Identification of an Alternatively Spliced Variant of Ca\(^{2+}\)-promoted Ras Inactivator as a Possible Regulator of RANKL Shedding*

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The receptor activator of NF-\(\kappa\)B ligand (RANKL), a critical regulator of osteoclastogenesis, is synthesized as a membrane-anchored protein and cleaved into a soluble form by ectodomain shedding. We developed an assay system to identify molecules regulating the RANKL shedding. Using this system, we found that a splice variant of Ca\(^{2+}\)-promoted Ras inactivator (CAPRI), \(\Delta\)CAPRI, which is expressed in primary osteoblasts, promoted the RANKL shedding. The wild type CAPRI is a member of the Ras GTPase-activating protein (GAP) family and suppresses Ca\(^{2+}\)-dependent Ras activation, whereas \(\Delta\)CAPRI, which lacks one exon in the GAP-related domain, activated the Ras pathway. Overexpression of \(\Delta\)CAPRI or a constitutive active form of Ras up-regulated the expression level of matrix-metalloproteinase 14 (MMP14), which directly cleaves the ectodomain of RANKL, whereas Erk activation by expressing the constitutive active Mek1 did not affect the MMP14 expression or RANKL shedding. These results suggest that \(\Delta\)CAPRI is a possible regulator of RANKL shedding by modulating MMP14 expression through Ras signaling cascades other than the Erk pathway.

The receptor activator of NF-\(\kappa\)B ligand, RANKL (also known as TNF-related activation-induced cytokine, TRANCE, osteoprotegrin ligand, OPGL, and osteoclast differentiation factor, ODF) is a type II transmembrane glycoprotein with a molecular mass of ~45 kDa, which belongs to the tumor necrosis factor (TNF)\(^\dagger\) ligand family (1–5). RANKL is expressed on the membrane of osteoblasts and bone marrow stromal cells and binds to and activates TNF family receptor RANK expressed on monocyte-macrophage lineage osteoclast precursors (3, 4). Upon binding to RANK, RANK activates the intracellular signaling pathways including NF-\(\kappa\)B, Erk, JNK, and NFATc1, and leads to osteoclast differentiation, activation, and survival (6, 7). The essential role of RANKL in normal bone turnover was further established by a series of knock-out mice, i.e. both RANKL- and RANK-deficient animals exhibited severe osteopetrosis because of the lack of osteoclast differentiation (8, 9), whereas the targeted disruption of osteoprotegerin, a natural inhibitor of RANKL, developed severe osteoporosis because of enhanced osteoclastogenesis (10).

Some transmembrane proteins are extracellularly cleaved and released into the surrounding environment. RANKL is also made as a membrane-bound protein, cleaved by some proteinases, and converted to the soluble RANKL (11, 12). This process, known as ectodomain shedding, has a diverse effect on a wide variety of membrane-bound proteins. For example, when TNF-\(\alpha\) is cleaved by the TNF-\(\alpha\) converting enzyme and released into the circulatory system, it exhibits strong systemic effects (13, 14). In contrast, the Fas ligand is a strong apoptosis inducer in its membrane-bound form, but the soluble Fas ligand has fewer effects on apoptosis induction (15).

Although some proteinases have been demonstrated to have the RANKL shedding activity, no definite RANKL sheddase(s) have yet been identified. TNF-\(\alpha\) converting enzyme was reported to be a candidate of RANKL sheddase (11); however, a more recent study has shown that TNF-\(\alpha\) converting enzyme had no apparent effect on the RANKL shedding and that the RANKL shedding in TNF-\(\alpha\) converting enzyme-deficient cells was indistinguishable (16). A disintegrin and metalloproteinase domain family (ADAM)19 has also been reported to exhibit RANKL shedding activity (17), but embryonic fibroblasts from ADAM19 knock-out mice showed almost the same RANKL shedding activity as the cells from the wild type animals (16). Matrix metalloproteinase 14 (MMP14), also called the membrane-type 1 matrix metalloproteinase, MT1-MMP can also cleave RANKL, although its cleavage site differs from that previously reported (16). These results suggest that there are other molecules implicated in RANKL shedding.

To identify molecules involved in the regulation of RANKL shedding, we developed a novel screening system, in which expression plasmids encoding secreted placental alkaline phosphatase (SEAP) fused with mouse C-terminally truncated RANKL (tRANKL-SEAP) were co-transfected with cDNA library pools of ST2 cells. Utilizing this screening system, we found that an alternatively spliced variant of Ca\(^{2+}\)-promoted Ras inactivator (CAPRI), \(\Delta\)CAPRI led to an increase in the RANKL shedding.

MATERIALS AND METHODS

Reagents—\(p\)-Nitrophenyl phosphate was purchased from Sigma-Aldrich. DNA polymerase, Pyrobust (for subcloning), was purchased from Takara biochemicals (Shiga, Japan). KOD plus (for reverse transcription-PCR) was from Toyobo (Osaka, Japan), and ionomycin was from Merck. Antibodies were purchased as follows: the His tag, Erk, phosphorylated Erk, integrin-\(\beta\) and HSP-90 from Santa Cruz Biotechnology,
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(+)-MeK1(CA), was constructed by insertion of the cDNA fragments digested from pGEM-MeK1(CA) (generous gift from K. Arai, Research Institute, National Rehabilitation Center for Persons with Disabilities) to NotI restriction site of pcDNA3.1(+) (Invitrogen). The rRANKL-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′-CGCTCGAGAACATCTCGCCAGGAGGAGA-3′ and 5′-GGGTCTAGAGTACCATGACCTGCAGTGA-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′-AAAGGTTGATGCGTTCAGCCCATCTCTGAG -3′ and 5′-GGGTCTAGAGTACCATGACCTGCAGTGA-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(+) (Invitrogen). The full-length RANKL was digested from pcDL-MMP14 at SalI sites and HindIII/XhoI sites of pcDNA3.1-V5HisA. Small interfering RNA plasmids for MMP14 were constructed using pGEM-U6 vector (iGENE Therapeutics Inc., Ibaraki, Japan) and was analyzed using the Mx3000PTM 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 C57BL6 mice and for formation of osteoclasts were previously described (19, 20). The total RNA was purified from primary osteoblasts and osteoclasts using ISOGEN (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′-GGTGCG-GCAAGAGCCTCTG-3′ and 5′-CTATGTTCGGACCGCCTG-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 Systems) 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 mM IL-1α (Gibco BRL, Gaithersburg, MD). The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Nonidet P-40, and subjected to SDS-PAGE. Membrane fraction was gained using Mem-PER mammalian protein extraction reagent (Pierce Chemical Co.).

Real Time PCR—The reaction mixture for the real time PCR was prepared using qPCR QuickGoldStar Plus for SYBR® Green I (Nippon Gene, Tokyo, Japan) and was analyzed using the Mx3000PTM QPCR System (Stratagene, La Jolla, CA). The set of primers used were 5′-GCTACCCCACTGTCATAGG-3′ and 5′-CTCTGGCCTTCATGATG-3′.

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
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 $1 \times 10^6$ 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 (ΔCAPRI) (Fig. 1, B and C). The other clones included full-length MMP14 (Fig. 1B), 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. 1D).

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 phosphatase 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 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-SR or pcDL-ΔCAPRI. Up-regulation of alkaline phosphatase activity in pcDL-ΔCAPRI-transfected cells was observed. *, significantly different, $p < 0.01$.
CAPRI 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 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(+)-MekCA. *, 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. 3A by immunocytochemistry and Fig. 3B 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. 3C, 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. 3D).

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. 3E, 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 (MekCA) did not increase the alkaline phosphatase activity released into the supernatant (Fig. 3E).
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. 1B). 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 RasDN. RasCA induced the up-regulation of MMP14, whereas MekCA did not affect the expression level of MMP14 (Fig. 4A). 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. 4B, 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 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 protein 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 R47S5 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 R47S5 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 knockout 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 RasCA increased the expression of MMP14, and MMP14 knockout 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 pathways 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.

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