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Akt Induces Osteoclast Differentiation through Regulating the GSK3β/NFATc1 Signaling Cascade

Jang Bae Moon,* Jung Ha Kim,* Kabsun Kim,* Bang Ung Youn,* Aeran Ko,* Soo Young Lee,† and Nacksung Kim*†

SHIP is an SH2-containing inositol-5-phosphatase expressed in hematopoietic cells. It hydrolyzes the PI3K product P(I)3, and blunts the PI3K-initiated signaling pathway. Although the PI3K/Akt pathway has been shown to be important for osteoclastogenesis, the molecular events involved in osteoclast differentiation have not been revealed. We demonstrate that Akt induces osteoclast differentiation through regulating the GSK3β/NFATc1 signaling cascade. Inhibition of the PI3K by LY294002 reduces formation of osteoclasts and attenuates the expression of NFATc1, but not that of c-Fos. Conversely, overexpression of Akt in bone marrow-derived macrophages (BMMs) strongly induced NFATc1 expression without affecting c-Fos expression, suggesting that PI3K/Akt-mediated NFATc1 induction is independent of c-Fos during RANKL-induced osteoclastogenesis. In addition, we found that overexpression of Akt enhances formation of an inactive form of GSK3β (phospho-GSK3β) and nuclear localization of NFATc1, and that overexpression of a constitutively active form of GSK3β attenuates osteoclast formation through downregulation of NFATc1. Furthermore, BMMs from SHIP knockout mice show the increased expression levels of phospho-Akt and phospho-GSK3β, as well as the enhanced osteoclastogenesis, compared with wild type. However, overexpression of a constitutively active form of GSK3β attenuates RANKL-induced osteoclast differentiation from SHIP-deficient BMMs. Our data suggest that the PI3K/Akt/GSK3β/NFATc1 signaling axis plays an important role in RANKL-induced osteoclastogenesis. The Journal of Immunology, 2012, 188: 163–169.

B one homeostasis is tightly regulated by bone-resorbing activity of osteoclasts and bone-forming activity of osteoblasts. Osteoclasts, which are multinucleated cells specialized in bone resorption, are differentiated from monocyte-macrophage lineage cells of hematopoietic origin. Their differentiation is triggered by two critical factors supplied by osteoblasts, M-CSF and receptor activator of NF-κb ligand (RANKL) (1–3). The essential role of these osteotropic factors in osteoclast formation is clearly demonstrated in genetic studies in which op/op mouse, which lacks M-CSF, shows osteopetrotic phenotype because of the defective osteoclast formation (4). RANKL-deficient mice also display a prominent osteopetrotic phenotype because of the complete absence of osteoclasts (5).

Upon binding of RANKL to its receptor RANK, downstream signaling pathways are activated including NF-κb, JNK, p38 MAPK, extracellular signal-related kinase, and Akt to induce the expression of osteoclastogenesis-related genes such as c-Fos, NFATc1, osteoclast-associated receptor (OSCAR), and tartrate-resistant acid phosphatase (TRAP) (3, 6). Among the induced genes, NFATc1 in particular acts as a key modulator of osteoclastogenesis. Ectopic expression of NFATc1 in the precursors efficiently induces differentiation into functional osteoclasts even in the absence of RANKL (7). Moreover, NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL (8), suggesting indispensable role of NFATc1 in osteoclastogenesis.

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that phosphorylates phosphoinositides at the 3′-OH position of the inositol ring. D3-phosphoinositides generated by PI3K recruit PH-domain-containing proteins such as Akt and mediates various cellular functions, including mitogenesis, survival, motility, and differentiation. However, the signaling pathways mediated by PI3K can be blunted by a phosphatase, such as phosphatase and tensin homolog (PTEN) and SHIP. SHIP is a Src homology (SH) 2-containing inositol-5-phosphatase widely expressed in hematopoietic cells. SHIP specifically hydrolyzes the 5′-phosphate group from phosphatidylinositol-3,4,5-trisphosphate (PIP3), the major product of PI3K and negatively regulates PI3K activity (9). Whereas PI3K initiates and regulates the signals promoting osteoclast precursors survival and differentiation, SHIP-/- mice show increased numbers of osteoclast precursors and enhanced osteoclastogenesis, resulting in severe osteoporosis (10). These data suggest that PI3K is involved in osteoclast differentiation.

PI3K and phospholipid dependent activation of the serine/threonine kinase Akt (also known as PKB) mediate the anti-apoptotic function in a variety of cell types (11). PI3K/Akt signaling pathway also has been shown to regulate osteoclasts survival and differentiation (12, 13). However, the precise mechanism by which Akt regulates the differentiation of osteoclasts remains unknown.
Upon stimulation by insulin or growth factors, Akt phosphorylates glycogen synthase kinase-3β (GSK3β) at serine residue and subsequently inhibits the kinase activity of GSK3β, suggesting that GSK3β is one substrate for Akt (14). It has been shown that GSK3β can modulate the activity of NFATc transcription factors (15, 16). NFAT family members are regulated primarily at the level of their subcellular localization (17). GSK3β directly phosphorylates NFATc1 at its conserved serine residues necessary for nuclear export and promotes nuclear exit (15). It also has been demonstrated that the phosphorylation of the Ser-Pro repeats by GSK3β inhibits the ability of NFATc1 to bind DNA and thereby inhibits NFATc1-dependent gene expression (16). These results prompted us to test the possibility that Akt could induce osteoclast differentiation through regulating the GSK3β/NFATc1 signaling cascade.

PI3K/Akt activation inhibits GSK3β by phosphorylation, and this inhibition of GSK3β leads to the nuclear localization of NFATc1, resulting in enhanced osteoclastogenesis. The enhanced osteoclastogenesis appears to be consistent in SHIP−/− mice, where PI3K/Akt signaling is up-regulated and the expression level of phospho-GSK3β (inactive form of GSK3β) is elevated. Our studies suggest that the PI3K/Akt/GSK3β/NFATc1 signaling axis plays an important role in RANKL-induced osteoclastogenesis.

Materials and Methods

Mice
SHIP-deficient mice were purchased from The Jackson Laboratory. Breeding and genotyping were performed as described previously (9, 18).

Reagents and plasmids
LY294002 was purchased from Calbiochem. The OSCAR reporter construct and expression construct encodung NFATc1 were described previously (19). A human constitutively active form of Akt (pECE-myr-Akt-D4-129) was provided by J.K. Jung (Seoul National University). Expression constructs encoding wild type (WT) of GSK3β (GSK3β-WT) and a kinase-inactive mutant of GSK3β (GSK3β-KD) were provided by K.Y. Lee (Chonnam National University) (20). A constitutively active form of GSK3β, GSK3β-S9A (serine 9 of human GSK3β was replaced with alanine), was generated by the QuickChange method of site-directed mutagenesis (Stratagene) (21).

Osteoclast formation
Murine osteoclasts were prepared from bone marrow cells as described previously (22). Bone marrow cells were cultured in α-MEM containing 10% FBS with M-CSF (5 ng/ml) for 2 days. Nonadherent cells were harvested and adherent cells were used as osteoclast precursors (bone marrow-derived macrophages [BMMs]). To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 days. Cultured cells were fixed and stained for TRAP. TRAP-positive multinuclear cells (TRAP+ mononuclear cells [MNCs]) containing more than three nuclei were counted as osteoclasts.

Retroviral infection

To generate retroviral stocks, retroviral vectors were transfected into the packaging cell line Plat E using FuGENE 6 (Roche Applied Sciences). Viral supernatant was collected from culture media 24–48 h after transfection. BMMs were incubated with viral supernatant for 8 h in the presence of M-CSF. BMMs were cultured further with M-CSF and RANKL in the absence or presence of LY294002, a specific inhibitor of PI3K. Consistent with previous results (12), treatment with LY294002 inhibited RANKL-induced osteoclast formation in a dose-dependent manner (Fig. 1A, 1B).

It has been shown that RANKL-induced activation of early signaling pathways is important for osteoclast formation (3, 6). Because treatment of LY294002 attenuated RANKL-induced osteoclastogenesis, we investigated whether LY294002 could affect other RANKL-induced signaling pathways. BMMs were starved and stimulated with RANKL for the indicated times. Consistent with previous results (23, 24), RANKL activated Akt, JNK, p38, and NF-kB in 10 min. The treatment with LY294002 inhibited RANKL-induced osteoclast differentiation (Fig. 1C).

Results

The role of Akt/GSK3β in RANKL-induced osteoclastogenesis

We first examined the effect of the PI3K signaling pathway on RANKL-induced osteoclast differentiation. BMMs were cultured with M-CSF and RANKL in the absence or presence of LY294002, a specific inhibitor of PI3K. Consistent with previous results (12), treatment with LY294002 inhibited RANKL-induced osteoclast formation in a dose-dependent manner (Fig. 1A, 1B).

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FIGURE 1. PI3K plays a role in RANKL-induced osteoclastogenesis. A and B, BMMs were derived from bone marrow cells by culturing for 3 days in the presence of M-CSF. BMMs were cultured for an additional 3 days in the presence of M-CSF and RANKL with increasing concentrations (0.5–2.5 μM) of LY294002, an inhibitor of PI3K, as indicated. A, Cultured cells were stained for TRAP. Original magnification ×100. B, TRAP-positive multinuclear cells (MNCs) were counted as osteoclasts. *p < 0.01, **p < 0.001 versus positive control. C, BMMs were starved and pretreated with 2.5 μM LY294002 or DMSO (vehicle) for 2 h before exposure to RANKL for the indicated times. D and E, BMMs were cultured with M-CSF and RANKL in the presence of 2.5 μM LY294002 or DMSO for the indicated times. F, Whole cell extracts were subjected to Western blot analysis with specific Abs as indicated.
strongly blocked RANKL-induced Akt activation, whereas other signaling pathways such as JNK, p38, and NF-κB were not affected by LY294002 (Fig. 1C). Thus, our data confirmed that LY294002 could specifically inhibit PI3K/Akt pathway among RANKL-induced early signaling pathways and that PI3K is important for RANKL-induced osteoclastogenesis.

To identify how PI3K regulates osteoclast differentiation, we examined the gene expression patterns during osteoclastogenesis. The expression of c-Fos, an AP-1 family member, was induced after 12 h exposure to RANKL and strongly increased till 24 h (Fig. 1D). This RANKL-induced expression of c-Fos was not affected by treatment with LY294002. However, NFATc1 induction mediated by RANKL was strongly attenuated by blockage of PI3K (Fig. 1E). We also observed the downregulation of OSCAR, an osteoclast-specific gene, by treatment with LY294002. As a result, PI3K can regulate NFATc1 gene expression, not c-Fos, during RANKL-induced osteoclastogenesis.

PI3K/Akt signaling cascade regulates NFATc1 expression during RANKL-induced osteoclastogenesis

To investigate the role of Akt in osteoclast differentiation, we overexpressed constitutively active Akt (Ca-Akt) in BMMs using retrovirus. Ectopic expression of Ca-Akt alone did not induce osteoclast differentiation in the presence of M-CSF (Fig. 2A, upper panel). However, RANKL-induced osteoclast formation was significantly enhanced by overexpression of Akt (Fig. 2A, 2B).

We have demonstrated that LY294002 treatment blocks Akt activation and attenuates RANKL-induced osteoclastogenesis. Therefore, we examined whether overexpression of Akt could rescue osteoclast formation in LY294002-treated cells. LY294002 treatment reduced the formation of TRAP+ MNCs from BMMs in empty vector-infected cells (Fig. 2C, 2D). Retroviral overexpression of Akt, however, overcame the inhibitory effect of LY294002 on osteoclastogenesis. These data suggest that a PI3K/Akt axis is important for RANKL-induced osteoclast formation.

Next, we investigated which genes can be regulated by overexpression of Akt during osteoclastogenesis. RANKL-mediated c-Fos induction was comparable in empty vector- and Akt-infected samples (Fig. 2E). However, overexpression of Akt strongly increased the expression of NFATc1 and OSCAR compared with control (Fig. 2F). Collectively, the PI3K/Akt signaling cascade plays a role in RANKL-induced osteoclastogenesis via regulating NFATc1 expression without affecting expression of c-Fos.

Overexpression of Akt enhances phosphorylation of GSK3β and nuclear localization of NFATc1

It has been shown that GSK3β is one of the known downstream targets of Akt and that GSK3β enhances nuclear export of NFAT proteins (14, 15). Therefore, we hypothesized that GSK3β might have a role in Akt-mediated NFATc1 induction during osteoclastogenesis. When Akt was transfected into 293T cells, overexpressed Akt increased the level of phosphorylated GSK3β, whereas total level of GSK3β was not changed (Fig. 3A). To investigate whether Akt regulates GSK3β in osteoclast cells, we first examined the effect of Akt on the phosphorylation level of GSK3β in BMMs. RANKL stimulation induced phosphorylation of GSK3β in 10 min, and the level of RANKL-induced GSK3β phosphorylation was further enhanced by overexpression of Akt (Fig. 3B). Next, we compared the status of GSK3β phosphorylation in empty vector-infected and Akt-overexpressed osteoclasts. Compared with control, the ratio of phosphorylated GSK3β/GSK3β was strongly increased by overexpression of Akt in osteoclasts (Fig. 3C, middle panel). We could observe the stronger induction of NFATc1 in Akt-overexpressed osteoclasts than control vector-infected osteoclasts (Fig. 3C, lower panel). When we examined the localization of NFATc1 in osteoclasts, overexpressed Akt strongly induced enrichment of NFATc1 in the nuclear region of osteoclasts, rather than in cytoplasmic region (Fig. 3D). Our data suggest that Akt can enhance the level of GSK3β phosphorylation and nuclear localization of NFATc1 during RANKL-induced osteoclastogenesis.

Overexpression of GSK3β attenuates osteoclast formation through downregulation of NFATc1

To investigate the effect of GSK3β on RANKL-induced osteoclastogenesis, we used the retroviral vector expressing the non-phosphorylatable constitutively active mutant of GSK3β (GSK3β-S9A) where the serine residue at position 9 was mutated to alanine. Compared with control vector-infected samples, overexpression of GSK3β-S9A significantly attenuated RANKL-induced osteoclast differentiation (Fig. 4A, 4B).

When we examined the NFATc1 expression by Western blot analysis, overexpression of GSK3β-S9A downregulated RANKL-induced NFATc1 expression in osteoclasts (Fig. 4C, lower panel). By fractionation analysis, we confirmed that overexpression of
mice was much stronger than that in BMMs from WT (Fig. 5D, SHIP Akt activation mediated by RANKL in BMMs from WT). Consistent with previous results (10), the formation of TRAP+ M-CSF and RANKL. A–C, pMX-IRES-EGFP (control) or Ca-Akt retrovirus and cultured for 2 d with RANKL for the indicated times. C, BMMs were transduced with pMX-IRES-EGFP (control) or Ca-Akt retrovirus and cultured for 2 d with M-CSF and RANKL. Whole cell extracts, cytoplasmic fractions, and nuclear fractions were harvested from cultured cells and subjected to Western blot analysis with specific Abs as indicated. Abs for actin and lamin B1 were used for the normalization of cytoplasmic and nuclear extracts, respectively.

SHIP in osteoclast formation using 293T cells was transfected with empty vector (Mock) or Akt. B, BMMs were transduced with pMX-IRES-EGFP (control) or Ca-Akt retrovirus. Cells were starved and stimulated with RANKL, for the indicated times. C, BMMs were transduced with pMX-IRES-EGFP (control) or Ca-Akt retrovirus and cultured for 2 d with M-CSF and RANKL. Whole cell extracts, cytoplasmic fractions, and nuclear fractions were harvested from cultured cells and subjected to Western blot analysis with specific Abs as indicated. Abs for actin and lamin B1 were used for the normalization of cytoplasmic and nuclear extracts, respectively.

GSK3β-S9A strongly induced nuclear export of NFATc1 in osteoclasts (Fig. 4C, upper panel).

Next, we investigated the effect of GSK3β on transcriptional activity of NFATc1 using a reporter assay. Consistent with previous results (19, 25), cotransfection of the OSCAR reporter construct and NFATc1 resulted in approx 4-fold increase in promoter activity (Fig. 4D). This NFATc1-mediated transactivation of OSCAR was strongly attenuated by wild type of GSK3β (GSK3β-WT), but not by a kinase-inactive mutant GSK3β (GSK3β-KD). Collectively, these data suggest that GSK3β attenuates RANKL-induced osteoclast differentiation by downregulation of NFATc1.

**Deficiency of SHIP enhances osteoclastogenesis through activating the Akt/GSK3β/NFATc1 signaling cascade**

It has been shown that SHIP negatively regulates growth factor receptor-mediated Akt activation in hematopoietic cells (9). Therefore, we investigated whether SHIP could regulate the Akt/GSK3β/NFATc1 signaling cascade during RANKL-induced osteoclast differentiation. We first examined the physiologic role of SHIP in osteoclast formation using SHIP knockout (KO) mice. Consistent with previous results (10), the formation of TRAP+ MNCs was significantly increased in SHIP KO mice compared with WT littermates (Fig. 5A, 5B). The RANKL-induced early signaling pathways such as activation of JNK, p38, and NF-κB were comparable in WT and SHIP KO mice (Fig. 5C). However, Akt activation mediated by RANKL in BMMs from SHIP KO mice was much stronger than that in BMMs from WT (Fig. 5D, upper panel). We could also observe that RANKL induced more phosphorylation of GSK3β in SHIP KO mice than that in WT littermates (Fig. 5D, middle panel). When we examined the expression patterns of important genes such as c-Fos and NFATc1 during osteoclastogenesis, RANKL-mediated c-Fos induction was comparable in WT and SHIP KO mice (Fig. 5E). However, induction of NFATc1 mediated by RANKL was stronger in SHIP KO mice than in WT littermates (Fig. 5F). Our data suggest that deficiency of SHIP enhances osteoclastogenesis through activating the Akt/GSK3β/NFATc1 signaling cascade.

**Overexpression of GSK3β attenuates osteoclast formation via downregulation of NFATc1 in SHIP KO mice**

Given our observation that inactivation of GSK3β was strongly induced by RANKL in BMMs from SHIP KO mice, we tested whether a constitutively active form of GSK3β (GSK3β-S9A) could attenuate osteoclast formation through downregulation of NFATc1 in SHIP KO mice. BMMs from SHIP KO mice were transduced with control or GSK3β-S9A retrovirus and cultured with M-CSF and RANKL for 3 d. Compared with controls,
overexpression of GSK3β-S9A in BMMs strongly attenuated RANKL-induced osteoclast formation (Fig. 6A, 6B). Next, we compared the expression levels of c-Fos and NFATc1 in both samples during osteoclastogenesis. As shown in Fig. 6C, the expression levels of c-Fos are comparable in control vector- or SHIP knockout mice. Overexpression of GSK3β-S9A, however, strongly downregulated expression of NFATc1 and OSCAR (Fig. 6D). When we examined the localization of NFATc1 in osteoclasts, overexpression of GSK3β-S9A strongly induced the export of NFATc1 from the nucleus (Fig. 6E). Collectively, our data suggest that overexpression of an active form of GSK3β attenuates osteoclast formation via downregulation of NFATc1 in SHIP KO mice.

Discussion
In osteoclasts, PI3K/Akt signaling cascade is activated as a critical downstream effector from at least three cell surface receptors, c-fms, α5β3 integrin, and RANK (13, 26, 27). The effector actions of PI3K/Akt signaling pathway are diverse; however, the pathway needs to be tightly controlled. This is done through the action of several PIP3 phosphatases such as PTEN (phosphatase and tensin homolog deleted on chromosome 10, a 3'-phosphatase) and two 5'-phosphatases, SHIP and SHIP2. SHIP is predominantly expressed in hematopoietic cells, and it blunts PI3K-initiated signaling pathway. Notably, Takeshita et al. (10) showed that the Akt/GSK3β/NFATc1 pathway may also pertain to osteoclasts and that Akt would induce osteoclasts differentiation through this pathway, which proved to be the case.

PI3K primarily generates PIP3 after activation of receptors for cytokines and growth factors. PIP3 is a major modulator of cell functions and as such is an important point where many physiologic effects diverge. Thus, the signaling capacity of this molecule needs to be tightly controlled. This is done through the action of several PIP3 phosphatases such as PTEN (phosphatase and tensin homolog deleted on chromosome 10, a 3'-phosphatase) and two 5'-phosphatases, SHIP and SHIP2. SHIP is predominantly expressed in hematopoietic cells, and it blunts PI3K-initiated signaling pathway. Notably, Takeshita et al. (10) showed that the attenuated PIP3 degradation in SHIP deficiency leads to enhanced precursor proliferation, more robust differentiation of osteoclasts, and thus severe osteoporosis. The enhanced osteoclastogenesis

FIGURE 6. Overexpression of GSK3β attenuates osteoclast formation via downregulation of NFATc1 in SHIP KO mice. A and B, BMMs from SHIP KO mice were transduced with pMX-IREs-EGFP (control) or GSK3β-S9A retrovirus and cultured for 3 d with M-CSF and RANKL. A. Cultured cells were fixed and stained for TRAP. Original magnification ×100. B, TRAP+ MNCs were counted as osteoclasts. *p < 0.01, **p < 0.001 versus positive control. C and D, BMMs from WT or SHIP KO mice were starved in 0.5% FBS for 4 h and stimulated with RANKL for the indicated times. Whole cell extracts were subjected to Western blot analysis with specific Abs as indicated. E and F, BMMs from WT or SHIP knockout mice were cultured with M-CSF and RANKL for the indicated times. Whole cell extracts were subjected to Western blot analysis with specific Abs as indicated.
of SHIP insufficiency was repeated in our study. Moreover, the experiments revealed that the accelerated osteoclasts differentiation in SHIP−/− was mediated by the hyperactivation of Akt and consequent inactivation of GSK3b. These results were recapitulated at the level of NFATc1 expression where SHIP−/− showed increased expression level. Consistent with these findings, PTEN mutant with no phosphatase activity also has been reported to increase RANKL-induced osteoclastogenesis (29). Ultimately, these studies implicate that the net activity of lipid kinases and phosphatase is an important regulatory factor in determining the degree of osteoclasts differentiation, at least, through regulating Akt/GSK3b/NFATc1 axis.

There are three Akt family members—Akt1, Akt2, and Akt3. Akt1 and Akt2, but not Akt3, are abundantly expressed in both bone cells: osteoblasts and osteoclasts (30). Although single KO mice of Akt isoform showed a mild phenotype, double KO mice of Akt1/2 showed severely impaired bone development and dwarfism (31). Kawamura et al. (30) reported that Akt1 is a crucial regulator of osteoblast and osteoclast by promoting their differentiation and survival. They showed that Akt1 deficiency caused impairment of bone resorption via cell autonomous dysfunction in osteoclasts and the cell nonautonomous inhibition of osteoclastogenesis because of reduced RANKL expression in osteoblasts. Sugatani et al. (32) showed that knockdown of Akt1 and Akt2 inhibited osteoclast differentiation because of downregulation of RANKL-induced NF-κB p50 DNA binding activity. In this study, however, PI3K inhibition by LY294002 abrogated RANKL-induced Akt activation and phosphorylation of GSK3β (Supplemental Fig. 1), but not other signaling pathways including NF-κB activation. We also observed the RANKL-induced hyperactivation of Akt and phosphorylation of GSK3β in SHIP KO osteoclasts, although activation of other signaling pathways such as NF-κB, p38, and JNK is comparable to WT osteoclasts. These data suggest that PI3K/Akt regulates osteoclast differentiation primarily through GSK3β rather than the NF-κB signaling cascade.

GSK3β is a critical downstream effector of the PI3K/Akt signaling pathway. GSK3β is ubiquitously expressed and constitutively active in resting cells. Thus, it is regulated through inhibition of its activity by Akt-mediated phosphorylation at serine 9 (14). The wide distribution of GSK3β in all cell types confers a Akt/GSK3β signaling pathway capable of controlling diverse cellular functions such as cell cycle regulation, glycogen and protein synthesis regulation, and transcription factor modulation (33). Whether the pathway is also involved in the regulation of osteoclasts differentiation and functions has not been elucidated; however, our observation that the overexpression of constitutively active form of Akt increased the expression of phospho-GSK3β suggests that GSK3β is a bona fide target of Akt in osteoclasts.

Although GSK3β is an important downstream target of Akt, we did not observe complete abrogation of RANKL-induced osteoclastogenesis by overexpression of a constitutively active form of GSK3β in BMMs from SHIP KO mice. This finding could be the result of imperfect retroviral infection efficiency, because we have demonstrated 50–70% infection in BMMs. Another possibility is that other kinases have a role in the Akt-NFATc1 signaling axis in osteoclasts.

GSK3β has been shown to regulate NFATc1 signaling pathway as demonstrated in several independent studies. Beals et al. (15) identified GSK3β as the principal cellular kinase responsible for the phosphorylation of NFATc at conserved serine residues in COS cells. Upon phosphorylation by GSK3β, NFATc was exported from the nucleus and its transcriptional activity was ceased. However, LiCl treatment, which acts as a GSK3β inhibitor, was shown to antagonize the nuclear export of NFATc (34). Several other studies with murine primary T cells and bone marrow-derived mast cells also support the notion that GSK3β is a potential regulator of NFATc (35, 36). In fact, our results also provide several lines of evidence to suggest GSK3β-dependent regulation of NFATc1 in osteoclasts. The overexpression of the constitutively active form of GSK3β (GSK3β-S9A) dramatically inhibited osteoclast formation through directly reducing the nuclear localization of NFATc1. GSK3β-S9A showed the same inhibitory effect on SHIP−/− osteoclasts, where GSK3β-S9A promoted nuclear exit of NFATc1 and thus reducing osteoclasts formation. Recently, it has been shown that transient genetic expression of GSK3β-S9A mutant show an osteopetrotic phenotype because of impaired osteoclast differentiation. Furthermore, osteoclast precursor cells from the transgenic mice showed defects in expression and nuclear localization of NFATc1 (37). These collective observations show that GSK3β has an important role in the regulation of NFATc1 during osteoclastogenesis.

The classic NFATc1 activation pathway requires an elevation of the intracellular calcium concentration. The calcium signals then activate calcium/calcmodulin-dependent protein phosphatase calcineurin, which in turn dephosphorylates cytosolic NFATc1 to lead to nuclear translocation. However, a number of protein kinases oppose calcineurin-mediated activation of NFATc1 by directly phosphorylating and promoting nuclear exit of NFATc1. This regulatory interplay between calcineurin and various NFATc1 kinases determines the subcellular localization of NFATc1. In our study, however, GSK3β appeared to be an important NFATc1 kinase in osteoclasts. GSK3β efficiently translocated NFATc1 from the nucleus of both WT and SHIP−/− osteoclasts, thus resulting in reduced osteoclasts formation. However, given the fact that there are large numbers of phosphorylation sites on the NFATc1 protein (38), it is possible that other NFATc1 kinases besides GSK3β participate in the regulation of NFATc1 localization in osteoclasts. The more detailed analysis of the regulatory network of NFATc1 may help us to better understand the kinase-phosphatase-mediated regulation of NFATc1 localization.

In this study, we demonstrated that Akt induces osteoclastogenesis through GSK3β/NFATc1 signaling cascade. Furthermore, our data revealed insights into the regulation of NFATc1 in osteoclasts, in which GSK3β regulated NFATc1 in the phosphorylation-dependent manner. However, future studies with GSK3β transgenic mice or GSK3β conditional KO mice will clarify and extend our understanding of the role of GSK3β and its regulation of NFATc1 nuclear presence in osteoclasts.

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Disclosures
The authors have no financial conflicts of interest.

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Supplementary Figure 1. PI3K inhibition by LY294002 attenuates RANKL-induced phosphorylation of Akt and GSK3β. BMMs were derived from bone marrow cells by culturing for 3 days in the presence of M-CSF. BMMs were starved and pretreated with 2.5 mM LY294002 or DMSO (vehicle) for 2 h before exposure to RANKL for the indicated times. Whole cell extracts were subjected to Western blot analysis with specific antibodies as indicated.