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Suppression of osteoclastogenesis by N,N-dimethyl-D-*erythro*-sphingosine: A SPHINGOSINE KINASE INHIBITION-INDEPENDENT ACTION*

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Running title: SPHK-independent inhibition of osteoclastogenesis by DMS

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[1]The abbreviations used are: RANKL, receptor activator of nuclear factor kappa B ligand; M-CSF, macrophage colony-stimulating factor; BMM, bone marrow-derived macrophage; DMS, N,N-dimethyl-D-*erythro*-sphingosine; SPHK, sphingosine kinase; TRAP, tartrate-resistant acid phosphatase; MNC, multinucleated cell.

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ABSTRACT

N,N-dimethyl-D-*erythro*-sphingosine (DMS) competitively inhibits sphingosine kinase (SPHK) and has been widely used to assess the role of SPHK during cellular events including motility, proliferation, and differentiation. In the present study, the effect of DMS on the differentiation of bone marrow macrophages (BMMs) to osteoclasts was investigated. When the osteoclast precursor cells were treated with DMS, the receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclastogenesis was completely blocked. Surprisingly, however, knock-down of SPHK by siRNA in BMMs did not reduce osteoclastogenesis. Furthermore, both overexpression of SPHK and exogenous addition of sphingosine-1-phosphate, the product of SPHK activity, failed to overcome the anti-osteoclastogenic effect of DMS. These results suggest that DMS inhibited osteoclastogenesis independently of SPHK. Subsequent characterization of the DMS-mediated suppression of osteoclastogenesis revealed that DMS did not affect RANKL-induced activation of JNK, p38, NF-κB, and Ca^{2+} oscillation. On the other hand, DMS strongly inhibited two separate signaling pathways, the RANKL-induced activation of ERK and Akt, which eventually converged on the transcription factors c-Fos and NFATc1. There was significant increase in the osteoclast formation in the presence of DMS when BMMs were overexpressed with c-Fos, suggesting that c-Fos was a critical downstream target of DMS for the inhibition of osteoclastogenesis. Taken together, our data demonstrate that DMS has an anti-osteoclastogenic function independently of its SPHK inhibitory activity. Considering previously reported anti-cancer properties of DMS, our study may also propose that DMS is an ideal drug candidate for bone metastases, for which osteoclastic bone-resorption is crucial.

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Introduction

Bone is a complex tissue composed of several cell types. The functions of bone are accomplished by continuous tissue renewal, termed 'bone remodeling', occurring throughout life in the adult skeleton. The two major cell types responsible for bone remodeling are osteoclasts and osteoblasts. The harmony between osteoclastic bone resorption and osteoblastic bone formation maintains skeletal homeostasis in adults (Walsh et al., 2006). In pathological bone resorption, this balance is tipped in favor of osteoclast formation, as seen in postmenopausal osteoporosis, autoimmune arthritis, periodontitis, and Paget's disease. Osteoclasts, the only type of cells responsible for bone resorption, are highly specialized cells originating from monocyte/macrophage lineage precursors. The single most important cytokine in the development of osteoclasts is receptor activator of nuclear factor kappa B ligand (RANKL)[2], whereas macrophage-colony stimulating factor (M-CSF), secreted by osteoblasts, provides the survival signal to the precursor and differentiating cells (Boyle et al., 2003).

RANKL, expressed by osteoblasts, stromal cells, and activated T cells, signals through its receptor RANK (receptor activator of nuclear factor kappa B), present on osteoclasts and their precursors (Boyle et al., 2003). Genetic experiments in mice have revealed that RANKL, RANK, TRAF6 (TNF receptor-associated factor 6) and the

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transcription factors c-Fos and NF-κB (nuclear factor kappa B) are essential for precursors to commit into osteoclast lineage. The RANK signaling pathway can activate three major groups of mitogen-activated protein kinases (MAPKs): c-Jun-*N*-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38. Association of TRAF6 with transforming growth factor-β-activated kinase 1 (TAK1) activates JNK, AP-1 and NF-κB (Boyle et al., 2003; Huang et al., 2006; and Lee et al., 2003). The transcription factor AP-1 is a heterodimeric protein composed of c-Fos proteins (c-Fos, Fos B, Fra-1, and Fra-2) and Jun proteins (c-Jun, Jun B, and Jun D). Recently, the activation of NFATc1 (nuclear factor of activated T-cells) was found to be critical to the osteoclast formation (Takayanagi et al., 2002). RANKL stimulates intracellular calcium oscillation, a requisite for calcineurin-mediated NFATc1 activation. NFATc1 then binds to its DNA response element via a ternary complex with AP-1 proteins, c-Fos/c-Jun, to transactivate osteoclastogenic genes (Takayanagi et al., 2002). It was reported that NFATc1 expression is abolished in $Fos^{-/-}$ precursors. Moreover, ectopically expressed NFATc1 could restore bone-resorbing activity in cells from $Fos^{-/-}$ precursors *in vitro* and can induce osteoclast differentiation in the absence of RANKL (Takayanagi et al., 2002; Matsuo et al., 2004). These findings have suggested that NFATc1 is both a key downstream target and a partner of c-Fos for efficient osteoclast differentiation.

Sphingolipids are ubiquitous membrane constituents of all eukaryotic cells. Intensive

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investigation in the past decade has established that sphingolipids, in addition to being structural components of cell membranes, play key roles as signaling molecules. Particularly, three of these sphingolipid metabolites, ceramide, sphingosine, and sphingosine-1-phosphate (S1P), have recently proved to be a new class of lipid messengers that regulate cell proliferation, migration, survival, and differentiation (Spiegel and Milstien 2003). The balance of these three lipid signaling molecules is fine-tuned by sphingosine kinase (SPHK), which is a key enzyme catalyzing the formation of S1P by phosphorylating sphingosine, in response to diverse stimuli. Two mammalian isozymes, namely SPHK1 and SPHK2, have been cloned and characterized (Kohama et al., 1998). Several studies have demonstrated that SPHK can be activated by diverse stimuli such as platelet-derived growth factor (Olivera and Spiegel 1993), tumor necrosis factor α (TNF-α) (Xia et al., 1999), vascular endothelial growth factor (Shu et al., 2002), and lipopolysaccharide (LPS) (Wu et al., 2004). However, the role of SPHK and S1P on osteoclastogenesis has not been studied. Based on our microarray data that showed increased SPHK1 expression in response to RANKL, we began to investigate the potential role of SPHK for osteoclastogenesis in primary osteoclast precursor cells. We observed a potent inhibition of RANKL-induced osteoclast differentiation by a specific SPHK inhibitor, N,N-dimethyl-D-*erythro*-sphingosine (DMS). Notably, this effect of DMS was found to be independent of its SPHK inhibitory activity. In fact,

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suppression of the MEK/ERK and the PI3K/Akt pathways, which converged on the expression of c-Fos and NFATc1, was responsible for the anti-osteoclastogenic function of DMS. Our study for the first time reveals osteoclastogenesis inhibitory effect of DMS and provides an example of SPHK-independent action of DMS.

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Materials and Methods

Reagents. N,N-dimethyl-D-*erythro*-sphingosine and sphingosine-1-phosphate were purchased from BIOMOL (Plymouth Meeting, PA). Antibodies against phospho-ERK (#9101), ERK (#9102), phospho-JNK (#9251), JNK (#9252), phospho-p38 (#9211), p38 (#9212), phospho-IκB (#9241) and IκB (#9242) were obtained from Cell Signaling (Beverly, MA). Anti-hemagglutinin (HA) antibody was purchased from COVANCE (Berkeley, CA). Anti-NFATc1 (sc-7294) and anti-actin (A5316) antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and Sigma Aldrich (St. Louis, MO), respectively. Anti-c-Fos antibody was obtained from Upstate Biotechnology, Inc (Waltham, MA). U0126 was from Calbiochem (La Jolla, CA) and Ionomycin was purchased from Sigma Aldrich (St. Louis, MO). [γ-^{32}p]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Preparation of Macrophages from Mouse Bone Marrow and In Vitro Osteoclastogenesis. Bone marrow macrophages (BMMs) were generated as described (Huang et al., 2006; Ha et al., 2003). Briefly, Bone marrow cells collected from long bones of 5-6 week-old ICR mice were plated on 100-mm petri dishes and cultured for 16-24 h in α-MEM (WelGENE Inc, Korea) supplemented with 10% fetal bovine serum (FBS) containing 5 ng/ml M-CSF in 5%

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CO_2 at 37□. Adherent cells were discarded and remaining nonadherent cells were cultured in the presence of 30 ng/ml M-CSF. After 3 days, adherent cells were used as BMMs after washing out the nonadherent cells including lymphocytes. These osteoclast precursor cells were further cultured for 3 days in the osteoclastogenic medium (medium containing 30 ng/ml M-CSF and 200 ng/ml RANKL) to generate osteoclasts unless otherwise indicated. Three days later, cells were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells ($TRAP^+$ MNC; ≥3 nuclei) were counted as osteoclasts.

Plasmid Construction. The full-length human SPHK1 cDNA was amplified from HeLa cell mRNA using sense primer 5'-GAATTCATGGATCCAGCGGGCGGCCCCCGG-3' and antisense primer 5'-GTCGACTCATAAGGGCTCTTCTGGCGGTGG-3' and cloned into pSRα-HA vector. A retroviral vector, pMX-HA-SPHK1 was constructed by inserting a 1.4 kb fragment of human full-length SPHK1 cDNA. To generate retroviral vectors for siRNA experiments, targeting oligonucleotides were annealed and ligated into the pSuper-retro vectors (Oligoengine, Seattle, WA) using BamH Ⅰ and HindⅢ sites. The sequences of used oligonucleotides are as follows: mouse SPHK1 siRNA, 5'-AGCTTAAAAATATGGAACTTGACTGTCCATCTCTTGAATGGACAGTCAAGTTCCATAGGG-3' and 5'-

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GATCCCCTATGGAACTTGACTGTCCATTCAAGAGATGGACAGTCAAGTTCCATATT TTTA-3'; mouse SPHK2 siRNA, 5'-GATCTCGATTGACCAATATGAGCAGCCTTGATATCCGGGCTGCTCATATTGGTCAAT CTTTTTTCCAAA-3' and 5'-AGCTTTTGGAAAAAAGATTGACCAATATGAGCAGCCCGGATATCAAGGCTGCTCAT ATTGGTCAATCGA-3'; luciferase siRNA, 5'-GATCTGTATAATACACCGCGCTACTTGATATCCGGTAGCGCGGTGTATTATACTTTTT TCCAAA-3' and 5'-AGCTTTTGGAAAAAAGTATAATACACCGCGCTACCGGATATCAAGTAGCGCGGTGT ATTATACA-3'.

Retroviral Gene Transfer. Retrovirus packaging was performed by transfection of pMX-HA-SPHK1 and pSuper-retro-siRNA plasmids into Plat-E cells. Transfection procedure was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation at 37℃ for 6 h, the DNA mixture was replaced with DMEM supplemented with 10% FBS. In the next day, the medium was changed with α-MEM/10% FBS and cells were further cultured for 48 h. Cell culture medium containing viral particles was collected and filtered with 0.45 μm syringe filter (Satorius). The filtered medium was

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stored at -70℃ until use. For infection with retroviruses, BMMs plated in 6-well plates (1×10^6 cells/well) were incubated with the virus-containing medium (2 ml/well), polybrene (10 μg/ml) and M-CSF (30 ng/ml) for one day. A portion of infected cells was assayed for infection efficiency, and the rest of the cells were further cultured in osteoclastogenic medium (30 ng/ml M-CSF plus 200 ng/ml RANKL). After 3 days, osteoclastogenesis was evaluated by TRAP staining.

Luciferase Reporter Assays. Raw264.7 cells were plated at 5×10^5 cells/well in 6-well plates. The next day, cells were transfected with 4 μg of NF-κB or NFATc1-dependent luciferase reporter vector using 10 μl Lipofectamin 2000 (Invitrogen) in DMEM. At 4 h after transfection, the medium was replaced by DMEM/10% FBS. After incubation for 14 h at 37℃ in 5% CO_2, cells were collected by scraping, resuspended in α-MEM/10% FBS, and replated in 96-well plates at 2×10^4 cells/well. Cells were stimulated with 200 ng/ml RANKL in the presence or absence of DMS for 8 h. Cells were lysed in Reporter Lysis Buffer (Promega) and luciferase activity was measured using a luminometer. The protein concentration of the cell lysates was also determined and the relative reporter activity/μg protein was calculated.

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Fluorescence Measurement of $[Ca^{2+}]_i$. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured using the fluorescent dye fura-2/AM (Molecular Probes). BMMs on non-coated glass coverslips were incubated with 30 ng/ml M-CSF and 200 ng/ml RANKL for 48 h in the presence or absence of 2 μM DMS. To load the calcium indicator, the cells were incubated for 40 min at room temperature in culture medium containing 5 μM fura-2/AM and 0.05% pluronic F127 (Molecular Probes). After the incubation, cells were washed three times with Hank's balanced salt solution (GIBCO). The cell ensembles were illuminated at wavelengths of 340 and 380 nm, and the emitted light passed through a 510 nm interference filter was detected with an intensifier charge coupled device camera (International Ltd., Sterling, VA). Images were recorded at every 500 ms and analyzed using image analysis software (Metafluor; Universal Imaging, West Chester, PA).

Immunoblotting. Cells were disrupted in a lysis buffer (20 mM pH 7.4 Tris-HCl, 150 mM NaCl, 50 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1% Triton X-100). Protein concentration of cell lysates was determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). For immunoblotting of NFATc1 and c-Fos, whole cell extracts were prepared by disrupting cells with boiling sample buffer. Twenty to thirty micrograms of cell lysates or

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equal portion of whole cell extracts were resolved by 8 to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, NJ). The membrane was blocked with 5% skim milk and probed with appropriate primary antibodies. After incubation with appropriate secondary antibodies, the immunoreactivity was detected with enhanced chemileuminescence reagents.

Quantitative PCR analysis. Total RNAs were isolated with TRIzol reagent (Invitrogen) and 2 μg of RNAs were reverse transcribed with Superscript Ⅱ (Invitgogen) according to the manufacturer's instructions. For quantitative real-time PCR analysis, 4 μg of cDNAs were amplified with SYBR green PCR master mix (Applied Biosystems, UK) in a MicroAmp optical tube using AB7500 instrument (Applied Biosystems, UK), for 40 cycles of 15 s denaturation at 95℃ and 1 min amplification at 60℃. The PCR primer sequences were: SPHK1, 5'-CTGGTTCATGTGCCCGTGGT-3' (forward) and 5'-CACTTGGCCCTGCACAGCTT-3' (reverse); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AGGTCATCCCAGAGCTGAACG-3' (forward) and 5'-CACCCTGTTGCTGTAGCCGTAT-3' (reverse). Results were analyzed by 7500 system sequence detection software (ver. 1.3, Applied Biosystems) and the mRNA expression level

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of SPHK1 was normalized using the level of GAPDH.

Sphingosine Kinase Activity Assay. Sphingosine kinase activity was measured as previously described (Ryu et al., 2006). Briefly, Cells were scraped in SPHK assay buffer (20mM Tris (pH 7.4), 20% glycerol, 1mM mercaptoethanol, 1mM EDTA, 1mM sodium orthovanadate, 40mM β-glycerophosphate, 15mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1mM phenylmethylsulfonyl fluoride, and 0.5mM 4-deoxypyridoxine) and disrupted by freeze–thawing. An 80 μg portion of cell extracts in 185 μl volume was mixed with 5 μl of [γ-32P]ATP (5 μCi) containing $MgCl_2$ (0.2 M) and 10 μl of 1mM sphingosine (dissolved in 5% Triton X-100) and then incubated for 30 min at 37℃. The reaction was terminated with 10 μl of 1N HCl. A 400 μl portion of chloroform/methanol/HCl (100:200:1, v/v) mixture was added and mixed. Then, 120 μl of chloroform and 120 μl of 2M KCl were added and phases separated by centrifugation. The organic phase was dried and resolved by thin-layer chromatography (TLC) on silica gel G60 with SPHK1-butanol/methanol/acetic acid/water (80:20:10:20, v/v). The radioactive spots corresponding to S1P were detected using phosphoimager, BAS-1500 (Fujifilm Co. Ltd).

Cytotoxicity Assay. Cytotoxicity of DMS was evaluated with the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). BMMs were plated in 96-well plates at a density

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of 2×10^4 cells/well in triplicates and treated with increasing concentrations of DMS. After 16 h incubation, 10 μl of the solution of Cell Counting Kit-8 was added to each well and the plate was incubated for an additional 2 h. The absorbance of each well was measured at 450 nm with a reference at 655 nm using Benchmark microplate reader (Bio-Rad).

Fluorescence Microscopy. BMMs on non-coated glass coverslips were infected with pMX-GFP or pMX-GFP-c-Fos retroviruses. At 24 h after infection, cells were washed twice with ice-cold PBS and fixed with 3.7% formaldehyde. Coverslips were mounted on glass slides, and images were photographed under Zeiss AxioImager D.1 fluorescence microscope.

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Results

DMS Suppresses Osteoclastogenesis in Primary BMMs Cultured with RANKL and M-CSF. In microarray experiments to investigate the changes in the gene expression profile during osteoclast differentiation, we found that the sphingosine kinase 1 (SPHK1) expression is increased in response to RANKL (data not shown). To confirm the SPHK1 up-regulation, we analyzed the mRNA levels of SPHK1 in mouse bone marrow-derived macrophages (BMMs), primary osteoclast precursor cells, by quantitative real-time PCR. Incubation of BMMs in the osteoclastogenic medium (containing RANKL plus M-CSF) increased the SPHK1 mRNA level (Fig. 1*A*, lower panel). Elevation in the protein level of SPHK1 was also observed (Fig. 1*A*, top two panels). The SPHK1 up-regulation was not detected in medium containing only M-CSF (data not shown), indicating that RANKL was responsible for the SPHK1 induction. M-CSF was included in long-term cultures as BMMs fail to survive in the absence of this factor. SPHK2 mRNA and protein levels were also increased by RANKL (data not shown). The increase in SPHK1 expression by RANKL raised the possibility of involvement of this lipid kinase and its product, sphingosine-1-phosphate (S1P), in osteoclast differentiation. To explore this possibility, we utilized DMS, a competitive inhibitor of SPHK. BMMs were induced to differentiate by culturing in osteoclastogenic medium containing RANKL and M-CSF and differentiated osteoclasts were identified by tartrate-resistant acid

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phosphatase (TRAP) staining. In the absence of RANKL, no TRAP-positive multinucleated cells ($TRAP^+$ MNC; osteoclasts) were generated (data not shown). Addition of DMS to the BMM culture in the presence of RANKL inhibited the formation of $TRAP^+$ MNC in a dose-dependent manner (Fig. 1*B* and 1*C*). No cytotoxicity of DMS was observed at the concentrations used (Fig. 1*D*), indicating the anti-osteoclastogenic effect of DMS was not due to toxic effects on the precursor cells to differentiate. At 1 μM, DMS profoundly reduced SPHK activity (Fig. 1*E*). DMS also inhibited the osteoclast formation in RANKL-stimulated RAW264.7 cells, an established myeloid line of cells used in osteoclastogenesis studies (data not shown).

DMS Suppression of Osteoclastogenesis Is Independent of SPHK. Since DMS significantly inhibited the RANKL-induced osteoclastogenesis, we evaluated the role for SPHK in osteoclast differentiation by introducing siRNA targeted to SPHK1 and SPHK2 to BMMs by retroviral transduction (Fig. 2*A*). Contrary to our expectation, the reduction in SPHK1 and 2 expression by SPHK1/2-siRNA did not cause a decrease in osteoclast formation, rather it significantly increased osteoclastogenesis (Fig. 2*B* and 2*E*). These data raised the possibility that DMS might function in a manner independent of its SPHK inhibitory activity during RANKL-induced osteoclastogenesis. To further clarify the

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relationship between SPHK and anti-osteoclastogenic effect of DMS, we overexpressed SPHK1 in BMMs by infecting with retroviruses harboring HA-tagged SPHK1. Overexpression of SPHK1 was confirmed by Western blotting with anti-HA antibody (Fig. 2*C*). When the osteoclastogenesis was evaluated from the SPHK1-overexpressing BMMs, DMS still potently suppressed the osteoclastogenesis (Fig. 2*D* and 2*E*). This result further supports the SPHK-independent anti-osteoclastogenic action of DMS.

SPHK catalyses the phosphorylation of sphingosine, and resulting S1P works as a bioactive molecule (Spiegel and Milstien 2003). It has become clear that the biological role of extracellular S1P is mediated by plasma membrane S1P receptors (Spiegel and Milstien 2003; Olivera and Spiegel 1993). BMMs expressed four members of S1P receptors– EDG1/$S1P_1$, EDG5/$S1P_2$, EDG3/$S1P_3$, and EDG8/$S1P_5$ (data not shown). To further confirm the SPHK-independent suppression of osteoclast formation by DMS, we treated BMMs with S1P. Exogenously added S1P (dissolved in methanol) could not rescue the DMS suppression of osteoclast formation even at doses as high as 10 μM (data not shown). The methanol/dodecane mixture, which facilitates intracellular delivery of sphingolipids has been described (Tauzin et al., 2007). Therefore, we used a dodecane delivery of S1P into the cells to elucidate whether S1P needs to be transported across the plasma membrane in osteoclastogenesis (Fig. 2*F* and 2*G*). However, exogenously added S1P, dissolved in

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methanol/dodecane mixture, also failed to reverse the anti-osteoclastogenic effect of DMS. The S1P concentrations used were not cytotoxic to BMMs (data not shown). Taken together, these data demonstrate that the anti-osteoclastogenic effect of DMS might be independent of SPHK.

DMS has no effect on RANKL-induced NF-κB Activation. The SPHK-independent mode of DMS activity for osteoclastogenesis suppression led us to investigate the effects of this inhibitor on the activation of transcription factors and signaling molecules that have been reported to be crucial for osteoclast differentiation. NF-κB activation composes an essential part of osteoclastogenic signaling pathways by stimulating induction of several osteoclastogenesis-associated genes such as TRAP, cathepsin K, and MMP9 (Boyle et al., 2003; Lee and Kim 2003). Furthermore, it was recently reported that NF-κB regulates the expression NFATc1, another key transcription factor for osteoclastogenesis (Takatsuna et al., 2005). Typically, NFκB activation starts from phosphorylation and subsequent degradation of the inhibitory subunit IκB in response to an extracellular signal. The freed NFκB then translocates to the nucleus to bind target gene promoters. To examine whether NFκB could be a target of DMS during the suppression of osteoclastogenesis, we stimulated BMMs with RANKL in the presence or absence of DMS and assessed the activation of NF-κB. DMS did

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not affect the RANKL-induced phosphorylation and subsequent degradation of IκB (Fig. 3*A*). Consistently, a promoter activity reporter assay for NF-κB revealed no effect of DMS on the RANKL-induced NF-κB activity (Fig. 3*B*). Therefore, NFκB is not the target of DMS for its inhibition of RANKL-induced osteoclastogenesis.

DMS Blocks the Expression of NFATc1 Induced by RANKL in BMMs. It has been shown by several reports that the NFATc1 transcription factor is greatly up-regulated by RANKL and its induction is critical to efficient osteoclast differentiation (Walsh et al., 2006; Takayanagi et al., 2002). In order to explore the potential effect of DMS on NFATc1 induction by RANKL, we treated BMMs with the osteoclastogenic medium (M-CSF plus RANKL) together with or without DMS for various time periods, and assessed the NFATc1 expression. The induction of NFATc1 protein level was clearly observed at 12 h and even higher levels of NFATc1 were detected at 24~72 h (Fig. 4*A*). This elevation in NFATc1 expression was not detected in the absence of RANKL (Fig. 4*B*, lane M). DMS treatment strongly reduced the induction of NFATc1 by RANKL in BMMs (Fig. 4*A*). The inhibition of RANKL induction of NFATc1 by DMS was dose-dependent (Fig. 4*B*). In line with the inhibitory effect on NFATc1 protein level, DMS treatment decreased NFATc1-dependent reporter activity in response to RANKL stimulation (Fig. 4*C*).

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DMS Does not inhibit RANKL-induced Ca^{2+} Oscillation in BMMs. It has been reported that the sustained Ca^{2+} oscillation induced by RANKL is essential for the auto-amplification of NFATc1 gene expression which is critical to the NFATc1-dependent osteoclastogenic gene transcription (Takayanagi et al., 2002). As DMS inhibited the NFATc1 induction by RANKL, we next explored the potential involvement of Ca^{2+} oscillation response in the anti-osteoclastogenic function of DMS. The intracellular Ca^{2+} levels were determined in BMMs cultured in the osteoclastogenic medium with or without DMS (Fig. 4*D*). Grossly, DMS did not alter the cell population showing Ca^{2+} oscillation (about 20~30%; data not shown) and the frequency of Ca^{2+} spikes in RANKL-treated cells. While it slightly decreased oscillation amplitude, it is likely that DMS did not block RANKL-induced Ca^{2+} oscillation.

DMS Inhibits c-Fos Expression in RANKL-Stimulated BMMs. Another important factor in NFATc1 induction by RANKL in osteoclast precursor cells is c-Fos. It has been reported that expression of NFATc1 is induced in *c-Fos*$^{+/+}$ mice, but not in *c-Fos*$^{-/-}$ mice during osteoclast differentiation (Takayanagi et al., 2002; Matsuo et al., 2004; and Matsuo and Ray, 2004). As a component of the AP-1 transcription factor, c-Fos was suggested to regulate the NFATc1 induction by RANKL (Takayanagi et al., 2002; Matsuo et al., 2004; and Matsuo and

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Ray, 2004). We therefore examined whether DMS could interfere with the c-Fos regulation by RANKL. BMMs were incubated in RANKL together with either DMS or vehicle and analyzed c-Fos expression levels. The c-Fos protein started to slightly increase from 30 min after stimulation and reached to a greatly elevated level from 6 h (Fig. 5*A*). DMS strongly inhibited the RANKL induction of c-Fos protein (Fig. 5*A*). The inhibitory effect of DMS was also observed in cells stimulated for up to 3 days (Fig. 5*B*). In BMMs treated with various concentrations of DMS, dose-dependency of the inhibition was observed (Fig. 5*C*). Notably, the induction of c-Fos occurred faster than that of NFATc1 (Fig. 5*A* and Fig. 4*A*). This observation is congruent with the current understanding that c-Fos plays a critical role for NFATc1 induction. Thus, the decreased level of c-Fos may explain the ablation of NFATc1 induction by DMS. Together with data shown in Fig. 4*D*, It could be concluded that DMS blocks the induction of NFATc1 by interfering with the RANKL signaling for c-Fos expression, but not that for Ca^{2+} oscillation.

DMS Inhibits the Activation of ERK, but not That of JNK and p38 MAPKs by RANKL in BMMs. The activation of MAPK signaling pathways by RANKL is an important mechanism involved in osteoclastogensis (Lee and Kim, 2003; Teitelbaum, 2004). The activation of ERK, JNK, and p38 MAPKs by RANKL and their contribution to the

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osteoclastogenesis has been demonstrated with pharmacological inhibitors and dominant-negative forms (Matsumoto et al., 2000; Wei et al., 2002). In addition, ERK has been well-documented to induce and activate c-Fos (Treisman, 1996; Muller et al., 1997). So, we next focused on the effect of DMS on the activation of ERK and the other two MAPK family member proteins. When the MAPK activity was assessed by Western blotting with phosphorylated form-specific antibodies, it was found that DMS strongly suppressed the activation of ERK by RANKL in BMMs (Fig. 6*A*). In contrast, the RANKL-stimulated activation of JNK and p38 was not affected by DMS (Fig. 6*B* and 6*C*). Therefore, it is possible that DMS suppressed c-Fos and NFATc1 induction by inhibiting the ERK activation by RANKL, resulting in a blockade in osteoclastogeneis.

DMS Suppresses the Activation of MEK by RANKL in BMMs. The activation of ERK occurs through phosphorylation of both threonine and tyrosine residues by upstream MAP kinase kinases, MEK1/2 (Payne et al., 1991). The activation of MEK1/2 occurs through phosphorylation of two serine residues by Raf-like molecules (Pearson et al., 2001). As DMS inhibited ERK activation by RANKL, we next investigated the activation of the upstream kinase MEK1/2 by RANKL and effects of DMS on MEK1/2 activity. As shown in Fig. 7*A*, DMS greatly reduced MEK1/2 phosphorylation in response to RANKL in BMMs. Therefore,

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the MEK-ERK pathway to c-Fos induction is likely to be the target in the DMS-mediated anti-osteoclastogenesis effect. So, we next explored the relationship between the MEK/ERK pathway, osteoclast differentiation, and the osteoclastogenic transcription factors c-Fos and NFATc1. For this investigation, a specific MEK inhibitor, U0126, was employed and its effects on osteoclastogenesis and the induction of c-Fos and NFATc1 were evaluated. U0126 blocked the activation of ERK1/2 at concentrations 0.1-10 μM (Fig. 7*B*). When BMMs were cultured in the osteoclastogenic medium in the presence of U0126, osteoclast formation was profoundly suppressed (Fig. 7*C* and 7*D*). Consistent with its osteoclastogenesis-inhibitory effect, U0126 inhibited the induction of c-Fos by RANKL in BMMs (Fig. 7*E*, upper panel). Similarly, the induction of NFATc1 by RANKL was suppressed by U0126 in a dose-dependent manner (Fig. 7*E*, third panel).

DMS Inhibits PI3K and MEK1/2 Activation Independently. The PI3K (phosphatidylinositol 3-kinase/Akt pathway is also an important component of RANKL signaling in osteoclast precursor cells (Wong et al., 1999). Interestingly, DMS has been documented to impede the PI3K/Akt signaling pathway in epithelial cells (Monick et al., 2004). On the other hand, PI3K has been shown to stimulate ERK1/2 activation in response to TNF-α, insulin, and NGF (Gronning et al., 2002; Zhuang et al., 2004; and Lee et al., 2005).

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With these previous findings, we wondered whether DMS would affect the PI3K/Akt activation by RANKL in BMMs and, if it does, whether the PI3K/Akt inhibition is related to the DMS-mediated suppression of MEK-ERK activation by RANKL. When the activation of Akt was examined by Western blotting with anti-phospho Akt, Akt activation by RANKL was detected from 8 min after stimulation in BMMs (Fig. 8*A*, lanes 1-5). This Akt activation was greatly inhibited in the presence of DMS (Fig. 8*A*, lanes 6-9). Next, we investigated whether the Akt inhibition mediates the DMS-induced suppression of ERK activation by RANKL. BMMs were treated with the PI3K inhibitor LY294002 to block Akt activation and the phosphorylation of ERK in response to RANKL was assessed. As shown in Fig. 7*B*, LY294002 abolished Akt activation by RANKL (Fig. 8*B*, top panel). However, the ERK1/2 activation by RANKL was not affected by LY294002 (Fig. 8*B*, third panel), demonstrating that PI3K/Akt is not an upstream target of DMS for the inhibition of ERK1/2 activation by RANKL. Furthermore, LY294002 inhibited the induction of c-Fos and NFATc1 by RANKL (Fig. 8*C*). These results are congruent with previous works indicated that the PI3K/Akt pathway is required for AP-1 activation through c-fos expression (Huang et al., 1996). Therefore, it appears that Akt inhibition by DMS also contributes to the blockade of NFATc1 induction, independently of its ERK inhibition.

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Effect of c-Fos Overexpression on Osteoclastogenesis in DMS-treated BMMs. If the inhibition of RANKL-induced ERK/Akt activation and c-Fos expression is the critical cause of NFATc1 down-regulation, one would expect that the DMS-mediated inhibition of osteoclastogenesis would be overcome by ectopic c-Fos overexpression. Thus, we examined whether transient overexpression of c-Fos would recover osteoclastogenesis in the presence of DMS. BMMs were infected with recombinant retroviruses harboring GFP alone or GFP plus c-Fos. When the infection efficiency was evaluated by scoring GFP-positive cells, it was 80-90% (Fig. 9*A*). The overexpression of c-Fos was verified by Western blotting with anti-c-Fos (Fig. 9*B*). In cultures of c-Fos-overexpressing BMMs, there was a significant increase in $TRAP^+$ osteoclast numbers in the presence of DMS compared to the control virus-infected BMMs (Fig. 9*C* and 9*D*). These data suggest that c-Fos was a critical downstream target of DMS inhibition in the RANKL signaling for osteoclast differentiation.

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Discussion

During an approach to identify genes involved in the osteoclastogenesis using DNA microarrays, we found that SPHK1 expression was significantly increased upon RANKL treatment. SPHK has been implicated in the differentiation of various types of cells. In addition, the proinflammatory factors such as LPS and TNF-α, which augment RANKL-induced osteocastogenesis, stimulate SPHK in macrophages and epithelial cells (Xia et al., 1999; Shu et al., 2002). These backgrounds led us to test the potential role of SPHK for the differentiation of precursor cells into mature osteoclasts. The function of SPHK is usually estimated in *in vitro* studies by treating cells with N,N-dimethyl-D-*erythro*-sphingosine (DMS), a well-established competitive inhibitor of SPHK (Igarashi et al., 1989). In the present study, treatment of osteoclast precursors with DMS resulted in an almost complete inhibition of RANKL-induced osteoclast differentiation. Considering that the expression of SPHK was significantly increased upon RANKL treatment, the activity of SPHK might have been crucial for the RANKL-induced osteoclstogenesis. To test this possibility, SPHK in the osteoclast precursor BMMs were knocked-down using a retroviral gene transfer system for siRNA. Surprisingly, when these cells were stimulated to differentiate into osteoclasts by RANKL treatment, SPHK1 and SPHK2 siRNA-expressing cells generated higher number of osteoclasts than the control siRNA-infected cells. Furthermore, DMS efficiently inhibited the

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RANKL-induced osteoclastogenesis in SPHK1-overexpressed BMMs. In line with these observations, exogenous addition of S1P to the culture medium could not recover osteoclast differentiation in the presence of DMS. In addition, we could not find anti-osteoclastogenic effect using another inhibitor of SPHK, DL-threo-dihydrosphingosine (DHS). Taken together, these results indicate that DMS blocks RANKL-induced osteoclastogenesis independent of its SPHK inhibitory activity. We have previously suggested that SPHK1 and intracellular S1P may act as a negative feedback regulator during osteoclastogenesis (Ryu et al., 2006). On the other hand, secreted S1P stimulated osteoblast migration and survival, thus playing an osteoclast-osteoblast communication factor. The observation in this paper that the siRNA against SPHK1/2 potentiated osteoclastogenesis further supports a negative role of SPHK during osteoclast differentiation. However, further studies are required to clearly delineate the role of SPHK during osteoclastogenesis in the context of co-operation with osteoblasts or T cells.

As DMS was first reported as an inhibitor of protein kinase C (PKC) (Hannun et al., 1987), we considered the possibility of involvement of PKC pathways in the DMS-mediated osteoclastogenesis inhibition. It has been reported that the activation of PKC by 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited osteoclastogenesis (Wang et al., 2003) and that a PKC-activating synthetic peptide fragment significantly decreased osteoclast activity

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(Moonga et al., 1998). In our culture system, we could not find any differences in PKC activation after the treatment of cells with DMS at concentrations that significantly inhibited osteoclastogenesis (data not shown). Moreover, the concentration of DMS required for PKC inhibitory effect is much higher than the DMS dose at which osteoclast differentiation was inhibited in our study (Edsall et al., 1998; Hamaguchi et al., 2003). Therefore, it is likely that PKC was not involved in DMS inhibition of osteoclastogenesis in our experimental conditions. Since it seemed that the SPHK and PKC were not involved, we tried to delineate the mechanism by which DMS exerts its inhibitory effect on RANKL-induced osteoclastogenesis. First of all, the effect of DMS on the expression of transcription factors crucially involved in the osteoclast differentiation was investigated. NFATc1 is the master transcription factor for osteoclastogenesis and the distinguishing feature of NFATc1 induction by RANKL is its regulation by Ca^{2+} oscillation and c-Fos (Takayanagi et al., 2002). Controversial results have been reported concerning DMS, SPHK and Ca^{2+} signals. Recently, it was reported that DMS increases cytosolic calcium in human T lymphocytes (Alfonso et al., 2003) and monocytes (Lee et al., 2006). On the contrary, some studies have shown the opposite results. Melendez *et al.* showed that C5a-triggered cytosolic Ca^{2+} signals are inhibited by DMS (Melendez and Ibrahim, 2004) and Yadav *et al.* demonstrated SPHK mediates cytosolic Ca^{2+} increase upon mycobacterial infection in mouse BMMs (Yadav et al.,

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2006). In our expriments, DMS had little effect on the RANKL-induced Ca^{2+} oscillation. On the other hand, enforced c-fos expression reversed the DMS inhibition of osteoclastogenesis and restored NFATc1 expression, suggesting that c-fos might be a critical downstream target of DMS for the inhibition of RANKL-induced osteoclastogenesis. The RANKL-induced phosphorylation and subsequent degradation of IκB and the RANKL-stimulated transcription activity of NFκB were not significantly affected by DMS treatment, suggesting that the inhibitory effects of DMS on osteoclast differentiation do not involve NFκB signaling pathway. Thus, the inhibition of osteoclastogenesis by DMS was mediated by controlling specific signaling pathways, not by cytotoxicity.

RANKL is known to activate three major MAPK subfamilies (ERK, JNK, and p38) and inhibition of these MAPKs suppresses osteoclast differentiation (Boyle et al., 2003; Lee and Kim, 2003; and Matsumoto et al., 2000). We found that the effect of DMS on the RANKL-induced MAPK activation in BMMs was very selective, i.e. while the ERK activation was prominently inhibited by DMS, the activation of JNK and p38 was nearly unaffected. Since it was previously reported that the ERK1/2 activation led to the stimulation of c-Fos expression by acting on transcription factors bound at the c-Fos promoter in various cell types (Triesman, 1996; Muller et al., 1997), the involvement of ERK pathway singling in the inhibition of osteoclastogenesis and c-Fos expression by DMS was investigated. The

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ERK upstream activators that have been mostly well characterized are MEK1/2. We found that DMS exerts inhibitory effects on the activation of MEK1/2 by RANKL in osteoclast precursor BMMs. When BMMs were treated with an MEK inhibitor U0126, these cells failed to differentiate into osteoclasts. Furthermore, U1026 had similar inhibitory effect on the RANKL-induced c-Fos and NFATc1 expression. Although further study is required to fully define the direct molecular target for the inhibition of MEK by DMS, it is clear that the inhibition of the MEK/ERK pathway and subsequent hampering of the RANKL-stimulated c-Fos/NFATc1 expression is crucial for the anti-osteoclastogenic effect of DMS.

In our study, DMS also significantly inhibited RANKL-induced Akt phosphorylation. PI3K has been shown to stimulate ERK1/2 activation in response to some stimuli (Gronning et al., 2002; Zhuang et al., 2004; and Lee et al., 2005). Thus the possibility that DMS inhibited ERK by reducing PI3K activity was tested by treating cells with a PI3K inhibitor LY294002. The inhibition of PI3K resulted in the reduction of RANKL-induced expression of c-Fos and NFATc1, which was also observed after DMS treatment. These results are congruent with previous works that have indicated PI3K activation is required for c-fos expression and AP-1 activation (Huang et al., 1996). However, LY294002 did not inhibit RANKL-induced ERK phosphorylation, indicating that these two pathways are distinct. Consistent with our data, Wilden *et al*. showed that smooth muscle cell proliferation requires

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independent ERK and PI3K activation (Wilden et al., 1998) and Monick *et al*. demonstrated that DMS blocks through separate mechanisms the activation of both ERK and Akt upon viral infection in epithelial cells (Monick et al., 2004). Thus, DMS is likely to exert its anti-osteoclastogenic effect through inhibitory action on the two separate signaling pathways crucial for RANKL-induced osteoclastogenesis, i.e. the MEK/ERK and the PI3K/Akt pathways (Fig. 10).

The anti-osteoclastogenic property of DMS may have very important clinical implication. Recently, anti-cancer effects of DMS such as inhibition of tumor cell growth and migration have been reported, triggering intensive studies for application of DMS as an anti-cancer drug (Endo et al., 1991). Importantly, patients with advanced breast and prostate cancers almost always develop bone metastasis, because these cancer cells find bone to be a fertile soil on which to grow (Mundy, 2002). One of the cues that attract cancer cells to the bone is the bone-derived growth factors such as transforming growth factor β, insulin-like growth factor 1, and fibroblast growth factor, which are released during osteoclastic bone resorption. Consistent with this notion, it has been shown that osteoprotegerin, a natural antagonist of RANKL, inhibited bone metastasis in animal model (Mundy, 2002). These findings suggest the possibility that osteolysis inhibitors might also decrease bone tumor burden. Thus, the anti-cancer properties previously reported and the anti-osteoclastogenic

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activity shown in our present study place DMS to a uniquely interesting drug candidate for treatment of tumor bone metastases. In summary, our study provides the first evidence that DMS suppresses osteoclastogenesis from primary precursors and points out the potential usefulness of this sphingolipid for the treatment of bone-associated tumors.

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Footnotes

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Legends for figures

Fig. 1. DMS inhibits RANKL-induced osteoclastogenesis from primary precursor cells. *A,* Mouse bone marrow-derived macrophages (BMMs) were incubated in the osteoclastogenic medium (containing 30 ng/ml M-CSF plus 200 ng/ml RANKL) or in medium containing only M-CSF (30 ng/ml) for 3 days. Cell lysates were prepared and the protein levels of SPHK1 and actin were examined by Western blotting (top two panels). Also, SPHK1 mRNA expression at day 0 (BMM), day 2 (pre-osteoclast, pOC) or day 3 (osteoclast, OC) were analyzed by quantitative-PCR. Results are presented as fold induction of SPHK1 mRNA (lower panel). *B* and *C*, BMMs were cultured in the osteoclastogenic medium for 3 days in the presence of DMS (0.25~2 μM) or vehicle (V; DMSO). Cells were stained for TRAP and numbers of TRAP-positive multinuclear cells containing more than three nuclei ($TRAP^+$ MNC) were counted. *D*, BMMs were cultured in the osteoclastogenic medium for 24 h together with DMS (0.1~2 μM) or vehicle (V). Cell viability was evaluated as described in *Materials and Methods*. *E*, BMMs were serum-starved for 5 h, pretreated with or without DMS (2 μM) for 2 h, and stimulated with RANKL (500 ng/ml) for 15 min. After RANKL stimulation, SPHK activity was measured in cell extracts using [γ-^{32}P]ATP and sphingosine. The reaction products were resolved by thin layer chromatography and the radioactive spots corresponding to S1P were detected using phosphoimager.

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Fig. 2. SPHK knock-down and overexpression reveal a SPHK-independent effect of DMS on osteoclastogeneis. *A* and *B*, BMMs were infected with retroviruses harboring SPHK1 siRNA plus SPHK2 siRNA (SPHK1/2-si) or luciferase siRNA (Luc-si). Then, cells were cultured for 3 days in the osteoclastogenic medium. One of the duplicate cultures was lysed and equal amounts of proteins (30 μg) were immunoblotted with anti-SPHK1 or anti-SPHK2 antibody. The other set of cells was TRAP-stained. *C* and *D*, BMMs were infected with retroviruses for pMX or pMX-HA-SPHK1. Cells were cultured for 3 days in the osteoclastogenic medium in the presence of DMS (2 μM). The overexpression of SPHK1 was confirmed by immunoblotting with anti-HA antibody and the osteoclast differentiation was examined by TRAP staining. *E*, The number of $TRAP^+$ MNCs generated in *B* and *D* was scored. *F* and *G*, BMMs were cultured for 3 days in the osteoclastogenic medium with DMS (2 μM) together with S1P dissolved in methanol or dissolved in methanol/dodecane mixture (used at v/v 99:1~96:4). Cells were stained for TRAP and the number of $TRAP^+$ MNCs was counted.

Fig. 3. DMS has no effect on RANKL-induced NFκB activation. *A*, BMMs were serum-starved for 5 h, pretreated with or without DMS (2 μM) for 2 h, and stimulated with RANKL (500 ng/ml) for the indicated time. Cells were lysed and subjected to immunoblotting with an

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antibody for p-IκB or IκB. *B*, Raw264.7 cells were transfected with an NFκB reporter luciferase plasmid. At 14 h after transfection, cells were serum-starved for 5 h, pretreated with or without DMS (2 μM) for 2 h, and stimulated with RANKL (200 ng/ml) for 24 h. Cells were lysed and the luciferase activity was determined. The relative NFκB activity was presented by luciferase activity per μg protein.

Fig. 4. DMS blocks the expression of NFATc1 induced by RANKL in BMMs. *A*, BMMs were cultured in the osteoclastogenic medium (M+R; 30 ng/ml M-CSF+200 ng/ml RANKL) for the indicated hours (left) and days (right) in the absence or presence of DMS (2 μM). NFATc1 expression was determined by immunoblotting with an anti-NFATc1 antibody. *B*, BMMs were cultured in the osteoclastogenic medium (M+R) or medium containing M-CSF (M) for 24 h with or without DMS (0.5~2 μM). NFATc1 expression was determined by immunoblotting with an anti-NFATc1 antibody. *C*, Raw264.7 cells were transfected with an NFATc1 reporter luciferase plasmid. At 14 h after transfection, cells were stimulated with 200 ng/ml RANKL together with indicated concentrations of DMS or vehicle (DMSO) for 24 h. Cells were lysed and the luciferase activity was determined. The relative NFATc1 activity was presented by lucuferase activity per μg protein. *D*, BMMs on glass coverslips were grown in the medium containing M-CSF (M) or M-CSF+RANKL (M+R) for 48 h in the presence or

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absence of DMS (2 μM). Cells were loaded with fura-2/AM (5 μM) and imaging was performed as described in *Material and Methods*. Maximum ratio increase was obtained with the addition of 10 μM ionomycin at the end of each experiment. Each color indicates individual cell in the same field, and black color indicates the baseline status.

Fig. 5. DMS blocks c-Fos induction by RANKL in BMMs. *A*, BMMs were incubated with 200 ng/ml RANKL for the indicated hours in the absence or presence of DMS (2 μM). The c-Fos expression was determined by immunoblotting with an anti-c-Fos antibody. *B*, BMMs were cultured in the osteoclastogenic medium (M+R) for the indicated days with or without DMS (2 μM). M-CSF was included in long-term cultures as BMMs fail to survive in the absence of this factor. The c-Fos expression was analyzed by immunoblotting. *C*, BMMs were cultured in the medium containing M-CSF (30 ng/ml) or M-CSF plus RANKL (200 ng/ml) for 24 h in the presence or absence of DMS (0.5~2 μM). c-Fos expression was determined by immunoblotting.

Fig. 6. DMS inhibits RANKL-induced ERK activation. BMMs were serum-starved for 5 h, pretreated with or without DMS (2 μM) for 2 h, and stimulated with RANKL (500 ng/ml) for the indicated time. The phosphorylated forms of ERK1/2 *(A)*, JNK1/2 *(B)* and p38 *(C)* were

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detected with phospho-specific antibodies. Blots were stripped and re-probed with the control antibodies to demonstrate comparable loading. Protein band intensities were quantified by densitometry and the levels of phosphorylated MAPKs were normalized to the total levels of corresponding MAPKs. The ratio of phospho/total MAPKs in the RANKL-stimulated cells was compared with that of untreated cells.

Fig. 7. Inhibition of RANKL-induced MEK activation mediates the anti-osteoclastogenic effect of DMS. *A*, BMMs were serum-starved for 5 h, pretreated with or without DMS (2 μM) for 2 h, and stimulated with RANKL (500 ng/ml) for the indicated time. Cell lysates were analyzed by immunoblotting with a phospho-MEK1/2 antibody. The same blot was stripped and re-probed with an anti-MEK1/2 antibody. *B*, BMMs were serum-starved for 5 h, pretreated with or without U0126 (0.1~10 μM) for 2 h, and stimulated with RANKL (500 ng/ml) for the indicated time. Phospho-ERK1/2 levels were determined by immunoblotting. *C* and *D*, BMMs were cultured in the osteoclastogenic medium for 3 days in the presence or absence of U0126 (0.1~10 μM). Cells were stained for TRAP *(C)* and the numbers of $TRAP^+$ MNC were counted *(D)*. *E*, BMMs were incubated in medium containing M-CSF (30 ng/ml) or M-CSF plus RANKL (200 ng/ml) for 24 h with indicated dose of U0126 (0.1~10 μM). The whole cell lysates were analyzed by immunoblotting with anti-c-Fos and anti-NFATc1

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antibodies.

Fig. 8. DMS also inhibits the PI3K/Akt pathway. *A*, BMMs were serum-starved for 5 h, pretreated with or without DMS (2 μM) for 2 h, and stimulated with RANKL (500 ng/ml) for the indicated time. Cell lysates were analyzed by immunoblotting for phospho-Akt or total Akt level. *B*, Serum-starved BMMs were pretreated with indicated concentration of LY294002 (5~20 μM) or DMS (2 μM) for 2 h and stimulated with RANKL (500 ng/ml) for 5 min. Phosphorylation of Akt and ERK1/2 was analyzed by immunoblotting. *C*, BMMs were incubated in medium containing M-CSF (30 ng/ml) or M-CSF plus RANKL (200 ng/ml) for 24 h with indicated dose of LY294002 (5~20 μM) or DMS (2 μM). The whole cell lysates were analyzed by immunoblotting with anti-c-Fos and anti-NFATc1 antibodies.

Fig. 9. c-Fos overexpression overcomes the anti-osteoclastogenic effect of DMS. *A*, BMMs were infected with c-Fos or the control retroviruses. At 24 h after infection, cells were subjected to fluorescence microscopy to examine the efficiency of infection. Representative pictures of infected cells are shown (left panel) and the percent GFP^+ cells was quantified (right panel). *B*, Infected BMMs were cultured for 24 h and cell extracts were analyzed for c-Fos expression levels by immunoblotting. *C* and *D*, BMMs uninfected or infected with either

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c-Fos or the control retroviruses were cultured in the osteoclastogenic medium for 3 days in the presence or absence of DMS (2 μM). Cells were TRAP-stained (*C*) and $TRAP^+$ MNC numbers were counted (*D*).

Fig. 10. A schematic model for the osteoclastogenesis inhibitory function of DMS. RANKL engagement to RANK results in the activation of both MEK/ERK and PI3K/Akt pathways. Combined inhibition of the two pathways by DMS significantly reduces the induction of c-Fos and NFATc1 transcription factors by RANKL. The blockade of these transcription factors leads to suppression of osteoclastogenic gene expression and subsequent osteoclast formation.

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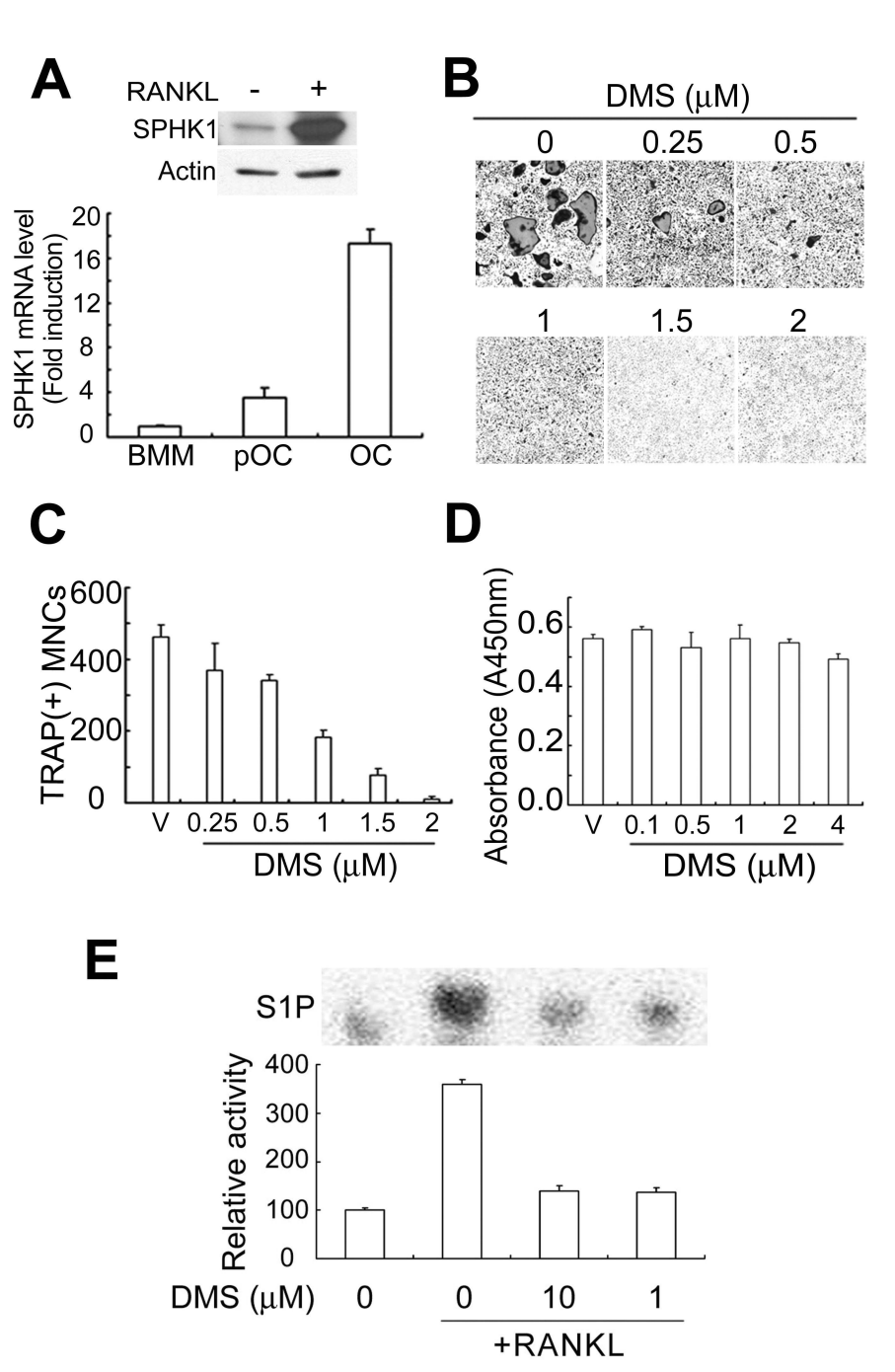

A
RANKL - +
SPHK1
Actin
SPHK1 mRNA level (Fold induction)
20
16
12
8
4
0
BMM
pOC
OC
B
DMS (μM)
0
0.25
0.5
1
1.5
2
C
TRAP(+) MNCs
600
400
200
0
V
0.25
0.5
1
1.5
2
DMS (μM)
D
Absorbance (A450nm)
0.6
0.4
0.2
0.0
V
0.1
0.5
1
2
4
DMS (μM)
E
S1P
Relative activity
400
300
200
100
0
DMS (μM)
0
0
10
1
+RANKL

Fig. 1

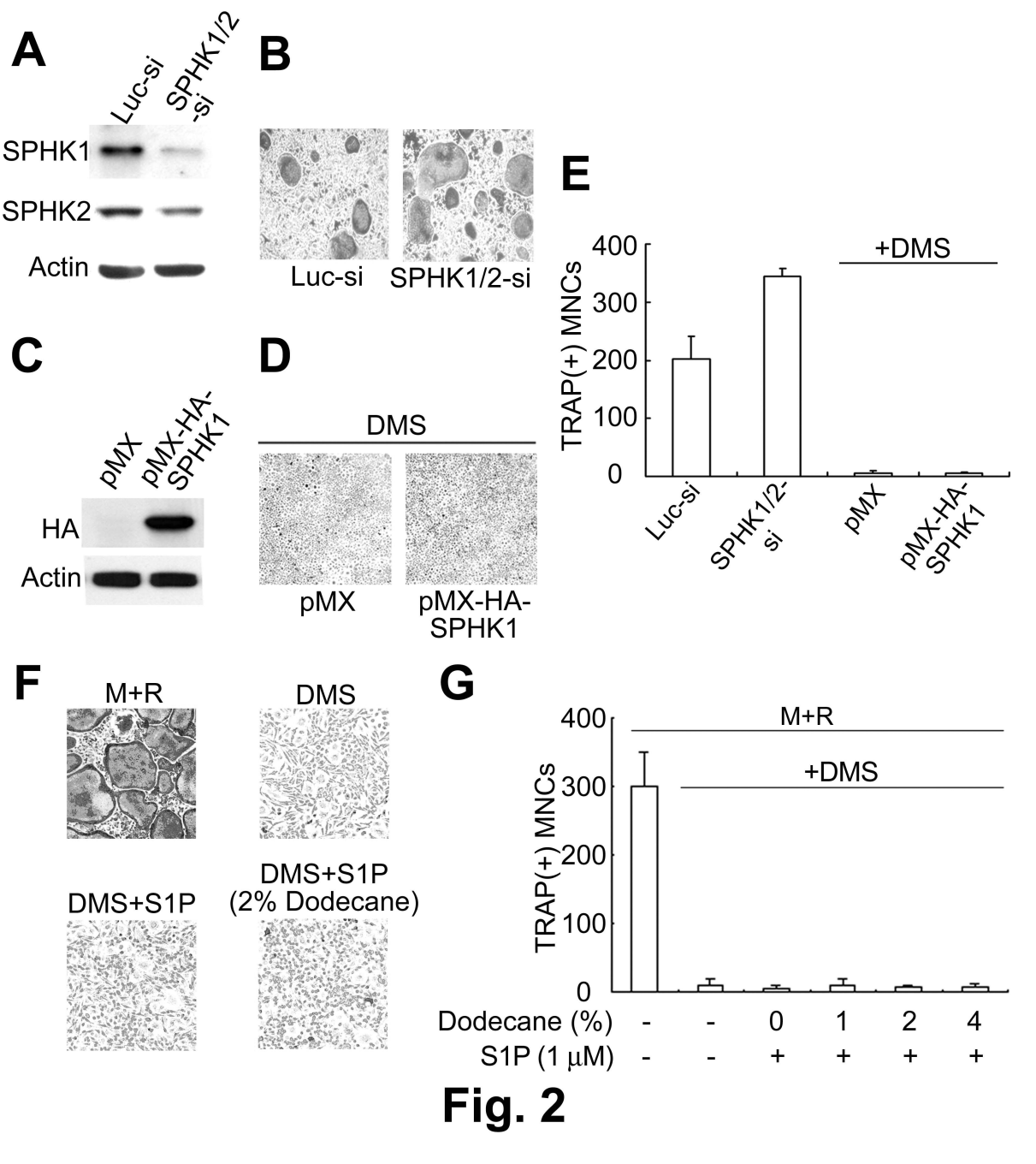
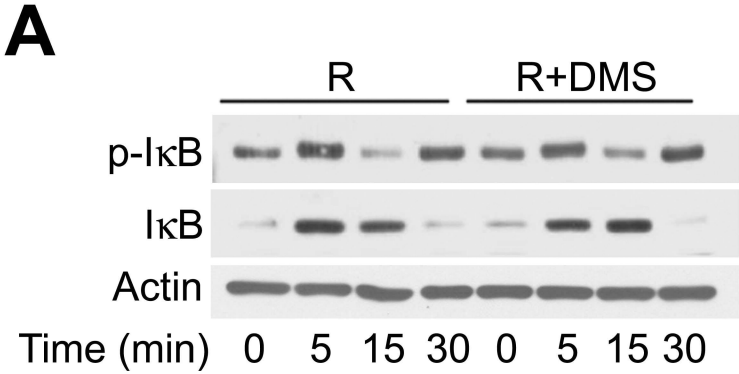

A
Luc-si
SPHK1/2 -si
SPHK1
SPHK2
Actin
B
Luc-si
SPHK1/2-si
C
pMX
pMX-HA-SPHK1
HA
Actin
D
DMS
pMX
pMX-HA-SPHK1
E
+DMS
TRAP(+) MNCs
400
300
200
100
0
Luc-si
SPHK1/2-si
pMX
pMX-HA-SPHK1
F
M+R
DMS
DMS+S1P
DMS+S1P (2% Dodecane)
G
M+R
+DMS
TRAP(+) MNCs
400
300
200
100
0
Dodecane (%) - - 0 1 2 4
S1P (1 μM) - - + + + +

Fig. 2


A
R
R+DMS
p-IκB
IκB
Actin
Time (min) 0 5 15 30 0 5 15 30

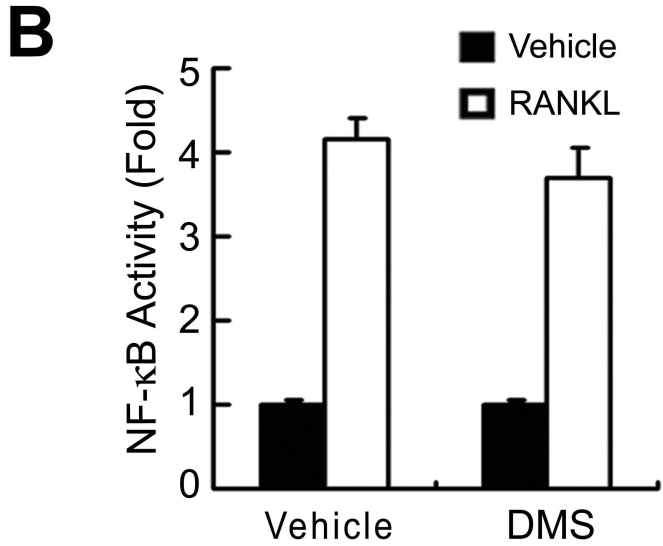

B
Vehicle
RANKL
NF-κB Activity (Fold)
0
1
2
3
4
5
Vehicle
DMS

Fig. 3

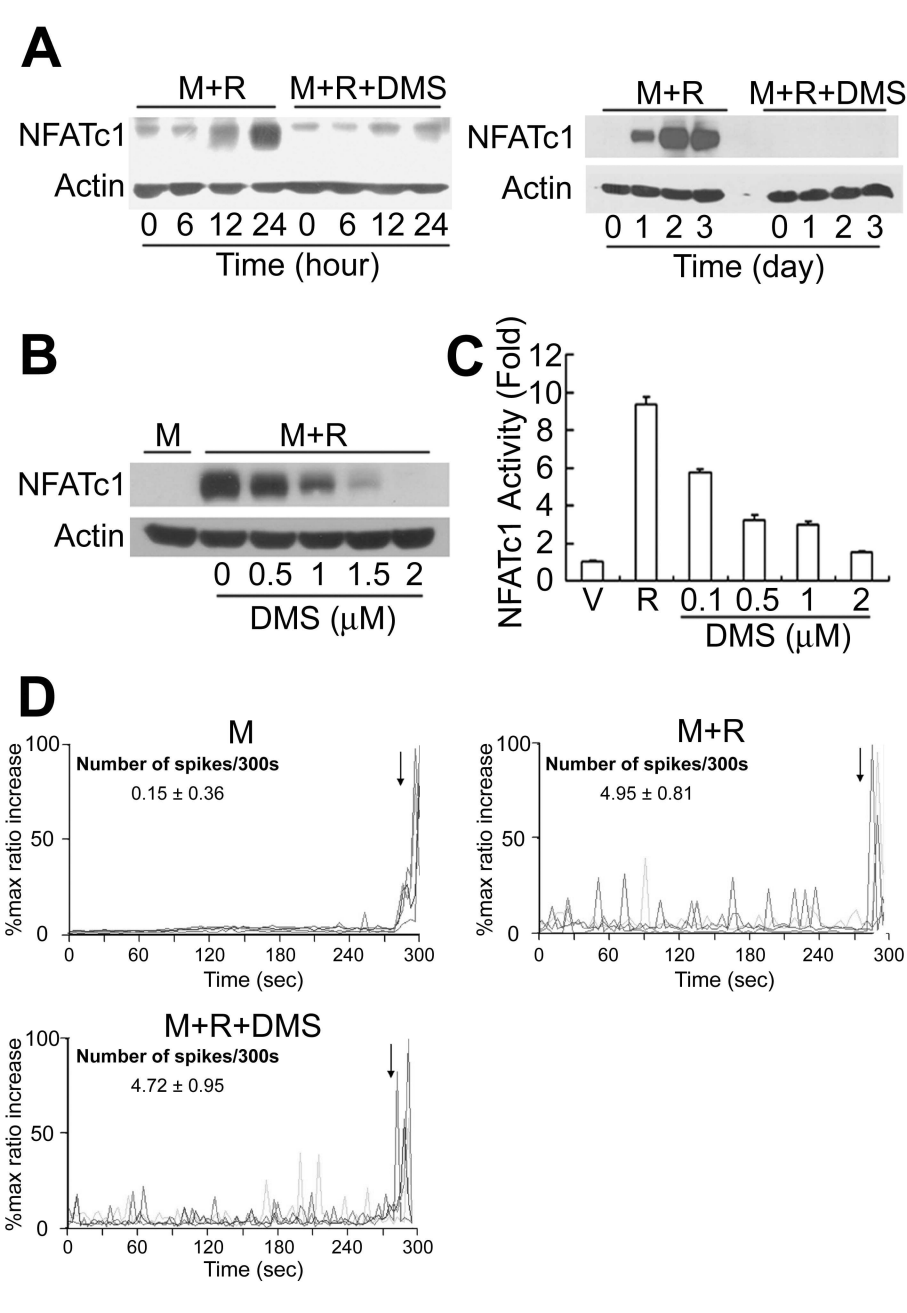

A
M+R
M+R+DMS
NFATc1
Actin
0 6 12 24 0 6 12 24
Time (hour)
M+R
M+R+DMS
NFATc1
Actin
0 1 2 3 0 1 2 3
Time (day)
B
M
M+R
NFATc1
Actin
0 0.5 1 1.5 2
DMS (μM)
C
NFATc1 Activity (Fold)
0 2 4 6 8 10 12
V R 0.1 0.5 1 2
DMS (μM)
D
M
Number of spikes/300s
0.15 ± 0.36
%max ratio increase
0 50 100
0 60 120 180 240 300
Time (sec)
M+R
Number of spikes/300s
4.95 ± 0.81
%max ratio increase
0 50 100
0 60 120 180 240 300
Time (sec)
M+R+DMS
Number of spikes/300s
4.72 ± 0.95
%max ratio increase
0 50 100
0 60 120 180 240 300
Time (sec)

Fig. 4

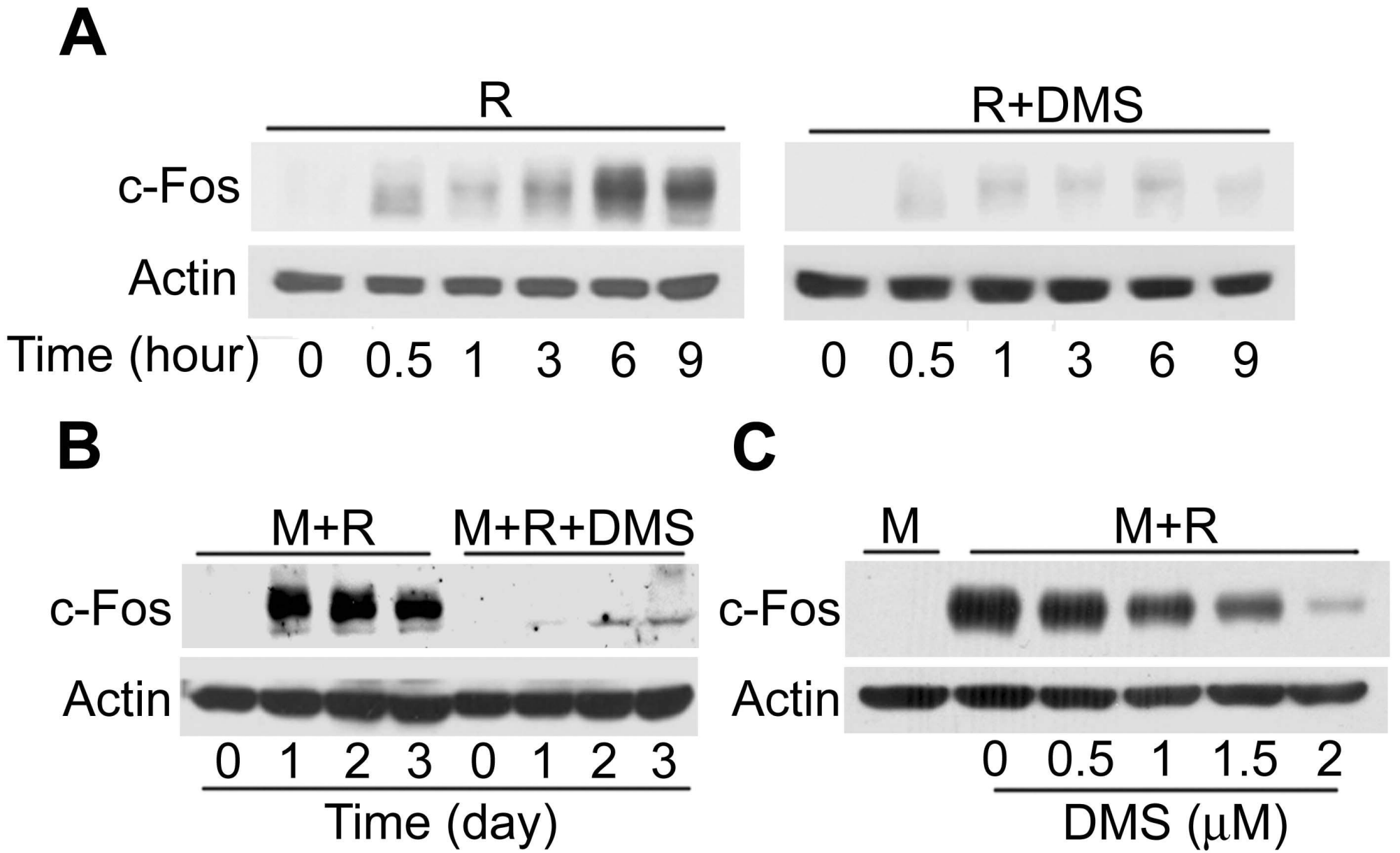

A
R
R+DMS
c-Fos
Actin
Time (hour) 0 0.5 1 3 6 9
0 0.5 1 3 6 9
B
M+R
M+R+DMS
c-Fos
Actin
0 1 2 3 0 1 2 3
Time (day)
C
M
M+R
c-Fos
Actin
0 0.5 1 1.5 2
DMS (μM)

Fig. 5

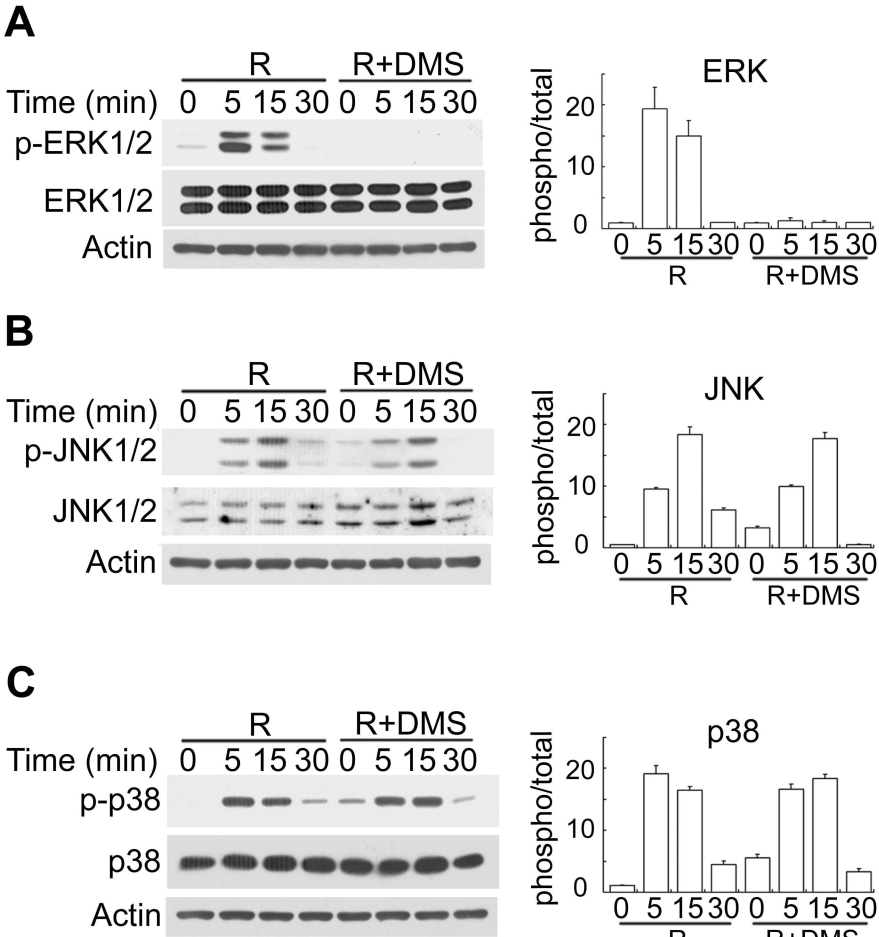

A
R
R+DMS
Time (min)
0 5 15 30 0 5 15 30
p-ERK1/2
ERK1/2
Actin
ERK
phospho/total
20
10
0
0 5 15 30 0 5 15 30
R
R+DMS
B
R
R+DMS
Time (min)
0 5 15 30 0 5 15 30
p-JNK1/2
JNK1/2
Actin
JNK
phospho/total
20
10
0
0 5 15 30 0 5 15 30
R
R+DMS
C
R
R+DMS
Time (min)
0 5 15 30 0 5 15 30
p-p38
p38
Actin
p38
phospho/total
20
10
0
0 5 15 30 0 5 15 30
R
R+DMS

Fig. 6

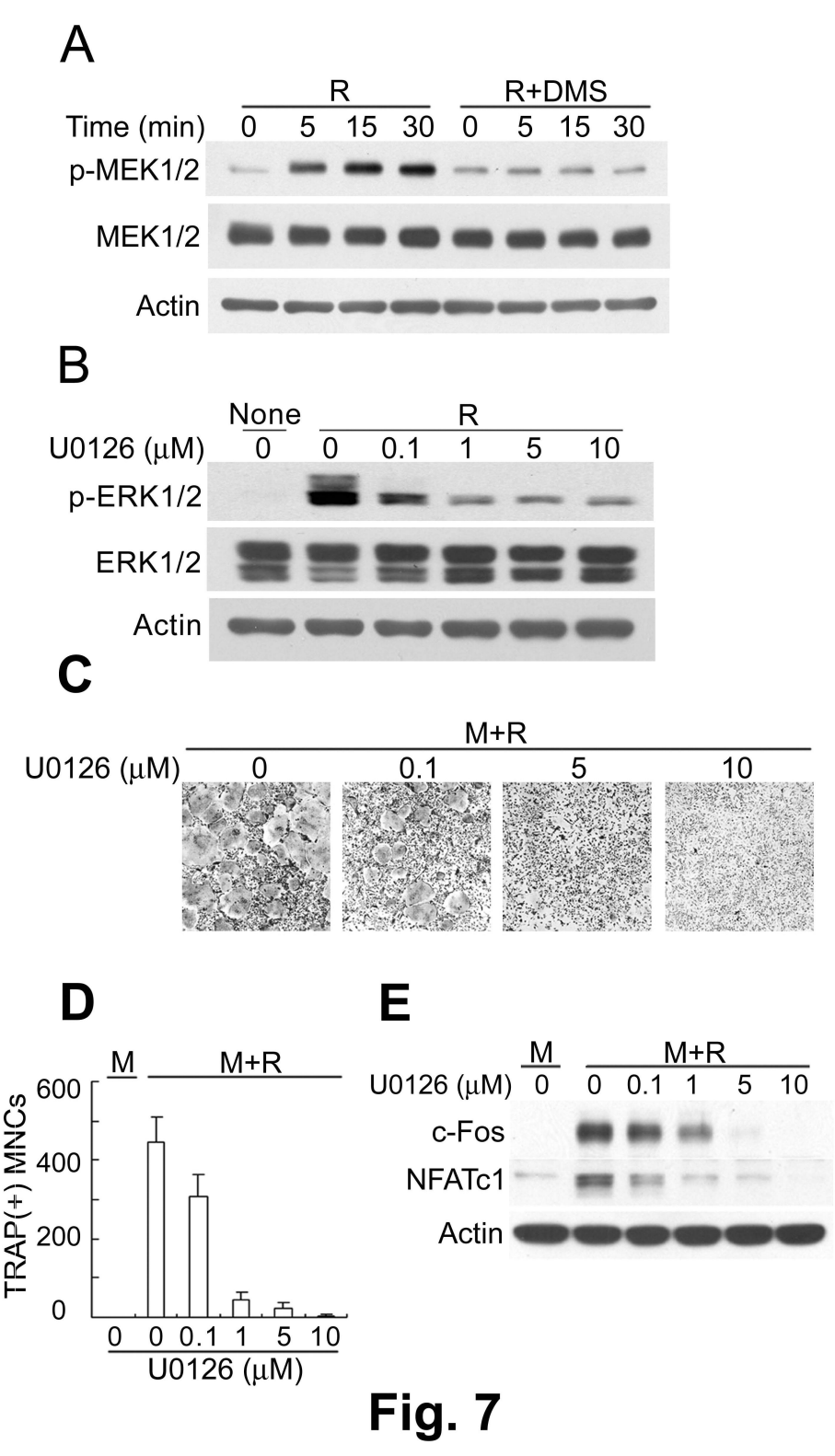

A
R
R+DMS
Time (min)
0 5 15 30 0 5 15 30
p-MEK1/2
MEK1/2
Actin
B
None
R
U0126 (μM)
0 0 0.1 1 5 10
p-ERK1/2
ERK1/2
Actin
C
M+R
U0126 (μM)
0 0.1 5 10
D
M
M+R
TRAP(+) MNCs
600
400
200
0
0 0 0.1 1 5 10
U0126 (μM)
E
M
M+R
U0126 (μM)
0 0 0.1 1 5 10
c-Fos
NFATc1
Actin

Fig. 7

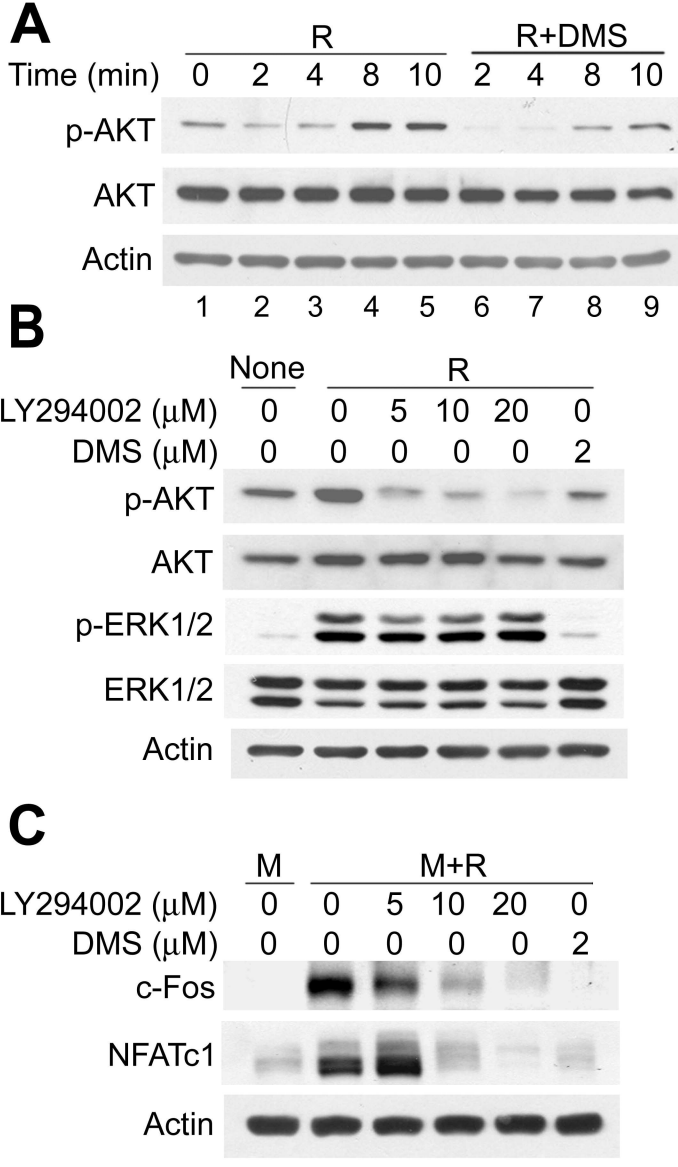

A
R
R+DMS
Time (min) 0 2 4 8 10 2 4 8 10
p-AKT
AKT
Actin
1 2 3 4 5 6 7 8 9
B
None
R
LY294002 (μM) 0 0 5 10 20 0
DMS (μM) 0 0 0 0 0 2
p-AKT
AKT
p-ERK1/2
ERK1/2
Actin
C
M
M+R
LY294002 (μM) 0 0 5 10 20 0
DMS (μM) 0 0 0 0 0 2
c-Fos
NFATc1
Actin

Fig. 8

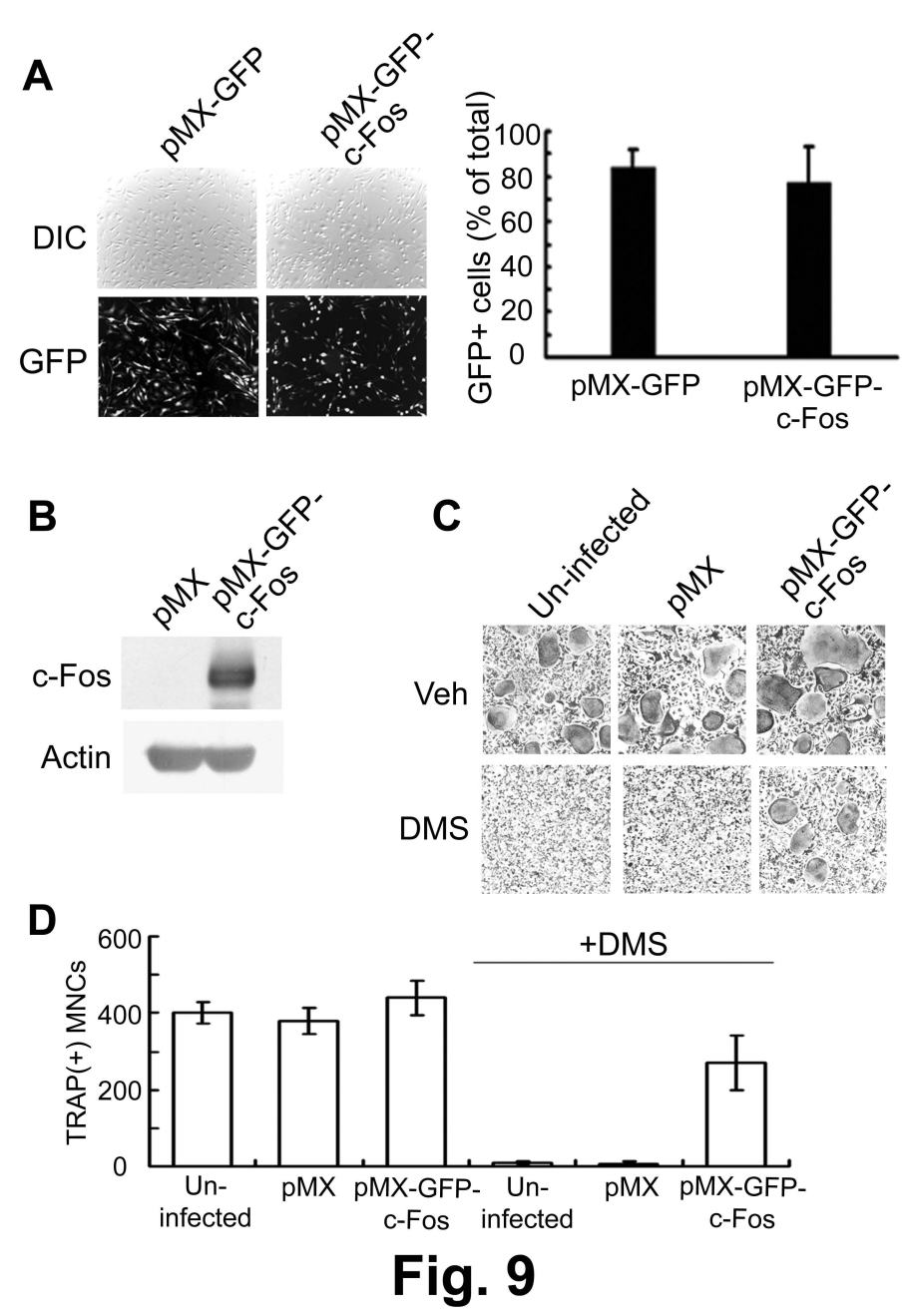

A
pMX-GFP
pMX-GFP-c-Fos
DIC
GFP
GFP+ cells (% of total)
100
80
60
40
20
0
pMX-GFP
pMX-GFP-c-Fos
B
pMX
pMX-GFP-c-Fos
c-Fos
Actin
C
Un-infected
pMX
pMX-GFP-c-Fos
Veh
DMS
D
TRAP(+) MNCs
600
400
200
0
+DMS
Un-infected
pMX
pMX-GFP-c-Fos
Un-infected
pMX
pMX-GFP-c-Fos

Fig. 9

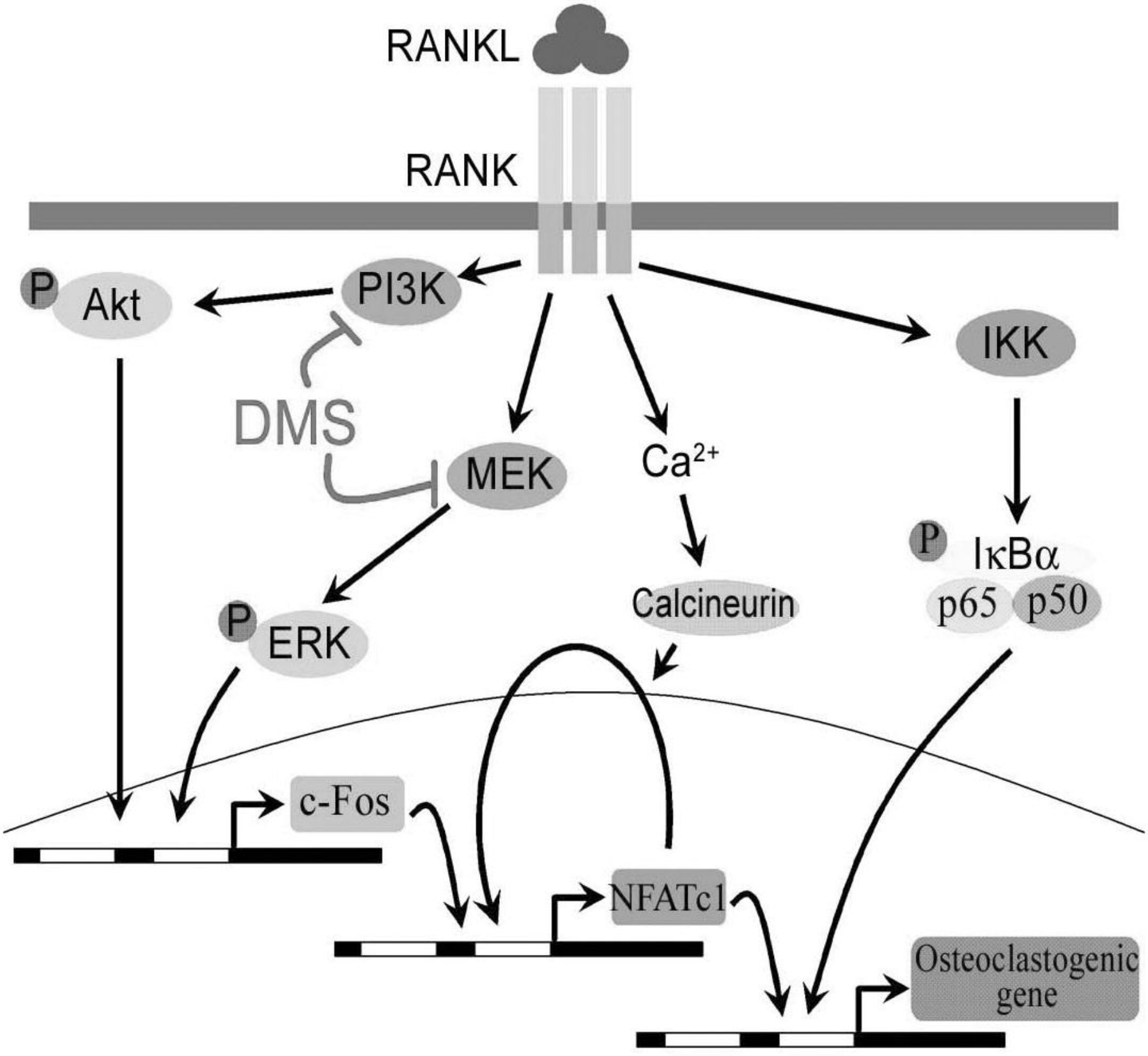

RANKL
RANK
P
Akt
PI3K
DMS
MEK
Ca2+
IKK
P
IκBα
p65
p50
Calcineurin
P
ERK
c-Fos
NFATc1
Osteoclastogenic gene

Fig. 10