Arch. Pharm. Res. (2019) 42:712–721
https://doi.org/10.1007/s12272-019-01163-8

Online ISSN 1976-3786
Print ISSN 0253-6269

Archives of Pharmacal Research
www.springer.com/12272

RESEARCH ARTICLE

Idelalisib inhibits osteoclast differentiation and pre-osteoclast migration by blocking the PI3Kδ-Akt-c-Fos/NFATc1 signaling cascade

Jeong-Tae Yeon[1] · Kwang-Jin Kim[2] · Young-Jin Son[2] · Sang-Joon Park[3] · Seong Hwan Kim[4]

Received: 11 June 2018 / Accepted: 21 May 2019 / Published online: 3 June 2019

Abstract Since increased number of osteoclasts could lead to impaired bone structure and low bone mass, which are common characteristics of bone disorders including osteoporosis, the pharmacological inhibition of osteoclast differentiation is one of therapeutic strategies for preventing and/or treating bone disorders and related facture. However, little data are available regarding the functional relevance of phosphoinositide 3-kinase (PI3K) isoforms in the osteoclast differentiation process. To elucidate the functional involvement of PI3Kδ in osteoclastogenesis, here we investigated how osteoclast differentiation was influenced by idelalisib (also called CAL-101), which is p110δ-selective inhibitor approved for the treatment of specific human B cell malignancies. Here, we found that receptor activator of nuclear factor kappa B ligand (RANKL) induced PI3Kδ protein expression, and idelalisib inhibited RANKL-induced osteoclast differentiation. Next, the inhibitory effect of idelalisib on RANKL-induced activation of the Akt-c-Fos/NFATc1 signaling cascade was confirmed by western blot analysis and real-time PCR. Finally, idelalisib inhibited pre-osteoclast migration in the last stage of osteoclast differentiation through down-regulation of the Akt-c-Fos/NFATc1 signaling cascade. It may be possible to expand the clinical use of idelalisib for controlling osteoclast differentiation. Together, the present results contribute to our understanding of the clinical value of PI3Kδ as a druggable target and the efficacy of related therapeutics including osteoclastogenesis.

Keywords Phosphoinositide 3-kinases · Idelalisib · Osteoclast differentiation

Introduction

Bone homeostasis requires a tightly maintained balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption (Takayanagi 2005). Skeletal disorders, such as osteoporosis, Paget's disease, rheumatoid arthritis, and periodontal disease, are characterized by low bone density, which is mainly caused by excessive activity and/or increased numbers of osteoclasts rather than impaired osteoblastic bone formation (Rodan and Martin 2000; Khosla and Riggs 2005; Manolagas and Parfitt 2010). Therefore, most presently available therapeutics have been developed to mitigate the extent of bone loss and reduce bone loss-related fractures through the inhibition of osteoclast differentiation and/or osteoclastic activity (Marie and Kassem 2011).

Bone resorbing osteoclasts are multinucleated giant cells—also called multinucleated osteoclast cells

Jeong-Tae Yeon and Kwang-Jin Kim have equally contributed to this study.

✉ Sang-Joon Park
psj26@knu.ac.kr

✉ Seong Hwan Kim
hwan@krict.re.kr

[1] Research Institute of Basic Science, Sunchon National University, Suncheon, Republic of Korea

[2] Department of Pharmacy, Sunchon National University, Suncheon, Republic of Korea

[3] Department of Histology, College of Veterinary Medicine, Kyungpook National University, 80 Daehakro, Bukgu, Daegu 41566, Republic of Korea

[4] Innovative Target Research Center, Bio & Drug Discovery Division, Korea Research Institute of Chemical Technology, 141 Gajeong-ro, Yuseong-gu, Daejeon 334114, Republic of Korea

(MNCs)—derived from hematopoietic stem cells. Osteoclast development is a complex multi-step process involving differentiation, migration and fusion, which is triggered by two critical factors: macrophage/monocyte colony-forming factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) (Feng 2005). Notably, RANKL induces the expression of osteoclastogenesis-related genes by activating several essential signaling molecules and transcription factors, including c-Fos and nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) (Feng 2005; Takayanagi 2005; Takayanagi 2007a). The resulting MNC formation is essentially responsible for mineralized matrix degradation (Nakamura et al. 2012).

Phosphoinositide 3-kinases (PI3Ks) belong to a large family of lipid signaling kinases that are considered potential drug targets. PI3Ks are divided into three classes (I, II, and III) based on their sequence homology and substrate specificity (Vanhaesebroeck et al. 2001; Cantley 2002; Foster et al. 2003). Moreover, class I PI3Ks are subcategorized into two groups: class IA, which comprises three isoforms, PI3Kα (p110α), PI3Kβ (p110β), and PI3Kδ (p110δ); and class IB, which includes only PI3Kγ (p110γ) (Foster et al. 2003). Class I PI3Ks are implicated in the maintenance of various diseases—including cancer, inflammation, and autoimmunity—prompting pharmaceutical companies to focus on the development of class I PI3K inhibitors (Stark et al. 2015; Vanhaesebroeck et al. 2016). Interestingly, the PI3Kδ pathway is overactivated in types of B-cell malignancies (Herman et al. 2010; Ikeda et al. 2010; Lannutti et al. 2011; Meadows et al. 2012; Pauls et al. 2012). These findings have directed research focus towards the development of p110δ-selective inhibitors (Fruman and Rommel 2011; Norman 2011).

While several pharmacological studies have reported the functional importance of PI3Ks in the function of mature osteoclasts, relatively little data are available regarding the functional relevance of PI3K isoforms in the process of osteoclast differentiation. Therefore, in our present study, we investigated the effect of idelalisib on osteoclast differentiation with the aim of elucidating the functional involvement of PI3Kδ in the osteoclastogenesis.

Materials and methods

Reagents

Mouse soluble RANKL and M-CSF were purchased from R&D Systems (Minneapolis, MN, USA). Penicillin, streptomycin, cell culture medium, and fetal bovine serum (FBS) were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Idelalisib was purchased from Selleckchem (Houston, TX, USA). Antibodies against c-Fos, NFATc1, actin, PI3K-β and PI3K-δ were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against p-Akt (Ser 473), p-Akt (Thr 308), Akt, p-ERK, ERK, p-p38, p38, p-JNK, JNK, PI3K-α, and PI3K-γ were obtained from Cell Signaling Technology (Danvers, MA, USA).

Osteoclast differentiation

Isolation of bone marrow cells (BMCs) from 5-week-old male ICR mice (Damool Science, Daejeon, Korea) was carried out in strict accordance with the recommendations in 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. Mice were euthanized by cervical dislocation, and then BMCs were obtained by flushing isolated femurs and tibias with α-MEM supplemented with antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). BMCs were cultured for 1 day on a culture dish in α-MEM supplemented with 10% FBS and 10 ng/mL of M-CSF. Non-adherent BMCs were plated on a Petri dish and cultured in humidified 5% CO_2 at 37 °C 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). When BMMs were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) for 3 days, most of cells differentiated into tartrate-resistant acid phosphatase-positive ($TRAP^+$)-mononuclear osteoclasts, and $TRAP^+$-MNCs generated by the fusion between each mononuclear cells were observed in the differentiation day 4. Therefore, for the complete formation of $TRAP^+$-MNCs, BMMs (1×10^4 cells/well in a 96-well plate or 3×10^5 cells/well in a 6-well plate) were cultured with M-CSF and RANKL for 4 days, and most of small round mononuclear $TRAP^+$-cells generated from BMMs incubated with M-CSF and RANKL for 3 days were considered to pre-osteoclasts (Takeshita et al. 2000).

TRAP staining and activity assay

Mature osteoclasts were visualized by staining for TRAP, a biomarker of osteoclast differentiation. Briefly, BMMs-derived MNCs 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, St. Louis, MO, USA). $TRAP^+$-MNCs with three or more nuclei were counted as mature osteoclasts. To measure TRAP activity, the permeabilized

cells were incubated 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 transferred into 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 (PerkinElmer, Turku, Finland).

Cytotoxicity assay

Cytotoxicity was evaluated by quantitatively measuring lactate dehydrogenase (LDH). Briefly, BMMs (1×10^4 cells/well) were seeded in a 96-well plate and incubated for 24 h. Then, cells were incubated with idelalisib in the presence of M-CSF (30 ng/mL) for 3 days, and released LDH in culture supernatants was detected using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The absorbance was measured at 492 nm using Wallac EnVision microplate reader.

Western blot analysis

Western blot analysis was performed as described previously (Yeon et al. 2014). Briefly, cells were washed, lysed, and centrifuged at 10,000 × *g* for 15 min. After protein quantification, proteins were denatured, separated on SDS-PAGE gels, and transferred onto PVDF membranes (EMD Millipore, Burlington, MA, USA). After probing with antibody, the membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Fisher Scientific) and visualized with LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). Actin was used for the loading control. Densitometric analysis was performed using ImageJ software (https://imagej.nih.gov/ij/download.html).

Real-time PCR

Real-time PCR was performed as described previously (Yeon et al. 2014). Primers were chosen with the online Primer3 design program (Rozen and Skaletsky 2000). The primer sets used in this study were listed in Table 1. Briefly, total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific), and the first-strand cDNA was synthesized with the Omniscript RT kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. SYBR green-based QPCR was performed in Stratagene Mx3000P Real-Time PCR system (Thermo Fisher Scientific) by using Brilliant SYBR Green Master Mix (Thermo Fisher Scientific). All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Gene encoding hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as the internal standard, and the statistical significance was determined with *HPRT1*-normalized $2^{-\Delta\Delta CT}$ values.

Cell migration assay

The migratory ability of pre-osteoclasts was measured in Boyden chamber with modifications (Yeon et al. 2014). Briefly, after generating pre-osteoclasts, cells were resuspended with α-MEM medium containing 0.1% FBS, M-CSF (30 ng/mL), RANKL (10 ng/mL), and/or idelalisib. α-MEM medium (30 μL) containing 10% FBS and/or idelalisib was added into the bottom chamber, and after placing over the gelatin-coated membrane filter, the silicone gasket, and the top chamber, cell suspension (2×10^4 cells/50 μL) was added into the top chamber, followed by culture in humidified 5% CO_2 at 37 °C for 12 h. Then, cells in the upper surface of the membrane were carefully removed with a cotton swab, and pre-osteoclasts that had migrated across the membrane to the lower surface of the membrane were fixed and stained with Diff-Quik stain kit (Siemens Healthcare, Erlangen, Germany). The number of migrated cells were counted in random areas of membrane.

Statistical analysis

All experiments were performed in triplicate and all quantitative values were presented as mean ± SD. Statistical differences were analyzed using Student's t-test or ANOVA with post hoc analysis using GraphPad Prism 5

Table 1 Primer sequences used in this study

Target gene	Forward Primer (5′–3′)	Reverse primer (5′–3′)
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
TRAP	GATGACTTTGCCAGTCAGCA	ACATAGCCCACACCGTTCTC
OSCAR	AGGGAAACCTCATCCGTTTG	GAGCCGGAAATAAGGCACAG
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
ATP6vOd2	AGACCACGGACTATGGCAAC	CGATGGGTGACACTTGGCTA
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
HPRT1	TGCTCGAGATGTCATGAAGG	AGAGGTCCTTTTCACCAGCA

(GraphPad Software, San Diego, CA, USA), and a value of $P < 0.05$ was considered significant.

Results

PI3K isoforms are increased during osteoclast differentiation

To investigate how PI3K isoforms are involved in osteoclast differentiation, we evaluated their protein expression levels during RANKL-induced commitment of BMMs into osteoclasts. As shown in Fig. 1, western blot analysis confirmed expression of all isoforms in BMMs, and revealed that the expression levels of PI3Kα, γ, and δ were temporally increased by RANKL treatment. In addition, PI3Kα, β, and γ were strongly induced during the late stage of osteoclast differentiation, while PI3Kδ was strongly induced in the early stage compared to the other isoforms.

The RANKL-mediated induction of c-Fos has been known to be required for the auto-amplification of NFATc1, enabling the robust induction of NFATc1 during osteoclastogenesis (Asagiri and Takayanagi 2007). To confirm the RANKL-induced commitment of BMMs into osteoclasts, we evaluated transcription factors related to osteoclast differentiation, c-Fos and NFATc1, and found that they were temporally induced by RANKL, as expected; RANKL strongly induced the protein expression of c-Fos 1 ~ 2 days after its treatment, and then the subsequential induction of NFATc1 was observed.

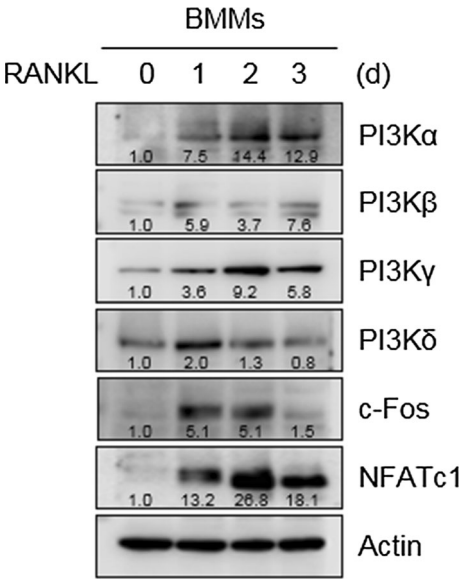

Fig. 1 Increased protein expression patterns of PI3K isoforms during osteoclast differentiation. In the presence of M-CSF (30 ng/mL), BMMs were cultured with RANKL (10 ng/mL) for the indicated number of days, and then the protein expression levels of the PI3K isoforms were evaluated by western blot analysis. The relative ratios of PI3K isoforms/actin, c-Fos/actin and NFATc1/actin were presented. The numbers are the intensity compared with that of the control

Pharmacological PI3Kδ inhibition by idelalisib inhibits RANKL-induced osteoclast differentiation

Idelalisib (Fig. 2a) is one of p110δ-selective inhibitors. Although PI3Kα, β, γ, and δ were all induced during RANKL-induced osteoclast differentiation of BMMs, the following experiments mainly focused on the use of idelalisib to investigate the biological relevance of PI3Kδ inhibition in osteoclast differentiation. Interestingly, idelalisib dose-dependently inhibited $TRAP^+$-MNC formation (Fig. 2b). We confirmed its inhibitory effect on osteoclast differentiation by counting the number of $TRAP^+$-MNCs (Fig. 2c) and measuring TRAP activity (Fig. 2d).

We could not exclude the possibility that the RANKL-induced commitment of BMMs into osteoclasts might be inhibited by cytotoxicity of idelalisib towards BMMs. Therefore, we further evaluated idelalisib's effect on BMM survival by measuring the activity of LDH, a stable cytosolic enzyme that is released upon cell lysis. As shown in Fig. 2e, idelalisib exhibited no cytotoxicity in BMMs, indicating that its anti-osteoclastogenic activity was not due to cytotoxicity.

Idelalisib inhibits RANKL-induced activation of the Akt-c-Fos/NFATc1 signaling cascade

To elucidate idelalisib's anti-osteoclastogenic mechanism, we performed western blot analysis to evaluate how idelalisib affected the activation of osteoclastogenesis-related signaling molecules, including Akt, ERK, p38, and JNK. As shown in Fig. 3a, idelalisib attenuated the RANKL-induced phosphorylation of Akt at serine 473, while the other molecules were not changed.

We further examined idelalisib's effects on the RANKL-induced protein expressions of c-Fos and NFATc1, which are master transcriptional regulators of complete osteoclastogenesis. The IC_{50} of idelalisib on TRAP activity was approximately 3 μM (Fig. 2d); therefore, the following experiments were performed using 3 μM idelalisib. In BMMs treated with idelalisib during their differentiation into osteoclasts, we observed inhibition of the RANKL-mediated induction of c-Fos and NFATc1 at 24 and 48 h after RANKL treatment, respectively (Fig. 3b). Real-time PCR analysis confirmed that idelalisib strongly inhibited NFATc1 expression (Fig. 3c).

To additionally confirm that idelalisib inhibited osteoclastogenesis-related transcription factors, we evaluated the mRNA expression levels of the osteoclastogenesis-related genes encoding TRAP, osteoclast-associated receptor

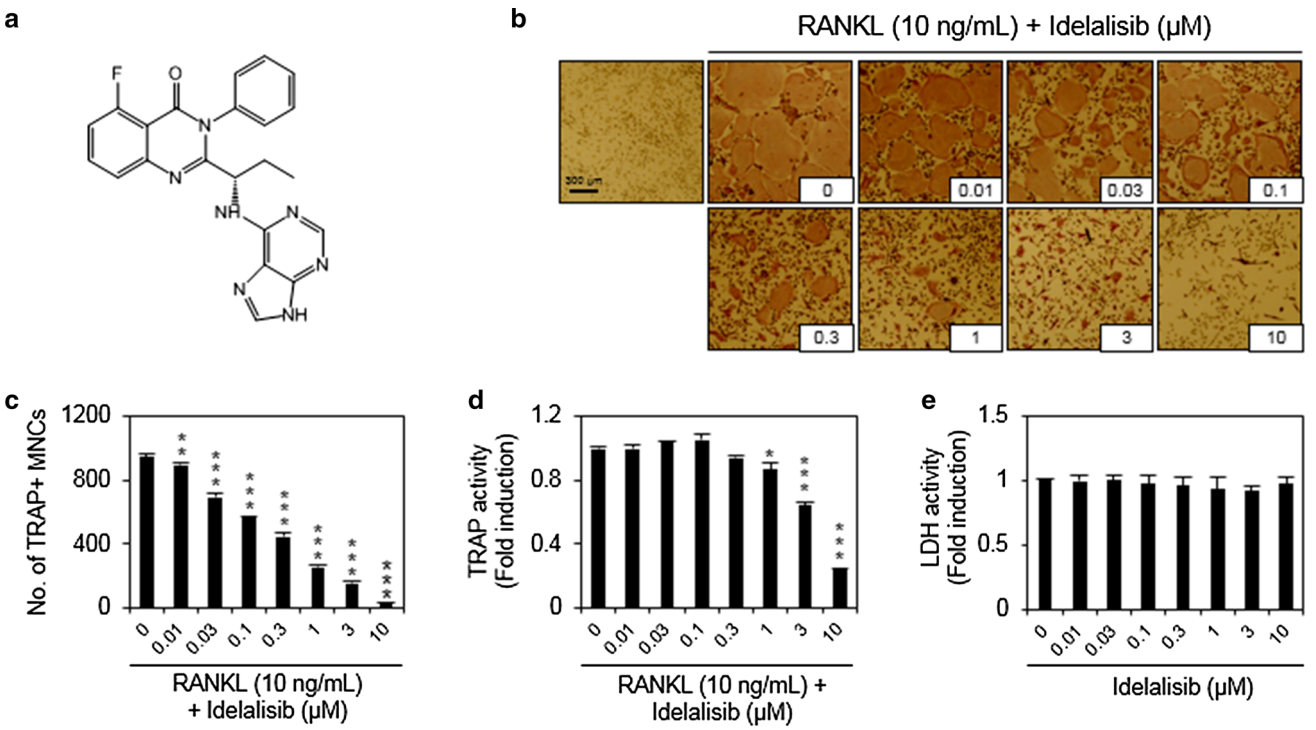

Fig. 2 Idelalisib inhibits the osteoclast differentiation of BMMs. **a** Chemical structures of idelalisib. **b–d** In the presence of M-CSF (30 ng/mL), BMMs were pretreated with the vehicle control (0.1% DMSO) or idelalisib for 5 min and then cultured with RANKL (10 ng/mL) for 4 days. Then, the effect of idelalisib on the RANKL-induced differentiation of BMMs into osteoclasts was evaluated by TRAP staining (b; magnification, 100 ×), counting the $TRAP^+$-MNCs with ≥ 3 nuclei (**c**), and measuring TRAP activity (**d**). $TRAP^+$-MNCs were photographed under a light microscope with 100 × magnification. **e** The cytotoxicity of idelalisib towards BMMs was evaluated by measuring LDH activity. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

(OSCAR), dendritic cell-specific transmembrane protein (DC-STAMP), d2 isoform of vacuolar (H^+) ATPase V_0 domain (ATP6v0d2), and cathepsin K. As shown in Fig. 3d, idelalisib significantly inhibited the mRNA expression levels of each of these genes. Overall, these results suggested that idelalisib's anti-osteoclastogenic activity could be due to its specific inhibition of PI3Kδ, which subsequently downregulates the expression of osteoclastogenesis-related genes by inhibiting the RANKL-induced activation of the Akt-c-Fos/NFATc1 signaling axis.

Idelalisib inhibits the last stage of osteoclast differentiation

To determine what stages of osteoclast differentiation were affected by idelalisib, we reevaluated idelalisib's effects on RANKL-induced osteoclast differentiation by treating cells at eight time-points, (b)-(i), as shown in Fig. 4a. Interestingly, idelalisib strongly inhibited $TRAP^+$-MNC formation when BMMs were incubated with idelalisib from differentiation day 3 to 4, (b–e) in Fig. 4b, c. Additionally, idelalisib moderately inhibited $TRAP^+$-MNC formation when BMMs were continuously incubated with idelalisib from day 0 to day 3, (i) in Fig. 4b, c. Anti-osteoclastogenic activity of idelalisib was clarified by visualizing the accumulation of mononuclear cells in the microscopic images, (b–e) and (i) in Fig. 4b, as well as measuring the number of TRAP + -MNCs (Fig. 4c). On the other hand, osteoclast differentiation was not impacted by incubation with idelalisib for only 1 day within days 0 to 3, (f–h) in Fig. 4b, c.

Idelalisib inhibits pre-osteoclast migration by blocking activation of the Akt-c-Fos/NFATc1 signaling cascade

At the last stage of osteoclast differentiation, pre-osteoclasts migrate and fuse to each other to generate MNCs. Thus, our results suggested that the pharmacological inhibition of PI3Kδ by idelalisib might affect pre-osteoclast migration during osteoclast differentiation. To investigate this possibility, we used the Boyden chamber migration assay to examine the effect of idelalisib on the migration of pre-osteoclasts. As shown in Fig. 5a, b, the migration of pre-osteoclasts was significantly and dose-dependently inhibited by idelalisib.

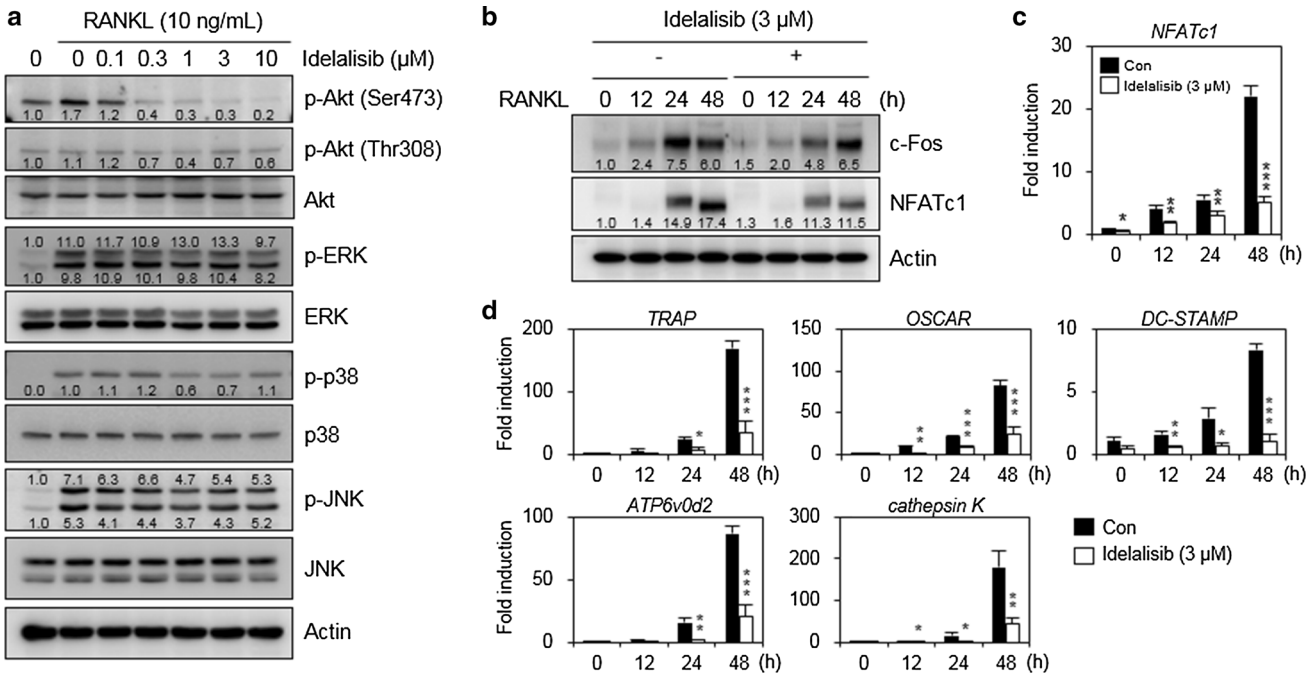

Fig. 3 Idelalisib inhibits RANKL-mediated activation of the Akt-NFATc1 signaling cascade. **a** In the presence of M-CSF (30 ng/mL), BMMs were pretreated with idelalisib for 5 min, and then incubated with RANKL (10 ng/mL) for 5 min. Next, western blot analysis was performed to evaluate the effect of idelalisib on the RANKL-induced activation of signaling molecules. The relative ratios of p-Akt/Akt, p-ERKs/ERK, p-p38/p38 and p-JNKs/JNKs were presented. The numbers are the intensity compared with that of the control. **b–d** In the presence of M-CSF (30 ng/mL), BMMs were pretreated with idelalisib (3 μM) for 5 min, and then incubated with RANKL (10 ng/mL) for the indicated time. Then, western blot analysis was performed to evaluate the effect of idelalisib on the RANKL-mediated induction of c-Fos and NFATc1. The relative ratios of c-Fos/actin and NFATc1/actin were presented. The numbers are the intensity compared with that of the control (**b**). Real-time PCR analysis was also performed to evaluate the effects of idelalisib on the RANKL-mediated mRNA inductions of NFATc1 (**c**) and osteoclastogenesis-related genes (**d**). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

We additionally performed western blot analysis to confirm the inhibitory effect of idelalisib on downstream molecules of PI3Kδ, such as Akt, c-Fos, and NFATc1, in pre-osteoclasts. As shown in Fig. 5c, idelalisib inhibited the RANKL-induced phosphorylation of Akt at serine 473, but not at threonine 308, in pre-osteoclasts. Moreover, idelalisib inhibited the RANKL-mediated induction of c-Fos and NFATc1 protein expression in pre-osteoclasts.

Discussion

Several pharmacological studies have investigated the contributions of specific PI3K isoforms to mature osteoclast's function. Wortmannin is a pan-PI3K inhibitor that shows little selectivity within the PI3K family (IC_{50}:-PI3Kα, 1 nM; IC_{50}:PI3Kβ, 10 nM; IC_{50}:PI3Kγ, 9 nM; and IC_{50}:PI3Kδ, 5 nM) and reports have described its inhibitory effects on bone resorptive activity, attachment, spreading, and chemotaxis of osteoclasts (Nakamura et al. 1995; Lakkakorpi et al. 1997; Pilkington et al. 1998; Juss et al. 2012). On the other hand, GS-9820 is a selective PI3Kδ inhibitor with an IC_{50}:PI3Kα of 5.44 μM, IC_{50}:PI3Kβ of 3.38 μM, IC_{50}:PI3Kγ of 1.40 μM, and IC_{50}:PI3Kδ of 12.7 nM. A recent pharmacological inhibition study used wortmannin and GS-9820 to verify that PI3Kδ plays a critical role in regulating the osteoclast cytoskeleton and the resorptive activity of mature osteoclasts (Shugg et al. 2013).

Research groups have also examined how specific PI3K isoforms contribute to osteoclast differentiation by assessing the direct actions of pharmacological inhibitors on osteoclast precursors. Interestingly, one report demonstrated that osteoclastogenesis was significantly decreased by treatment with PI3Kα inhibitors, but not with PI3Kβ inhibitors or PI3Kδ inhibitors such as IC87114 (Grey et al. 2010). IC87114 shows an IC_{50}:PI3Kα of > 100 μM, IC_{50}:PI3Kβ of 5 μM, IC_{50}:PI3Kγ of 1 μM, and IC_{50}:PI3Kδ of 100 nM, and is thus certainly more selective for PI3Kδ than for other PI3K class I enzymes (Hawkins et al. 2015). However, it is possible that its IC_{50}:PI3Kδ value of 100 nM may be insufficient for it to show specificity to PI3Kδ. Thus, in our present study, we reexamined the functional relevance of PI3Kδ in osteoclast differentiation by using idelalisib, which is the only p110δ-selective inhibitor approved by the FDA and EMA. A previous in vitro cell-

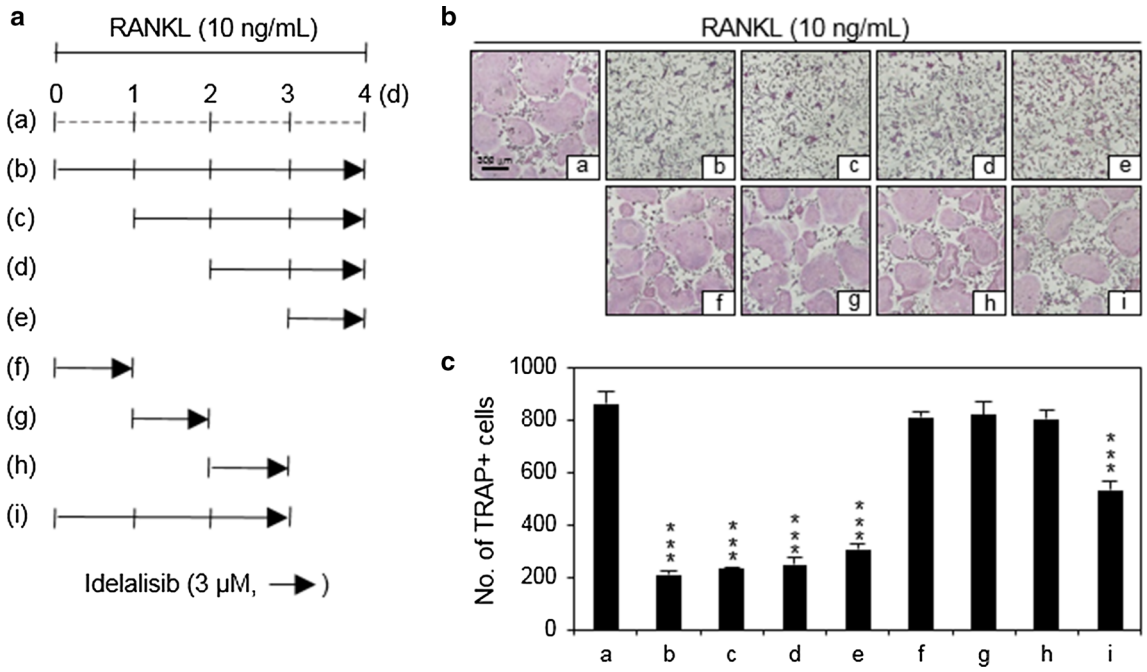

Fig. 4 Idelalisib inhibits the last stage of osteoclast differentiation. **a** Idelalisib exposure schedule. BMMs cultured in the presence of M-CSF (30 ng/mL) and RANKL (10 ng/mL) were treated with idelalisib (3 μM) or vehicle (0.1% DMSO) for the time periods indicated by black arrows. After BMMs differentiated into osteoclasts, $TRAP^{+}$-MNCs were photographed under a light microscope (b; magnification, 100 ×), and the number of $TRAP^{+}$-MNCs were counted (**c**). *** $P < 0.001$

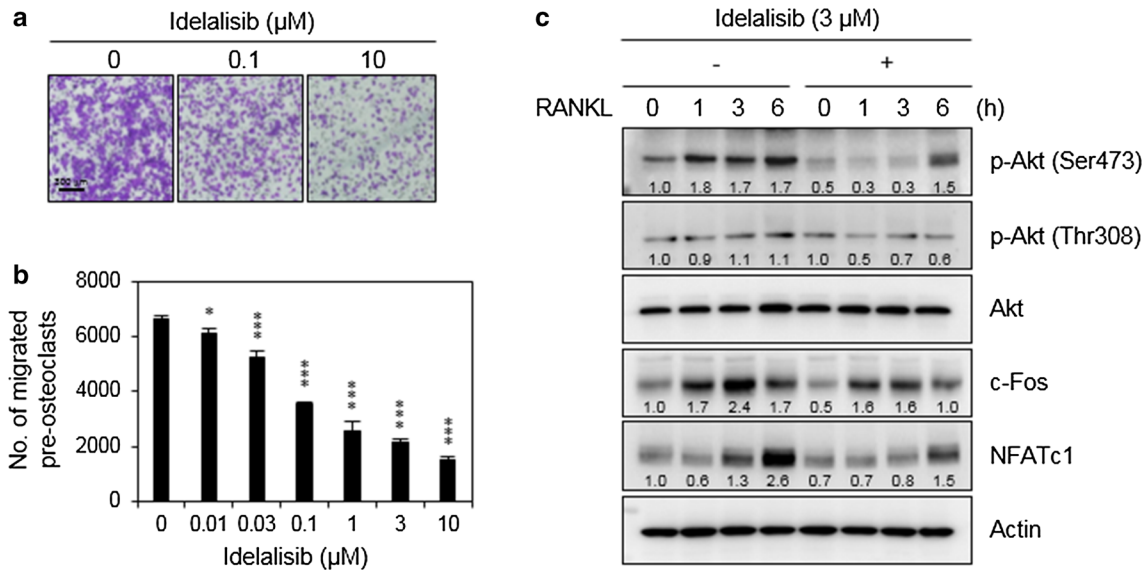

Fig. 5 Idelalisib inhibits pre-osteoclast migration and activation of the Akt-c-Fos/NFATc1 signaling cascade in pre-osteoclasts. **a** The effect of idelalisib on pre-osteoclast migration was evaluated using the Boyden chamber migration assay. The migrated pre-osteoclasts were fixed and stained, and representative images are presented (magnification, 100 ×). **b** The number of migrated pre-osteoclasts were counted. *$P < 0.05$; ***$P < 0.001$. **c** Western blot analysis was performed to evaluate the effects of idelalisib on the induction of p-Akt/Akt, c-Fos and NFATc1 in pre-osteoclasts. The relative ratios of p-Akt/Akt, c-Fos/actin, and NFATc1/actin were presented. The numbers are the intensity compared with that of the control

free assay demonstrated that idelalisib was more selective for PI3Kδ than for other PI3K class I enzymes (IC_{50}:-PI3 Kα, 820 nM; IC_{50}:PI3 Kβ, 565 nM: IC_{50}:PI3Kγ, 89 nM; and IC_{50}:PI3Kδ, 2.5 nM) (Lannutti et al. 2011).

PI3Kδ is predominantly expressed in cells of hematopoietic origin (Kok et al. 2009). Therefore, at the start of this study, we confirmed the expression of PI3Kδ protein in the BMMs used as precursor cells of mature

osteoclasts. Triggering the commitment of BMMs into osteoclasts with RANKL resulted in increased PI3Kδ protein expression, suggesting that this protein might be functionally relevant to osteoclast differentiation. All other PI3K isoforms (α, β, and γ) were also expressed in BMMs, and those proteins were expressed at increased levels following RANKL treatment, suggesting that all PI3K isoforms may play critical roles in osteoclast differentiation. In fact, the evidence showing the involvement of PI3Kβ to the osteoclast-mediated bone resorption in mice and humans has been reported (Győri et al. 2014), and PI3K/Akt signaling pathway has been also suggested to play a role in RANKL-independent osteoclastogenesis (Xing et al. 2016), but in our present study, we focused on the functional relevance of PI3Kδ to the RANKL-mediated osteoclast differentiation.

Here, idelalisib significantly and dose-dependently inhibited $TRAP^+$-MNC formation, confirming the functional relevance of PI3Kδ to osteoclast differentiation. Moreover, idelalisib showed no cytotoxicity towards BMMs, but rather inhibited activation of the RANKL-mediated Akt-c-Fos/NFATc1 signaling cascade. This suggested that idelalisib's anti-osteoclastogenic activity might be due to its specific potential to inhibit PI3Kδ, leading to subsequent suppression of RANKL-induced activation of the Akt-c-Fos/NFATc1 signaling axis. In addition to Akt, MAP kinases (e.g., ERK, p38, and JNK) have been reported to play roles in the early stage of RANKL-induced osteoclast differentiation by controlling the activity and/or expression of c-Fos and NFATc1 (Lee et al. 2002; Huang et al. 2006; Takayanagi 2007a, b; Yamanaka et al. 2013). However, in this study, idelalisib did not affect the RANKL-induced activation of those three MAP kinases. Our data indicated that the idelalisib-mediated direct inhibition of PI3Kδ dominantly blocked the RANKL-induced phosphorylation of Akt on Ser473, but did not influence MAP kinases in the osteoclastogenesis. It has been previously demonstrated that the RANKL-induced phosphorylation of Akt on Ser473 is PI3K-dependent (Kim et al. 2003).

Akt induces osteoclast differentiation through regulation of the NFATc1 signaling cascade (Moon et al. 2012), and both c-Fos and NFATc1 are well-known transcription factors controlling the expression of osteoclastogenesis-related genes (Takayanagi 2007a, b). In Akt1 deficiency, osteoclastogenesis is markedly inhibited, with reduced accumulation of specific osteoclast mRNAs and proteins, and impaired fusion to form MNCs (Mukherjee and Rotwein 2012). In the present study, to confirm that idelalisib's anti-osteoclastogenic effect was exerted through inhibiting the actions of c-Fos and NFATc1, we also evaluated the mRNA expression levels of osteoclastogenesis-related genes, including *TRAP*, *OSCAR*, *DC-STAMP*, *ATP6v0d2*, and *cathepsin K* (Song et al. 2009), and found that idelalisib significantly inhibited the RANKL-induced mRNA expression levels of these genes, which have been considered biomarkers for osteoclastogenesis. Notably, both *DC-STAMP* and *ATP6v0d2* contain multiple NFATc1-binding sites in their promoter regions, which reportedly play critical roles in pre-osteoclast fusion (Kim et al. 2008; Song et al. 2009). In addition, the products of these genes have been shown to function in the complete fusion of the migrating pre-osteoclasts to form functionally activated MNCs.

Osteoclast precursors migrate to the bone surface and fuse with each other to form fully differentiated and functionally activated MNCs (Kikuta and Ishii 2013). Although this process is incompletely understood, it is considered that targeting cell behavior (e.g., migration) may be a potential therapeutic strategy (Millar et al. 2017). Interestingly, our present results demonstrated that idelalisib inhibited pre-osteoclast migration by blocking activation of the Akt-c-Fos/NFATc1 signaling cascade, suggesting the functional importance of PI3Kδ in pre-osteoclast migration during osteoclast differentiation.

The PI3K-Akt pathway has been implicated in osteoclast precursor migration (Munugalavadla et al. 2008; Boudot et al. 2010), and PI3Kδ has been reported to play an important role in controlling cell migration via Akt activity in macrophages (Vanhaesebroeck et al. 1999; Papakonstanti et al. 2008). Akt can control the transcriptional activity of NFATc1, which subsequently regulates the expression of fusion-related genes such as *DC-STAMP* and *ATP6v0d2*. Thus, the idelalisib-mediated direct inhibition of the PI3Kδ-Akt signaling pathway might decrease the expression of c-Fos and NFATc1, resulting in subsequent downregulation of fusion-related genes. This suggests that the anti-osteoclastic action of idelalisib could phenotypically present in blocked pre-osteoclast migration.

In summary, our results suggest that idelalisib may inhibit pre-osteoclast migration, and that its anti-osteoclastogenic action could result from blockade of the PI3Kδ-Akt-c-Fos/NFATc1 signaling cascade. Idelalisib was recently approved for the treatment of specific human B-cell malignancies, and several clinical trials are currently investigating its possible future use in the treatment of a range of malignancies (Yap et al. 2015). Importantly, micro-osteoclast resorption has been suggested as a characteristic feature of B-cell malignancies in clinics (Rossi et al. 1990), and furthermore, significant bone erosion has been found in all clinical stages of chronic lymphocytic leukemia (Marini et al. 2017). Therefore, drug repositioning to expand the clinical use of idelalisib could be a cost-efficient strategy for obtaining new treatment options for a variety of diseases including skeletal disorders and disease-related skeletal problems (Choi et al. 2015). Finally, our

present findings could improve our understanding of the clinical value of PI3Kδ as a druggable target and the efficacy of related therapeutics.

Acknowledgements This work was supported by project grants from National Research Foundation of Korea (KN-1331) and Korea Research Institute of Chemical Technology (KK1703-F02, KK1803-F00 & KK1932-20).

Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

References

Asagiri M, Takayanagi H (2007) The molecular understanding of osteoclast differentiation. Bone 40(2):251–264

Boudot C, Saidak Z, Boulanouar AK, Petit L, Gouilleux F, Massy Z, Brazier M, Mentaverri R, Kamel S (2010) Implication of the calcium sensing receptor and the Phosphoinositide 3-kinase/Akt pathway in the extracellular calcium-mediated migration of RAW 264.7 osteoclast precursor cells. Bone 46:1416–1423

Cantley LC (2002) The phosphoinositide 3-kinase pathway. Science 296(5573):1655–1657

Choi SW, Yeon JT, Ryu BJ, Kim KJ, Moon SH, Lee H, Lee MS, Lee SY, Heo JC, Park SJ, Kim SH (2015) Repositioning potential of PAK4 to osteoclastic bone resorption. J Bone Miner Res 30(8):1494–1507

Feng X (2005) RANKing intracellular signaling in osteoclasts. IUBMB Life 57(6):389–395

Foster FM, Traer CJ, Abraham SM, Fry MJ (2003) The phosphoinositide (PI) 3-kinase family. J Cell Sci 116(Pt 15):3037–3040

Fruman DA, Rommel C (2011) PI3 Kδ inhibitors in cancer: rationale and serendipity merge in the clinic. Cancer Discov 1(7):562–572

Grey A, Chaussade C, Empson V, Lin JM, Watson M, O'Sullivan S, Rewcastle G, Naot D, Cornish J, Shepherd P (2010) Evidence for a role for the p110-alpha isoform of PI3 K in skeletal function. Biochem Biophys Res Commun 391(1):564–569

Győri D, Csete D, Benkő S, Kulkarni S, Mandl P, Dobó-Nagy C, Vanhaesebroeck B, Stephens L, Hawkins PT, Mócsai A (2014) The phosphoinositide 3-kinase isoform PI3 Kβ regulates osteoclast-mediated bone resorption in humans and mice. Arthritis Rheumatol 66(8):2210–2221

Hawkins PT (1851) Stephens LR (2015) PI3 K signalling in inflammation. Biochim Biophys Acta 6:882–897

Herman SE, Gordon AL, Wagner AJ, Heerema NA, Zhao W, Flynn JM, Jones J, Andritsos L, Puri KD, Lannutti BJ, Giese NA, Zhang X, Wei L, Byrd JC, Johnson AJ (2010) Phosphatidylinositol 3-kinase-δ inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. Blood 116(12):2078–2088

Huang H, Chang EJ, Ryu J, Lee ZH, Lee Y, Kim HH (2006) Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway. Biochem Biophys Res Commun 351(1):99–105

Ikeda H, Hideshima T, Fulciniti M, Perrone G, Miura N, Yasui H, Okawa Y, Kiziltepe T, Santo L, Vallet S, Cristea D, Calabrese E, Gorgun G, Raje NS, Richardson P, Munshi NC, Lannutti BJ, Puri KD, Giese NA, Anderson KC (2010) PI3 K/p110δ is a novel therapeutic target in multiple myeloma. Blood 116(9):1460–1468

Juss JK, Hayhoe RP, Owen CE, Bruce I, Walmsley SR, Cowburn AS, Kulkarni S, Boyle KB, Stephens L, Hawkins PT, Chilvers ER, Condliffe AM (2012) Functional redundancy of class I phosphoinositide 3-kinase (PI3 K) isoforms in signaling growth factor-mediated human neutrophil survival. PLoS ONE 7(9):e45933

Khosla S, Riggs BL (2005) Pathophysiology of age-related bone loss and osteoporosis. Endocrinol Metab Clin North Am 34(4):1015–1030

Kikuta J, Ishii M (2013) Osteoclast migration, differentiation and function: novel therapeutic targets for rheumatic diseases. Rheumatology 52(2):226–234

Kim HH, Shin HS, Kwak HJ, Ahn KY, Kim JH, Lee HJ, Lee MS, Lee ZH, Koh GY (2003) RANKL regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. FASEB J 17(14):2163–2165

Kim K, Lee SH, Ha Kim J, Choi Y, Kim N (2008) NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). Mol Endocrinol 22(1):176–185

Lakkakorpi PT, Wesolowski G, Zimolo Z, Rodan GA, Rodan SB (1997) Phosphatidylinositol 3-kinase association with the osteoclast cytoskeleton, and its involvement in osteoclast attachment and spreading. Exp Cell Res 237(2):296–306

Lannutti BJ, Meadows SA, Herman SE, Kashishian A, Steiner B, Johnson AJ, Byrd JC, Tyner JW, Loriaux MM, Deininger M, Druker BJ, Puri KD, Ulrich RG, Giese NA (2011) CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3 K signaling and cellular viability. Blood 117(2):591–594

Lee SE, Woo KM, Kim SY, Kim HM, Kwack K, Lee ZH, Kim HH (2002) The phosphatidylinositol 3-kinase, p38, and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation. Bone 30(1):71–77

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25(4):402–408

Manolagas SC, Parfitt AM (2010) What old means to bone. Trends Endocrinol Metab 21(6):369–374

Marie PJ, Kassem M (2011) Osteoblasts in osteoporosis: past, emerging, and future anabolic targets. Eur J Endocrinol 165(1):1–10

Marini C, Bruno S, Fiz F, Campi C, Piva R, Cutrona G, Matis S, Nieri A, Miglino M, Ibatici A, Maria Orengo A, Maria Massone A, Neumaier CE, Totero D, Giannoni P, Bauckneht M, Pennone M, Tenca C, Gugiatti E, Bellini A, Borra A, Tedone E, Efetürk H, Rosa F, Emionite L, Cilli M, Bagnara D, Brucato V, Bruzzi P, Piana M, Fais F, Sambuceti G (2017) Functional activation of osteoclast commitment in chronic lymphocytic leukaemia: a possible role for RANK/RANKL pathway. Sci Rep 7(1):14159

Meadows SA, Vega F, Kashishian A, Johnson D, Diehl V, Miller LL, Younes A, Lannutti BJ (2012) PI3 Kδ inhibitor, GS-1101 (CAL-101), attenuates pathway signaling, induces apoptosis, and overcomes signals from the microenvironment in cellular models of Hodgkin lymphoma. Blood 119(8):1897–1900

Millar FR, Janes SM, Giangreco A (2017) Epithelial cell migration as a potential therapeutic target in early lung cancer. Eur Respir Rev 26(143):160069

Moon JB, Kim JH, Kim K, Youn BU, Ko A, Lee SY, Kim N (2012) Akt induces osteoclast differentiation through regulating the GSK3β/NFATc1 signaling cascade. J Immunol 188(1):163–169

Mukherjee A, Rotwein P (2012) Selective signaling by Akt1 controls osteoblast differentiation and osteoblast-mediated osteoclast development. Mol Cell Biol 32(2):490–500

Munugalavadla V, Vemula S, Sims EC, Krishnan S, Chen S, Yan J, Li H, Niziolek PJ, Takemoto C, Robling AG, Yang FC, Kapur R

(2008) The p85alpha subunit of class IA phosphatidylinositol 3-kinase regulates the expression of multiple genes involved in osteoclast maturation and migration. Mol Cell Biol 28(23):7182–7198

Nakamura I, Takahashi N, Jimi E, Udagawa N, Suda T (2012) Regulation of osteoclast function. Mod Rheumatol 22(2):167–177

Nakamura I, Takahashi N, Sasaki T, Tanaka S, Udagawa N, Murakami H, Kimura K, Kabuyama Y, Kurokawa T, Suda T, Fukui Y (1995) Wortmannin, a specific inhibitor of phosphatidylinositol-3 kinase, blocks osteoclastic bone resorption. FEBS Lett 361(1):79–84

Norman P (2011) Selective PI3 Kδ inhibitors, a review of the patent literature. Expert Opin Ther Pat 21(11):1773–1790

Papakonstanti EA, Zwaenepoel O, Bilancio A, Burns E, Nock GE, Houseman B, Shokat K, Ridley AJ, Vanhaesebroeck B (2008) Distinct roles of class IA PI3 K isoforms in primary and immortalised macrophages. J Cell Sci 121(Pt 24):4124–4133

Pauls SD, Lafarge ST, Landego I, Zhang T, Marshall AJ (2012) The phosphoinositide 3-kinase signaling pathway in normal and malignant B cells: activation mechanisms, regulation and impact on cellular functions. Front Immunol 3:224

Pilkington MF, Sims SM, Dixon SJ (1998) Wortmannin inhibits spreading and chemotaxis of rat osteoclasts in vitro. J Bone Miner Res 13(4):688–694

Rodan GA, Martin TJ (2000) Therapeutic approaches to bone diseases. Science 289(5484):1508–1514

Rossi JF, Chappard D, Marcelli C, Laplante J, Commes T, Baldet P, Janbon C, Jourdan J, Alexandre C, Bataille R (1990) Micro-osteoclast resorption as a characteristic feature of B-cell malignancies other than multiple myeloma. Br J Haematol 76(4):469–475

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386

Shugg RP, Thomson A, Tanabe N, Kashishian A, Steiner BH, Puri KD, Pereverzev A, Lannutti BJ, Jirik FR, Dixon SJ, Sims SM (2013) Effects of isoform-selective phosphatidylinositol 3-kinase inhibitors on osteoclasts: actions on cytoskeletal organization, survival, and resorption. J Biol Chem 288(49):35346–35357

Song I, Kim JH, Kim K, Jin HM, Youn BU, Kim N (2009) Regulatory mechanism of NFATc1 in RANKL-induced osteoclast activation. FEBS Lett 583(14):2435–2440

Stark AK, Sriskantharajah S, Hessel EM, Okkenhaug K (2015) PI3 K inhibitors in inflammation, autoimmunity and cancer. Curr Opin Pharmacol 23:82–91

Takayanagi H (2005) Inflammatory bone destruction and osteoimmunology. J Periodontal Res 40(4):287–293

Takayanagi H (2007a) Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 7(4):292–304

Takayanagi H (2007b) The role of NFAT in osteoclast formation. Ann N Y Acad Sci 1116:227–237

Takeshita S, Kaji K, Kudo A (2000) Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res 15(8):1477–1488

Vanhaesebroeck B, Jones GE, Allen WE, Zicha D, Hooshmand-Rad R, Sawyer C, Wells C, Waterfield MD, Ridley AJ (1999) Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. Nat Cell Biol 1(1):69–71

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD (2001) Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem 70:535–602

Vanhaesebroeck B, Whitehead MA, Piñeiro R (2016) Molecules in medicine mini-review: isoforms of PI3 K in biology and disease. J Mol Med 94(1):5–11

Xing R, Zhang Y, Li C, Sun L, Yang L, Zhao J, Liu X (2016) Interleukin-21 promotes osteoclastogenesis in RAW264.7 cells through the PI3 K/AKT signaling pathway independently of RANKL. Int J Mol Med 38(4):1125–1134

Yamanaka Y, Clohisy JC, Ito H, Matsuno T, Abu-Amer Y (2013) Blockade of JNK and NFAT pathways attenuates orthopedic particle-stimulated osteoclastogenesis of human osteoclast precursors and murine calvarial osteolysis. J Orthop Res 31(1):67–72

Yap TA, Bjerke L, Clarke PA, Workman P (2015) Drugging PI3 K in cancer: refining targets and therapeutic strategies. Curr Opin Pharmacol 23:98–107

Yeon JT, Ryu BJ, Choi SW, Heo JC, Kim KJ, Son YJ, Kim SH (2014) Natural polyamines inhibit the migration of preosteoclasts by attenuating Ca2 + -PYK2-Src-NFATc1 signaling pathways. Amino Acids 46(11):2605–2614