PTEN Regulates RANKL- and Osteopontin-stimulated Signal Transduction during Osteoclast Differentiation and Cell Motility*

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Toshifumi Sugatani‡§, Ulises Alvarez‡, and Keith A. Hruska¶‡

From the ¶Department of Pediatrics, Cell and Molecular Biology Unit, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Department of Oral and Maxillofacial Surgery, Menninger University School of Medicine, New York, NY 10037, Japan

PTEN (also known as MMAC-1 or TEP-1) is a frequently mutated tumor suppressor gene in human cancer. PTEN functions have been identified in the regulation of cell survival, growth, adhesion, migration, and invasiveness. Here, we characterize the diverse signaling networks modulated by PTEN in osteoclast precursors stimulated by RANKL and osteopontin (OPN). RANKL dose-dependently stimulated transient activation of Akt before activation of PTEN, consistent with a role for PTEN in decreasing Akt activity. PTEN overexpression blocked RANKL-activated Akt stimulated survival and osteopontin-stimulated cell migration while a dominant-negative PTEN increased the actions of RANKL and OPN. PTEN overexpression suppressed RANKL-mediated osteoclast differentiation and OPN-stimulated cell migration. The PTEN dominant-negative constitutively induced osteoclast differentiation and cell migration. Our data demonstrate multiple roles for PTEN in RANKL-induced osteoclast differentiation and OPN-stimulated cell migration in RAW 264.7 osteoclast precursors.

The tumor suppressor gene PTEN1 (phosphatase and tensin homolog deleted from chromosome 10), also known as MMAC1 (mutated in multiple advanced cancers) or TEP1 (TGF-β-regulated and epithelial cell-enriched phosphatase), is located on chromosome 10q23, a genomic region that suffers loss-of-heterozygosity in many human cancers (1–7). Recent studies have demonstrated that PTEN plays an essential role in regulating signaling pathways involved in cell growth, adhesion, migration, invasion, and apoptosis, and mutations in the PTEN gene cause tumorigenesis in a number of human tissues (1–5, 7). Germline mutations of PTEN cause Cowden disease, a multiple hamartoma condition associated with high incidence of breast, brain, and thyroid neoplasia (1–7). Biochemical studies of the PTEN phosphatase have revealed a molecular mechanism by which tumorigenesis may be caused in individuals with PTEN mutations. The protein product, PTEN, has homology to dual-specificity phosphatases, and PTEN functions not only as a protein phosphatase, but also as a lipid phosphatase (1–7). As a lipid phosphatase, PTEN functions during dephosphorylation of the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), a product of phosphatidylinositol 3-kinase (PI3K) activity, and as a protein phosphatase by negatively regulating survival signaling mediated by protein kinase B/Akt (PKB/Akt) (1–7). PTEN converts the biologically active lipid PtdIns(3,4,5)P3 to PtdIns(4,5)P2 (1–7), and it dephosphorylates PtdIns(4,5)P2 to PtdIns(3,4)P2 and PtdIns(3,4,5)P3, which regulates Akt and survival signaling pathways, leading to inhibition of apoptosis, hyperplasia, and tumor formation (1–5, 7).

RANKL is expressed by osteoblastic lineage cells and stimulates its specific receptor, the receptor activator of NFκB (RANK), which located on osteoclasts, to promote differentiation, survival, fusion, and activation of osteoclasts and to prevent osteoclast apoptosis. The signaling cascade of RANK activation involves stimulation of the c-Jun, NFκB, and serine/threonine kinase Akt pathways (13–18). RANKL also activates the antiapoptotic serine/threonine kinase Akt through a signalling complex involving c-Src and TRAF 6 in primary osteoclasts (17, 19–21).

Osteopontin (OPN) is one of the major noncollagenous bone matrix proteins produced by osteoblasts and osteoclasts (22–25). It also stimulates PI3K activity (10–24, 26, 27), which is a target of PTEN (28) and induces cell migration (26). In addition, a recent study indicates that Akt is essential for cell migration (29).

The major function of PTEN appears to be down-regulation of the PI3K product PtdIns(3,4,5)P3, which regulates Akt and complex downstream pathways affecting cell growth, survival, and migration. In addition, PTEN has weak protein tyrosine phosphatase activity, which may target focal adhesion kinase (FAK) and Shc, and thereby modulate other complex pathways (1, 7). In this report, we show that PTEN regulates the RANKL-activated Akt survival signaling pathway and the OPN-stimulated cell migration in RAW 264.7 osteoclast precursors. Moreover, we found that PTEN also regulates RANKL-induced osteoclast differentiation from RAW 264.7 osteoclast precursors. In addition, we suggest that RANKL may
regulate balance of activated Akt and activated PTEN and have influence osteoclast differentiation. Thus, it is likely that PTEN plays multiple roles involving osteoclast formation, survival, and migration.

**EXPERIMENTAL PROCEDURES**

**Materials**—RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA). Polyclonal anti-Akt, anti-phospho-Akt (Thr-308), anti-IκBα, anti-phospho-IκBα (Ser-32), anti-Bad, anti-

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**Fig. 1.** PTEN regulates RANKL-activated Akt survival signaling pathway in RAW 264.7 osteoclast precursors. RAW 264.7 osteoclast precursors were transiently cotransfected with GFP, GFP-PTEN wild-type (WT), GFP-PTEN (C124A) mutant, Myc, or Myc-Akt (K179M) dominant-negative. Cells were treated with RANKL (100 ng/ml) for the indicated time, and whole cell extracts or nuclear extracts (for NFκB) were electrophoresed and analyzed by immunoblotting with antibodies against Akt (A), phospho-Akt (A), Bad (B), phospho-Bad (B), IκBα (D), phospho-IκBα (D), and NFκB (E). C, the GFP- or GFP-PTEN (WT)-expressing cells treated with or without RANKL (5 min) were fixed and stained for TRAP (reddish brown).
phospho-Bad (Ser-136), and anti-phospho-Rac/cdc42 (Ser-71) were pur-
chased from New England Biolabs (Beverly, MA). Polyclonal anti-
NFκB/p50 is obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal anti-Rho is purchased from Upstate Biotechnology (Lake Placid, NY). Green fluorescent protein (GFP), GFP-PTEN wild-
type (WT), and GFP-PTEN point mutant C124A (Cys-124 → Ala) cDNA plasmid were kindly provided by Dr. Kenneth M. Yamada (Craniofacial Developmental Biology and Regeneration Branch, NIH, Bethesda, MD). Myc (empty) and Myc-Akt (K179M) dominant-negative (Lys-179 → Met) cDNA plasmid were purchased from Upstate Biotechnology. The cDNA encoding Rho (V14Rho), Rac (L61Rac), and cdc42 (V12cdc42) were kindly provided by Dr. Alan Hall (MRC Laboratory for Molecular Cell Biology, Department of Biochemistry, University College of Lon-
don, London, UK).

Cell Cultures and Transient Transfection—RAW 264.7 cells (a mu-
rine macrophage line capable of RANKL-mediated osteoclastogenesis) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitro-
g) supplemented with 10% heat-inactivated fetal bovine serum (FBS). After 24 h, GFP, GFP-PTEN (WT), GFP-PTEN (C124A; phospho-
phatase dead mutant), Myc (empty), or Myc-Akt (K179M) dominant-
negative expression vector were transiently cotransfected into the cells using the LipofectAMINE reagent System (Invitrogen) according to the manufacturer's instructions. Then, the GFP- or GFP-PTEN (WT)- ex-
pressing cells treated with or without RANKL were fixed and stained for tartrate-resistant acid phosphatase (TRAP) to observe cell morphol-
ogy.

Mouse bone marrow macrophages (BMMs) were prepared from the femur and tibia of 4–6-week-old C57BL/6 mice and incubated in tissue culture dishes (100-mm dishes) in the presence of recombinant mouse macrophage-colony-stimulating factor (20 ng/ml). After 24 h in culture, the non-adherent cells were collected and layered on Histopaque gra-
dient, and the cells at the gradient interface were collected. The cells were replated (60-mm dishes) at 65,000/cm² in DMEM medium (DMEM; Invitro-
g) supplemented with 10% heat-inactivated FBS in the presence of M-CSF (100 ng/ml). After 3 days in culture, cells were harvested for immunoblotting.

Preparation of Cell Lysates and Immunoblotting—24–36 h after transfection, medium was removed, and cells were washed two times with phosphate-buffered saline (PBS) and then cultured in DMEM serum-free medium for 24 h. For RANKL (100 ng/ml) or OPN (25 µg/ml) stimulation experiments, RANKL or OPN were added to the culture medium and incubated for 5, 10, 15, 30, and 60 min. The cells were then washed once with ice-cold PBS and lysed in a cell lysis buffer (New England Biolabs) to prepare whole cell lysates. Lysates were clarified by centrifugation at 14,000 × g for 10 min, and protein concentrations in the supernatants were measured using the Bio-Rad protein assay reagent kit (Bio-Rad). Proteins were resolved by SDS-PAGE, electro-
blotted to polyvinylidene difluoride membrane (Millipore, Bedford,
MA), blocked in 5% skim milk, 0.05% Tween 20, and probed with primary antibodies. For the detection of NFκB/p50, nuclear ex-
tracts were used instead of whole cell lysates. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (New England Biolabs), bound immunoglobulins were detected using en-
hanced chemiluminescence (Pierce).

Apoptosis Assay—After cotransfected, cells were treated with RANKL (100 ng/ml). Then, whole cell lysates were prepared as above. Lysates were clarified by centrifugation at 14,000 × g for 10 min, and the supernatant fractions were harvested. Caspase-3 activity assay of cell extracts were measured using a kit (CaspACE™ assay system; Promega) according to the manufacturer's instructions.

Osteoclast Formation Assay—Cells were cultured in a 60-mm dish (40 × 10⁴ cells/5-ml dish) in DMEM containing 10% FBS overnight. Cells were then transfected with five expression vectors, respectively. After 24 h, media was removed, and cells were washed two times with PBS and then cultured in the above-mentioned medium with RANKL (100 ng/ml). After culturing for 2 days, cells were added to fresh me-
dium and RANKL. Then, after culturing for 2 days, cells were fixed and stained for TRAP (30). TRAP-positive multinucleated cells (MNCs) containing more than three nuclei were counted as osteoclasts under microscopic examination (31).

Affinity Precipitation of Cellular Rho—The cells (RAW cells or RAW cells with GFP-PTEN-WT) were washed once with ice-cold PBS and lysed in Rho-binding lysis buffer (Upstate Biotechnology) to prepare whole cell lysates. Lysates were clarified by centrifugation at 14,000 × g for 10 min, and equal volumes of lysates were incubated with the Rhotekin Rho binding domain (20 µg; Upstate Biotechnology) beads at 4 °C for 45 min. The beads were washed three times with wash buffer (Tris buffer containing 1% Triton X, 150 mM NaCl, 10 mM MgCl₂, 10 µg/ml each of leupeptin and aprotinin, and 0.1 mM phenylmethylsulfo-
nyl fluoride). Bound Rho proteins were detected by immunoblotting using polyclonal anti-Rho.

Protein Purification—Constitutively active Rho (V14Rho), Rac (L61Rac), and cdc42 (V12cdc42) were cloned in-frame into a bacterial expression vector, pTAT-HA, to produce TAT fusion proteins. The vector pTAT-HA has an N-terminal His₆ leader followed by the 11-amino acid sequence, a hemagglutinin (HA) tag, and a polylinker. The cDNAs encoding V14Rho, L61Rac, and V12cdc42 were cloned into the Tat-HA plasmid. High copy number plasmids were obtained by transformation of the pTAT-HA vector with V14Rho, L61Rac, and V12cdc42 in BL21. The purification protocol was adapted from the published procedure using a Ni-NTA column (32, 33). Briefly, bacterial pellets were resuspended in a buffer containing 100 mM NaCl, 20 mM Hepes (pH 8.0), and 8 M urea and sonicated and
centrifuged at 12,000 rpm for 10 min at 4 °C. Imidazole was added to the supernatant to a final concentration of 10–20 mM and purified in the Ni-NTA column as described (32, 33). Addition of 8 M urea to the reaction buffer allows for the isolation of insoluble protein in bacterial inclusion bodies and efficient transduction into cells. Bound proteins were eluted with stepwise addition of 5–10 ml each of 100, 250, and 500 mM imidazole in the above buffer. Urea was removed by rapid display by using the Slide-A-Lyzer cassette (Pierce) or by the use of desalting PD-10 columns (Sephadex G-25; Amersham Biosciences).

Migration Assay—Cell migration assays were performed using transwell migration chambers (Corning Inc., Corning, NY) (10). Membranes with a pore size of 8 μm (Corning Inc.) were coated with OPN (25 ng/ml) at 4 °C overnight (haptotaxis) and dried under air. Approximately 5 × 10^5 cells (RAW cells with or without GFP, GFP-PTEN WT, GFP-PTEN C124A, Myc, or Myc-Akt K179M) were added to the upper chamber in DMEM containing 1% FBS and 2% bovine serum albumin (100 μl) and allowed to adhere for 1–2 h. After cells with GFP-PTEN (WT) attached to the membrane, TAT fusion protein was added to a final concentration of 100 nm in the upper chamber in the above medium (100 μl). Substrates such as OPN (25 μg/ml) were added to the lower chamber in DMEM containing 1% FBS and 2% bovine serum albumin (600 μl; chemotaxis). The cells were allowed to migrate for 12–14 h at 37 °C in a tissue culture incubator with 5% CO_2. After the incubation period, nonmigrated cells on the upper side on the membrane were removed with a cotton swab. Wells were fixed with an alcohol/formaldehyde/acetic acid mixture (20:2:1) for 15 min. Filters were stained with hematoxylin stain (Sigma), rinsed well with water, and dried. Dried filters were cut out and mounted with permount solution (Thomas Scientific, Swedesboro, NJ) on a glass slide. Cells were viewed under a ×40 objective in an inverted microscope and counted (Zeiss microscope). Data are presented as the number of cell-migrated fields (mean ± S.D.), and all assays were performed in triplicate.

PTEN Activity Assay—For the generation of whole cell lysates prepared from cells with or without RANKL (100 ng/ml) treatment, cells were washed once with ice-cold PBS and lysed in a cell lysis buffer (New England Biolabs) to prepare whole cell lysates. The lysates were centrifuged and supernatants analyzed using PTEN malachite green assay kit (Upstate Biotechnology) according to the manufacturer’s instructions.

RESULTS

Expression of PTEN in RAW 264.7 Osteoclast Precursors Regulates RANKL-stimulated Signal Transduction—RANKL activates PI3K/Akt survival signaling and activity of osteoclasts (17, 19–21). It also prompts macrophages to develop the osteoclast phenotype. PTEN, a tumor suppressor, is frequently mutated in human cancers and is a negative regulator of PI3K/Akt survival signaling (1–7). However, the role of PTEN in osteoclast precursors is unknown. Therefore, we examined whether PTEN regulates Akt survival signaling pathway in RANKL-treated RAW 264.7 osteoclast precursors. Activation of Akt and Bad has been implicated in anti-apoptotic signaling pathways (8, 9). As shown in Fig. 1, RANKL activated Akt and Bad in 5 min in GFP-expressing cells (peaks at 5 min and 10 min, respectively), and the effects were blocked by GFP-PTEN (WT) expression (Fig. 1, A and B). Moreover, RANKL altered cell morphology in RAW 264.7 osteoclast precursors with and without GFP (Fig. 1C). RANKL-treated GFP-PTEN (WT)-expressing cells revealed no obvious morphological change (Fig. 1C). In most cell types, mobilization of NFκB involves phosphorylation IκB-α serine residues 32/36, resulting in ubiquitination and rapid proteasomal degradation of the phosphorylated inhibitory protein (34–36). RANKL stimulation also stimulated IκB-α phosphorylation by 5 min (peak 5 min), leading to

FIG. 4. PTEN regulates OPN-activated Akt and Rac/cdc42, but not Rho, in RAW 264.7 osteoclast precursors. RAW 264.7 osteoclast precursors were transiently cotransfected with GFP, GFP-PTEN wild-type (WT), GFP-PTEN (C124A) mutant, Myc, or Myc-Akt (K179M) dominant-negative, and cells were treated with OPN (25 μg/ml) for the indicated time. Whole cell extracts were electrophoresed and analyzed by immunoblotting with antibodies against Akt (A), phospho-Akt (A), and phospho-Rac/cdc42 (C). B, lysates from transfected cells were incubated with Rhotekin Rho binding domain beads, the beads washed, and the bound protein analyzed by immunoblotting with a monoclonal antibody against RhoA.
a decrease of total cellular IκB-α beginning at 10 min in GFP-expressing cells, events required for NFκB activation (Fig. 1D) (13–18). In contrast, in GFP-PTEN (WT) transfectants, RANKL failed to activate IκB-α (Fig. 1D). NFκB is essential for osteoclast differentiation and anti-apoptosis (13–18, 37, 38). RANKL stimulated nuclear translocation of NFκB at 5 min (peak 5 and 10 min) in GFP-expressing cells, and the effect was delayed (peak 15 min) by GFP-PTEN (WT) expression (Fig. 1E). On the other hand, in transfectants expressing the GFP-PTEN (C124A) mutant enhanced Akt, Bad, and IκB-α phosphorylation, and nuclear translocation of NFκB in the absence of RANKL compared with GFP-expressing cells. (Fig. 1, 0 min). To further confirm the above results, we measured GFP-PTEN (WT) induced apoptosis and its suppression by GFP-PTEN

Fig. 5. PTEN and Akt regulate OPN-stimulated cell migration in RAW 264.7 osteoclast precursors. A, RAW 264.7 osteoclast precursor migration was assessed in both haptotaxis and chemotaxis assays. Data represent means ± S.D. of three experiments in triplicate. B and C, transfected RAW 264.7 osteoclast precursors were added to the upper chamber and assessed in both haptotaxis and chemotaxis assays. Data represent means ± S.D. of three experiments in triplicate. *, p < 0.01 compared with control or GFP-, GFP-PTEN (C124A) mutan-, or Myc-expressing cells in haptotaxis assay and control, or GFP- or Myc-expressing cells in chemotaxis assay. D, after GFP-PTEN wild-type (WT)-expressing RAW 264.7 osteoclast precursors attached to the membrane, TAT fusion protein (V14Rho, L61Rac, V12cdc42) added to the upper chamber and assessed chemotaxis assays. Data represent means ± S.D. of three experiments in triplicate. *, p < 0.01 compared with GFP-PTEN (WT)- and GFP-PTEN (WT) + HA-TAT-expressing cells.
Regulates Osteoclast Differentiation in Vitro—GFP-PTEN (WT)-expressing cells (Fig. 1, essential for osteoclasts differentiation, and it was delayed in phage lineage (13—RAW 264.7 osteoclast precursors. Our results indicate that PTEN regulates the RANKL-activated survival signaling pathway and apoptosis in RAW 264.7 osteoclast precursors. Our results indicate that PTEN regulates the RANKL-activated survival signaling pathway and apoptosis in RAW 264.7 osteoclast precursors. Our results indicate that PTEN regulates the RANKL-activated survival signaling pathway and apoptosis in RAW 264.7 osteoclast precursors.

Expression of PTEN in RAW 264.7 Osteoclast Precursors Regulates Osteoclast Differentiation in Vitro—Osteoclasts are derived from hematopoietic progenitors of the monocyte macrophage lineage (13—18). Therefore, we examined whether GFP-PTEN (WT) suppresses osteoclast differentiation from RAW 264.7 osteoclast precursors. Nuclear translocation of NFκB is essential for osteoclasts differentiation, and it was delayed in GFP-PTEN (WT)-expressing cells (Fig. 1E). GFP-PTEN (WT) suppressed TRAP-positive MNCs number compared with control and other vector-expressing cells. In contrast, GFP-PTEN (C124A) stimulated TRAP-positive MNC number compared with other conditions (Fig. 3).

Expression of Akt Dominant-negative in RAW 264.7 Osteoclast Precursors Induces Apoptosis, but Does Not Influence Osteoclast Differentiation—Recent studies have demonstrated that Akt regulates apoptosis at multiple sites and identified direct Akt targets including Bad, caspase 9, the forkhead family of transcription factors, and the NFκB regulator IKK, each of which plays a critical role in mediating cell death (8, 9, 39, 40). We showed that Myc-Akt (K179M) dominant-negative delayed nuclear translocation of NFκB (Fig. 1E) compared with Myc-expressing cells and induced apoptosis (Fig. 2). In contrast, TRAP-positive MNC numbers were not influenced by Myc-Akt (K179M) dominant-negative-expressing cells (Fig. 3). These data indicate that the suppression of osteoclast differentiation may be attributed to a decreased number of RAW 264.7 osteoclast precursors by GFP-PTEN (WT)-induced apoptosis and that delayed nuclear translocation of NFκB did not affect osteoclast production. Indeed, GFP-PTEN (WT) more strongly induced apoptosis, compared with the Myc-Akt (K179M) dominant-negative (Fig. 2).

Expression of PTEN in RAW 264.7 Osteoclast Precursors Regulates the OPN Signaling Pathway—OPN stimulates monocyte/macrophage migration, and it also stimulates osteoclasts migration via Rho activation (10, 24). Rho and Rac, which are members of the Rho-GTPase family, play an important role in the organization of the actin cytoskeleton in osteoclasts (10, 41—46). Recent studies have reported that Akt is essential for endothelial cell chemotaxis, whereas PTEN reconstitution or overexpression inhibits cell migration (29, 47, 48). Therefore, we examined whether PTEN regulates OPN-activated Akt, Rho, and Rac signaling pathway in RAW 264.7 osteoclast precursors. OPN activated Akt (at 5 min; peak 10 min), Rho (at 5 min), and Rac/cdc42 (at 5 min; peak 10 and 15 min) in GFP-expressing cells, and the effects of OPN on Akt and Rac/cdc42 were completely blocked by GFP-PTEN (WT) expression (Fig. 4). However, GFP-PTEN (WT) did not inhibit activated Rho expression (Fig. 4B). OPN also activated Rho (at 5 min) in GFP-PTEN (C124A) mutant-expressing cells (Fig. 4B). Myc-Akt (K179M) dominant-negative also blocked OPN-stimulated Rac/cdc42 phosphorylation (Fig. 4C) compared with Myc-expressing cells. In contrast, GFP-PTEN (C124A) mutant enhanced Akt (peak 10 min) and Rac/cdc42 phosphorylation (peak 10 min) compared with GFP-expressing cells, but not Rho, and these effects were continued until the end of the assays (60 min) (Fig. 4). These results demonstrate that PTEN regulates OPN-activated cell survival and migration signaling pathways in RAW 264.7 osteoclast precursors.

Expression of PTEN and Akt in RAW 264.7 Osteoclast Precursors Regulates OPN-stimulated Migration—PTEN suppresses migration of a variety of cell types, including primary human fibroblasts, non-transformed mouse fibroblasts, and tumor cells (47, 49). PTEN—null mouse fibroblasts also show enhanced rates of migration, which are reduced by reintroduction of PTEN (48). Since the role of PTEN in OPN-activated signaling pathway has not been studied, we investigated whether PTEN and Akt regulate OPN-stimulated migration in RAW 264.7 osteoclast precursors using migration assays. We have previously demonstrated that OPN stimulates osteoclast migration (10). RAW 264.7 osteoclast precursor migration was also strongly stimulated by OPN (Fig. 5A). GFP-PTEN (WT) and Myc-Akt (K189M) dominant-negative suppressed OPN-stimulated cell migration in haptotaxis and chemotaxis assays (Fig. 5, B and C). In contrast, GFP-PTEN (C124A) mutant stimulated OPN-stimulated cell migration in chemotaxis assays, but not significantly in haptotaxis assays (Fig. 5, B and C). These data support the hypothesis that OPN-activated Rac and cell migration were suppressed by GFP-PTEN (WT) and Myc-Akt (K189M) dominant-negative in RAW 264.7 osteoclast precursors.

FIG. 6. RANKL may regulate PTEN activity in RAW 264.7 osteoclast precursors and BMMs. A and C, RAW 264.7 osteoclast precursors and BMMs were treated with RANKL (100 ng/ml) for the indicated time, and whole cell extracts were electrophoresed and analyzed by immunoblotting with antibodies against phospho-Akt, phospho-PTEN, and PTEN. B and D, whole cell extracts were prepared from RAW 264.7 osteoclast precursors with or without RANKL (100 ng/ml)-treated, and PTEN activity was measured using PTEN malachite green assay kit. Data represent means ± S.D. of three experiments in duplicate. *, p < 0.01 compared with control.
Rac Rescues the Suppression of Migration by GFP-PTEN (WT) in RAW 264.7 Osteoclast Precursors—Rho and Rac are essential for osteoclast migration (10, 41, 44, 45). Rho acts upstream of PI3K in osteoclasts (10). Rac also acts upstream of PI3K in breast carcinoma epithelial cells (43), whereas Rac acts downstream of Akt in endothelial cells (50). Therefore, the Rac signaling pathway in osteoclast migration is not clear. We found that Rac is downstream of Akt in RAW 264.7 osteoclast precursors since GFP-PTEN (WT) and Myc-Akt (K179M) dominant-negative blocked Rac phosphorylation (Fig. 4C). To further examine this issue, we performed migration assays following transduction of constitutive active Rho, Rac, and cdc42 in GFP-PTEN (WT)-transfected Raw cells. Constitutively active Rac, but not Rho and cdc42, rescued the suppression of migration in GFP-PTEN (WT)-expressing cells (Fig. 5D). We conclude that Rac acts downstream of Akt, and PTEN has influence on cell migration by Rac, not Rho, in RAW 264.7 osteoclast precursors.

RANKL May Regulate PTEN Activity in RAW 264.7 Osteoclast Precursors and BMMs—Little is known about modes of PTEN regulation. Recent studies have reported that PTEN transcription is regulated by p53 in immortalized mouse embryonic fibroblasts (51). However, it is not clear how PTEN activity is regulated in osteoclast precursors. To determine whether RANKL regulates PTEN activity, we examined expression of PTEN phosphorylation and its activity in RANKL-treated RAW 264.7 osteoclast precursors and BMMs. RANKL-activated Akt (RAW: 5 min, BMMs: 5 and 10 min) and suppressed PTEN activity (RAW: 5 min, BMMs: 5 and 10 min) (Fig. 6). After activation by RANKL, activated Akt gradually decreased (Fig. 6, A and C). In contrast, PTEN activity was stimulated by RANKL at 15 and 30 min (Fig. 6, B and D). PTEN also activated by RANKL at 10 min (peak 15 min) in RAW 264.7 osteoclast precursors and 30 min in BMMs (Fig. 6, A and C). These data indicate that RANKL directly or indirectly may regulate PTEN activity in RAW 264.7 osteoclast precursors and BMMs.

**DISCUSSION**

RANKL produced by osteoblastic lineage cells and activated T lymphocytes is an essential factor for osteoclast differentiation, fusion, activation, and survival, thus resulting in bone resorption and bone loss (13–18). We demonstrate that RANKL activates Akt, which is essential for cell survival in osteoclasts in agreement with others (17, 19–21). Although RANKL is known to stimulate phosphorylation of a serine residue at position 473 in Akt (Ser-473), we have shown stimulation of threonine phosphorylation at position 308 in Akt (Thr-308) (data not shown) in agreement with Wong et al. (19). However, RANKL did not stimulate Akt (Ser-473) phosphorylation (data not shown) in our hands except in GFP-PTEN (C124A) mutant-expressing cells (data not shown). Akt requires phosphorylation at positions Ser-473 and Thr-308 for full activation (8, 9, 39). Therefore, the mechanism of RANKL-stimulated activation of Akt demonstrated here is not yet clear.

Bad is a Bcl-2 family member regulated through phosphorylation at Ser-136 by activated Akt resulting in its inactivation and cell survival (9). We demonstrated that RANKL phosphorylates Bad, and the GFP-PTEN (C124A) mutant enhances the effect of RANKL, whereas the RANKL effect is negatively regulated by GFP-PTEN (WT). These results indicate that PTEN activity down-regulates both RANKL-activated Akt and Bad phosphorylation in RAW 264.7 osteoclast precursors, regulating RANKL-stimulated survival signals.

We demonstrated that GFP-PTEN (WT) negatively regulates RANKL-activated Akt survival signaling and suppresses osteoclast differentiation of RAW 264.7 osteoclast precursors. Mice deficient in NFκB develop severe osteoporosis because of failed osteocalcification (37). Therefore, NFκB is essential for osteoclasts differentiation. GFP-PTEN (WT) and Myc-Akt (K179M) dominant-negative delayed nuclear translocation of NFκB compared with GFP-expressing cells. However, the Myc-Akt (K179M) dominant-negative failed to suppress osteoclast differentiation in RAW 264.7 osteoclast precursors. We suggest that the number of RAW 264.7 osteoclast precursors were decreased by GFP-PTEN (WT) because of its strong induction of apoptosis compared with Myc-Akt (K179M) dominant-negative-expressing cells. Moreover, GFP-PTEN (WT) may directly suppress RANKL-activated TRAF6/NFκB signaling pathway, but how it does this is unclear (Fig. 7). In contrast, the GFP-PTEN (C124A) mutant enhanced RANKL-activated Akt survival signaling pathway and markedly induced nuclear translocation of NFκB. It also stimulated osteoclast differentiation. Our data suggest that PTEN regulates the RANKL-activated Akt survival signaling pathway and RANKL-induced osteoclast differentiation in RAW 264.7 osteoclast precursors (Fig. 7).

OPN has been shown to stimulate macrophage and osteoclast migration (10, 24). It is not known, however, whether OPN stimulates cell migration via PI3K/Akt pathway activation. It has been previously reported in LNCap cells, that αvβ3...
mediates cell migration by the PI3K/Akt pathway activation. However, adhesion to OPN did not support $\alpha_{v}\beta_{3}$-mediated cell migration or activate the PI3K/Akt pathway (11). We have demonstrated that OPN stimulates production of phosphoinositides, including phosphatidylinositol trisphosphate in osteoclasts and activates gelsolin-associated PI3K (10, 26). Here, we have demonstrated that OPN stimuli cell migration via the Akt pathway activation including Rac, and the effect is regulated by PTEN in RAW 264.7 osteoclast precursors (Fig. 7). Moreover, we demonstrated that Rac acts downstream of Akt. Because, GFP-PTEN (WT) negatively regulated OPN-activated Rac, but not activated Rho. In addition, L61Rac, but not V14Rho, rescued cell migration in GFP-PTEN-expressing cells.

It has been reported that PTEN inhibits cell migration (1–7). One recently identified mechanism is through its effects on PtdIns (3, 4, 5) P3 levels, which have downstream effects on Rac and cdc42 signaling (48). However, tyrosine-phosphorylated FAK can bind to and activate PI3K (52), and a contributing pathway affecting PtdIns (3, 4, 5) P3 through FAK and PI3K has also been identified (53). This is a second mechanism by which PTEN may inhibit phosphotyrosine-based signaling pathways that have proven useful for dissecting the signaling pathways that regulate cell migration, although they do not prove that PTEN normally regulates these pathways. In the studies reported here, we found that PTEN inhibition of OPN-stimulated cell migration by PTEN through negative regulation of OPN-activated Akt and Rac, but not Rho.

As shown in Fig. 7, it is likely that PTEN plays multiple roles in RAW 264.7 osteoclast precursors. One question that remains is how PTEN activity is regulated. There is very little information on the regulation of PTEN expression, localization, or activity. In this study, we demonstrate that one possibility is RANKL regulation of PTEN activity. RANKL activates the PI3K/Akt survival signaling pathway and osteoclast differentiation, which PTEN also regulates (Fig. 7). Our data support that RANKL may regulate the balance between activated Akt and PTEN, which influences osteoclast differentiation.

In summary, we provide the first evidence that PTEN regulates the RANKL- and OPN-activated signaling pathways. Furthermore, PTEN activity influences osteoclast differentiation, survival, and migration in osteoclast precursors. The molecular target of PTEN is PtdIns(3,4,5)P3 (28), but whether PTEN directly or indirectly regulates the RANK-activated TRAF6/NFkB signaling pathway is not yet clear (Fig. 7).

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