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ORIGINAL ARTICLE

Molecules and signaling pathways involved in the expression of OC-STAMP during osteoclastogenesis

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Abstract The receptor activator of nuclear factor-κB ligand (RANKL) is a key factor in regulating osteoclastogenesis and in maintaining the survival of mature osteoclasts. We screened differentially expressed genes in RAW264.7 cells in response to RANKL and found osteoclast stimulatory transmembrane protein (OC-STAMP) as one of the RANKL-induced genes of interest. Recently, OC-STAMP has been identified as the RANKL-induced protein that promotes osteoclast differentiation, but the mechanism that regulates its expression is not understood. Therefore, the tissue distribution of OC-STAMP and the signaling pathways that regulate its expression were studied here. Similar to osteoclasts, OC-STAMP was expressed in most tissues, suggesting its involvement in the function of other tissues. Interestingly, OC-STAMP was downregulated by 17β-estradiol at high concentrations, suggesting the potential relationship between OC-STAMP and estrogen. Importantly, the knockdown of OC-STAMP at the transcript level resulted in the inhibition of multinucleated osteoclast formation and the decreased expression of genes including transcription factor (such as c-Jun), receptors (such as RANK and c-Fms), a signaling molecule (such as TRAF6), and a cell fusion-related molecule (such as meltrin-α), suggesting that the osteoclast differentiation needs the coordinated expression of OC-STAMP with several molecules required for transcription, signaling transduction, and cell fusion. Additionally, the treatment of its specific antibody inhibited the formation and bone resorptive activity of mature osteoclasts, suggesting its involvement in the function of mature osteoclasts. Furthermore, studies with pharmacological inhibitors suggested PKCβ or Akt might be the major signaling molecules to regulate the expression of OC-STAMP during osteoclastogenesis.

Keywords OC-STAMP · Osteoclast · Differentiation

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Introduction

Bone maintains its strength and integrity by the dynamic process involving the synthesis of bone matrix and its resorption, referred to as bone remodeling. However, an imbalance in bone remodeling caused by excessive osteoclastic bone resorption relative to osteoblastic bone formation results in the reduction of bone mass that leads to several bone disorders such as osteoporosis (Boyle et al. 2003; Sipos et al. 2009). Apparently, the loss of bone mass increases the risk of fractures followed by serious problems such as substantial skeletal deformity, pain, increased mortality, and severe economic burden (NIH Consensus 2001). Therefore, a clear understanding of the sequential, but complex process of osteoclast differentiation (osteoclastogenesis) will contribute to the prevention or treatment of osteoclast-mediated bone loss (Yavropoulou and Yovos 2008).

In the early 1990s, unexpected bone phenotypes in gene disruption studies led to the identification of the genes or their products involved in osteoclastogenesis, and together with the success of the Human Genome Project, the experimental advances in molecular biology provided us

several strategies to identify novel genes or to profile gene expression. Large-scale screening methods such as microarray technology led to the identification of previously unfamiliar gene products such as the receptor activator of nuclear factor-κB ligand (RANKL)-inducible genes (Rho et al. 2002; Yang et al. 2008a; Zhang et al. 2008). Because RANKL is a key factor in regulating osteoclastogenesis and in maintaining the survival of mature osteoclasts (Fuller et al. 1998), the identification of RANKL-regulated molecules and further elucidation of mechanisms that regulate their expressions could extend our understanding of these complex processes.

In the present study, we have screened differentially expressed genes (DEGs) in RAW264.7 cells in response to RANKL by an annealing control primer (ACP)-based PCR method and have identified the osteoclast stimulatory transmembrane protein (OC-STAMP) as a RANKL-induced gene of interest. Recently, OC-STAMP has been identified as the RANKL-induced protein that promotes osteoclast differentiation (Yang et al. 2008b), but the mechanism that regulates its expression has not been studied. Therefore, we undertook an investigation of the tissue distribution of OC-STAMP and the signaling pathways that regulate its expression. Furthermore, genes related to OC-STAMP expression were also identified for predicting its functional role in osteoclastogenesis.

Materials and methods

Chemicals and materials

All chemicals to be used for evaluating the regulatory mechanisms of OC-STAMP expression were purchased from Calbiochem (La Jolla, CA, USA); PKCβ inhibitor (Cat. No. 539654), NFAT activation inhibitor III (Cat. No. 480403), p38 inhibitor (SB203580; Cat. No. 559389), JNK inhibitor II (SP600125; Cat. No. 420119), NF-κB activation inhibitor (Cat. No. 481406), Ras inhibitor (FTI-277; Cat. No. 344555), Raf1 kinase inhibitor (Cat. No. 553008), MEK inhibitor (PD98059; Cat No. 513000), PI3K inhibitor (LY294002; Cat No. 440202), and Akt inhibitor (Cat. No. 124005). 17β-Estradiol (E2) was purchased from Sigma (St. Louis, MO, USA). OC-STAMP specific antibody was kindly provided from Prof. Paul R. Odgren (Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA, USA).

Cell culture and induction of osteoclastogenesis

All the materials for cell culture were purchased from HyClone (Logan, UT, USA). Osteoclast generation was achieved using either mouse monocyte/macrophage RAW264.7 cells or primary cultures of mouse bone marrow-derived macrophages (BMMs) as described previously (Kim et al. 2009). RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. Medium was changed every 3 days. For differentiation, RAW264.7 cells were suspended in α-minimal essential medium (MEM) supplemented with 10% FBS in the presence of 100 ng/ml RANKL (R&D Systems, Minneapolis, MN, USA) and plated at a density of 1×10^3 cells/well in a 96-well plate (differentiation day 0). TRAP-positive multinucleated cells (number of nuclei > 4) were observed on differentiation day 4. For the generation of BMM-derived osteoclasts, monocytes were isolated from the femurs and tibias of ICR mice (Central Lab Animal, Seoul, Korea), seeded and cultured in α-MEM with 10% FBS and 10 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems) for 1 day. Suspended cells were then transferred into new Petri dish plates and cultured in α-MEM with 10% FBS and 30 ng/ml M-CSF for 3 days. Adherent cells at this stage were considered to be M-CSF-dependent BMMs and were used as osteoclast precursors. Differentiation of BMMs into osteoclasts was achieved after plating them into a 96-well plate at a density of 3×10^5 cells/well in α-MEM with 10% FBS, 100 ng/ml RANKL and 30 ng/ml M-CSF (differentiation day 0). The medium was replaced with fresh medium every other day and multinucleated osteoclasts derived from BMMs were observed from differentiation day 6 onward.

ACP-based GeneFishing PCR

Differentially expressed genes were identified by ACP-based GeneFishing PCR (Kim et al. 2004a) using the GeneFishing DEG kits (Seegene, Seoul, Korea). In brief, total RNA was isolated using TRIzol reagent (Life technologies, Rockville, MD, USA) according to the manufacturer's protocol. The concentration and purity of total RNA were calculated with absorbance at 260 and 280 nm. Then, the first-strand cDNA was synthesized by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume of 20 μl containing 3 μg of the purified total RNA, 4 μl of 5× reaction buffer (Promega, Madison, WI, USA), 5 μl of dNTPs (each 2 mM), 2 μl of 10 μM dT-ACP1, 0.5 μl of RNasin RNase Inhibitor (40 U/μl; Promega, Madison, WI, USA), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl; Promega, Madison, WI, USA). First-strand cDNAs were diluted by the addition of 80 μl of ultra-purified water. Second-strand cDNA synthesis was conducted at 50°C

Table 1 Primer sequences used in this study

Target gene	Forward (5′–3′)	Reverse (5′–3′)
OC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
TRAP	ACACAGTGATGCTGTGTGGCAACTC	CCAGAGGCTTCCACATATATGATGG
MMP-9	AGTTTGGTGTCGCGGAGCAC	TACATGAGCGCTTCCGGCAC
c-Src	CCAGGCTGAGGAGTGGTACT	CAGCTTGCGGATCTTGTAGT
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
c-Jun	TCC CCT ATC GAC ATG GAG TC	TGA GTT GGC ACC CAC TGT TA
JunD	CGACCAGTACGCAGTTCCTC	AACTGCTCAGGTTGGCGTAG
Fra-1	AGAGCTGCAGAAGCAGAAGG	CAAGTACGGGTCCTGGAGAA
Fra-2	ATCCACGCTCACATCCCTAC	GTTTCTCTCCCTCCGGATTC
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
Mitf	AGCGTGTATTTTCCCCACAG	CCTTAGCTCGTTGCTGTTCC
RANK	CACAGACAAATGCAAACCTTG	GTGTTCTGGAACCTATCTTCC
c-Fms	GACCCTCGAGTCAACAGAGC	TGTCAGTCTCTGCCTGGATG
OSCAR	ACTCCTGGGATCAACGTGAC	CTACGCGGTACAGTGCAAAA
TRAF6	GCTCAAACGGACCATTCGGA	GGGATTGTGGGTCGCTGAAA
ATP6v0d2	AGACCACGGACTATGGCAAC	CGATGGGTGACACTTGGCTA
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
CD44	GAGACCTCAGATTCCAGAATGG	AATGCACCATTTCCTGAGACTT
CD47	AACTGACTTCCATCCAGGAAAA	TAACCCTCAGGTTAGTGCTTGG
Meltrin-α	AGACCCTTAAGATGACCAAGTACG	ATAGAGCATTTGTCGATGTCATTC
E-cadherin	GAGTATAATGACGCAGCTCAAGAA	GTTCTCCTCCGTAGAAACAGTAGG
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

during one cycle of first-stage PCR in a final reaction volume of 20 µl containing 3–5 µl (about 50 ng) of diluted first-strand cDNA, 1 µl of dT-ACP2 (10 µM), 1 µl of 10 µM arbitrary ACP, and 10 µl of 2× Master Mix (Seegene, Seoul, Korea). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide.

Direct sequencing

Differentially expressed genes were re-amplified and extracted from the gel using the Genclean II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly sequenced with ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Evaluation of mRNA expression levels

Primers were chosen with an online primer design program (Rozen and Skaletsky 2000); primer nucleotide sequences are presented in Table 1. Isolation of total RNA, synthesis of cDNA, and real-time quantitative PCR (QPCR) were carried out as described previously (Kim et al. 2009). In brief, total RNA from cells or tissues that was isolated from ICR mice and ground under liquid nitrogen to a fine powder was isolated with TRIzol reagent. Then, the first strand cDNA was synthesized with Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). Next, SYBR green-based real-time QPCR was performed with the Brilliant SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) in the Stratagene Mx3000P Real-Time PCR system. All reactions were run in triplicate and the data were analyzed by the $2^{-\Delta\Delta CT}$ method as described previously (Livak and Schmittgen 2001; Kim et al. 2009). GAPDH was used as a control for gene amplification. For semi-quantitative RT-PCR, Taq PCR Master Mix (Qiagen, Valencia, CA, USA) was used as described previously (Kim et al. 2004b).

Western blot analysis

Western blot was performed as described previously (Kim et al. 2009). Tissues were isolated from ICR mice and ground under liquid nitrogen to a fine powder. OC-STAMP antibody was diluted 1:100 in the blocking buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 3% nonfat dry milk).

Estrogen treatment

Cells were plated at 1×10^5 cells/well in a 6-well plate and then 1 day after, cells were incubated with the phenol red-free DMEM with 10% CD-FBS in the presence or absence of RANKL or E2 for 6 days with the medium change every 2 days.

Knock-down of OC-STAMP with its specific shRNA

Cells were transfected with SureSilencing shRNA plasmids for OC-STAMP (SABiosciences, Frederick, MD, USA) according to the manufacturer's protocol. In this study, the SureSilencing shRNA plasmid for OC-STAMP (inserted sequence: ACC TGC GTT TCG ACA ATA TCT) and negative control shRNA plasmid (inserted sequence: GGA ATC TCA TTC GAT GCA TAC) were used. After the transfected cell population was enriched by selection for neomycin resistance, the extent of knock-down was evaluated by real-time PCR.

TRAP staining and its activity assay

Cells were fixed with 10% formalin for 10 min and ethanol/acetone (1:1) for 1 min, and then stained with the Leukocyte Acid Phosphatase kit 387-A (Sigma, St. Louis, MO, USA). Images of TRAP-positive multinucleated cells (number of nuclei > 4) were captured with a microscope with a DP Controller (Olympus Optical, Tokyo, Japan). To measure TRAP activity, cells were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, and then 100 µl of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate (Sigma, St. Louis, MO, USA) was added to the fixed cells-containing wells of 96-well plates. After incubation for 1 h, the enzyme reaction mixtures in the wells were transferred into new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 410 nm with a Wallac EnVision HTS microplate reader (Perkin Elmer, Waltham, MA, USA). The experiment was performed in triplicate.

Pit formation assay

Pit formation assay was carried out as described previously (Kim et al. 2009). M-CSF-dependent BMMs (3×10^5 cells/well) were plated on BioCoat Osteologic multitest slides (BD Biosciences, MA) and cultured in α-MEM with 10% FBS, 100 ng/ml RANKL and 30 ng/ml M-CSF. The medium was replaced with fresh medium every other day and on the differentiation day 6, multinucleated osteoclasts derived from BMMs were observed. On the differentiation day 7, OC-STAMP antibody was treated everyday or every other day. On the differentiation day 10 or 13, the slides were washed with 6% sodium hypochlorite solution to remove the cells. Resorbed areas on the slides were observed with a microscope.

Cell viability

Cells were suspended in α-MEM with 10% FBS and plated in 96-well plates at a density of 1×10^3 cells/well. After 1 or 4 days, cell viability was measured with the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's protocol.

Statistics

Significance was determined with the Student's *t* test and differences were considered to be significant at $P < 0.05$.

Results

To identify the RANKL-regulated genes, we compared DEGs identified in untreated control RAW264.7 cells with those found in cells treated with RANKL (100 ng/ml) for 24 h using GeneFishing technology. Using 120 GeneFishing Primers (GPs), 40 DEGs were identified (data not shown). Densitometry analysis revealed RANKL treatment caused an eightfold greater rate of induction of DEG19 as compared to the control cells (arrow indicated in Fig. 1a). Using direct sequencing and sequence homology search in BLAST (http://www.ncbi.nlm.nih.gov), DEG19 was identified as a 285-bp cDNA fragment that had 99% homology with the *Mus musculus* RIKEN cDNA 4833422F24 gene (4833422F24Rik) mRNA sequence (GenBank NM_029021) (Fig. 1b). The product of the gene encoding this sequence has been recently designated as OC-STAMP (Yang et al. 2008b).

To ascertain if OC-STAMP mRNA is up-regulated in response to RANKL, the induction of its mRNA expression was evaluated together with four osteoclastogenesis-related genes, *TRAP, matrix metalloproteinase (MMP)-9, c-Src,* and *cathepsin K*. Osteoclastogenesis-related genes were significantly induced by RANKL as expected and, interestingly, OC-STAMP mRNA was up-regulated ~4-fold and ~130-fold by RANKL on the differentiation day 1 and 4, respectively (Table 2). The time-dependent induction of OC-STAMP mRNA expression was also observed in BMMs (data not shown). Additionally, the RANKL dose-dependent induction of OC-STAMP mRNA expression as well as TRAP was also observed (data not shown). Apparently, the induction of OC-STAMP protein during osteoclastogenesis of BMMs was confirmed by its specific antibody (Fig. 1c).

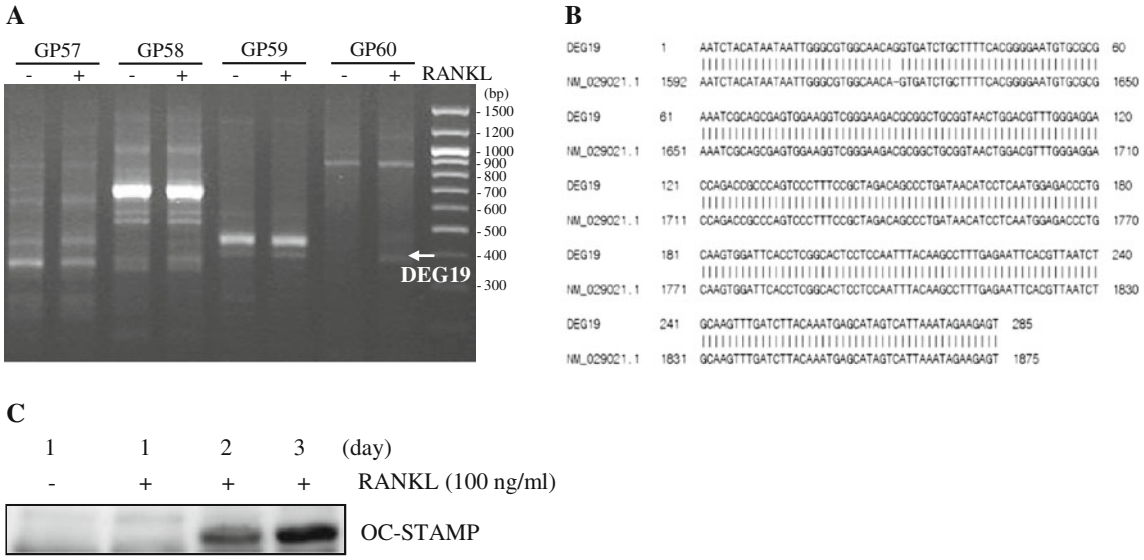

Fig. 1 Identification of OC-STAMP and its induction by RANKL. **a** *Arrow* indicated DEG19 that was highly expressed in cells treated with RANKL for 24 h compared to the control. **b** DEG19 was 285-bp cDNA fragment that has 99% homology with Mus musculus RIKEN cDNA 4833422F24 gene (4833422F24Rik) mRNA (accession no. NM_029021.1). **c** RANKL-induced OC-STAMP was evaluated by western blot analysis. In the absence or presence of RANKL (100 ng/ml), BMMs (7×10^5 cells) were suspended with α-MEM with 10% FBS and M-CSF (30 ng/ml), seeded in a 35-mm^2 plate and incubated for 1–3 days

Table 2 RANKL-induced expression levels of osteoclastogenesis-related genes and *OC-STAMP* in RAW264.7 cells

Target gene	1 day		2 day		4 day	
	Control	RANKL treated	Control	RANKL treated	Control	RANKL treated
TRAP	1.00 ± 0.10	2.21 ± 0.08[b]	1.09 ± 0.54	42.31 ± 0.59[b]	1.00 ± 0.07	82.25 ± 8.51[b]
MMP-9	1.00 ± 0.04	18.26 ± 1.70[b]	1.07 ± 0.49	109.66 ± 3.56[b]	1.04 ± 0.32	156.65 ± 8.22[b]
c-Src	1.00 ± 0.11	4.17 ± 0.70[a]	1.17 ± 0.67	25.50 ± 2.77[b]	1.00 ± 0.09	6.92 ± 1.08[b]
Cathepsin K	1.01 ± 0.16	3.00 ± 0.35[b]	1.17 ± 0.67	105.16 ± 25.48[a]	1.00 ± 0.08	39.48 ± 3.03[a]
OC-STAMP	1.00 ± 0.07	4.08 ± 0.26[b]	1.19 ± 0.90	22.11 ± 2.64[b]	1.00 ± 0.08	131.89 ± 21.20[b]

[a] $P < 0.01$, [b] $P < 0.001$

OC-STAMP protein expression in osteoclasts and its induction by RANKL have been also reported by its specific antibody (Yang et al. 2008b), but its tissue distribution has not been studied. Therefore, we evaluated the expression level of OC-STAMP in nine tissues and in BMMs isolated from female mice. As shown in Fig. 2a, OC-STAMP mRNA and protein were strongly induced by RANKL in BMMs and its mRNA was also expressed in all tissues tested with the exception of ovary. Although western blot analysis revealed that OC-STAMP protein was detected in ovary, OC-STAMP mRNA expression level was lower in ovary than other tissues, suggesting that it might be down-regulated by estrogen. Therefore, we further evaluated the effect of E2 on the mRNA expression of OC-STAMP. OC-STAMP mRNA expression was up-regulated by low concentrations of E2, but down-regulated at high concentrations (Fig. 2b). Additionally, the down-regulation of OC-STAMP protein expression by E2 at high concentrations was also confirmed by western blot analysis.

The involvement of OC-STAMP in osteoclastogenesis was confirmed in RAW264.7 cells using its specific shRNA. On differentiation day 4, OC-STAMP mRNA was significantly decreased in RAW264.7 cells transfected with the OC-STAMP-specific shRNA as compared to cells treated with negative control shRNA (Fig. 3a). Additionally, the OC-STAMP-specific shRNA-transfected cells were less differentiated into TRAP-positive multinucleated osteoclasts by RANKL treatment (Fig. 3b). Consistent with an earlier report showing that the inhibition of OC-STAMP expression did not prevent TRAP expression (Yang et al. 2008b), we also found that TRAP mRNA expression was not affected by OC-STAMP shRNA even in cells treated with RANKL (Fig. 3a). We also observed that the growth rates of control RAW264.7 cells and of cells transfected with OC-STAMP shRNA were not significantly different

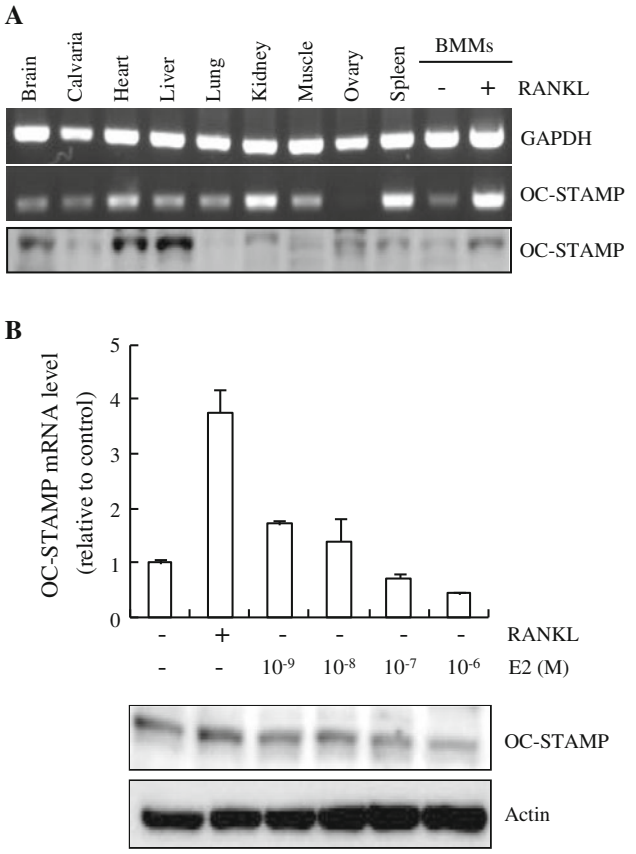

Fig. 2 Tissue distribution of OC-STAMP (**a**) and effect of E2 on its expression (**b**). Tissue distribution of OC-STAMP in nine tissues and in BMMs isolated from female mice was evaluated by semi-quantitative PCR and western blot analysis. The effect of E2 on its expression was evaluated by real-time PCR and western blot analysis as described in "Materials and methods"

(data not shown), thus ruling out the possibility that OC-STAMP shRNA-mediated cytotoxicity may account for the inhibition of osteoclast differentiation.

The involvement of OC-STAMP in the formation and bone resorptive activity of mature osteoclasts derived from BMMs was confirmed using its specific antibody. As shown in Fig. 3c, OC-STAMP antibody inhibited the TRAP activity and the formation of TRAP-positive multinucleated osteoclasts. Additionally, the pit formation assay revealed that OC-STAMP antibody inhibited the bone resorptive activity of mature osteoclasts when it was treated into multinucleated osteoclasts (Fig. 3d).

We examined the effect of OC-STAMP expression knock-down on the mRNA expression levels of osteoclastogenesis-related molecules (TRAP, MMP-9, and c-Src), transcription factors (AP-1 members, NFATc1, and Mitf), receptors (RANK, c-Fms, and OSCAR), a signaling molecule (TRAF6), and fusion-related molecules (ATP6v0d2, DC-STAMP, MFR, CD44, CD47, meltrin-α, and E-cadherin) required for osteoclast differentiation, bone resorption, and cell fusion. Real-time PCR revealed that the mRNA expression levels of c-Jun, RANK, c-Fms, and meltrin-α were reduced, but that of TRAF6 was induced in cells transfected with the OC-STAMP-specific shRNA (Table 3).

We carried out the pharmacological inhibition study to identify the candidates of signaling molecules regulating the RANKL-induced mRNA expression of OC-STAMP using specific inhibitors for osteoclastogenesis-related signaling molecules. The treatment concentration of each inhibitor was determined based on its effect on TRAP activity in the RANKL-treated RAW264.7 cells; the concentration of each inhibitor used in each study was similar with its IC_{50} value on TRAP activity with the exception of Ras and MEK inhibitors (Table 4). As shown in Fig. 4a, we evaluated the effects of five inhibitors directed against signaling molecules related to mainly the early stage of osteoclastogenesis (i.e., PKCβ, NFAT, p38, JNK, and NF-κB) on the mRNA expression of OC-STAMP and also of three well-known biomarkers for osteoclastogenesis, TRAP, MMP-9, and c-Src. The RANKL-induced mRNA expressions of TRAP, MMP-9, and c-Src were dramatically inhibited by these inhibitors. Importantly, the RANKL-induced mRNA expression of OC-STAMP was almost completely prevented by inhibitors for PKCβ, p38, and NF-κB. JNK inhibitor had a slight preventive effect on the RANKL-induced mRNA expression of OC-STAMP, but NFAT activation inhibitor III displayed no inhibitory action. Furthermore, the effects of five other inhibitors for signaling molecules mainly related to the survival of mature osteoclasts (such as Ras, Raf1, MEK, PI3K, and Akt) on the OC-STAMP mRNA expression were also evaluated (Fig. 4b). The Akt inhibitor generally prevented the RANKL-induced mRNA expression levels of osteoclastogenesis-related biomarkers as well as that of OC-STAMP, but the RANKL-induced mRNA expression of OC-STAMP was enhanced by inhibitors in the order Ras inhibitor (FTI-277) > MEK inhibitor (PD98059) > PI3K inhibitor (LY294002). Although there was no significant change in TRAP activity by the treatment with 20 μM of Ras inhibitor FTI-277, it uniformly enhanced the RANKL-induced mRNA expression levels of osteoclastogenesis-related biomarkers (Fig. 4b).

The involvement of PKCβ, p38, JNK, NF-κB, and Akt in the osteoclastogenesis of BMMs was also confirmed as shown in Fig. 5a; when BMMs were treated with each compound at around its IC_{50} (Table 5), the multinucleated osteoclasts were less observed by the pharmacological inhibition of PKCβ, p38, and NF-κB, and additionally, the protein expression of OC-STAMP was decreased by PKCβ or Akt inhibitor (Fig. 5b).

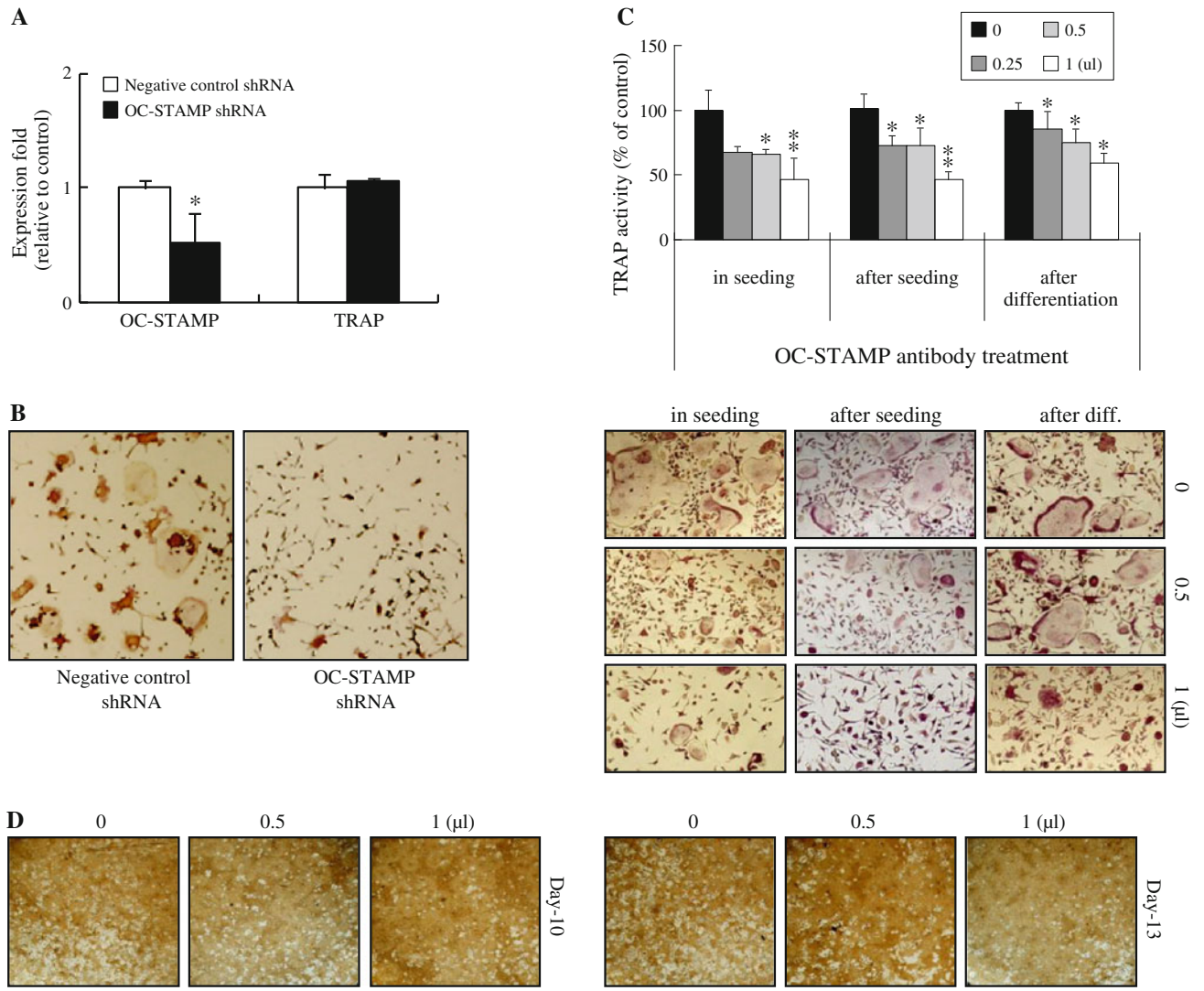

Fig. 3 Effects of OC-STAMP on formation and bone resorptive activity of mature osteoclasts. Effects of OC-STAMP shRNA clones on the mRNA expression levels of OC-STAMP and TRAP (**a**) and the formation of TRAP-positive multinucleated osteoclasts (**b**) in RAW264.7 cells. The mRNA levels were evaluated by the real-time QPCR 4 days after RANKL treatment. TRAP activity assay and its staining were also carried out 4 days after RANKL treatment. $*P < 0.05$. Effect of OC-STAMP antibody on the formation (**c**) and bone resorptive activity of mature osteoclasts derived from BMMs (**d**) were evaluated by TRAP activity, its staining and pit formation assay. OC-STAMP antibody was treated everyday from cell seeding (*left*), everyday from 1 day after seeding (*middle*) or everyday from 9 days after seeding (*right*). On the differentiation day 10, TRAP activity and its staining were carried out. $*P < 0.05$; $**P < 0.01$. For pit formation assay, BMMs were plated on BioCoat Osteologic multitest slides and cultured for 6 days. Next day, OC-STAMP antibody was treated into multinucleated osteoclasts everyday or every other day and the slides were washed and observed with a microscope on the differentiation day 10 or 13, respectively

Discussion

RANKL is a key factor in regulating the process of osteoclastogenesis and in maintaining the survival of mature osteoclasts (Fuller et al. 1998). The binding of RANKL to its tumor necrosis factor (TNF)-family receptor RANK triggers the activation of cytoplasmic tumor necrosis factor receptor-associated factor 6 (TRAF6), which subsequently induces the activation of several signaling molecules such as mitogen-activated protein (MAP) kinases and transcription factors such as NF-κB and NFATc1 (Boyle et al. 2003; Lee and Kim 2003; Kim et al. 2008a). These signaling molecules and transcription factors play a critical role in the successful expression of genes required for the osteoclastogenesis and the bone resorptive activity of mature osteoclasts (Ikeda et al. 2004; Takayanagi et al. 2002; Kim et al. 2005). Therefore, the identification of RANKL-induced genes and further elucidation of regulatory mechanism of their expressions are important for understanding the complex process of osteoclastogenesis.

Table 3 Effect of OC-STAMP knock-down on the mRNA expression of genes required for osteoclastogenesis

Target gene	mRNA expression level (relative to negative control)	
	Negative control shRNA	OC-STAMP shRNA
OC-STAMP	1.00 ± 0.03	0.60 ± 0.06[c]
TRAP	1.00 ± 0.01	1.05 ± 0.07
MMP-9	1.01 ± 0.24	0.96 ± 0.16
c-Src	1.01 ± 0.10	0.92 ± 0.13
c-Fos	1.03 ± 0.30	0.80 ± 0.19
c-Jun	1.03 ± 0.30	0.52 ± 0.09[a]
JunD	1.01 ± 0.15	1.32 ± 0.23
Fra-1	1.01 ± 0.16	1.25 ± 0.02
Fra-2	1.00 ± 0.07	1.30 ± 0.26
NFATc1	1.02 ± 0.22	0.76 ± 0.12
Mitf	1.03 ± 0.05	0.90 ± 0.04
RANK	1.00 ± 0.01	0.82 ± 0.14[a]
c-Fms	1.02 ± 0.24	0.30 ± 0.04[b]
OSCAR	1.02 ± 0.27	1.10 ± 0.12
TRAF6	1.00 ± 0.10	1.43 ± 0.17[a]
ATP6v0d2	1.01 ± 0.19	1.16 ± 0.26
DC-STAMP	1.03 ± 0.32	0.74 ± 0.10
MFR	1.00 ± 0.01	1.30 ± 0.20
CD44	1.00 ± 0.10	1.12 ± 0.09
CD47	1.01 ± 0.12	1.28 ± 0.08
Meltrin-α	1.00 ± 0.12	0.32 ± 0.10[c]
E-cadherin	1.00 ± 0.12	0.85 ± 0.29

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$

Table 4 IC_{50} of chemicals on TRAP activity and its concentration used in RAW264.7 cells

Chemical	IC_{50} (μM) on TRAP activity	Conc. (μM) used in Fig. 4
PKCβ inhibitor	17.57 ± 1.58	20
NFAT activation inhibitor III	21.27 ± 0.83	20
p38 inhibitor (SB202190)	4.87 ± 0.93	5
JNK inhibitor II (SP600125)	8.30 ± 0.23	10
NF-κB activation inhibitor	0.18 ± 0.01	0.2
Ras inhibitor (FTI-277)	>30[a]	20
Raf1 kinase inhibitor I	28.74 ± 0.83	30
MEK inhibitor (PD98059)	>30[b]	20
PI3K inhibitor (LY294002)	8.68 ± 0.45	10
Akt inhibitor	23.69 ± 0.67	20

[a] At 30 μM, TRAP activity relative to control was 88.01 ± 2.19%

[b] At 30 μM, TRAP activity relative to control was 60.60 ± 1.69%

In this study, using GeneFishing technology, we identified OC-STAMP as one of the RANKL-induced genes of interest. OC-STAMP in osteoclasts has been designated as a key molecule in the regulation of osteoclastogenesis (Yang et al. 2008b) and the induction of its expression by RANKL was confirmed in this study. Interestingly, similar to osteoclasts, OC-STAMP was expressed in most tissues. Although western blot analysis revealed that OC-STAMP protein was detected in ovary, OC-STAMP mRNA expression level was lower in ovary than other tissues. Since estrogen has been known to control osteoclastogenesis by inhibiting the over-activation of osteoclasts (Sipos et al. 2009), we evaluated the effect of E2 on the mRNA expression of OC-STAMP. We observed a biphasic effect of E2 on OC-STAMP mRNA expression, with up-regulation occurring at low concentrations of E2, but down-regulation at high concentrations. Although its protein level was also down-regulated by E2 at high concentrations, future studies are needed to elucidate the functional role of estrogens in the mRNA expression of OC-STAMP because of the complicated relationship between estrogens and the whole process of bone metabolism. Additionally, considering that OC-STAMP mRNA or protein was expressed in most tissues, OC-STAMP might play a critical house-keeping role in the whole body.

With respect to the function of OC-STAMP in osteoclasts, OC-STAMP can promote osteoclast differentiation (Yang et al. 2008b). The involvement of OC-STAMP expression in osteoclast differentiation was also confirmed in this study. We observed that OC-STAMP mRNA was induced in a dose-dependent manner by RANKL and its induction by RANKL was observed from the early-to-late stages of osteoclastogenesis in RAW264.7 cells and in BMMs. These results suggested that OC-STAMP expression could be required for the whole process of multinucleated osteoclast formation. Apparently, siRNA and overexpression techniques supported the involvement of OC-STAMP on osteoclastogenesis (Yang et al. 2008b). Consistent with these results, we also found that the mRNA level of OC-STAMP was significantly decreased in RAW264.7 cells transfected with the OC-STAMP-specific shRNA compared to those with negative control shRNA. Concurrently, OC-STAMP knock-down did not affect cell viability, but did inhibit the formation of multinucleated osteoclasts, suggesting the functional involvement of OC-STAMP expression per se in the process of multinucleated osteoclast formation.

The involvement of OC-STAMP in the formation and bone resorptive activity of mature osteoclasts derived from BMMs was confirmed using its specific antibody; OC-STAMP antibody inhibited the formation of TRAP-positive multinucleated osteoclasts. This was consistent with the result shown in the previous report (Yang et al. 2008b). Additionally, the pit formation assay revealed that OC-STAMP antibody inhibited the bone resorptive activity of mature osteoclasts without any induction of caspase-3 or

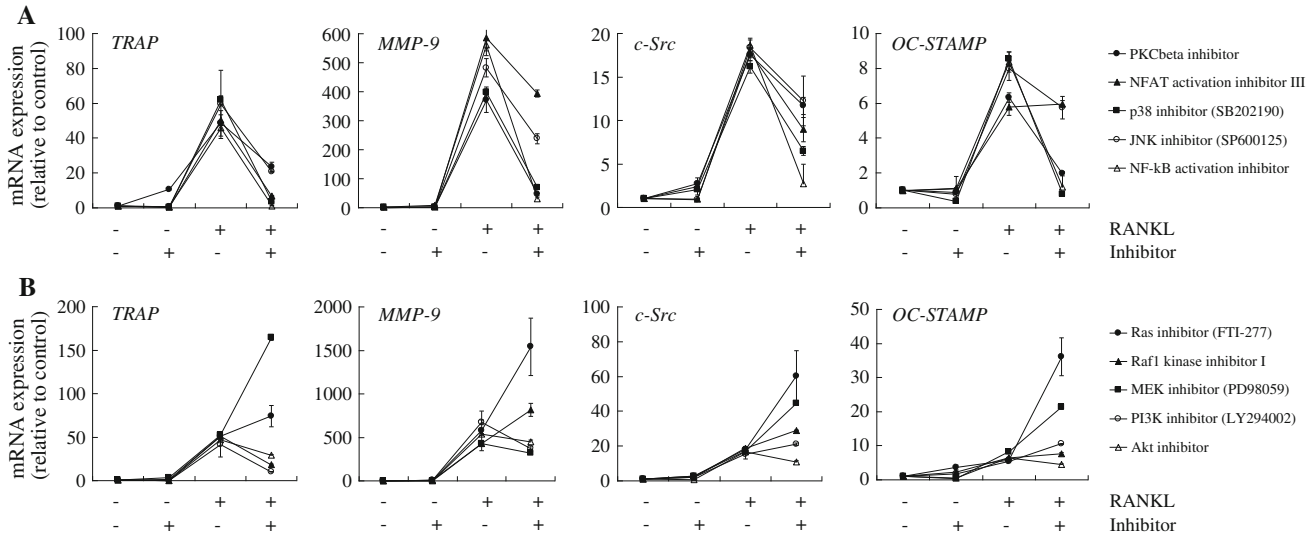

Fig. 4 Effects of RANKL or inhibitors on the mRNA expressions of biomarkers for osteoclastogenesis and OC-STAMP. The mRNA expression levels in response to RANKL and/or inhibitors for signaling molecules mainly related to the early (**a**) or late stage of osteoclastogenesis (**b**) were evaluated in RAW264.7 cells by the real-time QPCR. In brief, cells were incubated with RANKL and/or inhibitor for 1 day and then the real-time QPCR was performed as described in "Materials and methods"

LDH activity, suggesting that the inhibitory effect of OC-STAMP antibody on bone resorption might not be due to the apoptosis of mature osteoclasts (Kim et al. 2009). Further studies are needed to elucidate the functional involvement of OC-STAMP in mature osteoclasts and its precise mechanism.

Several lines of evidence have suggested a role for OC-STAMP in pre-osteoclast fusion (Yang et al. 2008b). (a) The amino acid sequence of OC-STAMP was significantly similar to the DC-STAMP (dendrite cells-specific transmembrane protein) family consensus sequence and the predicted secondary structures and membrane topology of both were remarkably similar. (b) DC-STAMP was reported to be essential for osteoclastogenesis (Kukita et al. 2004) and (c) in its knock-out mice, the complete abrogation of osteoclast cell fusion and foreign body giant cell formation by macrophage cell fusion suggested its role in cell fusion (Yagi et al. 2005). Aside from their structural similarities, both OC-STAMP and DC-STAMP were induced by RANKL and inhibited multinucleated osteoclast formation when blocked by siRNA or antibody, whereas overexpression of both proteins promoted multinucleated osteoclast differentiation (Kukita et al. 2004; Yang et al. 2008b). Considering these results, OC-STAMP might play a role in the fusion stage in the process of osteoclastogenesis.

To determine if OC-STAMP influences the fusion stage of osteoclastogenesis and to identify genes related with OC-STAMP gene expression, we evaluated the effect of OC-STAMP expression knock-down on the mRNA expression levels of osteoclastogenesis-related biomarkers, transcription factors required for osteoclast differentiation, receptors, signaling molecules, and fusion-related molecules. Real-time PCR revealed that the knock-down of OC-STAMP at the transcript level significantly reduced the mRNA expression levels of RANK, c-Fms, c-Jun, and meltrin-α, but induced that of TRAF6, suggesting that the expression of these genes could be functionally involved in the mechanism of OC-STAMP's action. RANK and c-Fms are the receptors for RANKL and macrophage colony-stimulating factor (M-CSF), respectively. In the commitment and differentiation of osteoclast precursor cells, the sequential expressions of c-Fms and RANK have been reported (Arai et al. 1999). Interestingly, TNF-α directly stimulated the genesis of bone marrow osteoclast precursors by enhancing c-Fms expression, suggesting that c-Fms expression might be needed in the early stage of osteoclastogenesis (Yao et al. 2006). A critical role for c-Jun signaling in the RANKL-regulated osteoclast formation has been revealed by its transgenic mice with severe osteopetrosis due to impaired osteoclastogenesis (Ikeda et al. 2004) and meltrin-α has been reported to be a fusion protein involved in multinucleated giant cells and osteoclast formation (Abe et al. 1999). Considering the biological function of those molecules in osteoclasts, the decreased expressions of RANK and c-Fms by the down-regulation of OC-STAMP expression might reduce the sensitivity of pre-osteoclasts for RANKL that consequently leads to the decrease of its potential to trigger and activate the RANKL-mediated osteoclastogenesis and/or osteoclast fusion via down-regulation of the expression and/or activities of c-Jun and meltrin-α. Since TRAF6 has been

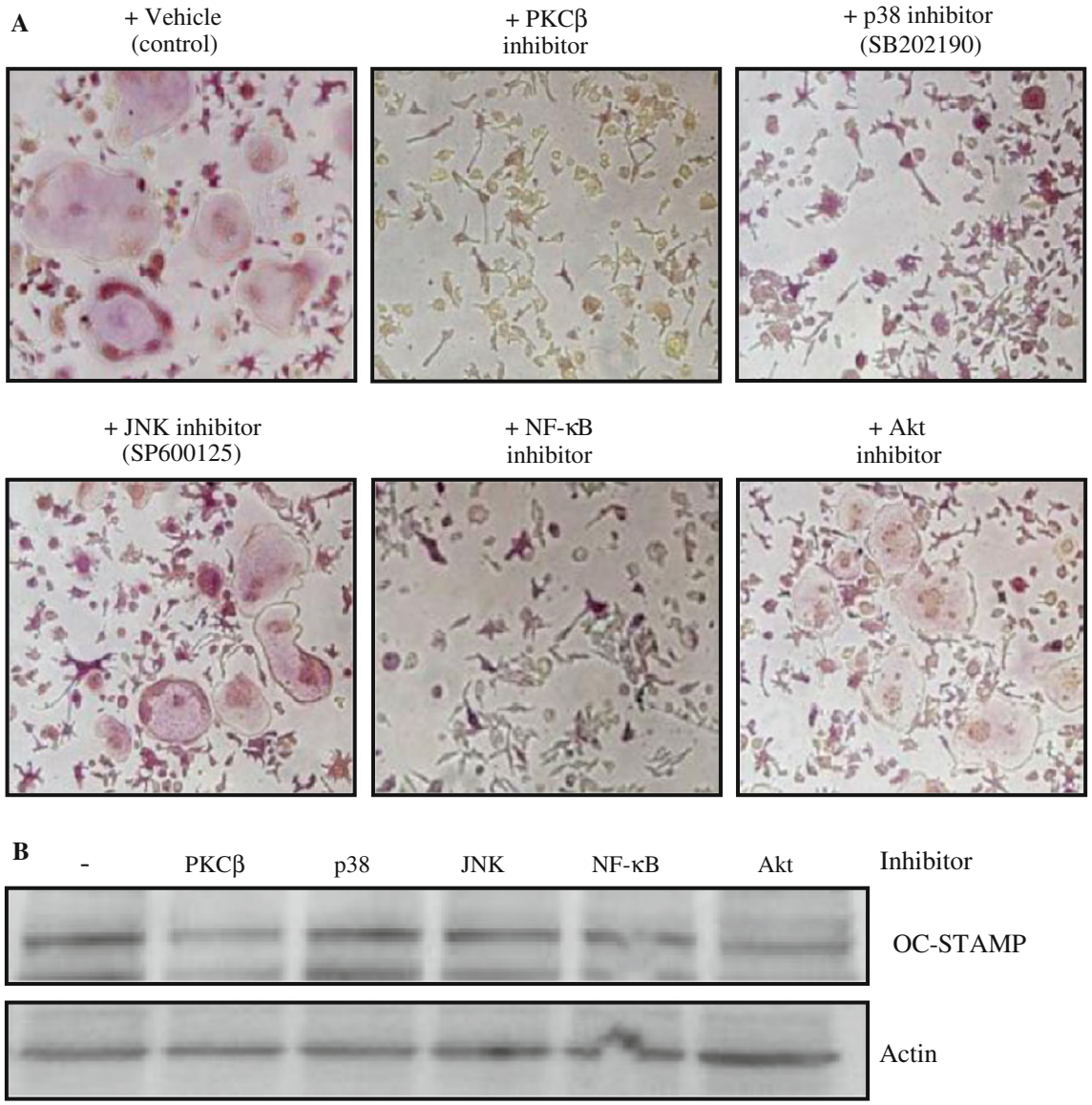

Fig. 5 Effects of inhibitors on the formation of multinucleated osteoclasts (**a**) and the protein expression of OC-STAMP in BMMs (**b**). For TRAP staining, cells (1×10^4 cells/well) were suspended with α-MEM with 10% FBS, 100 ng/ml RANKL, and 30 ng/ml M-CSF, plated into a 96-well plate and cultured for 1 day. Cells were incubated with each inhibitor for 3 days and then TRAP staining was performed. For western blot analysis, cells (7×10^5 cells/well) were cultured in a 6-well plate. Each inhibitor was treated on the differentiation days 1 and 3 and then cells were homogenized for western blot analysis on the differentiation day 4

Table 5 IC_{50} of chemicals on TRAP activity and its concentration used in BMMs

Chemical	IC_{50} on TRAP activity	Conc. used in Fig. 5
PKCβ inhibitor	2.20 ± 0.05 μM	2.50 μM
p38 inhibitor (SB202190)	1.70 ± 0.05 μM	1.25 μM
JNK inhibitor II (SP600125)	4.90 ± 0.56 μM	2.50 μM
NF-κB activation inhibitor	3.80 ± 0.40 nM	3.00 nM
Akt inhibitor	19.90 ± 6.66 μM	10.00 μM

shown to be involved in TNF-α-induced osteoclastogenesis even in the absence of RANKL (Kaji et al. 2001; Hotokezaka et al. 2007), the significant induction of TRAF6 mRNA by OC-STAMP down-regulation might compensate for the reduced sensitivity of pre-osteoclasts for RANKL. Conversely, its induction by OC-STAMP down-regulation per se could negatively regulate osteoclast formation by intracytoplasmic sequestration of FHL2 (four and half LIM domain 2) to blunt RANK activation (Bai et al. 2008). FHL2 has been reported to be the anti-osteoclastogenic

molecule exerting its effect by attenuating TRAF6-mediated RANK signaling (Bai et al. 2005). The precise relationship between OC-STAMP and TRAF6 requires further study. In addition, consistent with our results, the mRNA expression levels of DC-STAMP, ATP6v0d2, and NFATc1, which are considered to be a requisite for cell fusion of osteoclasts (Kim et al. 2008a), have been reported to be unchanged during osteoclast fusion (Hotokezaka et al. 2007). Therefore, the change in the mRNA expression of several molecules in the stream of the signaling cascade for osteoclastogenesis by the down-regulation of OC-STAMP expression suggests that the coordinated expression of receptors, signaling molecules, transcription factors, and cell fusion-inducing factors might be required for osteoclast fusion.

Since the usefulness of the annotated compounds (or employing inhibitory compounds) has been reported in several studies (Root et al. 2003; Kim et al. 2008b), we examined the effects of several pharmacological inhibitors on OC-STAMP mRNA expression to elucidate the regulatory mechanism for the RANKL-mediated induction of its mRNA expression in RAW264.7 cells. These inhibitors targeted signaling molecules related to both the early or late stages of osteoclastogenesis. When cells were treated with inhibitors against early stage osteoclastogenesis factors, we observed that the RANKL-induced mRNA expression of OC-STAMP was almost completely prevented by inhibitors against PKCβ, p38, JNK, and NF-κB. However, the inhibitor III against NFAT activation had no effect, suggesting that regulation of OC-STAMP mRNA expression might be independent of the NFAT signaling cascade. RANK signaling transduction during osteoclast development and activation has been well reviewed (Boyle et al. 2003). Several studies have presented experimental evidence for the involvement of p38, JNK, and NF-κB (Darnay et al. 1998; Wong et al. 1998; Matsumoto et al. 2000; David et al. 2002; Lee et al. 2002). Additionally, the specific inhibition of PKCβ activity suppressed the differentiation and fusion of osteoclasts (Lee et al. 2003).

When cells were treated with inhibitors of signaling molecules related to the survival of mature osteoclasts, Akt inhibitor was effective in blocking the RANKL-induced mRNA expression level of OC-STAMP and also of osteoclastogenesis-related biomarkers. Thus, Akt could also play a role in the early stage of osteoclastogenesis by regulating the expression of genes that are strongly induced by RANKL. Ras and its down-stream molecules (Raf, PI3K, and MEK/ERK) were shown to positively regulate the M-CSF-mediated osteoclast survival (Bradley et al. 2008) and osteoclast fusion (Lee et al. 2009), but we observed here that the treatment with inhibitors for those signaling molecules with RANKL in RAW264.7 cells enhanced the RANKL-induced expression of the OC-STAMP gene as well as osteoclast-specific genes. PD98059 has been reported to accelerate differentiation of RAW264.7 cells into osteoclast-like cells depending on cell density. Additionally, the MEK inhibitors increased phosphorylation of p38, whereas the phosphorylation of ERK was increased by treatment with p38 inhibitors suggesting that a seesaw-like balance exists between ERK and p38 phosphorylation (Hotokezaka et al. 2002). The RANKL-induced mRNA expression of OC-STAMP was shown to be enhanced by the inhibitors for Ras, MEK, and PI3K inhibitor (LY294002), but it might not be specific in osteoclasts because Ras and MEK inhibitors had no inhibitory effect on the RANKL-induced osteoclastogenesis at the used concentration, 20 μM. Further study is required to understand the effects of inhibition of signaling molecules mainly related to the late stage of osteoclast differentiation on the expression of OC-STAMP. The effects of those inhibitors on cell viability, differentiation into macrophages, and apoptosis (or cell death) should be carefully considered in the context of the experimental differences related to the type, density of used cells, the culture methods, and protocols.

The involvement of PKCβ, p38, JNK, NF-κB, and Akt in the osteoclastogenesis of BMMs was also confirmed; the multinucleated osteoclasts were less observed by the pharmacological inhibition of PKCβ, p38, and NF-κB, and additionally, the protein expression of OC-STAMP was decreased by PKCβ or Akt inhibitor. Together with the results observed in RAW264.7 cells, these results suggested that PKCβ or Akt might be the major signaling molecules to regulate the expression of OC-STAMP during osteoclastogenesis.

In conclusion, OC-STAMP was induced by RANKL during osteoclastogenesis and it might be involved in the bone resorptive activity of mature osteoclasts. Additionally, OC-STAMP expression was not limited in osteoclasts, suggesting its involvement in the function of other tissues. OC-STAMP expression could be regulated by estrogen and its knockdown at the transcript level resulting in the inhibition of multinucleated osteoclast formation and the decreased expressions of genes related to transcription, receptors, signal transduction, and cell fusion, suggesting that osteoclast differentiation requires the coordinated expressions of OC-STAMP with several molecules involved in these processes. Studies with pharmacological inhibitors suggested PKCβ or Akt might be the major signaling molecules to regulate the expression of OC-STAMP during osteoclastogenesis. Future investigations of promoter identification and characterization of the OC-STAMP gene and in vivo functional studies should elucidate the precise regulatory mechanism and function of OC-STAMP in the body.

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