



Acid sphingomyelinase regulates osteoclastogenesis by modulating sphingosine kinases downstream of RANKL signaling

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ABSTRACT

Acid sphingomyelinase (ASM) was identified as a gene induced by NFAT2 activation in osteoclasts. Suppression of ASM expression in bone marrow macrophages by knockdown enhanced c-Fos/NFAT2 expression, increasing the number of TRAP-positive multinucleated cells *in vitro*. SphK1 was upregulated during the late stage of osteoclastogenesis, while SphK2 expression remained constant. SphK1 was downregulated following ASM knockdown, while SphK2 levels were unchanged. Experiments using shRNA and catalytically-inactive form demonstrated inhibitory and stimulatory activities on osteoclast formation of SphK1 and SphK2, respectively. These results suggest that ASM regulates osteoclastogenesis by modulating the balance between SphK1 and SphK2 downstream of RANKL signaling.

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1. Introduction

Osteoclasts are multinucleated giant cells with bone-resorbing activity. Using a large-scale gene expression analysis using an *in vitro* osteoclastogenesis system, we identified NFAT2/c1 as a key regulator in the formation of TRAP-positive multinucleated (MN) cells [1]. We subsequently identified L-Ser as a factor contained within differentiation medium that is necessary for the induction of NFAT2 expression [2]. L-Ser can be converted into sphingolipids through a condensation reaction with palmitoyl-CoA mediated by serine palmitoyltransferase (SPT) [3,4]. We, therefore, identified an L-Ser analog, H-Ser(tBu)-OMe HCl, that suppresses the production of 3-ketodihydrosphingosine by SPT to function as an inhibitor of osteoclastogenesis *in vitro*. When administered *in vivo* to mice, the analog suppressed bone turnover [5]. These results implicated sphingolipid metabolism in osteoclastogenesis and bone remodeling. The detailed mechanism, including the molecules involved in this process, remains unclear.

We therefore searched for genes functioning downstream of NFAT2 [6], identifying acid sphingomyelinase (ASM) as one such gene. ASM catalyzes the cleavage of sphingomyelin to generate ceramide [3,5]. Ceramide is a bioactive sphingolipid implicated in the regulation of multiple fundamental cellular processes, including cell cycle arrest, apoptosis, senescence, and stress responses [3]. Multiple metabolic pathways converge upon ceramide; the

de novo, sphingomyelinase, and salvage pathways, in which SPT, ASM, and sphingosine kinase (SphK) play important roles, respectively, have been the subject of intense study [3,4,7]. Ceramide is broken down further by ceramidase to sphingosine, which is used to reproduce ceramide through reacylation. Sphingosine can also be phosphorylated by SphK to generate sphingosine 1-phosphate (S1P), thus modulating cellular ceramide concentrations [4]. The interconversion of sphingosine-ceramide is thus controlled by multiple pathways.

SphK, of which two distinct SphK isoforms, SphK1 and SphK2, have been cloned and characterized [8,9]. Although both SphKs are highly similar in amino acid sequence and possess five evolutionarily-conserved domains, SphK1 and SphK2 have been demonstrated to play distinctive functions in sphingolipid metabolism, cell proliferation, and apoptosis [4,10,11]. The mechanisms by which the expression and function of SphK1 and SphK2 are regulated, however, has been largely unclear. We determined that SphK1 and SphK2 play opposing roles in osteoclastogenesis and ASM differentially modulates the expression of SphK1 and SphK2. The significance of ASM as a NFAT2-inducible gene is also addressed.

2. Materials and methods

2.1. Osteoclastogenesis *in vitro* and materials

Mouse RAW264 cells and bone marrow macrophages (BMM) were maintained and stimulated to undergo osteoclastogenesis as described previously [2,5]. Purified RANKL (250 ng/ml) or

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purified GST (200 ng/ml) was used to stimulate osteoclastogenesis as described [2,5]. Medium was replaced every 3 days with fresh medium containing RANKL or GST. Sphingosine kinase inhibitor (SKI) was purchased from Calbiochem (CA, USA).

2.2. Immunoprecipitation and Western blotting

Cells were lysed in lysis buffer (20 mM Tris–HCl pH 7.5, 1% NP-40, 150 mM NaCl, and 1 mM EDTA) containing 2 mM Na_3VO_4 , 20 mM NaF, and protein inhibitor mix (Roche). Immunoprecipitation and Western blotting were performed as described previously [1,5] using the antibodies listed in Table S2.

2.3. Preparation of RNA and real-time RT-PCR

Quantification of ASM mRNA levels was performed by quantitative RT-PCR as follows. Total RNA was purified using an Isogen kit per the manufacturer's instructions (Nippon Gene). One microgram of purified total RNA was then used as a template for cDNA synthesis using a PrimerScript[®] II kit (Takara Bio). Target DNA amplification of the synthesized cDNA was performed using LightCycler 480[®] I Master Mix (Roche); the resulting products were characterized on a LightCycler 480[®] system (Roche) using GAPDH as an internal control. Primers used are shown in Table S1.

2.4. Generation of ASM knockdown cells

To suppress ASM expression, retroviral vectors encoding specific shRNA and negative control shRNA (Table S1) were prepared as described in supplementary information. To generate retrovirus, plasmids were first introduced into the platinum-E (Plat-E) packaging cell line with the pE-eco and pGp vectors (Takara Bio, Kyoto, Japan) and cultured for 72 h. Retroviral supernatants were harvested and filtered through a 0.45 μm pore filters before use as retrovirus stocks. BMM were infected with virus for 24 h, then selected in the presence of puromycin for two days prior to use in experiments as osteoclast precursors.

2.5. Construction of plasmids encoding SphK1/2 and expressing SphK2 shRNA

All of the primers used are listed in Table S1. The full-length mouse cDNA encoding SphK1 [8,9] was amplified by RT-PCR using cDNA prepared from RAW264 cells pre-stimulated with RANKL for 96 h, then cloned into the pBluescript II SK+ vector (Agilent Technologies). Point mutations in plasmids encoding SphK1 (G81D) were introduced by PCR-based mutagenesis. To generate retroviral vectors harboring HA-tagged SphK1, we sub-cloned DNA fragments encoding wild-type or mutant SphK1 into retroviral vectors, pCX4HM6-puro and pCX4H-N1-bsr, respectively. To construct retroviral vectors facilitating shRNA expression, targeting shRNA cassettes were prepared using a PCR-based method and ligated into the pCX6#PR vector between the XhoI and EcoRI sites. Additional details concerning plasmid construction and oligonucleotide sequences are provided in the Supplementary information.

2.6. Assay of ASM enzyme activity

BMM were lysed in 50 mM Tris (pH 7.4) using a probe sonicator. Cellular debris was removed by centrifugation at 3000 \times g for 10 min. Twenty-five micrograms of total cellular proteins were resuspended in 50 μl ; reactions were initiated by the addition of 50 μl reaction mixture containing 1 mM EDTA, 250 mM sodium acetate (pH 5.0), 40 μM [choline-methyl-¹⁴C]-sphingomyelin, and 0.1% Triton X. ASM activity was measured by quantitating the release of radioactive phosphocholine as described previously [12].

3. Results

3.1. ASM is NFAT2-inducible

Our previous microarray analysis searching for genes functioning downstream of NFAT2 [1,6] confirmed a clone encoding ASM to be a CsA-suppressible gene. As ASM has been implicated in the modulation of sphingolipid metabolism [7], we sought to investigate the significance of its induction in osteoclasts. We first examined the expression profile of ASM in mouse BMM. Quantitative RT-PCR analysis clearly revealed ASM expression during the late stage in M-CSF- and RANKL-stimulated BMM (Fig. 1A). This pattern of expression was confirmed at the protein level by Western blotting analysis (Fig. 1B). In contrast, CsA significantly suppressed these expression levels.

3.2. Effects of ASM expression in osteoclasts

To understand the significance of ASM induction downstream of NFAT2 in the late stages of osteoclastogenesis, we generated cells suppressing ASM and examined the resultant effect on osteoclastogenesis. The efficacy of ASM knockdown was confirmed first by Western blotting analysis and the decreased enzyme activity in ASM knockdown (KD) cells (Fig. 2A). Knockdown of ASM resulted in an increase in TRAP-positive MN cells with a significantly enlarged cell shape seen after RANKL treatment of bone marrow cells (Fig. 2A). These results suggested that ASM may negatively regulate the development of mature osteoclasts. Moreover, we observed the increased expression of both c-Fos and NFAT2 in ASM KD cells (Fig. 2B). c-Fos has been reported to be required for NFAT2 induction by RANKL [13]. To determine the molecular pathways involved, we also examined the expression/activation status of MAP kinases, known upstream effectors of c-Fos in osteoclasts; p38 appeared to be upregulated, suggesting that the p38-c-Fos-NFAT2 axis may mediate signals downstream of ASM.

To examine a possible correlation between intracellular ceramide levels and osteoclast induction, we prepared sphingolipid-free medium for our *in vitro* differentiation system. We then added

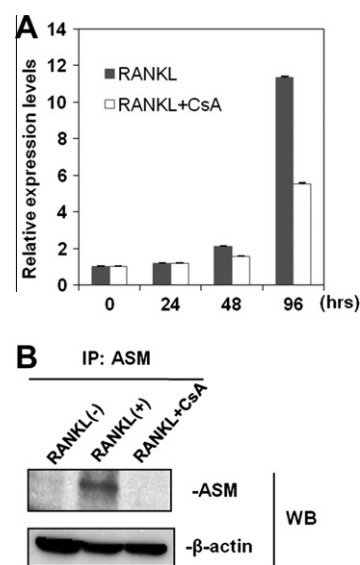


Fig. 1. Expression profile of ASM in osteoclastogenesis *in vitro*. BMM were induced to become osteoclasts for 96 h in the absence or presence of 1 $\mu\text{g}/\text{ml}$ cyclosporine A (CsA). (A) Quantitative RT-PCR analysis. Total RNA was prepared at the indicated time points and examined by quantitative PCR. (B) BMM were cultured as indicated for 96 h, after which ASM protein expression was assessed by immunoblot. We used β -actin-specific antibodies as a control.

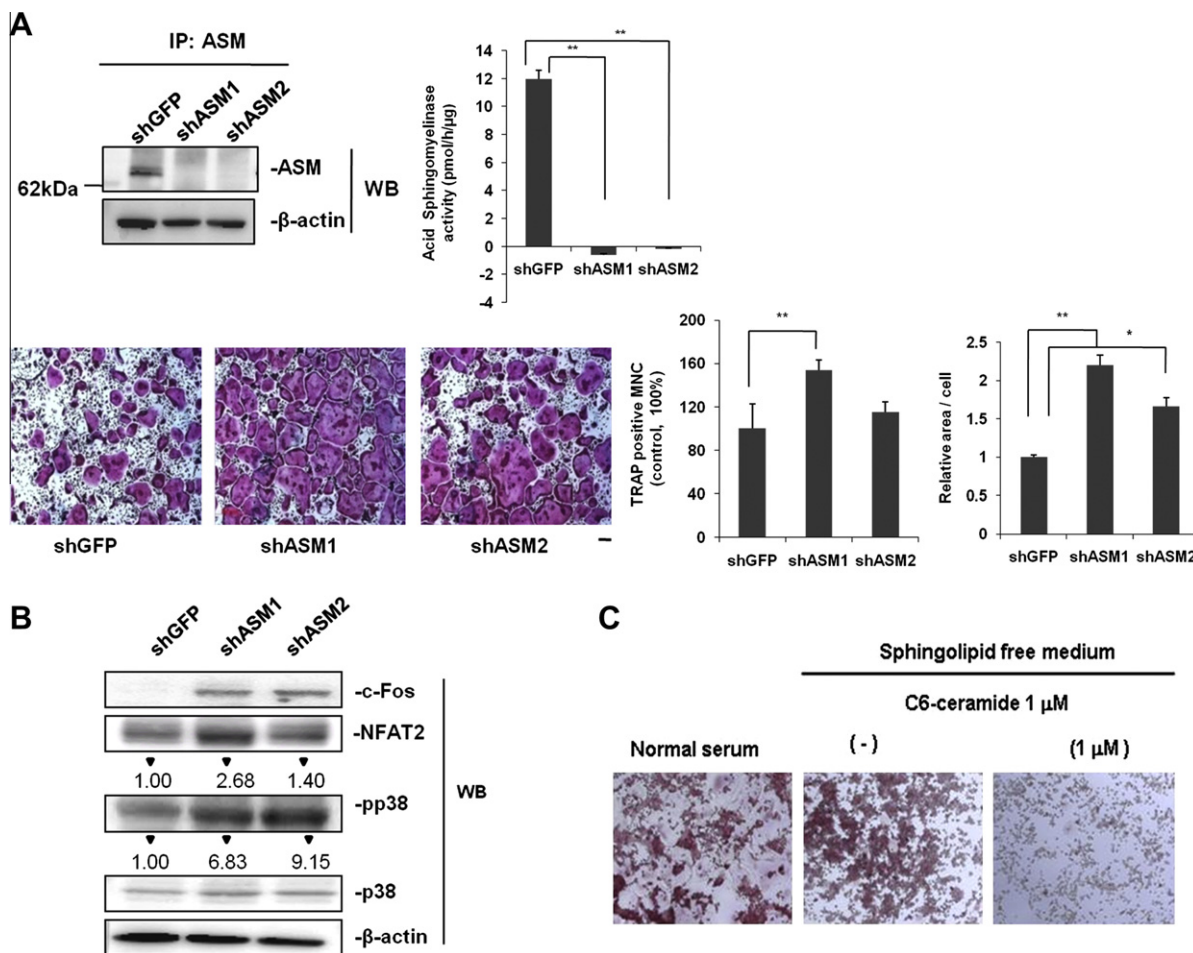


Fig. 2. Effect of ASM knockdown during osteoclastogenesis. (A) Knockdown of ASM. BMM infected with retroviral vectors expressing control shRNA (shGFP) or two kinds of shASM were cultured for 4 days with RANKL and M-CSF. Expression of ASM (upper left panel) and ASM activity (upper right panel) in two kinds of knockdown (KD) osteoclasts were confirmed. Cell morphology (lower left panel) and quantification of TRAP-positive MN cells and cell surface area (lower right panel). Bar, 50 μm. (B) Effects of ASM on intracellular signaling. Cell extracts were prepared from control (shGFP)-infected and two kinds of ASM KD osteoclasts. We examined c-Fos, NFAT2, and pp38/p38 expression by Western blotting using appropriate antibodies, including a positive control for β-actin. Densities in each row were quantified and intensities of NFAT2 and pp38 were normalized as relative values against β-actin and total p38, respectively (bottom numbers). (C) Effect of exogenously-added ceramide on RAW264 cells. Cells were cultured for 96 h in regular medium containing normal serum or sphingolipid-free medium in the presence or absence of 1 μM C6-ceramide. The formation of TRAP-positive multinucleated cells was evaluated by TRAP-staining. ** $p < 0.001$ and * $p < 0.05$.

exogenous C6-ceramide to examine the effect on osteoclast formation in our differentiation system [14]. The addition of ceramide to sphingolipid-free differentiation media almost completely suppressed the formation of TRAP-positive MN cells (Fig. 2C), supporting an inhibitory effect of ceramide on osteoclastogenesis and confirming the results of ASM knockdown experiments.

3.3. ASM differentially regulates SphK1 and SphK2 expression

Our results suggested that ceramide levels may be a determining factor in osteoclast formation. As ceramide levels are also regulated by SphK, as well as ASM [15], we examined the possible involvement of SphK in the regulation of osteoclastogenesis. First, we examined the expression profiles of SphK1 and SphK2 at both the mRNA and protein levels. We observed a significant induction of SphK1 mRNA expression at the late stages of osteoclastogenesis (after 48–96 h), while SphK2 mRNA levels remained relatively constant through the process (Fig. 3A). Similar expression patterns were observed at the protein level with moderate induction of SphK2 (Fig. 3A).

To further understand the function of SphK1 and SphK2 in osteoclasts, we examined the expression of SphK1 and SphK2 in

ASM KD cells. The protein levels of SphK2 were similar in the parental and KD cells, while SphK1 protein levels were significantly decreased after knockdown (Fig. 3B). Administration of 1 μM SKI for the first 24 h suppressed the formation of TRAP-positive multinucleated cells, which was accompanied by the downregulation of c-Fos and NFAT2 (Fig. 3C). Given its expression at the early stage and sensitivity to SKI [16], these results suggest that SphK2 activity plays an important role in the initiation of osteoclastogenesis.

3.4. SphK1 and SphK2 show opposing effects on osteoclast formation

We then investigated the roles of SphK1 and SphK2 in osteoclastogenesis. Expression of shRNA specific for SphK2 reduced the number of TRAP-positive MN cells, which was accompanied by the downregulation of c-Fos (Fig. 4A), further supporting a role for SphK2 as a positive regulator of osteoclast formation. In contrast, while SphK1 overexpression completely suppressed osteoclast formation, the expression of a catalytically-inactive form of SphK1 (G81D) [17] showed no such effect (Fig. 4B), suggesting SphK1 may function as a negative regulator of osteoclastogenesis through its enzyme activity. These results suggest that Sphk1

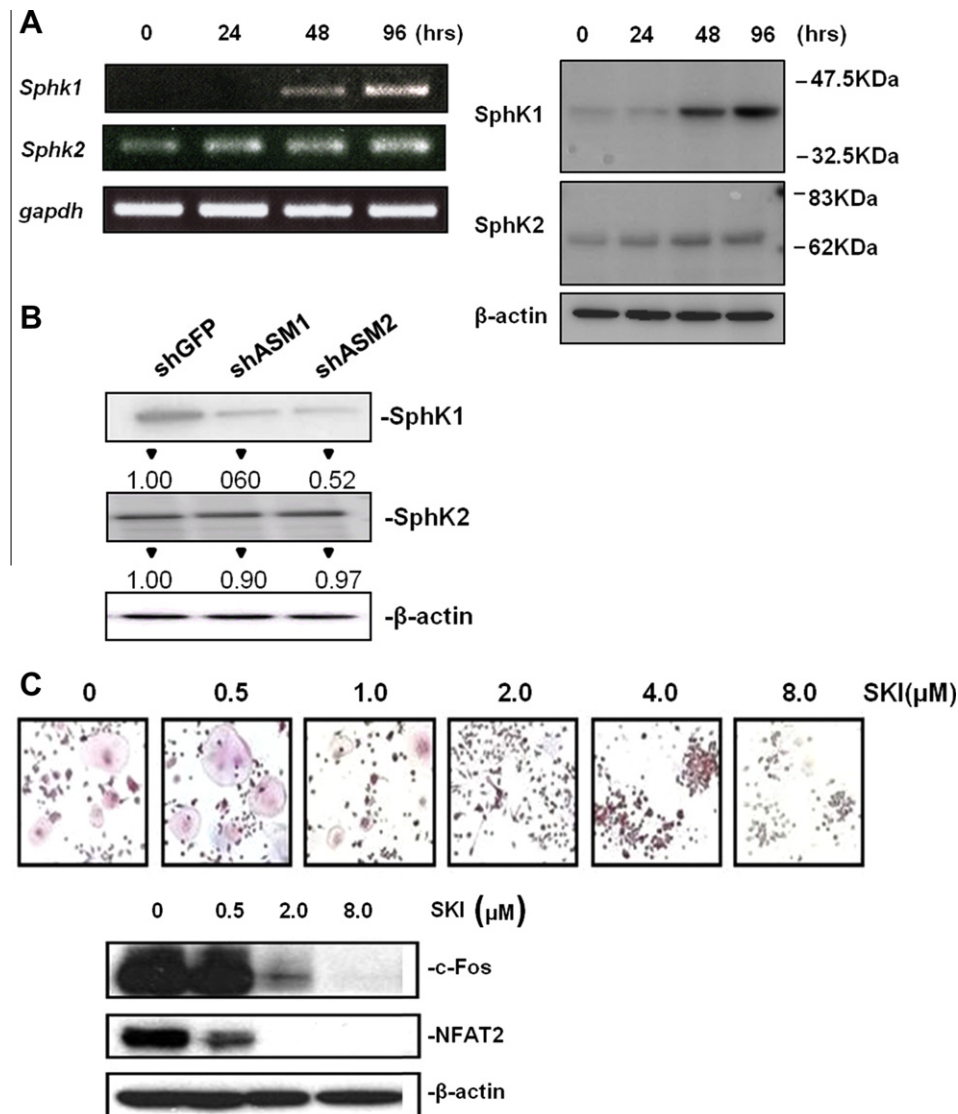


Fig. 3. SphK1 and SphK2 expression profiles. (A) Expression profiles of SphK1 and SphK2 in BMM. mRNA and protein expression was determined by RT-PCR (left panel) and Western blotting (right panel), respectively, at the indicated time points. (B) SphK1 and SphK2 expression in ASM KD osteoclasts. Cell extracts were prepared from control (shGFP)-infected cells as well as two kinds of ASM KD cells (shASM1 and shASM2). The expression of SphK1 and SphK2 was examined by Western blotting using the respective antibodies. Densities in each row were quantified (bottom numbers). (C) Effect of sphingosine kinase inhibitor (SKI) on osteoclast formation. Osteoclast differentiation of RAW264 cells was induced for 96 h in the presence of the indicated concentrations of SKI for the first 24 h. We observed the effect on cell morphologies (upper panel) and the expression of c-Fos and NFAT2 at 24 h (lower panel).

and SphK2 may act as negative and positive regulators of osteoclastogenesis, respectively, and their unique expression profiles in osteoclastogenesis may reflect these distinctive roles in osteoclastogenesis. Thus, ASM may participate in the control of osteoclastogenesis in conjunction with SphK1/2.

4. Discussion

Here, we identified ASM as a gene induced by NFAT2 during the late stages of osteoclastogenesis. Knockdown experiments revealed ASM to be a negative regulator of osteoclast formation. One prominent effect of ASM was to differentially affect SphK1 and SphK2 expression at the protein level; SphK1 expression was downregulated, while SphK2 remained unaffected. SphK1 and SphK2 appeared to exert opposing roles on the regulation of ceramide biosynthesis [10,18]. This study further implicated that the effect of these enzymes on sphingolipid metabolism mediated the negative and positive regulation of SphK1 and SphK2 on osteoclastogenesis, respectively. The exact molecular mechanism by

which sphingolipids, including ceramide, sphingosine, and sphingosine-1-phosphate [4], influence osteoclast formation remain unclear at present. Regardless, the differences in SphK1 and SphK2 expression profiles and functions suggest that relative SphK1 and SphK2 levels may regulate the progression of osteoclastogenesis downstream of ASM.

The crucial role of the c-Fos-NFAT2 axis in osteoclastogenesis is well established [1,13,19]; we confirmed the induction of c-Fos expression in ASM KD cells. Ryu et al. reported a role for SphK1 in osteoclastogenesis and the involvement of c-Fos/NFAT2 expression in this process [20]. SphK2 was recently identified as a positive inducer of c-Fos via HDAC inhibition of the c-fos promoter in MCF7 cells [11]. In this study, we confirmed the involvement of ASM in this regulatory machinery; the ASM-SphK axis appears to regulate the progression of osteoclastogenesis through the c-Fos/NFAT2 pathway. In conclusion, we revealed that ASM may regulate osteoclastogenesis downstream of RANKL signaling by modulating SphK1 and SphK2 regulation of sphingolipid metabolism.

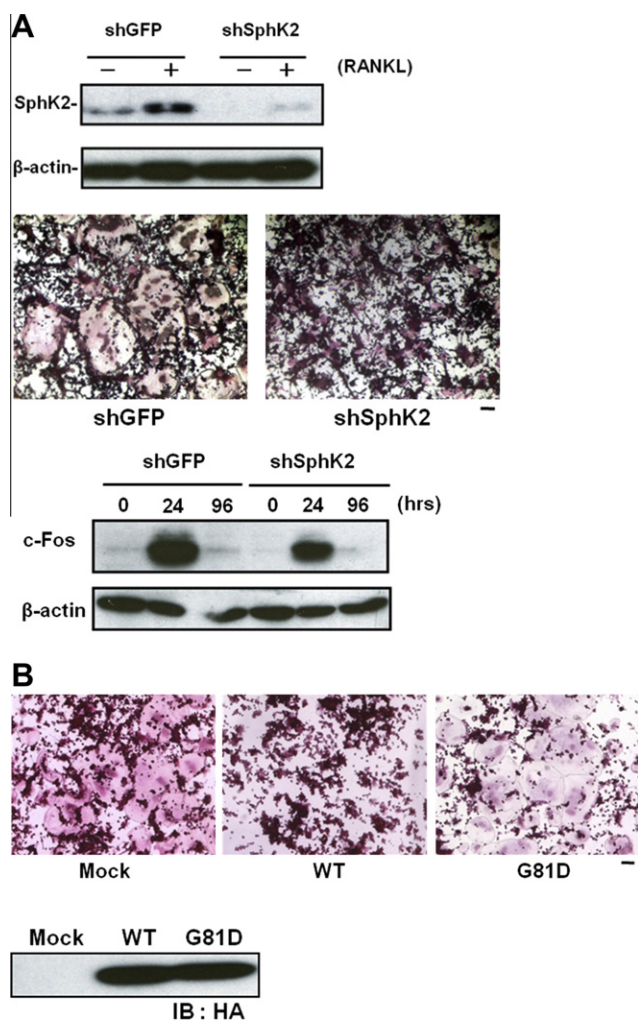


Fig. 4. Opposing functions of SphK1 and SphK2 in osteoclastogenesis. (A) Introduction of shSphK2 into RAW264 cells. After introducing a retroviral vector encoding a shRNA specific for SphK2 into RAW264 cells, we examined the expression level of SphK2 (upper panel) and its effect on the formation of TRAP-positive multinucleated cells (middle panel). We also examined the expression of c-Fos in those cells (lower panel). (B) Overexpression of SphK1 in RAW264 cells. Two forms of SphK1, wild-type and a variant containing encoding a catalytically-inactive form of SphK1 (G81D) were expressed in RAW264 cells. We then evaluated the formation of TRAP-positive multinucleated cells (upper panel) and the expression of each protein by Western blot with anti-HA antibody (lower panel). Bar, 200 μ m.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.061.

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