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Distinctive and selective route of PI3K/PKCα-PKCδ/RhoA-Rac1 signaling in osteoclastic cell migration

Jin-Man Kim, Mi Yeong Kim, Kyunghee Lee**, Daewon Jeong*

Department of Microbiology, Laboratory of Bone Metabolism and Control, Yeungnam University College of Medicine, Daegu, 42415, South Korea

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ABSTRACT

Cell migration during specialized stages of osteoclast precursors, mononuclear preosteoclasts, and multinucleated mature osteoclasts remain uncertain. M-CSF- and osteopontin-induced osteoclastic cell migration was inhibited by function-blocking monoclonal antibodies specific to the integrin αv and β3 subunits, suggesting that integrin αvβ3 mediates migratory signaling induced by M-CSF and osteopontin. M-CSF and osteopontin stimulation was shown to regulate two branched signaling processes, PI3K/PKCα/RhoA axis and PI3K/PKCδ/Rac1 axis. Interestingly, inactivation of RhoA or Rac1 blocked preosteoclast and mature osteoclast migration but not osteoclast precursor migration in a transwell-based cell migration assay. Moreover, the inhibitory effect on preosteoclast and mature osteoclast migration induced by Rac1 inactivation was more effective than that by RhoA inactivation. Collectively, our findings suggest that osteoclast precursor migration depends on PI3K/PKCα-PKCδ signaling mediated via integrin αvβ3 bypassing RhoA and Rac1, whereas preosteoclast and mature osteoclast migration relies on PI3K/PKCα-PKCδ/RhoA-Rac1 axis signaling mediated via integrin αvβ3 with increased dependency on PKCδ/Rac1 signaling route as differentiation progresses.

1. Introduction

Osteoclast differentiation is a multiple process requiring cell adhesion, proliferation, podosome formation, migration, and fusion to form multinuclear cells (Boyle et al., 2003). Thus, molecules involved in cell-extracellular matrix interactions, including integrins, play diverse functional roles in osteoclast differentiation (Feng et al., 2001; Ross and Teitelbaum, 2005). In particular, binding of integrin αvβ3 to specific bone matrix proteins, such as osteopontin and bone sialoprotein II, induces cell adhesion and survival, cytoskeletal reorganization during osteoclast differentiation, as well as bone resorption (Chellaiah et al., 2000; Feng et al., 2001; Ross et al., 1993; Sugatani et al., 2003). Integrin-mediated cell adhesion to its ligands stimulates the so-called "outside-in" signal transduction pathway (Schwartz et al., 1995). Engagement of integrin αvβ3 by its ligands induces activation of a number of intracellular signaling pathways, including extracellular signal-regulated kinase 1 and 2 (ERK1/2), Rho family GTPases, phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC) (Chellaiah et al., 2000; Grey et al., 2000; Ory et al., 2000; Rucci et al., 2005). Conversely, "inside-out" signaling was shown to be induced by conformational changes mediated through cytoplasmic and transmembrane domains of integrin αvβ3 by growth factors and cytokine stimulation, thereby modulating affinity of integrins for their ligands (Geiger et al., 2001). Several growth factors and cytokines control integrin-dependent cellular functions through "inside-out" signaling. For example, macrophage colony-stimulating factor (M-CSF) and hepatocyte growth factor (HGF) induce osteoclast adhesion, migration, and bone resorption in an integrin β3-dependent manner (Faccio et al., 2003). Transforming growth factor β1 (TGFβ1) and vascular endothelial growth factor receptor-2 (VEGF-2) promote adhesion of human lung fibroblasts and human endothelial cells via integrin αvβ3 signaling, respectively (Pechkovsky et al., 2008; Soldi et al., 1999).

Osteoclasts attach to bone surfaces through specialized discrete structures called podosomes, which are composed of polymerized actin (F-actin) and other cytoskeletal proteins, including integrin αvβ3, vinculin, talin, and paxillin (Chellaiah, 2006; Ory et al., 2008). Binding of integrin αvβ3 to ligands and stimulation of growth

* Corresponding author. Department of Microbiology, Yeungnam University College of Medicine, 170 Hyunchung-Ro, Nam-Gu, Daegu, 42415, South Korea.
** Corresponding author. Department of Microbiology, Yeungnam University College of Medicine, 170 Hyunchung-Ro, Nam-Gu, Daegu, 42415, South Korea.
E-mail addresses: kyungheelee@ynu.ac.kr (K. Lee), dwjeong@ynu.ac.kr (D. Jeong).

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factors promote osteoclast adhesion, migration, and bone resorption through podosomes assembly and disassembly (Kanehisa et al., 1990; Saltel et al., 2004). Numerous factors have been reported to be involved in the process of osteoclast adhesion, migration, and bone resorption. Activation of Rho GTPases has been known to stimulate podosome organization, migration, and bone resorption in osteoclasts. Treatment with exoenzyme C3 and ADP-ribosylating protein, which are known to inhibit Rho (A, B, and C) activity, was shown to reduce bone resorption in osteoclasts (Zhang et al., 1995). Activation of RhoA and Rac GTPases in response to growth factor stimulation induces cytoskeletal reorganization and lamellipodia formation of osteoclasts, respectively (Faccio et al., 2003; Ory et al., 2000). Inhibition of Rac1 by introducing anti-Rac antibodies into permeabilized osteoclasts promotes cell retraction and reduces bone resorption activity (Razzouk et al., 1999). PKC activation by stimulators, such as phorbol ester, integrins, and growth factors, has been shown to induce adhesion, podosome formation, and migration in endothelial cells, macrophages, and osteoclasts (Guo et al., 2009; Rucci et al., 2005; Tatin et al., 2006). It has been also reported that PKCα activation stimulated by integrin αvβ3 induces osteoclastic cell migration and bone resorption (Rucci et al., 2005), and PKC activation by growth factors promotes macrophage migration (Furundzija et al., 2010; Guo et al., 2009).

Despite reports that M-CSF and osteopontin mediate osteoclastic cell migration, the detailed molecular mechanisms of cell-migratory signaling in specialized stages during osteoclastogenesis remain largely unknown. We here analyzed a sequential link between migratory signal mediators in cell migration of osteoclast precursors, preosteoclasts, and mature osteoclasts derived from osteoclastgenesis.

2. Materials and methods

2.1. Materials

Chemical reagents and antibodies were purchased from the following sources: osteopontin (Sigma-Aldrich, St. Louis, MO, USA); bovine serum albumin (BSA, Sigma-Aldrich); fetal bovine serum (FBS, Hyclone, Logan, Utah); EDTA (Sigma-Aldrich); antibodies to integrin αv (RMV-7) and β3 (2C9.G2, BD Biosciences, San Jose, CA); PP1 (Src inhibitor, Biomol, Farmingdale, NY); LY294002 (PI3K inhibitor, Sigma-Aldrich); PD98059 (ERK inhibitor, Sigma-Aldrich); Gö6976 (PKCα inhibitor, Calbiochem, San Diego, CA); rottlerin (PKCδ inhibitor, Calbiochem); Y27632 (RhoA inhibitor, Sigma-Aldrich); and NSC23766 (Rac1 inhibitor, Calbiochem). All other commercial reagents were from Sigma Aldrich unless otherwise noted.

2.2. Preparation of osteoclast precursors, preosteoclasts, and mature osteoclasts

Bone marrow-derived monocytic osteoclast precursors were isolated from the tibia and femur bones of 6-week-old C57BL/6 male mice as described (Lee et al., 2006). Preosteoclasts and mature osteoclasts were prepared by further culturing osteoclast precursors in a humidified atmosphere of 5% CO_2 at 37 °C in bicarbonate-buffered minimum essential medium-alpha (α-MEM, Hyclone) containing M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 2 and 4 days, respectively. Fresh medium was changed on day 2. To assess osteoclast differentiation, cells were stained for tartrate-resistant acid phosphatase (TRAP) with the use of a leukocyte acid phosphatase staining kit (Sigma-Aldrich). TRAP-positive multinucleated cells [TRAP (+) MNCs] with more than three or 10 nuclei were counted under a light microscope. Osteoclast precursors and preosteoclasts can be defined as TRAP-negative mononuclear cells and TRAP-positive mononuclear cells, respectively.

2.3. Cell migration assay

Cell migration assays were performed using transwell migration chambers (pore size, 8 μm; Corning Inc., NY) according to the instructions of the manufacturer. For cell migration induced by M-CSF and RANKL, osteoclast precursors (2×10^5 cells/well), preosteoclasts (2×10^5 cells/well), and mature osteoclasts (4×10^4 cells/well) were seeded into the upper chamber of transwell filters in 100 μl of α-MEM containing 0.1% BSA and 0.5% FBS (osteoclast precursors and preosteoclasts) or 0.1% BSA and 10% FBS (mature osteoclasts). After M-CSF (30 ng/ml), RANKL (100 ng/ml), or M-CSF plus RANKL were added to the lower chamber in 600 μl of α-MEM containing 0.1% BSA and 0.5% FBS (osteoclast precursors and preosteoclasts) or 10% FBS (mature osteoclasts), cells were allowed to migrate for 5 h. For cell migration stimulated by osteopontin, the lower side of transwell filters was coated with osteopontin (10 μg/ml) for 12 h at 4 °C and then blocked with 1% BSA for 1 h at 37 °C. The prepared cells as described above were seeded into the upper chamber of transwell filters coated with osteopontin. The culture media in the lower chamber of transwell filters contained 0.1% BSA and 0.5% FBS for osteoclast precursor and preosteoclast migration or 0.1% BSA, 10% FBS, and M-CSF (30 ng/ml) for mature osteoclast migration. The cells were allowed to migrate for 5 h, fixed with 3.7% formalin for 15 min, and stained with crystal violet. Non-migrated cells in the upper side of transwell filters were removed with a cotton swab, after which migrated cells were counted under a light microscope.

2.4. Subcellular fractionation and immunoblot analysis

Cytosolic and membranous proteins were fractionated by using a plasma membrane protein extraction kit according to the instructions of the manufacturer (BioVision, Milpitas, CA). Briefly, osteoclast precursors, preosteoclasts, and mature osteoclasts were lysed with homogenization buffer. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatant was used as the cytosolic fraction. The pellets were solubilized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM glycerol phosphate, and 1× protease inhibitor cocktail) and sonicated for 30 s three times on ice. After centrifugation at 14,000 × *g* for 15 min at 4 °C, the resulting supernatants were used as the plasma membrane fraction. The protein concentration was determined using a BCA protein assay kit (Bio-Rad, Hercules, CA). The cytosolic and membranous proteins solubilized in RIPA buffer were denatured by adding an equal volume of SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 50 mM dithiothreitol) and subjected to 10% SDS-PAGE. The separated proteins were electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA) and probed with primary antibodies to $Thr^{638/641}$-phosphorylated PKCα (Cell Signaling, Beverly, MA), PKCα (BD Biosciences), $Ser^{643/676}$-phosphorylated PKCδ (Cell Signaling), PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Santa Cruz Biotechnology), RhoA (Cell Biolabs, San Diego, CA), and Rac1 (Cell Biolabs). All blots were then detected with HRP-conjugated secondary antibodies and ECL reagents (Abfrontier, Seoul, Korea).

2.5. RhoA/Rac1 GTPases assays

The assays for RhoA and Rac1 GTPase activity were performed using a RhoA/Rac1 assay kit according to the instructions of the manufacturer (Cell Biolabs). Osteoclast precursors, preosteoclasts,

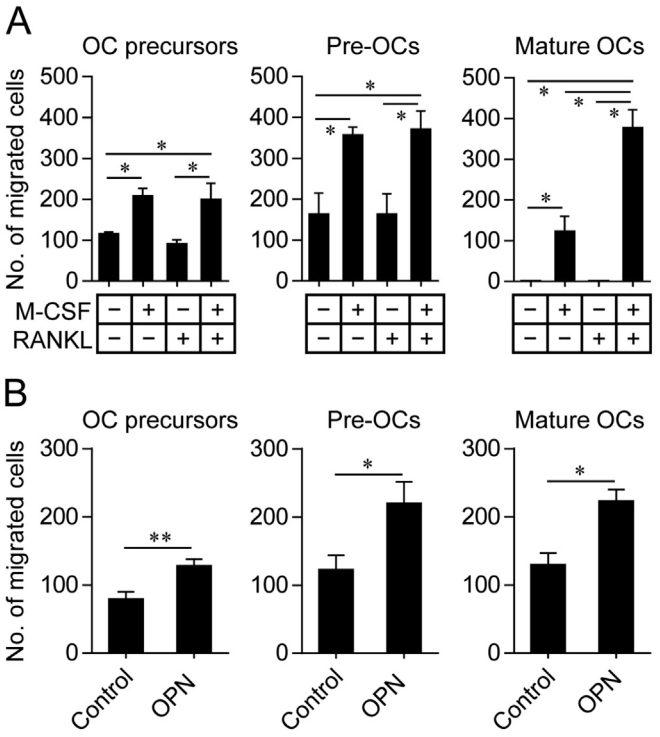

Fig. 1. M-CSF and osteopomtin facilitate osteoclastic cell migration. (A) Osteoclastic cell migration stimulated by M-CSF and RANKL. Migration of osteoclast precursors (OC precursors), preosteoclasts (Pre-OCs), and mature osteoclasts (mature OCs) in the absence or presence of M-CSF, RANKL, or M-CSF plus RANKL was analyzed using a transwell migration chamber. Cell migration was quantified by counting the number of cells that had migrated through the filter. Data between two groups were assessed by ANOVA analysis with post-hoc test. $^{*}P < 0.01$. (B) Osteopontin (OPN)-induced osteoclastic cell migration. Control indicates cell migration using transwell filters coated with BSA instead of OPN. Data are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.01$; $^{**}P < 0.05$.

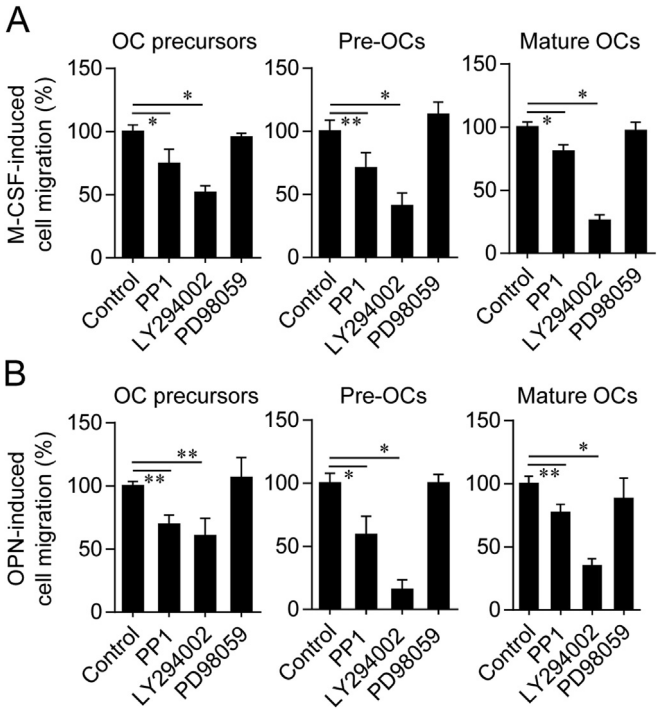

Fig. 2. Inactivation of Src and PI3K inhibits osteoclastic cell migration. (A) M-SCF-induced osteoclastic cell migration. Osteoclast precursors (OC precursors), preosteoclasts (Pre-OCs), and mature osteoclasts (mature OCs) were seeded into the upper chamber of transwell filters. After M-CSF (for OC precursor and Pre-OCs) or M-CSF plus RANKL (for mature OCs) was added to the lower chamber and allowed to adhere for 1 h, PP1 (Src inhibitor, 5 μM), LY294002 (PI3K inhibitor, 5 μM), or PD98059 (ERK inhibitor, 20 μM) was added to the lower chamber and allowed to further migrate for 4 h. (B) Osteopontin (OPN)-induced osteoclastic cell migration. OC precursors and pre-OCs were seeded into the upper chamber coated with OPN and incubated to induce cell adhesion for 1 h. Mature OCs were seeded into the upper chamber coated with OPN, and M-CSF was added to the lower chamber and allowed to adhere for 1 h. After cell adaptation, inhibitors as in (A) were added to the lower chamber and then allowed to further migrate for 4 h. Control indicates cell migration using transwell filters coated with BSA instead of OPN. Data on the number of migrated cells are expressed as the mean ± S.D. and percentages ($n = 3$). Data between two groups were assessed by ANOVA analysis with post-hoc test. $^{*}P < 0.01$; $^{**}P < 0.05$.

and mature osteoclasts were lysed with a lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 2% NP40, and 1× protease inhibitor cocktail). After lysates were centrifuged and the supernatants incubated with Rhotekin RBD agarose or PAK1 PBD agarose for 2 h at 4 °C, the incubated mixture was centrifuged and washed with lysis buffer. The resulting pellets were resuspended in lysis buffer and applied to immunoblot analysis.

2.6. Statistical analysis

Data are presented as mean values ± SD from at least three independent experiments, and differences between the two groups were analyzed using Student's two-tailed *t*-test. In addition, data with more than three groups were determined by analysis of variance (ANOVA) and the post-hoc tests were used to compare data between two groups using the SPSS 21.0 software package. A *P* value of <0.05 was considered to be statistically significant.

3. Results and discussion

3.1. M-CSF and osteopontin induce osteoclastic cell migration

M-CSF and RANKL are necessary and sufficient for differentiation of osteoclast precursors into multinucleated osteoclasts (Lacey et al., 1998; Yasuda et al., 1998). M-CSF has been shown to stimulate osteoclast migration (Felix et al., 1994; Ross and Teitelbaum, 2005), but the role of RANKL in osteoclastic cell migration has not been fully elucidated until now. Using a transwell-based cell migration assay, we first investigated whether or not RANKL mediates migration of osteoclast precursors, mononuclear preosteoclasts, and multinuclear osteoclasts produced from the serial processes of osteoclast differentiation. As shown in Fig. 1A. M-CSF enhanced cell migration of three different cell types, whereas RANKL did not induce any migratory action. On the contrary, mature osteoclast migration induced by M-CSF and RANKL was much greater than that induced by M-CSF alone. RANKL has been reported to mediate directed osteoclast migration in previous live cell migration studies (Jones et al., 2006). In addition, the stimulatory effect of M-CSF and RANKL on mature osteoclast migration can be attributed to the increased survival rate of mature osteoclasts (Supplementary Fig. 1). Therefore, increased osteoclast migration by RANKL could be explained by both the increased survival rate and direct stimulation of osteoclast migration. Reasoning that osteopontin has been reported to act as a key factor in macrophage and osteoclast migration (Chellaiah et al., 2000; Denhardt et al., 2001), we next analyzed osteopontin-mediated osteoclastic cell migration. Osteoclastic cell migration by osteopontin showed a result similar to that of M-CSF (Fig. 1B). The results indicate that M-CSF and osteopontin are involved in stimulating cell migration of osteoclast precursors, preosteoclasts, and mature osteoclasts derived from different stages of osteoclastogenesis.

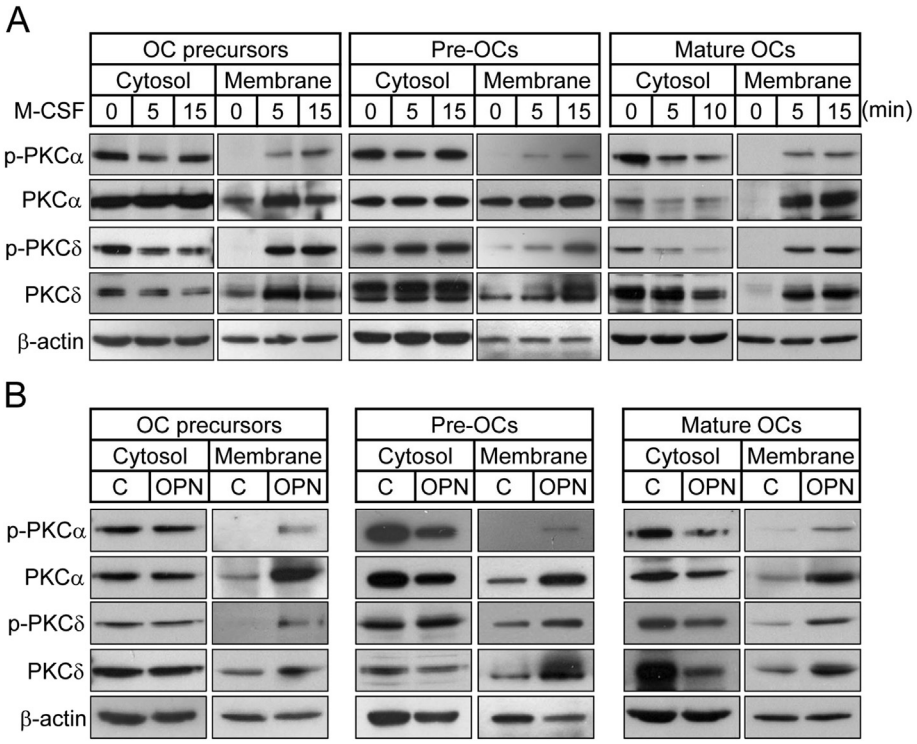

Fig. 3. M-CSF and osteopontin activate PKCα and PKCδ signals. (A) PKCα and PKCδ activation induced by M-CSF. Osteoclast precursors (OC precursors), preosteoclasts (Pre-OCs), and mature osteoclasts (mature OCs) were cultured in α-MEM with 0.5% FBS and without M-CSF for 4 h, followed by stimulation with M-CSF (30 ng/ml) for the indicated times. (B) PKCα and PKCδ activation induced by osteopontin (OPN). Cells prior to stimulation as in (A) were detached from culture dishes, centrifuged, and resuspended again in culture media with 0.5% FBS and without M-CSF. The suspended cells were used as a control and stimulated by inoculating cells onto OPN-coated culture dishes for 30 min. After subcellular fractionation from cells as in (A) and (B), cytosolic and membranous proteins were subject to immunoblot analysis with specific antibodies to phospho-PKCα, PKCα, phospho-PKCδ, and PKCδ. β-actin was used as the loading control.

3.2. Osteoclastic cell migration by M-CSF and osteopontin is dependent on integrin αvβ3/PI3K signaling

Osteoclastic cell migration is known to be achieved via bone matrix protein osteopontin/integrin αvβ3-mediated "outside-in" signaling and macrophage colony-stimulating factor (M-CSF)-mediated "inside-out" signaling depending on osteopontin/αvβ3 signaling (Chellaiah et al., 2000; Faccio et al., 2003; Geiger et al., 2001; Grey et al., 2000). Therefore, we analyzed whether or not M-CSF- and osteopontin-mediated migration in osteoclast precursors, preosteoclasts, and mature osteoclasts is dependent upon integrin αvβ3. Consistent with previous reports that a divalent cation is essential for binding of integrins to their ligands (Dransfield et al., 1992; Gachet et al., 1993), we observed that treatment with the divalent cation-chelating agent EDTA completely blocked osteoclastic cell migration induced by M-CSF and osteopontin (Supplementary Fig. 2). Further, function-blocking monoclonal antibodies to the integrin αv and β3 subunits suppressed cell migration induced by M-CSF and osteopontin in all osteoclastic stages, consistent with previous reports that M-CSF and osteopontin mediate cell migration through "inside-out" signaling linking αvβ3 and "outside-in" signaling directly depending on αvβ3 functional activity, respectively (Chellaiah et al., 2000; Faccio et al., 2003; Geiger et al., 2001; Grey et al., 2000).

Activation of integrin αvβ3 has been reported to induce multiple signaling pathways such as c-Src, PI3K, and ERK in osteoclasts (Hruska et al., 1995; Rucci et al., 2005; Teti et al., 1998). To analyze the downstream signal of αvβ3 in osteoclastic cell migration, we next investigated whether or not c-Src, PI3K, and ERK are involved in osteoclastic cell migration. As shown in Fig. 2, osteoclastic cell migration in response to M-CSF and osteopontin was blocked by c-Src inhibitor (PP1) and PI3K inhibitor (LY294002) but not ERK inhibitor (PD98059) in a transwell migration assay. The inhibitory effect of PI3K inactivation on osteoclastic cell migration was greater than that of c-Src inactivation. Thus, our findings indicate that cell migration induced by M-CSF and osteopontin is majorly dependent on integrin αvβ3/PI3K axis signaling.

3.3. PI3K mediates PKCα and PKCδ signaling in osteoclastic cell migration

Others have reported that PI3K activates PKC in osteoclasts (Bekhite et al., 2011; Frey et al., 2006), and our data show that M-CSF specifically stimulates PKCα and PKCδ among PKC isoforms in osteoclast precursors (Supplementary Fig. 3). Based on the results that PKC isoforms exist downstream of PI3K and M-CSF induces PKCα and PKCδ activation, we assessed the connection between PI3K and PKCα/δ in response to M-CSF and osteopontin. M-CSF and osteopontin stimulation induced translocation of total and phosphorylated forms of PKCα and PKCδ from the cytosolic compartment to the plasma membrane (Fig. 3). Further, PI3K inhibitor (LY294002) resulted in significant reduction in the levels of translocated PKCα and PKCδ from the cytosol to plasma membrane after M-CSF and osteopontin stimulation, indicating that activation of PKCα and PKCδ by M-CSF and osteopontin was regulated by PI3K (Fig. 4). We next investigated the involvement of PKCα and PKCδ in osteoclastic cell migration by using specific inhibitors for PKCα (Gö6976) and PKCδ (rottlerin). PKCδ inactivation significantly inhibited osteoclastic cell migration compared to PKCα inactivation (Fig. 5). These results imply that cell migration may be predominantly dependent on PI3K/PKCδ signaling compared with PI3K/PKCα signaling at all stages of osteoclastogenesis in a

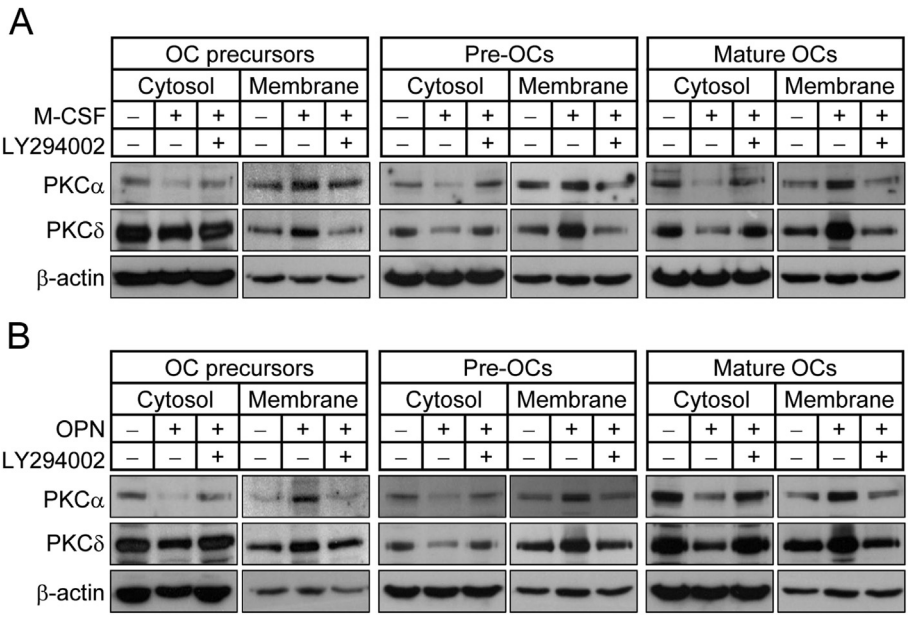

Fig. 4. PI3K regulates activities of PKCα and PKCδ induced by M-CSF and osteopontin. (A) Inhibitory effect of PI3K inactivation on membrane localization of PKCα and PKCδ after M-CSF stimulation. Osteoclast precursors (OC precursors), preosteoclasts (Pre-OCs), and mature osteoclasts (mature OCs) were serum-starved for 4 h, pretreated with LY294002 (5 μM) for 30 min, and then stimulated with M-CSF (30 ng/ml) for 5 min. (B) Inhibitory effect of PI3K inactivation on membrane localization of PKCα and PKCδ after OPN stimulation. Serum-starved osteoclastic cells (OC precursors, Pre-OCs, and mature OCs) were detached and pretreated with LY294002 (5 μM) for 15 min. Cells were seeded on OPN-coated plates and incubated for 30 min. The suspended cells were used as a control. Cytosolic and membrane fractions were prepared and immunoblotted with specific antibodies to PKCα and PKCδ. β-actin was used as the loading control.

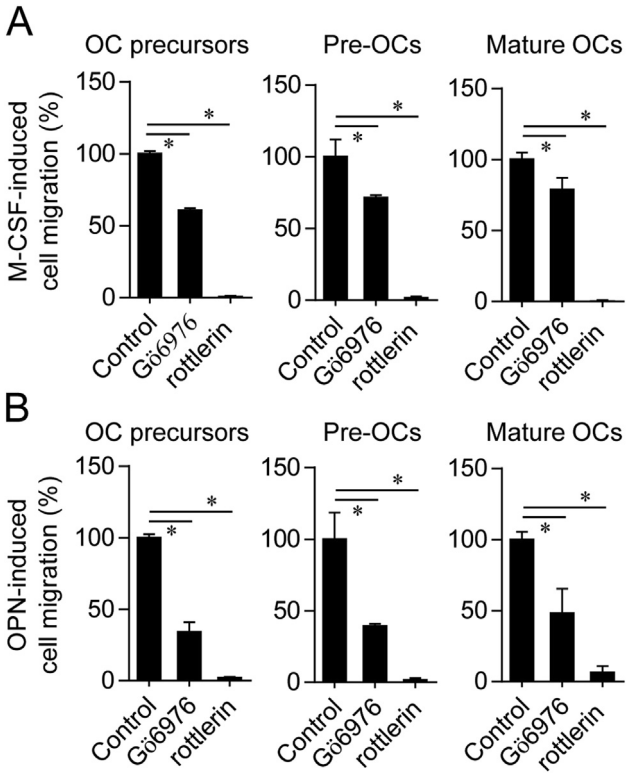

Fig. 5. PKCα and PKCδ signals mediate osteoclastic cell migration. (A and B) Effect of PKCα and PKCδ inactivation on M-CSF- and osteopontin (OPN)-induced osteoclastic cell migration. Osteoclastic cells (OC precursors, Pre-OCs, and mature OCs) as described in the Materials and Methods section and the legend of Fig. 2 were treated with Gö6976 (PKCα inhibitor, 1 μM) or rottlerin (PKCδ inhibitor, 5 μM), added to the lower chamber, and allowed to migrate for 4 h. In OPN-induced cell migration, control indicates cell migration using transwell filters coated with BSA instead of OPN. Data on the number of migrated cells are expressed as the mean ± S.D. and percentages ($n = 3$). Data between two groups were assessed by ANOVA analysis with post-hoc test. $^{*}P < 0.01$.

transwell-based cell migration assay. The inhibitory effect of PKCα and PKCδ inactivation on osteoclastic cell migration induced by M-CSF and RANKL was not significantly different compared to the effect on M-CSF-induced osteoclastic cell migration (see Fig. 5A versus Supplementary Fig. 4), suggesting that RANKL does not directly contribute to regulation of PKC isoforms in osteoclastic cell migration induced by M-CSF.

3.4. PKCα and PKCδ signaling controls RhoA and Rac1 activation in osteoclastic cell migration

The small GTPases Rho and Rac are known to regulate osteoclast migration upon M-CSF receptor signaling and are stimulated by PKCδ in a spastic porcine coronary artery model (Fukuda et al., 2005; Kandabashi et al., 2003). Thus, we examined the possibility that M-CSF and osteopontin stimulation could activate RhoA and Rac1 in a PKCα- and/or PKCδ-dependent manner. As shown in Fig. 6A and B, M-CSF and osteopontin stimulation resulted in a significant increase in the levels of GTP-bound RhoA and Rac1 in osteoclast precursors, preosteoclasts, and mature osteoclasts. The active form of GTP-bound RhoA stimulated by M-CSF and osteopontin was markedly suppressed by PKCα inhibitor (Gö6976), whereas the active form of GTP-bound Rac1 was not altered by Gö6976 treatment. Interestingly, PKCδ inhibitor (rottlerin) induced marked reduction of both RhoA and Rac1 activation in all stages of osteoclastogenesis. These results indicate that PKCα could selectively regulate RhoA activity, and PKCδ signaling could induce bidirectional activation of RhoA and Rac1 in the signaling processes stimulated by M-CSF and osteopontin. Finally, we determined the specific contribution of RhoA and Rac1 to osteoclastic cell migration by using RhoA inhibitor (Y27632) and Rac1 inhibitor (NSC23766). As shown in Fig. 6C and D, osteoclast precursor migration induced by M-CSF and osteopontin was not altered by treatment with NSC23766 or Y27632, whereas cell migration of preosteoclasts and mature osteoclasts was inhibited by NSC23766 or Y27632.

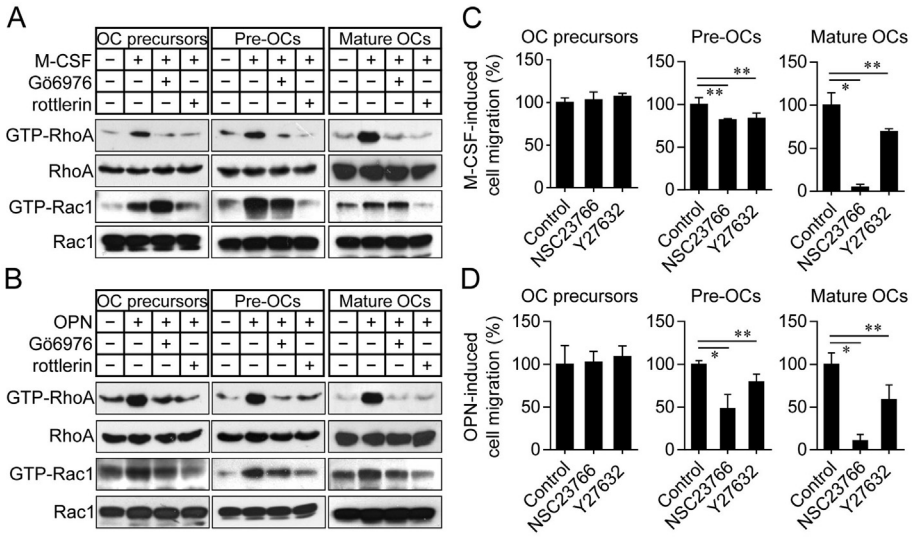

Fig. 6. PKCα and PKCδ signals regulate RhoA and Rac1 activities in osteoclastic cell migration. (A and B) Inhibitory effect of PKCα and PKCδ inactivation on RhoA and Rac1 activities. Osteoclastic cells (OC precursors, Pre-OCs, and mature OCs) as described in the legend of Fig. 3 were pretreated with Gö6976 (PKCα inhibitor, 1 μM) or rottlerin (PKCδ inhibitor, 5 μM) for 15 min and then stimulated with M-CSF for 5 min or with osteopontin (OPN) for 30 min. For OPN-mediated signal analysis, the suspended cells were used as a control. Cell extracts were applied to immunoblot analysis. (C and D) Inhibitory effect of RhoA and Rac1 inactivation on osteoclastic cell migration. Osteoclastic cells (OC precursors, Pre-OCs, and mature OCs) as described in the Materials and Methods section and the legend of Fig. 2 were treated with Y27632 (RhoA inhibitor, 10 μM) or NSC23766 (Rac1 inhibitor, 100 μM), added to the lower chamber, and allowed to migrate for 4 h. Control indicates cell migration using transwell filters coated with BSA instead of OPN. Data on the number of migrated cells are expressed as the mean ± S.D. and percentages ($n = 3$). Data between two groups were assessed by ANOVA analysis with post-hoc test. $^{*}P < 0.01$; $^{**}P < 0.05$.

Noticeably, the suppressive effect of mature osteoclast migration induced by Rac1 inhibition was much greater than that induced by RhoA inhibition. Together, these results indicate that migration of preosteoclasts and mature osteoclasts is tightly dependent on RhoA and Rac1 signaling, whereas osteoclast precursor migration has no association with RhoA and Rac1 activities. Moreover, our findings suggest that the role of PKCδ/Rac1 signaling is greater than that of PKCα/RhoA signaling as osteoclast differentiation progresses in a transwell-based cell migration assay.

Osteoclastogenesis includes serial processes via formation of osteoclast precursors derived from myeloid cells, differentiation of osteoclast precursors into TRAP (+)-mononuclear preosteoclasts, cell-cell fusion of preosteoclasts to form multinuclear mature osteoclasts, and maturation and activation to bone-resorbing osteoclasts (Boyle et al., 2003). Migration of mononuclear preosteoclasts is a prerequisite of cell-cell contact and fusion prior to multinuclear osteoclast formation, and mature osteoclasts migrate along the bone surface to remove old bone and repair damaged bone. This suggests osteoclastic cell migration is a critical event in homeostatic bone remodeling.

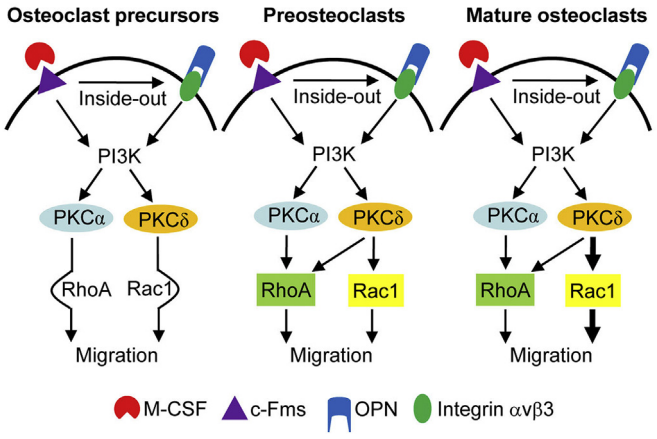

Fig. 7. Schematic representation of M-CSF- and osteopontin-mediated migratory signal transduction in the stages of osteoclast precursors, preosteoclasts, and mature osteoclasts.

In this study, we analyzed signaling processes in M-CSF- and osteopontin-mediated osteoclastic cell migration according to the stages of osteoclast differentiation. As shown in Fig. 7, M-CSF-mediated "inside-out" signaling via integrin αvβ3 signaling and osteopontin/αvβ3-mediated "outside-in" signaling was shown to mediate cell migration in the process of osteoclast differentiation. Osteoclast precursor migration proceeds via PI3K/PKCα and PI3K/PKCδ signaling bypassing RhoA and Rac1. Migration of pre-osteoclasts and mature osteoclasts shares two branched signals of the PI3K/PKCα/RhoA axis and PI3K/PKCδ/Rac1 axis. In the process of preosteoclast and mature osteoclast migration, PKCδ signaling regulates both RhoA and Rac1 activation, whereas PKCα signaling controls only RhoA activation. As compared with PI3K/PKCα/RhoA signaling, the effect of PI3K/PKCδ/Rac1 migratory signaling increased as osteoclast differentiation progressed from mononuclear preosteoclasts to multinuclear mature osteoclasts. Collectively, we report the distinctive and selective signaling pathway governing osteoclastic cell migration at all stages of osteoclast differentiation. Since PKCδ dominates both RhoA and Rac1 during migration of bone-resorbing mature osteoclasts and their migration is dependent on Rac1 compared to RhoA signaling, we suggest that PKCδ or Rac1 is a valuable therapeutic target for the development of anti-osteoporotic agents by inhibiting the migratory activity of bone-resorbing osteoclasts.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.08.042.

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