

Natural polyamines inhibit the migration of preosteoclasts by attenuating Ca^{2+} -PYK2-Src-NFATc1 signaling pathways

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Abstract Natural polyamines have numerous biological activities. Several studies have reported their beneficial role in bone metabolism, but their mode of action is not fully understood. Bone diseases such as osteoporosis, which is characterized by impaired bone structure and low bone mass, are caused by an increased number of osteoclasts and/or overactivation of osteoclastogenesis. Osteoclast differentiation is a multi-complex procedure involving the following sequential steps: differentiation–migration–fusion–resorption. In this study, we found that putrescine, spermidine or spermine inhibited the RANKL-mediated migration of preosteoclasts. Furthermore, the RANKL-mediated activation of the Src-PYK2 signaling axis and of transcription factors such as NF- κ B and NFATc1 was

prevented by each polyamine. Anti-osteoclastogenic and anti-migration activities of polyamines were confirmed by evaluating their potential to downregulate the mRNA expression levels of osteoclastogenesis-related genes such as *OSCAR*, *TRAP*, *cathepsin K* and *c-Src*, and genes related to fusion and/or migration of preosteoclasts. Moreover, ATP-mediated elevation of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was strongly inhibited by each polyamine, indicating the involvement of $[\text{Ca}^{2+}]_i$ in the anti-fusion activities of polyamines. In conclusion, polyamines could exhibit anti-osteoclastogenic activity by inhibiting the migration of preosteoclasts via the Ca^{2+} -PYK2-Src-NFATc1 signaling axis.

Keywords Polyamines · Preosteoclast · Fusion · Migration · NFATc1 · PYK2 · Calcium

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Introduction

Bone is dynamically maintained by the functional balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation (Boyle et al. 2003). However, an imbalance caused by the increased activity and/or number of osteoclasts leads to skeletal diseases characterized by low bone density, which is the major cause of bone fracture. Therefore, much attention has been focused on the pharmacologic control of osteoclasts for the treatment of osteoclast-related bone disorders including osteoporosis and arthritis (Broadhead et al. 2011; Kim and Moon 2013).

The representative natural polyamines, putrescine, spermidine, and spermine, (Fig. 1a), exhibit several biological activities in terms of cell growth, proliferation, and differentiation by regulating gene expression and intracellular signaling (Li et al. 2001; Zhao et al. 2012; Igarashi

and Kashiwagi 2010; Lefevre et al. 2011). Focusing on the pharmacological properties of polyamines in the musculoskeletal system, spermine has been shown to have pharmacological properties in cartilage and bone destruction in the joints of rats with collagen-induced arthritis (Iezaki et al. 2012). Furthermore, both spermidine and spermine have been shown to prevent bone loss in ovariectomized mice through inhibition of maturation and differentiation of osteoclasts (Yamamoto et al. 2012). Although the clinical benefits of natural polyamines for bone metabolism have been reported in several studies, the mode of action of their anti-osteoclastogenic activity has not been fully elucidated yet. Therefore, in this study, we have evaluated when and how polyamines can inhibit osteoclast differentiation using a bone marrow macrophages (BMMs)-based osteoclastogenesis model.

Materials and methods

Materials

Putrescine, spermidine, and spermine were purchased from Sigma-Aldrich (MO, USA).

Ethics statement

This study was carried out in strict accordance with the recommendations of the Standard Protocol for Animal Study of Korea Research Institute of Chemical Technology (KRICT; Permit No. 2012-7D-02-01). The protocol (ID No. 7D-M1) was approved by the Institutional Animal Care and Use Committee of KRICT (IACUC-KRICT). All efforts were made to minimize suffering, animal number and stress/discomfort.

Osteoclast differentiation

After cervical dislocation, bone marrow cells were obtained from 5-week-old male ICR mice (Daejeon Science, Daejeon, Korea) by flushing femurs and tibias with α -MEM supplemented with antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin; Invitrogen, NY, USA). Bone marrow cells were cultured for 1 day on a culture dish in α -MEM supplemented with 10 % fetal bovine serum (FBS; Invitrogen, NY, USA) and mouse macrophage-colony stimulating factor (10 ng/ml M-CSF; R&D Systems, MN, USA). Non-adherent bone marrow cells were plated on a Petri dish and cultured for 3 days in the presence of M-CSF (30 ng/ml). After non-adherent cells were washed out, adherent cells were used as bone marrow-derived macrophages (BMMs). For osteoclastogenesis, BMMs (1×10^4 cells/well in a 96-well plate or 3×10^5 cells/well

Fig. 1 Effect of polyamines on RANKL-induced osteoclast differentiation, and fusion and migration of preosteoclasts. **a** Chemical structures of polyamines including putrescine, spermidine, and spermine. **b** BMMs were cultured with/without polyamines at the indicated dose in the presence of M-CSF (30 ng/ml) for 3 days. Then the effect of polyamine on the viability of BMMs was evaluated by CCK-8 assay. PBS (phosphate-buffered saline) was used as vehicle or control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **c** After BMMs were co-treated with polyamines in the presence of RANKL (10 ng/ml) and M-CSF for 4 days, BMMs were derived to multinucleated cells (MNCs). MNCs were fixed, permeabilized, and stained with TRAP solution. Mature TRAP-positive MNCs were photographed under a light microscope. **d** TRAP-positive MNCs (nuclear number ≥ 3) were counted. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. To estimate the effect of polyamines on RANKL-induced preosteoclasts, BMMs were derived into preosteoclasts for 3 days in the presence of RANKL (10 ng/ml) and M-CSF (30 ng/ml). On day 3, after preosteoclasts were treated with polyamines at the indicated dose in the presence of M-CSF (30 ng/ml) for 2 h, RANKL (10 ng/ml) was added into each well. After culturing for an additional 1 day, BMMs were differentiated into MNCs. **e** MNCs were stained with TRAP solution. **f** TRAP-positive MNCs (nuclear number ≥ 3) were counted. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The effect of polyamines on migration of preosteoclasts was estimated by Boyden chamber assay as described in 'Materials and methods'. Briefly, preosteoclasts derived from BMMs by RANKL and M-CSF for 3 days were detached and incubated with polyamines at the indicated dose in the presence of M-CSF (30 ng/ml) and RANKL (10 ng/ml) for 2 h. The preosteoclasts were plated on Boyden chamber membrane in triplicate. After 18 h, the migrated preosteoclasts were fixed and stained with Diff-Quick (**g**) and counted (**h**). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

in a 6-well plate) were cultured in the presence of M-CSF (30 ng/ml) and mouse soluble receptor activator of nuclear factor- κ B ligand (10 ng/ml RANKL; R&D Systems, MN, USA) for 4 days. For preosteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (10 ng/ml) for 3 days.

Cytotoxicity assay

BMMs were plated at a density of 1×10^4 cells/well on a 96-well plate in triplicate. After treatment with M-CSF (30 ng/ml) and each polyamine, cells were cultured for 3 days. Then, cell viability was measured by CCK-8 assay kit (Dojindo Molecular Technologies, ML, Japan) according to the manufacturer's protocol.

Tartrate-resistant acid phosphatase (TRAP) staining and activity assay

Cells were fixed with 3.7 % formaldehyde for 5 min, permeabilized with 0.1 % Triton X-100 for 5 min, and stained with the Leukocyte Acid Phosphatase Kit 387-A (Sigma-Aldrich, MO, USA). TRAP-positive multinuclear cells with three or more nuclei were counted as osteoclasts. To measure TRAP activity, cells were treated with TRAP buffer (100 mM sodium citrate, pH 5.0, 50 mM sodium tartrate) including 3 mM *p*-nitrophenyl phosphate (Sigma-Aldrich) at 37 °C for 5 min. Reaction mixtures were

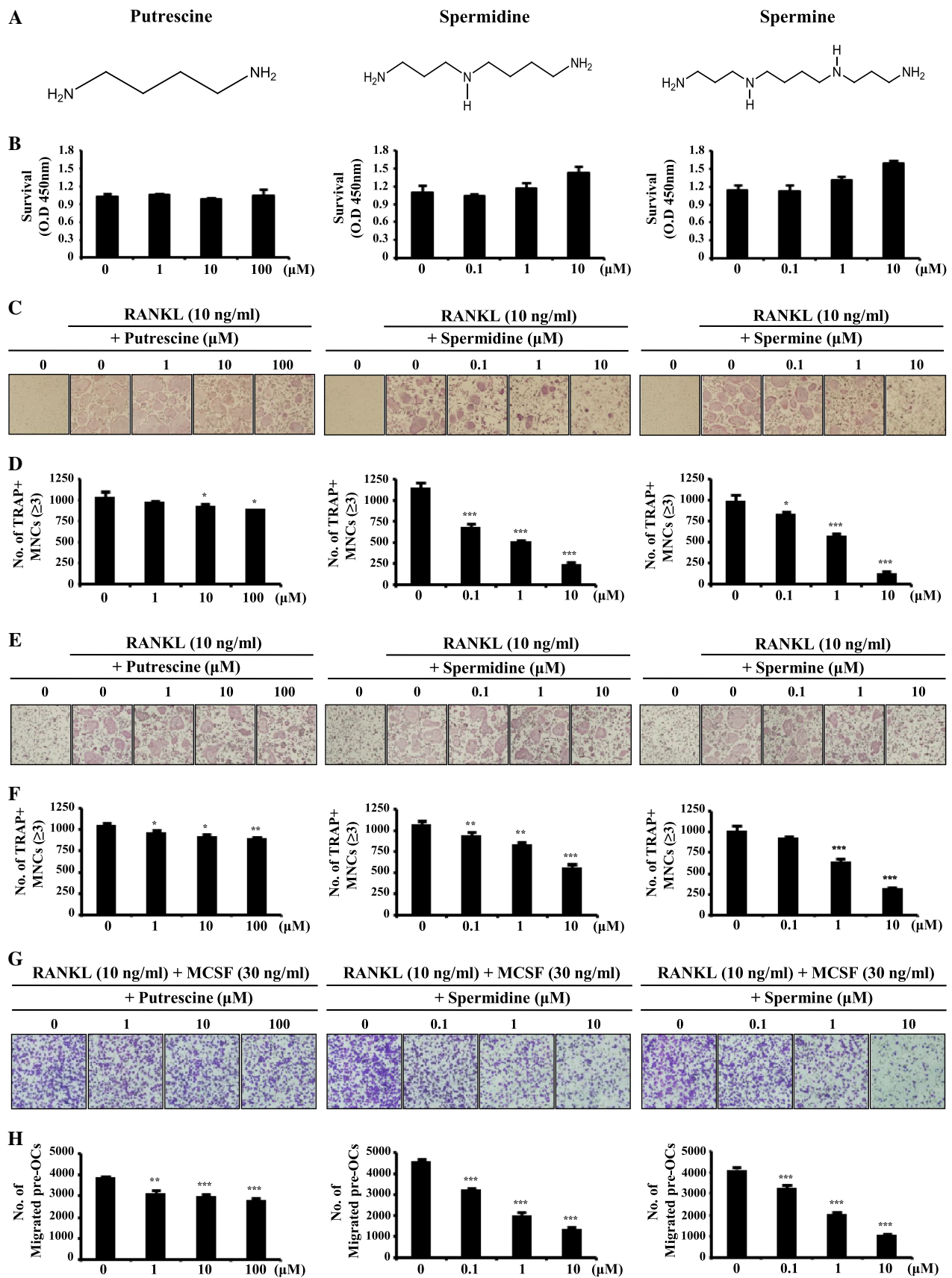


Table 1 Primer sequences used in this study

Target gene	Forward primer (5'–3')	Reverse primer (5'–3')
<i>NFATc1</i>	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
<i>OSCAR</i>	AGGGAAACCTCATCCGTTTG	GAGCCGGAAATAAGGCACAG
<i>TRAP</i>	GATGACTTTGCCAGTCAGCA	ACATAGCCCACACCGTTCTC
<i>Cathepsin K</i>	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
<i>c-Src</i>	CCAGGCTGAGGAGTGGTACT	CAGCTTGCGGATCTTGTAGT
<i>DC-STAMP</i>	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
<i>ATP6vOd2</i>	AGACCACGGACTATGGCAAC	CGATGGGTGACACTTGGCTA
α_v Integrin	CCTCAGAGAGGGAGATGTTACAC	AACTGCCAAGATGATCACCACAC
β_3 Integrin	GATGACATCGAGCAGGTGAAAGAG	CCGGTCATGAATGGTGATGAGTAG
<i>P85α</i>	CCTGAAGGAGCTGGTGCTAC	TGCAGCTCAGTTCACAGGTC
<i>CasR</i>	CCTGCTTCCCCTACAGTGTG	TCCCTCCATTAGGAGTGCAG
<i>HPRT1</i>	TGCTCGAGATGTCATGAAGG	AGAGGTCCTTTTCACCAGCA

transferred to a new plate containing an equal volume of 0.1 N NaOH, and optical density values were determined at 405 nm in Wallac EnVision microplate reader (Perkin Elmer, MA, USA).

Cell migration assay

The ability of preosteoclasts to pass through a gelatin-coated membrane was measured in a Boyden chamber with modifications (Kimachi et al. 2011; Brazier et al. 2009). Briefly, the preosteoclasts derived from BMMs cultured with RANKL (10 ng/ml) and M-CSF (30 ng/ml) for 3 days were detached with scraper (Corning, NY), resuspended, and incubated with each polyamine in α -MEM containing 0.1 % FBS, M-CSF (30 ng/ml) and RANKL (10 ng/ml) for 2 h. During incubation, α -MEM (30 μ L) containing 10 % FBS and each polyamine was added to the bottom of a Boyden chamber, which was then placed over the gelatin-coated membrane filter, the silicone gasket, and the top chamber. Then, polyamine-treated preosteoclasts (2×10^4 cells/50 μ L) were added to the top chamber, followed by incubation at 37 °C in 5 % CO₂ for 18 h. At the end of the incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab, and preosteoclasts that had migrated across the gelatin to the lower surface of the membrane were fixed and stained with Diff-Quick (Dade Behring, NJ). The density of migrated preosteoclasts on the lower surface of the membrane was calculated with NIH ImageJ 1.43u software. The numbers of migrated cells were counted in random areas of the membrane.

Real-time PCR

Real-time PCR was performed as previously described (Choi et al. 2012, 2013). Primers were chosen with the online Primer3 design program (Rozen and Skaletsky 2000). The primer sets used in this study are shown in Table 1.

Briefly, total RNA was isolated with TRIzol reagent, and first-strand cDNA was synthesized with the Omniscript RT kit (Qiagen, Germany) according to the manufacturer's protocol. SYBR green-based QPCR was performed with the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA). All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as an internal standard gene. The statistical significance was determined by Student's *t* test with HPRT1-normalized $2^{-\Delta\Delta CT}$ values; differences were considered significant at $p < 0.05$.

Western blot and densitometric analysis

Western blot analysis was performed as described previously (Choi et al. 2012, 2013). Briefly, cells were washed, lysed, and centrifuged at $10,000 \times g$ for 15 min. Cytoplasmic or nuclear protein fractions were prepared using a NucBuster Protein Extraction kit (Novagen, Germany) according to the manufacturer's protocol. After protein quantification of the supernatants by the BCA protein assay (Pierce, IL), proteins were denatured, separated on SDS-PAGE gels, and transferred onto PVDF membranes (Millipore, CA, USA). Expression of each protein was detected using the indicated primary antibody. Antibodies against p-Src and Src were purchased from Cell Signaling Technology (MA, USA). Antibodies against p-PYK2, PYK2, NF- κ B/p65, NFATc1, and Actin were purchased from Santa Cruz Biotechnology (CA, USA). Antibody against Lamin B1 was obtained from AbFrontier (Seoul, Korea). Actin and Lamin B1 were used for the loading control of cytosol and nuclear proteins, respectively. After incubation with antibody, the membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, IL, USA) and visualized with the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Each experiment was repeated at least three times and the data shown here represent them. ImageJ software-based quantification of the detected bands was performed, and the relative, normalized ratios between the density of phosphorylated form and that of the protein itself were presented. Also, the relative, normalized ratios of cytosolic protein to actin or nuclear protein to lamin B1 were also presented.

Intracellular Ca^{2+} measurement

Intracellular Ca^{2+} was measured using Fluo-4 NW Calcium assay kits (Molecular Probes, OR, USA). BMMs were seeded at a density of 2.5×10^4 cells in a 96-well assay plate (black with clear-bottom tissue culture plate; COSTAR, NY, USA) with α -MEM medium containing 10 % FBS in the presence of RANKL (10 ng/ml) and M-CSF (30 ng/ml) for 48 h. The media were then removed from the wells and replaced with 90 μL of assay buffer containing Fluo-4 NW and Probenecid solution for 45 min. While the fluorescence was measured by FlexStation II (Molecular Devices, USA), the assay buffer (10 μL) containing adenosine triphosphate (ATP) with/without each polyamine was applied into the 96-well plates. The excitation/emission/cut-off filter pair (485/525/515 nm) was used to measure signals every 2 s for 100 s. Of the total duration (100 s), co-treatment of ATP and/or polyamines was performed for 20 s.

Statistical analysis

All quantitative values are presented as mean \pm SD. Statistical differences were analyzed using Student's *t* test. A value of $p < 0.05$ was considered to be significant.

Result

Polyamines inhibit RANKL-induced osteoclast differentiation

First of all, the effect of polyamine on the survival of BMMs was evaluated. When BMMs were cultured with polyamines in the presence of M-CSF for 3 days, there was no cytotoxicity by the polyamines (Fig. 1b). In the same concentration range, the effect of polyamines on RANKL-induced osteoclast differentiation was evaluated. TRAP-positive multinucleated cells (MNCs) were observed in RANKL-treated BMMs, but polyamines inhibited the RANKL-induced formation of MNCs (Fig. 1c). Consistent with these data, the number of TRAP-positive MNCs (multinucleated cells ≥ 3) was significantly inhibited by polyamines in a dose-dependent manner (Fig. 1d). Compared to putrescine, both spermidine and spermine strongly suppressed the RANKL-induced formation of MNCs.

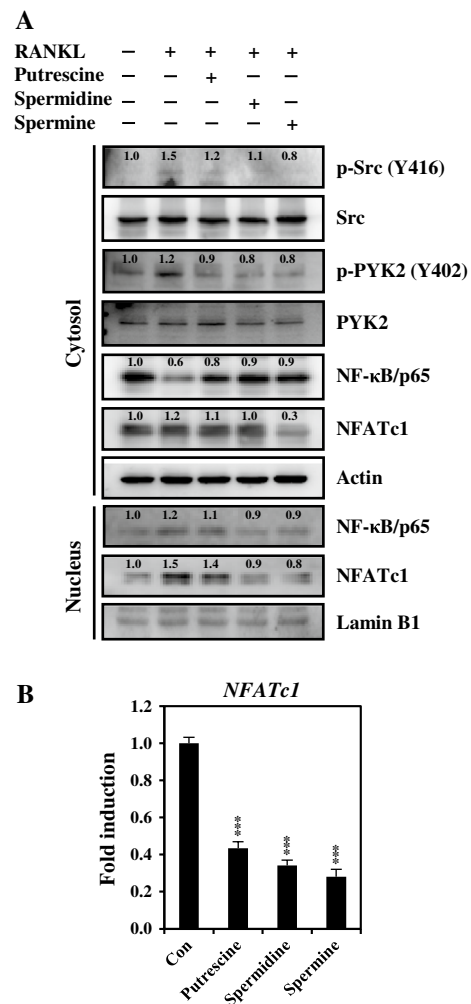


Fig. 2 Effect of polyamines on the RANKL-induced activation of PYK2, NF- κ B, and NFATc1, and the mRNA expression in preosteoclasts. BMMs were driven to preosteoclasts for 3 days in the presence of RANKL (10 ng/ml) and M-CSF (30 ng/ml). On day 3, after preosteoclasts were pre-treated with polyamines (putrescine, 100 μM ; spermidine, 10 μM ; putrescine, 10 μM , respectively) in the presence of M-CSF (30 ng/ml) for 2 h, BMMs were stimulated with RANKL for 15 min or 4 h. **a** The activity of migration-related signaling molecules in cytoplasmic or nuclear protein fractions was evaluated by Western blot analysis for 15 min as described in 'Materials and methods'. Actin and lamin B1 were used for the loading control of cytosolic and nuclear proteins, respectively. ImageJ software-based quantification was performed, and the relative, normalized ratios (p-Src/Src, p-PYK2/PYK2, NF- κ B and NFATc1/actin in cytosol; NF- κ B and NFATc1/lamin B1 in nucleus) were presented. **b** The effect of polyamines on the mRNA expression of NFATc1 by RANKL was analyzed by real-time PCR at 4 h after RANKL treatment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Polyamines inhibit RANKL-induced fusion and migration of preosteoclasts

Next, the effect of polyamines on the fusion of mononuclear preosteoclasts was evaluated. When preosteoclasts that were differentiated from BMMs by treatment with RANKL and

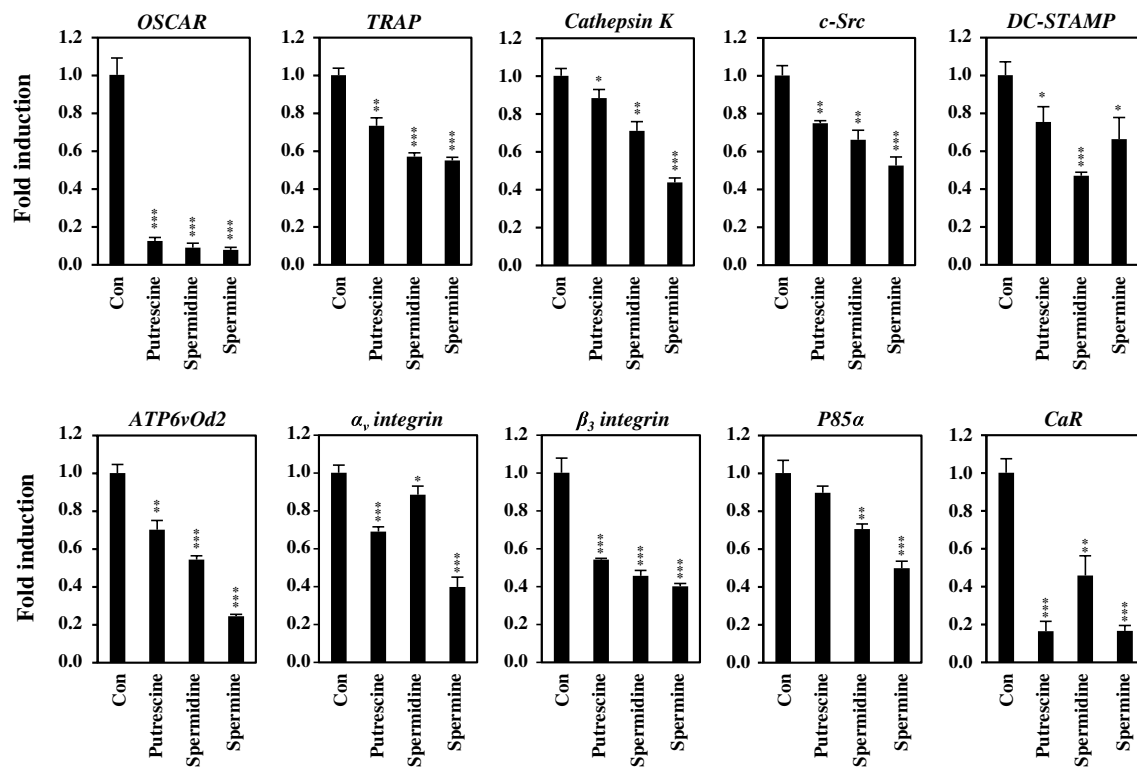


Fig. 3 Effect of polyamines on the RANKL-induced mRNA expression of osteoclastogenesis-related genes, and fusion and/or migration-related genes in preosteoclasts. After BMMs were cultured for 3 days with M-CSF (30 ng/ml) and RANKL (10 ng/ml), preosteoclasts were pre-treated with polyamines (putrescine, 100 μ M; spermidine, 10 μ M; putrescine, 10 μ M, respectively) in the presence of M-CSF

(30 ng/ml) for 2 h. Then RANKL (10 ng/ml) was added into the well for 4 h. The effect of polyamines on RANKL-induced mRNA expression of osteoclast-specific, fusion-mediating and migration-related genes was evaluated by real-time PCR as described in 'Materials and methods'. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

M-CSF for 3 days were treated with polyamines, the polyamines significantly suppressed the formation of TRAP-positive MNCs in a dose-dependent manner (Fig. 1e, f). Moreover, cell migration assay revealed the inhibitory effect of polyamines on the migration of preosteoclasts (Fig. 1g, h); consistent with the inhibitory effect of polyamines on the fusion of preosteoclasts, polyamines significantly and dose-dependently prevented the migration of preosteoclasts.

Polyamines inhibit RANKL-induced activation of Src, PYK2, NF- κ B, and NFATc1 in preosteoclasts

To elucidate how polyamines inhibit the migration of preosteoclasts, we further investigated the effect of polyamines on the activation of migration-related signaling molecules. BMMs differentiated into preosteoclasts for 3 days were treated with polyamines. As shown in Fig. 2a, RANKL-induced phosphorylation of Src at Y416 and proline-rich tyrosine kinase 2 (PYK2) at Y402 were prevented by the pre-treatment with polyamines. Furthermore, we investigated the effect of polyamines on the translocation of osteoclast-specific transcription factors in preosteoclasts. The

RANKL-induced nuclear translocation of NF- κ B/p65 and nuclear factor of activated T cells (NFATc1) was strongly prevented by spermidine and spermine, and weakly prevented by putrescine. The inhibitory effects of these polyamines on the RANKL-induced NF- κ B activation (Fig. S1) and nuclear translocation of NFATc1 (Fig. S2) were confirmed by measuring the NF- κ B reporter luciferase activity and by analyzing the immunofluorescence staining of NFATc1, respectively. The degradation of cytosolic NFATc1 by spermine was also observed. Additionally, polyamines significantly inhibited the mRNA expression of NFATc1 in preosteoclasts (Fig. 2b).

Polyamines inhibit the RANKL-induced mRNA expression of osteoclastogenesis-related genes and fusion and/or migration-related genes in preosteoclasts

We next explored the effect of polyamines on the RANKL-induced mRNA expression of osteoclastogenesis-related genes as well as fusion and migration-related genes. As shown in Fig. 3, the mRNA expression of these genes was significantly inhibited by polyamines.

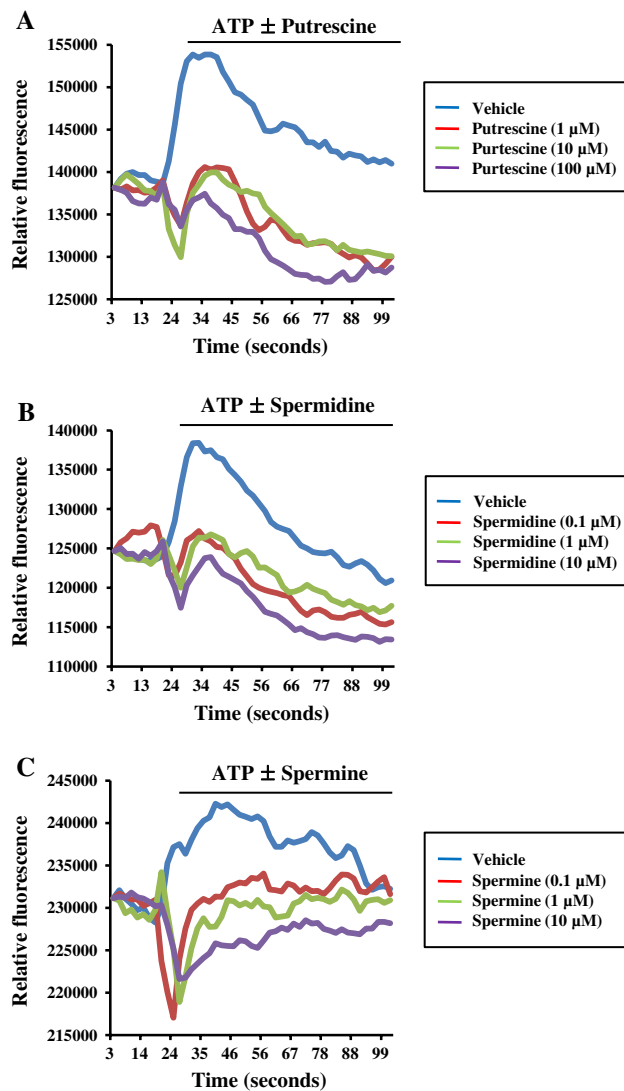


Fig. 4 Effect of polyamines on ATP-mediated induction of intracellular $[Ca^{2+}]_i$ in preosteoclasts. BMMs were cultured for 2 days in the presence of M-CSF (30 ng/ml) and RANKL (10 ng/ml). The media was replaced with buffer containing Fluo-4 NW for 45 min according to the manufacturer's method. Then ATP alone or ATP plus polyamines at the indicated dose was added by injector treatment at 20 s and the measuring time per data point was every 2 s as described in 'Materials and methods'. **a** The effect of putrescine on ATP-mediated induction of intracellular $[Ca^{2+}]_i$ was conducted with final concentration with 1, 10, or 100 μ M. Also, the effect of spermidine and spermine on ATP-mediated induction of intracellular $[Ca^{2+}]_i$ was evaluated with the final concentration of 0.1, 1, or 10 μ M, respectively (**b**, **c**)

Polyamines inhibit the ATP-mediated induction of $[Ca^{2+}]_i$ in preosteoclasts

Since polyamines have been reported to block and/or modulate a number of types of ion channel (Williams 1997), we evaluated the effect of polyamines on ATP-induced intracellular Ca^{2+} changes in preosteoclasts.

ATP was used for the induction of $[Ca^{2+}]_i$ in preosteoclasts, because it is known to induce a Ca^{2+} influx across the cell membrane in osteoclasts (Yu and Ferrier 1994), but the Ca^{2+} oscillation was not changed by additional treatment of RANKL in the RANKL-induced preosteoclasts (Fig. S3). In preosteoclasts derived from BMMs by RANKL and M-CSF for 2 days, the induction of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by ATP was observed, but its induction was strongly prevented by polyamines as shown in Fig. 4. Rather, the co-treatment of preosteoclasts with polyamines and ATP decreased the level of $[Ca^{2+}]_i$.

Discussion

Osteoclastogenesis is a multi-complex process including the sequential steps of differentiation–migration–fusion–resorption. M-CSF and RANKL trigger the differentiation of osteoclast precursors into mononuclear osteoclasts (pre-osteoclasts) and induce their migration for attachment onto the bone surface. They then fuse with each other to form giant multinucleated osteoclasts that consequently mediate bone resorption (Yavropoulou and Yovos 2008; Kikuta and Ishii 2013).

Recently, naturally occurring polyamines have been reported to significantly inhibit in vitro osteoclast differentiation and prevent in vivo bone loss in ovariectomized mice (Yamamoto et al. 2012). Furthermore, when RAW264.7 cells were cultured with either spermidine or spermine at 1 μ M in the presence of RANKL for 4 days, both polyamines significantly inhibited the formation of TRAP-positive multinucleated osteoclasts without affecting cell viability. Consistent with those results, we also found anti-osteoclastogenic activity of both spermidine and spermine at non-cytotoxic dosages in BMMs-based osteoclastogenesis model. Putrescine also significantly inhibited the RANKL-induced formation of multinucleated osteoclasts when it was co-treated with RANKL, even though its anti-osteoclastogenic activity was weak.

Additionally, the polyamines tested in this study significantly inhibited osteoclast differentiation in preosteoclasts derived from BMMs by RANKL treatment for 3 days. In RAW264.7 cells, both spermidine and spermine failed to inhibit osteoclast differentiation when added 1 day after RANKL-induced osteoclast differentiation (Yamamoto et al. 2012). This difference might be due to the presence of M-CSF in the model. M-CSF can stimulate osteoclast formation by enhancing the survival and fusion of preosteoclasts derived from bone marrow cells (Takami et al. 1999), but it was not used for differentiation of RAW264.7 cells into osteoclast-like cells. That is why both spermidine and spermine failed to inhibit osteoclast differentiation when

added 1 day after RANKL treatment (Yamamoto et al. 2012).

Furthermore, M-CSF has been known to induce the migration of osteoclasts (Owens and Chambers 1993; Fuller et al. 1993) and here we also found anti-migration activity of polyamines in preosteoclasts, suggesting that the anti-osteoclastogenic activity of polyamines could be due to their potential to inhibit the migration of preosteoclasts before their fusion. Furthermore, considering that there were no differences in the adhesion of preosteoclasts treated with polyamines, the anti-fusion activity of polyamines in preosteoclasts could not be adhesion dependent, but rather is associated with the change of intracellular signaling networks related to fusion and/or migration of preosteoclasts.

The Src-PYK2 signaling axis is related to osteoclast migration; the engagement of PYK2 with $\alpha_v\beta_3$ integrin leads to Src-dependent tyrosine phosphorylation (Y402) and activation of PYK2 (Lakkakorpi et al. 2003; Duong et al. 1998). The involvement of Src in osteoclast function has been validated by studying osteopetrotic Src-deficient mice (Soriano et al. 1991) and, interestingly, M-CSF-mediated activation of c-Src by phosphorylation of Y416 has been shown to induce osteoclast migration (Zou et al. 2007, 2008). In this study, the RANKL-mediated phosphorylations of Src (Y416) and PYK2 (Y402) in the presence of M-CSF were prevented by pre-treatment with polyamines, suggesting that the anti-fusion activity of polyamines involves preventing RANKL-induced activation of the Src-PYK2 signaling cascade.

RANKL-induced signaling cascades require the sequential expression and activity of transcription factors such as NF- κ B and NFATc1 to complete the osteoclast differentiation. Recently, spermidine and spermine have been shown to inhibit the transcriptional activity of NF- κ B, but not NFATc1, in RAW264.7 cells (Yamamoto et al. 2012). In this study, the inhibitory effects of polyamines on the activity of NF- κ B was confirmed and, furthermore, we found that the RANKL-induced nuclear translocation of NFATc1 as well as NF- κ B p65 subunit was inhibited in preosteoclasts derived from BMMs by polyamines. NF- κ B/p65 has been reported to enhance osteoclast differentiation (Vaira et al. 2008) and control cell survival during a critical stage of commitment to the osteoclast lineage (Novack 2011). Additionally, these polyamines significantly inhibited the mRNA expression of NFATc1 in preosteoclasts. Considering that NFATc1 has been recognized as a master regulator for osteoclastogenesis as well as osteoclast migration (Zhao et al. 2010), our results suggest that the inhibitory effects of polyamines on the expression and nuclear translocation of NFATc1 could cause their anti-fusion activities by regulating the expression of genes related to fusion and/or migration of preosteoclasts.

Anti-osteoclastogenic and anti-fusion activities of polyamines were confirmed by evaluating their potential to downregulate the mRNA expression levels of osteoclastogenesis-related molecules such as osteoclast-associated receptor (OSCAR), TRAP, cathepsin K and c-Src, and genes related to fusion and/or migration of preosteoclasts and/or osteoclasts. Dendrite cell-specific transmembrane protein (DC-STAMP) and d2 isoform of vacuolar (H^+) ATPase V_0 domain (ATP6vOd2) have been shown to contain multiple NFATc1 binding sites in their promoter regions (Kim et al. 2008), and both have been reported to play a role in the fusion of preosteoclasts (Kim et al. 2008; Song et al. 2009). Furthermore, overexpression of DC-STAMP and ATP6vOd2 rescued osteoclast fusion, even in conditions of reduced NFATc1 activity (Kim et al. 2008). Integrins and the p85 α subunit of phosphatidylinositol 3-kinase (PI3K) are involved in the migration of preosteoclasts (Nakamura et al. 1999; Munugalavadla et al. 2008). In particular, deficiency of p85 α osteoclasts leads to a significant reduction in the expression of several genes including TRAP, cathepsin K, and β_3 integrin (Munugalavadla et al. 2008). Additionally, the activation of calcium sensing receptor (CaR) produces an extracellular Ca^{2+} concentration gradient-dependent chemoattractant effect in the osteoclast precursors (Boudot et al. 2010).

In addition to downregulating CaR, the polyamines exhibit inhibitory effects on calcium mobilization in the bone, leading to further questions about whether calcium could be involved in the anti-fusion activities of polyamine (Ljunggren et al. 1991; Stern et al. 1991). Recently, elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) has been observed in the migrating osteoclasts for the uropod that precede retraction, suggesting that elevation of $[Ca^{2+}]_i$ is required for osteoclast migration (Wheal et al. 2014). They also confirmed the involvement of $[Ca^{2+}]_i$ in the osteoclast migration by a pharmacologic study; the Ca^{2+} chelator BAPTA abolished uropod retraction, and ionomycin-induced elevation of $[Ca^{2+}]_i$ initiated prompt uropod retraction. Here, we found that ATP-mediated elevation of $[Ca^{2+}]_i$ was strongly inhibited by treatment with each polyamine. Since ATP is known to induce a Ca^{2+} influx across the cell membrane in osteoclasts (Yu and Ferrier 1994), the inhibitory effects of polyamines on the ATP-induced increase of $[Ca^{2+}]_i$ indicated the involvement of $[Ca^{2+}]_i$ in the anti-fusion activities of polyamines.

Furthermore, the Ca^{2+} pathway may be correlated with the activity of NFATc1. Calcium oscillation can lead to the calcineurin-mediated activation of NFATc1 through autoamplification of NFATc1 (Negishi-Koga and Takayanagi 2009; Takayanagi 2007), and several calcineurin inhibitors have been shown to suppress osteoclastogenesis via inhibition of the Ca^{2+} -NFATc1 pathway (Negishi-Koga and Takayanagi 2009). These results suggest that the

mechanism of action for polyamines to abolish NFATc1 activity involves the blockade of Ca^{2+} -mediated signaling.

In conclusion, polyamines may exhibit anti-osteoclastogenic potential to inhibit the migration of preosteoclasts in the fusion step via the Ca^{2+} -PYK2-Src-NFATc1 signaling axis. Similarly, the phenotype of PYK2-deficient mice has macrophages that exhibit impaired migration as a result of cytoskeleton abnormalities induced by diminished Ca^{2+} mobilization and reduced activation of PI3K (Matsui et al. 2007). In the clinical aspect, targeting the migration of preosteoclasts might represent a new therapeutic approach for treating osteoclast-related disorders including osteoporosis, rheumatoid arthritis, and bone cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

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