix wing motif) domain-containing transcription factor PU.1 (Sp.1) plays an essential role in osteoclast development, based on the finding that the targeted deletion of PU.1 induced osteopetrosis in mice due to the defect in osteoclast differentiation. In PU.1$^{-/-}$ mice, mature macrophages cannot be observed, indicating that PU.1 is important for the differentiation of monocyte-macrophage lineage cells.

Microphthalmia transcription factor (MITF) is a basic helix-loop-helix-zipper type transcription factor, which is closely related to the TFE3, TFEB, and TFEC gene products, and MITF has been identified as the responsible gene product for the murine mi locus, which causes microphthalmia mutation (69). Homozygous mutation (mi/mi) is characterized by severe osteopetrosis as well as abnormalities in melanocytes and mast cells. The osteopetrosis in mi/mi mice is caused by the defective differentiation of mononuclear osteoclasts into mature multinucleated osteoclasts (70). MITF binds to a specific DNA sequence called E-box as a homodimer or by heterodimerizing with other TFE family members, and it transactivates TRAP and cathepsin K gene (71, 72). Sato et al. (73) demonstrated that mutated MITF observed in mi/mi mice acts in a DN fashion, binds specifically to c-Fos and PU.1, and blocks the nuclear localization of these transcription factors, which may result in the impaired osteoclastogenesis in mi/mi mutant mice. Recent studies have revealed that MITF is involved in M-CSF signaling, and M-CSF induces phosphorylation of MITF and TFE3 via a conserved MAPK consensus site, thereby triggering their recruitment of the coactivator

p300 (74). McGill et al. (75) demonstrated that an anti-apoptotic molecule, Bcl-2, is a target of MITF in osteoclasts, and Bcl-2 knockout mice showed a severe osteopetrosis, indicating a critical role of MITF in osteoclast development. Steingrimsson et al. (74) reported that the combined loss of MITF and TFE3 caused a severe osteopetrosis in mice, although deletion of one of these genes did not induce abnormal osteoclast phenotypes, suggesting a redundant role for these two genes in osteoclast development.

RANKL/RANK/OPG system and osteoclast apoptosis

Bisphosphonates

Recent studies have revealed that the rapid cell death of osteoclasts is caused by the programmed cell death. Apoptosis is a form of programmed cell death that is characterized by specific morphological and biochemical properties (77, 78). Morphologically, apoptosis is characterized by a series of structural changes in dying cells: blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies. Biochemically, apoptosis is characterized by the degradation of chromatin, initially into large fragments of 50–300 kilobases and subsequently into smaller fragments that are monomers and multimers of 200 bases (77, 79).

The first evidence that the death of osteoclasts is caused by the apoptotic process was shown by Hughes et al. (80), who reported that bisphosphonates (BPs) induced osteoclast apoptosis. BPs are pyrophosphate analogs in which the oxygen bridge between the two phosphorus atoms is replaced by a carbon with various side chains (81). They are potent inhibitors of bone resorption and are clinically applied for the treatment and prevention of osteoporosis, Paget's disease, tumor metastases in bone, and other skeletal disorders (81). They showed that risedronate, pamidronate, and clodronate strongly induced osteoclast apoptosis both in vitro and in vivo (80). They also exhibited that these BPs increased osteoclast apoptosis in vivo as determined morphologically in both normal mice and mice with increased bone resorption (80). Although the exact mechanism of action of BPs to induce osteoclast apoptosis is still unknown, recent studies have proposed a critical implication of the mevalonate pathways (Fig. 3). Amino-BPs (N-BPs), such as risedronate and alendronate, suppress farnesyl diphosphate synthase in the mevalonate pathways, which subsequently induces the apoptosis of osteoclasts by reducing the C-terminal prenylation of small G proteins (82). The effect of N-BPs on geranylgeranylation
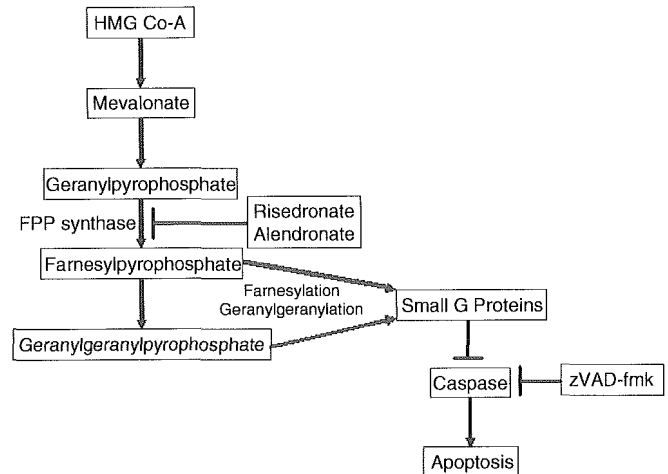
Fig. 3. Possible molecular mechanism of bisphosphonates action on osteoclast apoptosis. Amino-bisphosphonates such as risedronate and alendronate suppress farnesyl diphosphate (FPP) synthase and affect the farnesylation and the geranylgeranylation of small G proteins.

rather than farnesylation may be critical for the induction of osteoclast apoptosis, as geranylgeraniol rescues the inhibitory effects of alendronate on osteoclasts, and the action of these BPs can be mimicked by specific geranylgeranyl transferase inhibitors (83, 84). These observations suggest that the activity of small G proteins is important to maintain the integrity of osteoclasts. Recent studies also demonstrated that not only N-BPs but also non-nitrogen-containing BPs, such as clodronate, cause apoptosis in osteoclasts (83). The pro-apoptotic effect of these BPs is mediated by caspase activation, which can be blocked by an anti-apoptotic caspase inhibitor, zVAD-fmk (85). Rezka and coworkers (86) further demonstrated the cleavage of mammalian sterile 20-like kinase 1 to form the active 34-kDa species in association with apoptosis induced by BPs. Interestingly, they also showed that the BP-induced inhibition of bone resorption does not require osteoclast apoptosis, because zVAD-fmk maintained the osteoclast number by suppressing osteoclast apoptosis but did not prevent the inhibition of bone resorption (87).

Intracellular signal transduction pathways regulating osteoclast apoptosis

Using the adenovirus vector-mediated gene transduction system, we found that the activation of Erk by transducing a constitutively active mutant of Mek1 markedly promotes the survival of osteoclasts (88). Conversely, inhibiting Erk activation by overexpressing a DN ras gene mutant rapidly induced the apoptotic cell death of the cells (88). These results, combined with the fact that the anti-apoptotic factors such as RANKL, IL-1, and M-CSF induce Erk activation in osteoclasts,

suggest that the Ras/Erk pathway plays an essential role in osteoclast survival.

The critical role of phosphatidylinositol 3-kinase (PI3K)/Akt pathways in the survival of osteoclasts has also been suggested. Xing et al. (89) reported that the transgenic over-expression of Src251, which contains SH2 and SH3 domains of chicken c-Src but lacks the kinase domain, under the control of the TRAP promoter induced osteopetrosis in mice. The mice had reduced number of osteoclasts, and osteoclasts of Src251 transgenic mice showed apoptotic phenotypes. The activity of Akt in the Src251 transgenic mouse osteoclasts was significantly reduced, and RANKL-induced Akt activation was impaired in the cells, suggesting that Akt plays a critical role in osteoclast survival and that Akt activation is at least partly mediated by c-Src (89). Using the murine osteoclast formation system, Lee et al. (90) showed that TNF-α pro-longed the survival of osteoclasts, which was abrogated by either PI3K inhibitors or Mek/Erk inhibitors. They also revealed the involvement of Grb2 and ceramide in the TNF-α-induced Erk activation in osteoclasts (90). Similar effects were observed in IL-1α (91). Glantschnig et al. (85) exhibited the central role of mammalian target of rapamycin (mTOR)/S6K pathways in M-CSF- and RANKL-induced osteoclast survival, which is suppressed by rapamycin treatment. We also found that the adenovirus vector-mediated overexpression of constitutively active Akt promotes the survival of osteoclasts, and small G protein Rac1 is critically involved in this process (92). These results clearly suggest the important role of PI3K/Akt pathways on osteoclast survival. However, contrary to these previous observations, Sugatani and Hruska (93) recently reported that silencing of Akt1 and/or Akt2 by small interfering RNA (siRNA) suppressed the differentiation of osteoclasts but did not affect their survival. The reason of this discrepancy remains unknown, and further investigation is required to clarify the exact role of PI3K/Akt pathways in osteoclast survival.

Bcl-2 family members

The apoptotic process can be divided into two different path-ways, the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (94). In the death receptor pathway, activation of a receptor belonging to TNF receptor family leads to induction of apoptosis through activation of aspar-tate-specific cysteine proteases (caspases-8), and the anti- and pro-apoptotic Bcl-2 family members are critically involved in the intrinsic pathway by regulating cytochrome c release from the mitochondrial intermembrane into the cytosol, which leads to the activation of caspase-9 (94). The anti-apoptotic

members include mammalian Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and caenorhabditis elegans CED-9. So far, more than 20 members of pro-apoptotic Bcl-2 family proteins have been identified in mammals, and they can be further divided into two groups: multidomain members possessing homology in BH1-3 domains and BH3 domain-only members (95). Multidomain members include Bax, Bak, Bok, and Drosophila melanogaster DEBCL/DROB, and BH3-only members include mammalian Bad, Bik/Nbk, Bid, Hrk/DP5, Blk, Bim, Noxa, and C. elegans EGL-1 (95). BH3-only proteins fail to induce cytochrome c release and apoptosis in $bax^{-/-}bak^{-/-}$ cells, suggesting that Bax-like proteins mediate death signals from various BH3-only proteins (96). Bax-like proteins are ubiquitously expressed, while BH3-only members have more tissue-specific distribu-tion, indicating that the latter may play a role in tissue/cell-specific regulation of apoptosis.

The activity of BH3-only proteins is strictly regulated to prevent unorganized cell death. The expression of Noxa is induced by p53 or IRF-1, and DP5/Hrk is transcriptionally upregulated in the absence of growth factors or when the cells are exposed to β-amyloid protein. Their activity is also regulated by the post-translational modification. It is now widely recog-nized that Bad is phosphorylated at various sites and sequestrated away from Bcl-2 family members by binding to 14-3-3 (97, 98). Many kinases including Akt, mitochondria-anchored protein kinase A (PKA), Pak1, Rsk, Raf1, and the phosphatase calcineurin have been reported to modify Bad, although physio-logical importance of these regulations still remains unclear.

To analyze the involvement of mitochondrial pathways in osteoclast apoptosis, we examined the depolarization of mito-chondrial transmembrane potential. Depolarization of the mitochondrial transmembrane potential, chromatin condensa-tion, and cytochrome c release from mitochondria into cyto-plasm were observed in the apoptotic osteoclasts. These results show that the cytokine deprivation triggers osteoclast apopto-sis through the Bcl-2-regulated pathway.

A possible role of anti-apoptotic Bcl-2 family members on osteoclast survival has been reported. Okahashi et al. (99) showed that M-CSF treatment increased Bcl-2 mRNA expres-sion in osteoclasts, and we found that adenovirus vector-mediated overexpression of Bcl-xL markedly prolonged the survival of the cells in vitro (100). Roodman and coworkers (101) developed transgenic mice in which Bcl-xL together with simian virus 40 large T antigen were specifically over-expressed in osteoclasts under the control of TRAP promoter, and they successfully established an immortalized osteoclast precursor cell line from the mice. These results clearly showed that anti-apoptotic Bcl-2 family proteins play important roles

in osteoclast survival both in vitro and in vivo. The mechanism regulating Bcl-2 and Bcl-xL expression in osteoclasts has not been clarified yet, but McGill et al. (75) showed that Bcl-2 expression is regulated by MITF, a transcription factor essential for osteoclast and melanocyte development.

Pro-apoptotic BH3-only member Bim

Bim, one of the BH3-only proteins, was first identified as a Bcl-2 interacting protein by screening a λ phage expression library constructed from a mouse thymic lymphoma (102). Bim is expressed in hematopoietic, epithelial, neuronal, and germ cells (103), and alternative splicing generates various Bim isoforms, including BimS, BimL, and BimEL. Bim has been shown to play essential roles in the apoptosis of T lymphocytes, B lymphocytes, and neurons (104, 105), and its activity is also regulated both transcriptionally and post-transcriptionally (106). The expression of Bim is downregulated at the transcriptional level by IL-3 signaling through the Raf/Erk pathways and/or PI3K/mTOR pathways in IL-3-dependent Ba/F3 cells. Transcription of Bim is also upregulated in neonatal sympathetic neurons in response to nerve growth factor deprivation.

We demonstrated a novel and unique regulation of apoptosis by ubiquitylation-dependent degradation of Bim in osteoclasts (100) (Fig. 4). In the presence of M-CSF, Bim is constitutively ubiquitylated and degraded, and the cytokine deprivation induced a rapid upregulation of Bim due to the reduced level of its ubiquitylation (100). c-Cbl is a possible candidate of E3 ubiquitin ligase of Bim in osteoclasts, because Bim ubiquitylation was reduced in c-Cbl-deficient osteoclasts (100). Bim-deficient osteoclasts exhibited a prolonged survival both in vitro and in vivo; however, their bone-resorbing activity was significantly reduced, consistent with the in vivo observation that Bim$^{-/-}$ animals showed mild osteosclerosis (100). Consistent with our observation, Sugatani and Hruska reported that silencing bim gene by siRNA prolonged the survival of osteoclasts (93). These results clearly demonstrate that pro-apoptotic protein Bim plays a crucial role in osteoclast apoptosis and activation (Fig. 4). Further studies are required to elucidate the regulatory mechanism of Bim in osteoclasts and the role of Bim in skeletal disorders.

Pathological role of RANKL/RANK/OPG system

Osteoporosis

Accumulating evidence has shown that RANKL is not only important for normal bone development but also plays an important role in pathological bone destruction, such as in
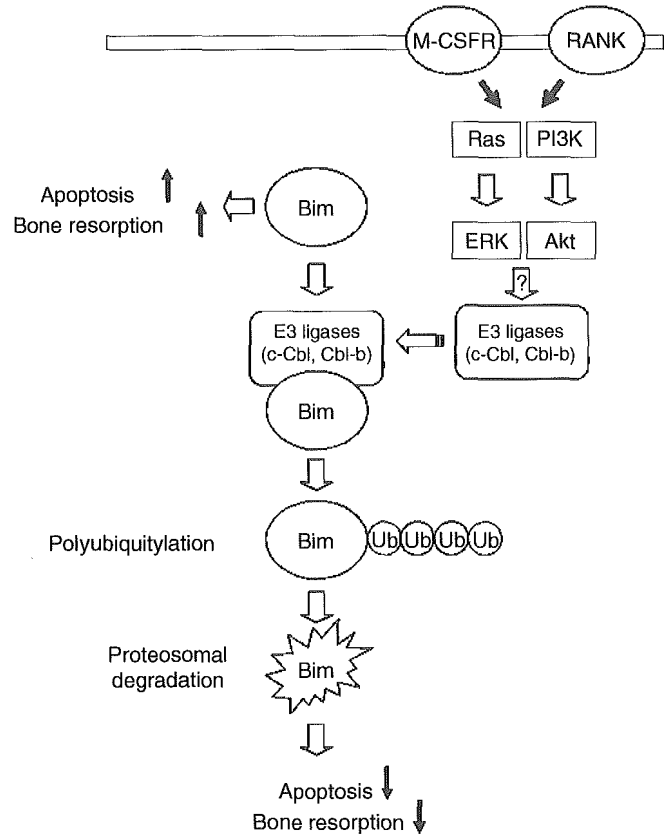

Fig. 4. Regulation of ubiquitin/proteasome-dependent degradation of Bim in osteoclasts. Cbl family E3 ligases are recruited to Bim in response to ERK and/or Akt activation, which subsequently causes polyubiquitylation and proteasome-dependent degradation of Bim. Bim is a critical regulator of apoptosis and an activator of osteoclasts.

postmenopausal osteoporosis, rheumatoid arthritis (RA), periodontal diseases, and tumor-induced osteolysis. Osteoporosis is a skeletal disorder characterized by low bone mass and loss of bone tissue integrity that causes weak and fragile bones (107). The weakness of bone strength leads to an increased risk of fragile fractures, particularly in the hip and the spine, which results in a huge social and economic problem. A hip fracture usually requires hospitalization and a major surgery and can impair a patient's quality of life and cause prolonged disability or even death. Spinal (or vertebral) fractures also have serious results, including loss of height, severe back pain, and kyphosis. Vertebral fractures also threaten human life. Cauley et al. (108) reported that the age-adjusted relative risk of mortality following a clinical vertebral fracture is 8.6, while that of a hip fracture is 6.7.

Although the exact etiology of osteoporosis remains unknown, it is now widely accepted that the normal balance between bone formation and bone resorption is impaired in the osteoporotic patient. The primary cause of postmenopausal osteoporosis in women is estrogen deficiency, and bone

destruction takes place at a faster rate after menopause. Recent evidence has shown that the increased remodeling frequency is a risk factor of fragile fracture independent of the change in bone mineral density (BMD), and Recker et al. (109) demonstrated that bone remodeling rates (activation frequency) were increased in postmenopausal women and osteoporotic patients. A new remodeling cycle is initiated by the novel osteoclast formation in the activation phase, and therefore, it is reasonable to speculate that the RANKL/RANK/OPG axis is critically involved in the pathogenesis of osteoporosis.

Consistent with this speculation, Xu et al. (110) reported that RANKL transcripts were highly expressed in bone tissues of ovariectomized rats. Estrogen has been demonstrated to upregulate the gene expression and protein synthesis of OPG both in vitro in human (111) and in rodent (112) osteoblastic cells and in vivo in elderly men (113). More recently, Riggs and coworkers (114) demonstrated that postmenopausal women express higher levels of RANKL on marrow stromal cells and lymphocytes than premenopausal women or postmenopausal women taking estrogen therapy. RANKL expression per cell correlated well with the bone resorption markers serum C-terminal telopeptide of type I collagen and urine N-telopeptide (NTX) of type I collagen and inversely with serum 17β-estradiol level (114). Taken together, RANKL/RANK/OPG pathways are critically involved in the pathogenesis of osteoporosis probably by recruiting osteoclasts and starting new remodeling cycles and therefore can be a good therapeutic target of the disease.

RA

RA is a chronic systemic inflammatory disorder with an unknown etiology characterized by the invasive synovial hyperplasia leading to the progressive joint destruction (112). Radiographic studies have shown that the bone erosion in RA begins at the early stage of the disease and gradually (and sometimes rapidly) exacerbates (116). Bone erosion results in the severe deformity of the affected joints and impairs the normal activity and the quality of life of the patients; preventing such devastating states is one of the most challenging issues in treating RA.

Because the exact etiology and pathology of RA is unknown, most treatments of RA just relieve symptoms of the disease. Non-steroidal anti-inflammatory drugs, including cyclooxygenase 2 inhibitors, are prescribed to reduce the painful symptoms of the disease, but they usually have little effect on stopping the progression of the joint destruction. Recent studies have revealed that some disease-modifying anti-rheumatic drugs and biological agents, such as anti-

TNF-α antibody and IL-1 receptor antagonist, ameliorate the clinical symptoms of RA and reduce the progression of the joint destruction (117). Anti-TNF-α therapy using anti-TNF-α antibody or soluble TNF receptor fusion protein almost completely suppressed the progression of bone destruction in some RA patients (118–120).

There are still a significant proportion of non-responders to the therapies, and the bone-protective effect of these reagents is limited in most cases. Additionally, their long-term efficacy has yet to be established, and long-term use of these medicines often causes severe adverse effects. Therefore, novel therapeutic interventions specifically targeting the joint destruction of RA are greatly anticipated.

Proliferating synovium produces an elevated amount of the pro-inflammatory cytokines IL-1, IL-6, IL-17, and TNF-α, and matrix-degenerating enzymes matrix metalloproteinases and cathepsins, which are involved in the bone and cartilage destruction (115). Bone erosion usually begins at the interface of the cartilage and the proliferating synovium, the so-called 'bare area,' and bone-resorbing osteoclasts are observed at the erosive synovium/bone interfaces (Fig. 5). It is not long time ago that it was recognized that osteoclasts are primarily involved in the bone destruction in RA. Bromley and Woolley (121) histologically analyzed the bone–pannus interface of RA joints by light microscopy and showed the presence of acid phosphatase-positive multinucleated cells with morphologic features of osteoclasts along the surface of mineralized subchondral bone and mineralized cartilage. We (122) and Fujikawa et al. (123) established an in vitro osteoclast formation system using synovial cells obtained from RA synovial tissues, in which macrophage-lineage osteoclast precursor cells in synovial tissues differentiate into mature osteoclasts in the presence of supporting cells, such as synovial fibroblastic cells or osteoblastic cells.

The important role of the RANKL/RANK/OPG system in the development of bone destruction in RA has been recently established (124). We and other groups reported that RANKL is highly expressed in synovial tissues of RA patients (125–128). The cells responsible for the upregulation of RANKL are considered to be synovial fibroblastic cells and T lymphocytes (125, 129). In the coculture system of synovial fibroblasts and peripheral monocytes, $1\alpha,25(OH)_2D_3$ treatment induced RANKL expression in synovial fibroblasts and induced osteoclast differentiation from monocytes (125). Although the detailed mechanism of upregulation of RANKL expression in the RA synovial cells remains elusive, our preliminary results showed that the treatment of synovial
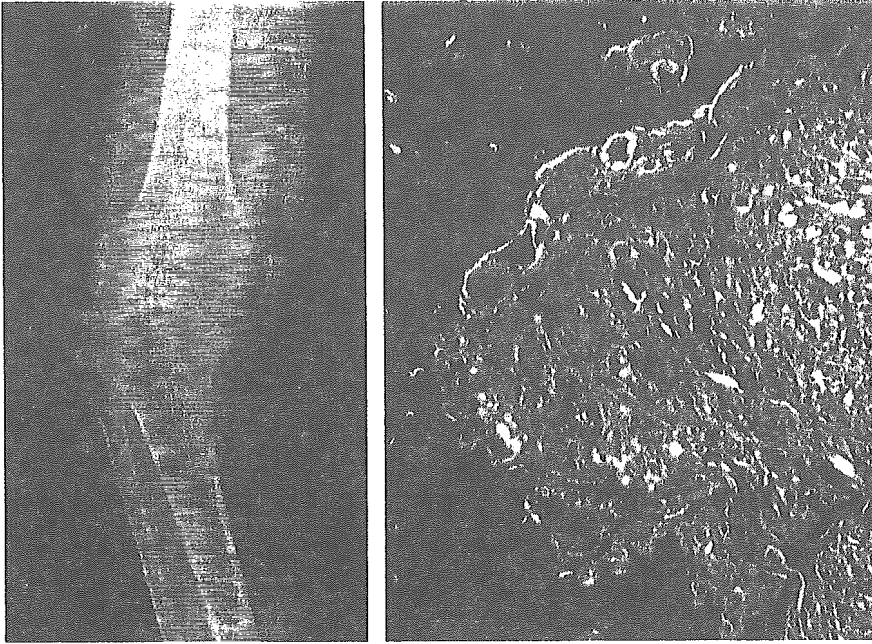
Fig. 5. Bone destruction in rheumatoid arthritis. (Left) X-ray of the knee joint of a patient with rheumatoid arthritis. Note the medial subluxation of the joint and remarkable bone destruction in the medial tibial plateau. (Right) Proliferating synovium stimulates osteoclast differentiation, and a number of osteoclasts are observed in the pannus-bone interface (arrows) (H&E staining).
Bar = 100 μm. Courtesy of Dr Yoshiaki Kuga (Tokyo Metropolitan Bokuto Hospital, Tokyo, Japan).

fibroblasts with pro-inflammatory cytokines such as IL-1 and TNF-α induced RANKL expression in the cells, indicating that cytokine network in the synovial milieu plays an essential role on the RANKL induction in the cells. These results strongly suggest that RANKL/RANK pathways are primarily involved in the pathological bone destruction in RA. In spite of a remarkable success of anti-TNF-α therapy in treating RA patients, there exist no in vivo data demonstrating the role of TNF-α signaling pathways on osteoclast development and bone destruction independently of RANKL/RANK pathways.

Tumor-induced bone diseases

Some primary tumors, benign or malignant, and metastatic malignant tumors, such as breast cancer and lung cancer, invade bone tissues (130). The typical tumor-induced bone lesion is focal osteolysis, although some tumors such as prostatic cancer metastases exhibit osteogenic bone lesions. The osteolysis induced by these tumors affects the quality of life of the patients by causing pathological fractures and severe bone pain and often exacerbates the prognosis of the patients. Recent studies have shown that osteoclasts are primarily responsible for such conditions, and the involvement of RANKL/RANK/OPG system has been indicated (131). Tumor cells produce various factors, such as PTH-related protein (PTHrP), TNF-α, and IL-1, that can stimulate RANKL expression in osteoblastic cells or bone marrow stromal cells and induce osteoclast formation in the bone microenvironment. PTHrP is a peptide hormone leading to humeral hypercalcemia of malignancy. Other factors produced by tumor

cells, including IL-6 and macrophage inflammatory protein-1-α, are capable of activating osteoclasts.

Grimaud et al. (132) reported that RANKL/OPG ratio is increased in patients with severe osteolysis. Among various bone tumors, the molecular mechanism of bone destruction in multiple myeloma is one of the most studied. Multiple myeloma is a plasma cell malignancy that develops in the bone marrow and causes marked skeletal destruction. It has been reported that myeloma cells induce an imbalance in the OPG/RANKL system in bone environments and that this imbalance is responsible for osteolysis observed in patients (133–136). Bone marrow biopsies from patients with myeloma showed an increase of RANKL and a reduction of OPG expression by immunohistochemical analysis (137). The RANKL/RANK/OPG system is also reported to be involved in bone destruction in breast cancer cells (138, 139), prostate cancer cells (140, 141), and other metastatic bone tumors. These results suggest that the RANKL/RANK/OPG system is also important for the tumor-induced bone destruction.

Periodontal diseases

Periodontal diseases are chronic infectious inflammatory diseases characterized by a heavy leukocyte infiltration into the periodontal lesions, resulting in the secretion of a variety of cytokines, which ultimately leads to the destruction of periodontal tissues including alveolar bone (142). Cell-to-cell interaction between T cells and monocytes/osteoclast progenitor cells appears to be important for osteoclast formation in periodontal diseases (142, 143). The expression of RANKL

mRNA was shown to be upregulated in gingiva from patients with severe periodontitis (143). Teng et al. (144) reported a novel animal model of periodontitis, in which peripheral blood leukocytes from patients with localized juvenile periodontitis were transplanted into NOD/SCID mice. Oral challenge of these humanized mice with *Actinomyces actinomycetemcomitans*, a Gram-negative bacterium that causes human periodontitis, leads to the local bone resorption in alveolar bone surrounding the teeth. In vitro stimulation of CD4$^+$ T cells from these mice with antigens from *A. actinomycetemcomitans* leads to the expression of RANKL, and OPG treatment reduced the number of periodontal osteoclasts and diminished alveolar bone destruction after microbial challenge (144). *Porphyromonas gingivalis*, another bacterium implicated in human periodontitis, can also induce RANKL expression in lymphocytes, and this expression is associated with increased osteoclast formation in spleen cell cultures (145). These observations suggest that bacteria stimulate osteoclast formation by activating cell-to-cell interactions in the infiltrating leukocytes. Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a strong bone-resorbing factor in periodontitis. Recent studies have revealed that Toll-like receptor (TLR) family members act as receptors for a variety of microbial ligands, and TLR4 is a signal-transducing receptor for LPS (146). The signaling cascade of TLR4 is quite similar to that of the IL-1 receptor, because both TLR4 and IL-1 receptors use common signaling molecules such as myeloid differentiation factor 88 (MyD88) and TRAF6 (146). In addition, Toll-IL-1 receptor domain-containing adapter inducing interferon-β (TRIF)-mediated signals are involved in TLR4-induced MyD88-independent pathways (146). LPS and IL-1 stimulated osteoclast formation in

coculture of osteoblasts and hemopoietic osteoclast progenitor cells (147). Kikuchi et al. (148) showed that LPS directly induced RANKL expression in osteoblasts through PKC and Mek/Erk signals. Using MyD88-deficient mice and TRIF-deficient mice, Sato et al. (149) reported that MyD88-mediated signals but not TRIF-mediated ones are involved in RANKL expression in osteoblasts treated with LPS as well as IL-1. These results suggest that RANKL expressed by either osteoblasts or infiltrating T cells in response to bacterial infection is involved in alveolar bone destruction in periodontal diseases.

Periprosthetic implant loosening

Since the initial report of total joint replacement by Sir John Charnley in the 1960s (150), both total hip and total knee arthroplasty have become a successful and reliable surgical procedure to achieve natural function lost in many destructive joint diseases including osteoarthritis, RA, osteonecrosis, and very severe pathologic fractures, and more than one million replacements have been performed per year in the world (151). Although joint replacement surgeries have been extremely successful in relieving painful symptoms and improving morbidity of the patients and their long-term results are satisfactory, the periprosthetic bone loss leading to aseptic loosening of the implants is one of the most challenging complications that may result in the failure of the procedure (152, 153). Histologically, the formation of synovium-like fibrous tissues surrounding the implants, also called pseudo-capsule, is frequently observed. The interface structure is rich in monocyte-macrophage lineage cells as well as fibroblastic cells, reminiscent of the inflammatory synovium observed in RA, and a number of osteoclast-like multinucleated cells can

Fig. 6. Periprosthetic osteolysis after total hip replacement. (Left) X-ray of the hip joint with periprosthetic osteolysis around the cemented acetabular component. (Right) Synovium-like fibrous tissues surrounding the implants contain a number of osteoclast-like multinucleated cells (arrows) (H&E staining). Bar = 100 μm.

be observed in the bone-implant interface of the loosened joints (154, 155) (Fig. 6). Recent studies have revealed that fibroblastic cells in periprosthetic tissues are capable of inducing the differentiation of normal human peripheral blood mononuclear cells to mature osteoclasts by a mechanism that involves both RANKL and TNF-α (156).

Paget's disease and inherited bone diseases

Sporadic Paget's disease is characterized by abnormal bone remodeling, leading to excessive and aggressive bone resorption by osteoclasts, the replacement of hematopoietic bone marrow by fibrous connective tissue, and the subsequent increase in bone formation; X-ray examination shows a 'mosaic' appearance (157). There exist abundant osteoclasts in the osteolytic area, and enhanced RANKL expression is observed in the area (158). It has been reported that the excessive activity of pagetic osteoclasts may be caused by hypersensitivity of osteoclast precursors to several osteoclastogenic factors, including $1\alpha,25(OH)_2D_3$ and RANKL (158–160). A number of studies have revealed the potential involvement of measles virus nucleocapsid protein in the pathogenesis in Paget's disease, although there is still a controversy whether this phenomenon is the cause of the disease (161–167).

Several genetic disorders were found to be associated with abnormal RANKL/RANK/OPG pathways (168). Familial expansile osteolysis (FEO) is characterized by osteolytic lesions in the long bones during early adulthood. Hughes et al. (169) demonstrated a mutation in the RANK gene in members of FEO families. There was an identical tandem duplication of bases 84–101 in exon 1 of the TNFRSF11A gene, which encodes RANK, in all affected individuals. In vitro gene transfection experiments has revealed that the mutated RANK protein is not sorted to the plasma membrane normally and is intracellularly sequestered, which leads to the constitutive activation of the RANK signaling. They also identified a similar mutation in the RANK gene in one familial Paget's disease family (169). A similar shorter tandem duplication (84dup15) in the RANK gene is the cause of expansile skeletal hyperphosphatasia, an inherited disease characterized by hyperostosis, deafness, hypercalcemia, and premature tooth loss (170). Several groups also revealed the mutations in SQSTM1/p62 gene, which encodes a ubiquitin-binding protein that is involved in the IL-1, TNF-α, and RANK signaling pathways, in both familial and non-familial Paget's diseases (171–174).

Some patients with idiopathic hyperphosphatasia (also known as juvenile Paget's disease) have mutations in the OPG gene (TNFRSF11B) (175). This disorder is a rare disease with an autosomal recessive inheritance pattern, and it displays various deformities of long bones and vertebral column, which increase in severity during adolescence. Deletion of the whole gene results in undetectable serum levels of OPG and marked increase in sRANKL levels (175). Cundy et al. (176) reported a 3-bp inframe deletion in exon 3 of the TNFRSF11B gene, which results in the formation of non-functional OPG protein. Chong et al. (177) reported a relationship between phenotype and genotype of the disease.

Vascular diseases

OPG-deficient animals developed arterial calcification of the renal arteries and the aorta, which was prevented by transgenic OPG expression from midgestation through adulthood but not by the intravenous injection of recombinant OPG after birth (30). In spite of such a protective effect of OPG on arterial calcification, clinical studies have shown that patients with coronary artery disease have significantly higher serum levels of OPG than healthy patients, and the relative risk of cardiovascular mortality was increased in patients with high OPG serum levels, which were associated with the severity of coronary artery disease and increased in elderly men and patients with diabetes mellitus (178–181). Further work is required to elucidate the role of the RANKL/RANK/OPG system in the physiological vascular integrity and the pathomechanism of cardiovascular disease.

Diagnostic implication of RANKL/RANK/OPG system

A number of clinical studies have been performed trying to clarify the relationship between the levels of RANKL or OPG and the pathological conditions. In spite of the potent inhibitory effect of OPG on bone resorption and the clinical observation that OPG deficiency results in the remarkable osteopenia, as shown in patients with juvenile Paget's disease, there is controversy about the relationship between serum concentration of OPG and BMD in postmenopausal women. Yano et al. (182) demonstrated a negative correlation with BMD in postmenopausal women, which could be due to the compensatory action to maintain the bone integrity by suppressing the abnormal bone resorption. Alternatively, Browner et al. (181) and Khosla et al. (113) did not find any significant relationship between serum OPG levels and BMD, and recent studies by Mezquita-Raya (183) showed that serum OPG levels are independently associated with bone mass and prevalent vertebral fractures in postmenopausal women. Serum OPG levels are significantly correlated with

age, and some age-related factors possibly regulate the expression of OPG in various tissues, which makes the interpretation of these contradictory results difficult. No definite correlation between serum levels of sRANKL and BMD has been established.

As mentioned above, there is a significant relationship between serum OPG levels and the risk of atherosclerosis and cardiovascular diseases. However, the exact mechanism and diagnostic value of these observations still remains elusive.

High RANKL serum levels and reduced OPG levels have been reported in multiple myeloma patients as compared with normal subjects (184, 185). OPG levels were decreased to a greater extent in patients with osteolytic lesions than those in patients without bone lesions. Moreover, Terpos et al. (186) demonstrated that the RANKL/OPG ratio may predict the prognosis in myeloma patients independently of other risk factors. Although these studies propose a possible diagnostic and a risk predicting value of the OPG/RANKL measurement, further study is required to establish its clinical significance.

Therapeutic strategies targeting RANKL/RANK/OPG system and RANK signaling

OPG, RANK-Fc, and anti-RANKL antibody

The crucial role of the RANKL/RANK/OPG system has been documented in various aspects of pathological bone resorption, and therefore, therapeutic strategies directly targeting this system have obtained a great deal of attention. The strategies include the usage of OPG, RANK-Fc, anti-RANKL antibody, modulators of intracellular signaling pathways of RANK, and RANKL vaccination.

OPG is a 401 amino acid protein with a short signal peptide (21 amino acids) that forms homodimers linked through a cysteine – cysteine disulfide bond at position 400 (22, 23). Unlike other members of the TNF receptor family, OPG lacks a transmembrane domain and is a secreted protein. OPG binds with the soluble and cell-bound forms of RANKL and suppresses their interaction with and therefore stimulation of RANK. Not only does OPG inhibit osteoclast formation in vitro, but transgenic expression of OPG in the liver induces osteopetrotic phenotypes in mice, and marked reduction in the osteoclast number was observed in the skeletal tissues of the mice (23). On the basis of these potent inhibitory actions of OPG on osteoclast differentiation and function, the therapeutic application of OPG has been extensively studied. Preclinical studies clearly showed the therapeutic effects of OPG on pathological bone loss. Recombinant OPG administration or adenovirus vector-mediated OPG gene expression prevents ovariectomy-induced bone loss in rodents (23, 187) and ameliorates bone destruction in animal models of arthritis (129), periodontitis (144), metastatic bone destruction (188), and periprosthetic osteolysis (189). Cheng et al. (190) reported that peptidomimetics of OPG that can interfere RANKL–RANK binding suppress bone loss in ovariectomized animals. RANK-Fc fusion protein also has an inhibitory effect on RANKL action; it suppresses arthritic bone destruction (191), wear debris-induced osteolysis (192), and myeloma-induced bone diseases (193, 194).

Clinical trials were conducted in postmenopausal women to determine the anti-resorptive activity and safety of recombinant Fc-OPG fusion protein, which is more stable than natural OPG by deleting the heparin-binding domain (195). They showed that subcutaneous administration of a single dose of Fc-OPG rapidly (within 12 h of the injection) decreased bone resorption markers in these patients, and the decrease was still observed after 6 weeks of the injection. The effect of Fc-OPG on patients with multiple myeloma or breast carcinoma-related bone metastases was also investigated, and Fc-OPG caused a rapid, sustained, and dose-dependent decrease in bone resorption marker levels, which was at least comparable with the results from patients treated with pamidronate (196).

The potent therapeutic potential of anti-RANKL antibody was more recently reported. A randomized, double blind, single-dose, placebo-controlled study of a fully humanized anti-RANKL monoclonal antibody (AMG 162) was conducted to evaluate its anti-resorptive effects on postmenopausal women (197). A single subcutaneous injection of AMG 162 induced dose-dependent reduction in the serum and urinary NTX/creatinine, which was detected as early as 12 h after injection. The maximum urinary NTX/creatinine reduction was observed at 2 weeks in the 0.01, 0.03, 0.3, and 1.0-mg/kg AMG 162 group, 1 month in the 0.1-mg/kg AMG 162 group, and 3 months in the 3.0-mg/kg AMG of 162 groups (84%). Surprisingly, although the treatment effect of the antibody was reversible, a significant decrease in urine NTX was still observed after 9 months of the injection in the 3.0-mg/kg AMG 162 group. No related serious adverse events were reported. These results suggest that both Fc-OPG and anti-RANKL antibody are promising therapeutic approaches with broad application in the treatment of bone disorders.

RANKL vaccine

The other anti-RANKL therapeutic strategy is the vaccination approach. The vaccination method is used to induce an

antibody response against disease-related proteins by immunization with modified pathological antigen as preventive therapy to acquire disease resistance. Mouritsen et al. (198) developed a novel therapeutic vaccine approach against disease-related self-protein such as TNF-α. These vaccines were generated by inserting a promiscuous T helper (Th) epitope into its sequence. The promiscuous Th epitope stimulates the Th cells, and then T cells induce the antibody production by B cells. The therapeutic vaccines can thus bypass immunological tolerance and elicit polyclonal antibodies that neutralize pathological self-protein. A RANKL vaccine was developed by modifying the soluble TNF-like domain of murine RANKL to incorporate a promiscuous Th epitope (199). Immunization with RANKL vaccine resulted in a rapid and sustainable polyclonal anti-RANKL antibodies in mice, which have neutralizing activity against murine RANKL. Anti-RANKL antibody titers declined to about 10% of the maximal level in 10 weeks when boosting is discontinued. No abnormality in lymph nodes, either macroscopically or microscopically, was observed in vaccinated animals, and no apparent macroscopic abnormality was observed in any other organs of the immunized animals. We first examined the effect of RANKL vaccine on ovariectomized mice. We found that RANKL vaccine normalized high-turnover osteoporosis, and mice immunized with RANKL vaccine were resistant to bone loss in response to ovariectomy. We also examined the effect of RANKL vaccine on a mouse model of RA (SKG mouse) established by Dr Shimon Sakaguchi (Kyoto University, Japan) (200). This strain of mice spontaneously develops RA-like symptoms at 2 months of age (200). Immunization with RANKL vaccine after the onset of arthritis ameliorated not only the synovitis but also the formation of osteoclasts and the bone destruction. These results clearly demonstrate that RANKL vaccination can prevent bone loss in murine model of pathological bone destruction, establishing a therapeutic use of RANKL vaccine.

Therapeutic approaches targeting intracellular signaling of RANK

Remarkable advances in the understanding of intracellular signaling pathways of RANK have enabled us to develop therapeutic drugs specifically targeting downstream signaling molecules. Interferon-γ markedly suppress the osteoclast differentiation in response to sRANKL and M-CSF stimulation, and Takayanagi et al. (201) reported that it promotes TRAF6 degradation via ubiquitin/proteasome pathways. They also showed that interferon-β suppresses osteoclast differentiation by downregulating c-Fos induction in response to RANKL (202). Inhibitors of p38 MAPK pathways (SB203580) and

JNK pathways (SB600125) also suppress osteoclast differentiation (59, 63, 203). Nishikawa et al. (204) also reported a therapeutic effect of p38 inhibitor FR167653 on bone destruction in collagen-induced arthritis rats and its inhibitory action on osteoclast formation in vitro. The immunosuppressive drugs cyclosporine A and macrolide tacrolimus (FK506) inhibit osteoclast differentiation by suppressing the calcineurin phosphatase activity and therefore downregulating NFATc1 activation (59, 64, 65). Ikeda et al. (59) also exhibited that a specific peptide inhibitor of NFAT, VIVIT, suppressed osteoclast differentiation in vitro. Inhibitors of PI3K pathways, such as wortmannin and LY290442, and a specific inhibitor of mTOR, rapamycin, markedly suppress bone resorption by inducing apoptotic cell death of osteoclasts (85, 86, 205). NF-κB activation is also a possible candidate of anti-RANKL therapy. Tomita et al. (206) demonstrated that synthetic double-stranded DNA with high affinity for NF-κB, which works as 'decoy' cis elements to bind the transcriptional factor, suppressed the bone destruction in collagen-induced arthritis rats. Jimi et al. (207) exhibited that a cell-permeable peptide inhibitor of the IKK complex (NBD peptide), which disrupts the association of NEMO with IKK1 and IKK2, inhibited RANKL-stimulated NF-κB activation and osteoclastogenesis both in vitro and in vivo.

The limitation of these approaches resides in the specificity of these compounds. Because these intracellular signaling pathways are also important for other cells or tissues, the development of a sophisticated drug delivery system or cell-specific or tissue-specific inhibitors is absolutely necessary to prevent or minimize significant adverse effects. Table 1 lists the candidates for anti-RANKL therapy.

Possible disadvantage of anti-RANKL therapy

The concerns of therapeutic strategies targeting RANKL/RANK system are mainly related to the extraskeletal adverse effects raised by suppressing the RANKL/RANK pathway. RANKL is expressed in activated T cells, while RANK is expressed on the surface of dendritic cells, mature T cells, and hematopoietic precursors. RANKL treatment induces Bcl-xL expression in dendritic cells and promotes their survival. Therefore, anti-RANKL therapy can potentially jeopardize the function of dendritic cells, although no abnormality in the dendritic cell function has been reported in clinical studies.

Mice deficient in RANKL or RANK exhibit complete absence of lymph nodes but intact splenic architecture and Peyer's patches. Studies have revealed that the RANK signaling is essential for the lymph node formation, while IL-7 receptor α is essential for Peyer's patch formation in the embryonic