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An Osteoclastic Transmembrane Protein-Tyrosine Phosphatase Enhances Osteoclast Activity in Part by Dephosphorylating EphA4 in Osteoclasts

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ABSTRACT

We have previously shown that PTP-oc is an enhancer of the functional activity of osteoclasts and that EphA4 is a suppressor. Here, we provide evidence that PTP-oc enhances osteoclast activity in part through inactivation of EphA4 by dephosphorylating key phosphotyrosine (pY) residues of EphA4. We show that EphA4 was pulled down by the PTP-oc trapping mutant but not by the wild-type (WT) PTP-oc and that transgenic overexpression of PTP-oc in osteoclasts drastically decreased pY602 and pY779 residues of EphA4. Consistent with the previous findings that EphA4 deficiency increased pY173-Vav3 level (Rac-GTP exchange factor [GEF]) and enhanced bone resorption activity of osteoclasts, reintroduction of WT-*Epha4* in *Epha4* null osteoclasts led to ~50% reduction in the pY173-Vav3 level and ~2-fold increase in bone resorption activity. Overexpression of Y779F-*Epha4* mutant in WT osteoclasts markedly increased in pY173-Vav3 and reduced bone resorption activity, but overexpression of Y602F-*Epha4* mutant had no effect, suggesting that pY779 residue plays an important role in the EphA4-mediated suppression of osteoclast activity. Deficient EphA4 in osteoclasts has been shown to up-regulate Rac-GTPase and down-regulate Rho-GTPase. PTP-oc overexpression in osteoclasts also increased the GTP-Rac level to 300% of controls, but decreased the GTP-Rho level to ~50% of controls. PTP-oc overexpression or deficient *Epha4* each also reduced pY87-Ephexin level, which is a Rho GEF. Thus, PTP-oc may differentially regulate Rac signaling versus Rho signaling through dephosphorylation of EphA4, which has shown to have opposing effects on Rac-GTPase versus Rho-GTPase through differential regulation of Vav3 versus Ephexin. J. Cell. Biochem. 116: 1785–1796, 2015.

KEY WORDS: PROTEIN-TYROSINE PHOSPHATASE; EphA4 RECEPTOR; OSTEOCLASTS; Vav3; EPHEXIN; Rho GTPASES

PTP-oc is a structurally unique non-receptor-associated transmembrane protein-tyrosine phosphatase (PTP). It is expressed predominantly in cells of monocyte-macrophage lineage, B-lymphocytes, and osteoclasts [Wu et al., 1996; Sheng and Lau, 2009]. With the exception of a short insert at the cytosolic juxtamembrane region, PTP-oc shares complete sequence identity with the transmembrane and cytosolic domains of PTPRO, a renal receptor-associated PTP (also known as, GLEPP1, PTP-Φ, CRYP2, or PTPBK) [Sheng and Lau, 2009]. However, PTP-oc is not a splicing variant of PTPRO. Its expression is driven by an alternative, cell-type-specific intronic promoter [Amoui et al., 2003; Yang et al., 2007b]. PTP-oc is a positive modulator of the functional activity of mature osteoclasts in vitro and in vivo [Suhr et al., 2001; Amoui et al., 2004, 2007; Lau et al., 2006; Sheng et al., 2009]. Overexpression of PTP-oc in osteoclasts increased cell size, spreading, and migration, and their enhanced bone resorption activity [Sheng et al., 2009].

We have previously demonstrated that the phosphorylated tyrosine-527 residue (pY527) of Src is a cellular substrate of PTP-oc in osteoclasts [Suhr et al., 2001; Amoui et al., 2004, 2007; Lau et al., 2006, 2012; Sheng et al., 2009]. The PTP-oc-mediated dephosphorylation of pY527-Src activates its protein-tyrosine kinase (PTK) activity. This in turns: 1) promotes osteoclast survival through the Src PTK-dependent activation of the JNK2/NFκB pathway [Amoui et al., 2007]; 2) up-regulates the β_3-integrin signaling via Src PTK-dependent phosphorylation of tyr-759

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residue of β_3-integrin and its downstream mediators [Lau et al., 2012]; and 3) stimulates the ITAM signaling via Src-dependent phosphorylation of tyr-525/tyr-526 of Syk [Lau et al., 2012]. The latter action acts in concert with the β_3-integrin signaling to promote osteoclast adhesion, spreading, and cytoskeleton re-organization. PTP-oc also acts through the Src PTK-dependent phosphorylation of Shp1 to inactivate Shp1 [Lau et al., 2012], which is an inhibitory mediator of osteoclast differentiation and activity [Umeda et al., 1999]. In addition to the aforementioned Src PTK-dependent actions, there is circumstantial evidence that some of the regulatory actions of PTP-oc on the functional activity of osteoclasts are not dependent of the Src-PTK signaling [Amoui et al., 2004; Yang et al., 2007a].

We have recently reported that the EphA4 signaling in mature osteoclasts functions as a negative regulator of the functional activity of osteoclasts in Src-independent manners [Stiffel et al., 2014]. EphA4 is a member of the Eph receptor multigene family, which is the largest receptor PTK family [Gale et al., 1996]. The Ephs are activated by the binding of membrane-bound ligand, ephrin (Efn), of neighboring cells. The binding of Efn ligand induces conformational change in the cytoplasmic portion of the Eph receptor [Wybenga-Groot et al., 2001], leading to auto-phosphorylation of two juxtamembrane tyrosines of the Eph receptor, which provide binding sites for SH2-containing signaling proteins. This then results in activation of its PTK activity and signaling mechanism [Zisch et al., 2000]. Ephs are divided into two subclasses, EphAs and EphBs, based on sequence similarity and ligand binding affinity [Zhou, 1998]. There are 10 EphAs (EphA1-10) and 6 EphBs (EphB1-6) in animals. The Efns are also divided into two structurally distinct multigene families: The glycosylphosphatidylinositol-linked EfnAs and the trans-membrane-bound EfnBs. There are currently five known EfnAs (EfnA1-5) and three EfnBs (EfnB1-3). Efn ligands are also capable of receptor-like signaling [Pasquale, 2008]. The Eph-mediated signal is known as the forward signaling and the Efn-mediated signal in neighboring cells is called the reverse signaling. Of all the members of Ephs, mature osteoclasts express predominantly EphA4 [Zhao et al., 2006; Irie et al., 2009; Stiffel et al., 2014]. Because activation of EphA4 signaling requires phosphorylation of several key tyrosine residues [Zisch et al., 2000], and because the EphA4 signaling is a negative regulator of the osteoclast activity, we postulate that PTP-oc upregulates the bone resorption activity of osteoclasts in part through the PTP-oc-mediated dephosphorylation of key phosphotyrosine residues of EphA4 that are essential for activation of its signaling. Accordingly, the objectives of this study were twofold: First, we sought to test the hypothesis that tyrosine-phosphorylated (pY)-EphA4 is a cellular substrate of PTP-oc in osteoclasts. We determined whether pY-EphA4 in osteoclastic cells could be pulled down by the phosphatase-dead (PD)-PTP-oc-glutathione-S-transferase (GST) trapping mutant and whether osteoclasts with overexpression of PTP-oc had reduced pY-EphA4 levels. The second objective was to determine whether the PTP-oc-dependent dephosphorylation of the key pY residues of EphA4 plays a key role in activation of the functional activity of mature osteoclasts in vitro.

MATERIALS AND METHODS

MATERIALS

RAW/C4 cells (a RAW246.7 cell clone) were originally obtained from Dr. A. Ian Cassady of the Griffith University (Gold Coast, Queensland, Australia). Culture media and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY) and Atlanta Biologicals (Lawrenceville, GA), respectively. Tissue culture supplies were from Falcon (Oxnard, CA). The enhanced chemiluminescence detection kit was obtained from Millipore (Billevica, MA). Nitrocellulose transfer membrane (Transblot Nitrocellulose) was a product of BioRad (Hercules, CA). Recombinant soluble receptor activator of NFκB ligand (RANKL) and macrophage colony stimulating factor (mCSF) were from Santa Cruz Biotech (Santa Cruz, CA) and CalBiochem (San Diego, CA), respectively. The anti-pY527-Src, anti-pY173-Vav3, anti-Vav3, anti-pY87-Ephexin, anti-Ephexin, and anti-phosphotyrosine (anti-pY) 4G10 antibodies were purchased from BioSource International (Camarillo, CA), UpState Biotechnology (Lake Placid, NY), Santa Cruz Biotechnology (SantaCruz, CA), R&D Systems (Minneapolis, MN), or BD Transduction Laboratories (San Diego, CA). The Rac Activation Assay Kit and the Rho Activation Assay Kit were purchased from Cell Biolab Inc., (San Diego, CA). All other reagents were obtained from either Sigma–Aldrich (St. Louis, MO) or Fisher (Los Angeles, CA).

CELL CULTURES

RAW/C4 cells were maintained in alpha-modified Eagle's medium (αMEM) supplemented with 10% (v/v) FBS. For osteoclastic differentiation, RAW/C4 cells were plated in αMEM containing 10% (v/v) FBS. RANKL (at 200 ng/ml) was added to initiate osteoclastic differentiation into tartrate-resistant acid phosphatase (TRACP)-positive, multinucleated (two or more nuclei) "osteoclast-like" cells. At day 3, the culture medium was replaced with fresh medium containing only 66 ng/ml RANKL.

To generate primary marrow-derived osteoclasts, bone marrow cells were flushed out of long bones of 10- to 12-week-old male transgenic (Tg) mice with targeted overexpression of PTP-oc in cells of osteoclastic lineage [Sheng et al., 2009], 10- to 12-weeks-old male *Epha4* null mice [Stiffel et al., 2014], or 10- to 12-weeks-old male WT littermates. The bone marrow cells were cultured in αMEM containing 10% (v/v) FBS for 24 h. Non-adherent cells containing osteoclast precursors were collected and plated at a density of 3.2×10^5 cells/cm^2 (in 24-well plates) or $3.7–4.25 \times 10^5$ cells/cm^2 (in 6-well plates) in αMEM with 10% (v/v) FBS in the presence of RANKL (66 ng/ml) and mCSF (50 ng/ml). Culture medium was changed at day 3 with fresh αMEM containing 10% (v/v) FBS and RANKL/mCSF. After 3 or 6 days, the total number of TRACP-positive osteoclast-like cells in 24-well culture wells was counted. Endpoint measurements were performed on day 6 of the RANKL treatment unless otherwise indicated. The animal use component was approved by the Animal Care and Use Committee of the Jerry L. Pettis Memorial VA Medical Center.

LENTIVIRUS TRANSDUCTION OF OSTEOCLAST PRECURSORS

Lentiviral-based vectors were used to re-introduce WT *Epha4* expression back into *Epha4* null osteoclast precursors or to overexpress various Y→F *Epha4* dominant negative mutants in

WT osteoclast precursors. The lentiviral-based vectors (lenti-*Epha4* or lenti-*gfp* control vectors) were generated as described previously [Yam et al., 2002]. Very briefly, freshly isolated non-attached marrow-derived osteoclast precursors were transduced with either lenti-*gfp* or lenti-*Epha4* vector with an MOI of 10 by a previously described procedure [Hall et al., 2007]. The transduced cells were then treated with RANKL and mCSF for 6 days to differentiate into multinucleated TRACP-positive osteoclasts. Cell lysate of each transduced cells was prepared and Western immunoblot against EphA4 was performed to confirm effective re-introduction of *Epha4*. Cellular level of actin was also measured for normalization against equal loading of extract proteins. Because Vav3 is an essential regulatory mediator of osteoclast activity and bone resorption [Faccio et al., 2005], and because PTP-oc [Lau et al., 2012] and EphA4 [Stiffel et al., 2014] both regulate osteoclast activity in part through modulation of the pY173-Vav3 levels, the effect of overexpression of WT or dominant negative mutants of Epha4 on the cellular level pY173-Vav3 (normalized against total Vav3) was measured.

PTP-oc TRAPPING MUTANT-GST FUSION PROTEIN PULL-DOWN ASSAY

The PD-PTP-oc trapping mutant-GST pull-down analysis was performed in RAW/C4 cells as described previously [Lau et al., 2012]. Briefly, RAW/C4 cells (5×10^5/well in 6-well plates) were transfected with 0.4 μg of WT-PTP-oc-GST, PD-PTP-oc-GST trapping mutant expression plasmid, or empty vector plasmid using Effectene (Qiagen, La Jolla, CA). The transfected cells, with or without 24-h pretreatment with 10 μM of the protein-tyrosine kinase inhibitor, PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo[3,4-*d*]pyrimidine), were lysed with the radioimmunoprecipitation assay (RIPA) buffer. Cellular proteins associated with WT- or PD-PTP-oc-GST-fusion proteins were "pulled down" by glutathione–sepharose affinity chromatography and eluted with 1 mM glutathione. The eluted proteins were then resolved on 7.5% (w/v) SDS–PAGE and transferred onto a nitrocellulose membrane, and EphA4 was identified by Western blots with a specific antibody against EphA4.

RESORPTION PIT FORMATION ASSAY

The resorption pit formation assay was performed as described previously [Suhr et al., 2001]. Because targeted overexpression of PTP-oc [Sheng et al., 2009] or deficient *Epha4* expression [Stiffel et al., 2014] in osteoclast precursors did not significantly alter the number of mature osteoclasts formed from marrow-derived precursors in response to RANKL/m-CSF treatment, we focused primarily on measurements of the average bone resorption activity of the derived osteoclasts. Very briefly, the total area of ~50 resorption pits per dentine slice was measured with the OsteoMeasure system. The average pit area per pit (determined by dividing total pit area by the number of pits measured) was reported as an index of the average bone resorption activity per osteoclast.

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qPCR) ASSAYS

qPCR was carried out using the Promega GoTag qPCR master mix by the SYBR Green method on the MJ Research DNA Engine Opticon® 2 System (Waltham, MA). Total RNA was extracted with Qiagen mini RNA kit. The purified RNA was used as template for synthesizing cDNA by reverse transcription using oligo dT(20) primers and Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA). An aliquot of the cDNA was subjected to qPCR amplification using the following gene-specific primer sets: for *Epha4*; forward primer: 5′-TAC ACC GAT CCG AAC CTA CC-3′, and reversed primer: 5′-CAG CGT TGC ATA GGC AGT T-3′; and for *Ppia* (cyclophilin); forward primer: 5′-AAT AGA AGC AGC AGG ACC TGG GAA-3′, and reverse primer: 5′-AAA GCA AAG CCT GCC AGA AGA CAC-3′. The reaction mixture (25 μl) consisted of 12.5 μl of 2X GoTag qPCR master mix (Promega, Madison, WI), the Hot Start *Taq* polymerase, 3 μM of primers, and 3 μl of cDNA template. The PCR amplification condition consisted of a 10-min hot start at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing and extension for 30 s at 60°C, and a final step of melting curve analysis from 60 to 72°C. The data (normalized against *Ppia* [*cyclophilin*] mRNA) were analyzed with Opticon® Monitor Software 2.0. The relative fold change was calculated by the threshold cycle (ΔC_T) method.

WESTERN IMMUNOBLOT ASSAYS

Relative levels of pY527-Src and total Src were determined by Western immunoblots as described previously [Suhr et al., 2001; Lau et al., 2006] using a polyclonal antibody against pY527 residue of Src and a polyclonal anti-Src antibody, respectively. Relative levels of other test signaling protein were also determined by Western immunoblots using respective specific antibodies against indicated pY species or total species of each protein-of-interest. Quantification was performed with the FluorChem Q imaging system (Proteinsimple Inc., Santa Clara, CA).

ACTIVATION OF Rac AND Rho GTPASES ASSAY

The activation status of Rac or Rho was determined by measuring the relative GTP-bound Rac or Rho levels in 350 μg or 1,000 μg, respectively, of extract proteins of marrow-derived osteoclasts of PTP-oc Tg mice or WT littermates using the Rac Activation Assay Kit or Rho Activation Assay Kit, respectively, as instructed by the supplier.

STATISTICAL ANALYSIS

Results are shown as mean ± SEM. Statistical significance was determined with two-tailed Student's *t*-test. The difference was considered significant when $P < 0.05$.

RESULTS

EphA4 is a potential cellular substrate of PTP-oc in osteoclastic cells. Our previous GST pull-down analysis in RAW/C4 osteoclastic cells has shown that at least nine cellular pY-proteins were pulled-down by the PD-PTP-oc-GST trapping mutant but not by the WT-PTP-oc-GST protein [Lau et al., 2012]. The major 60-kD pY-protein being pulled down with the PD-PTP-oc-GST was shown to be Src. To evaluate whether pY-EphA4 is a potential cellular substrate of PTP-oc in osteoclastic cells, we repeated the PD-PTP-oc-GST trapping mutant pull-down assay. Figure 1A shows that the 120-kD pY-

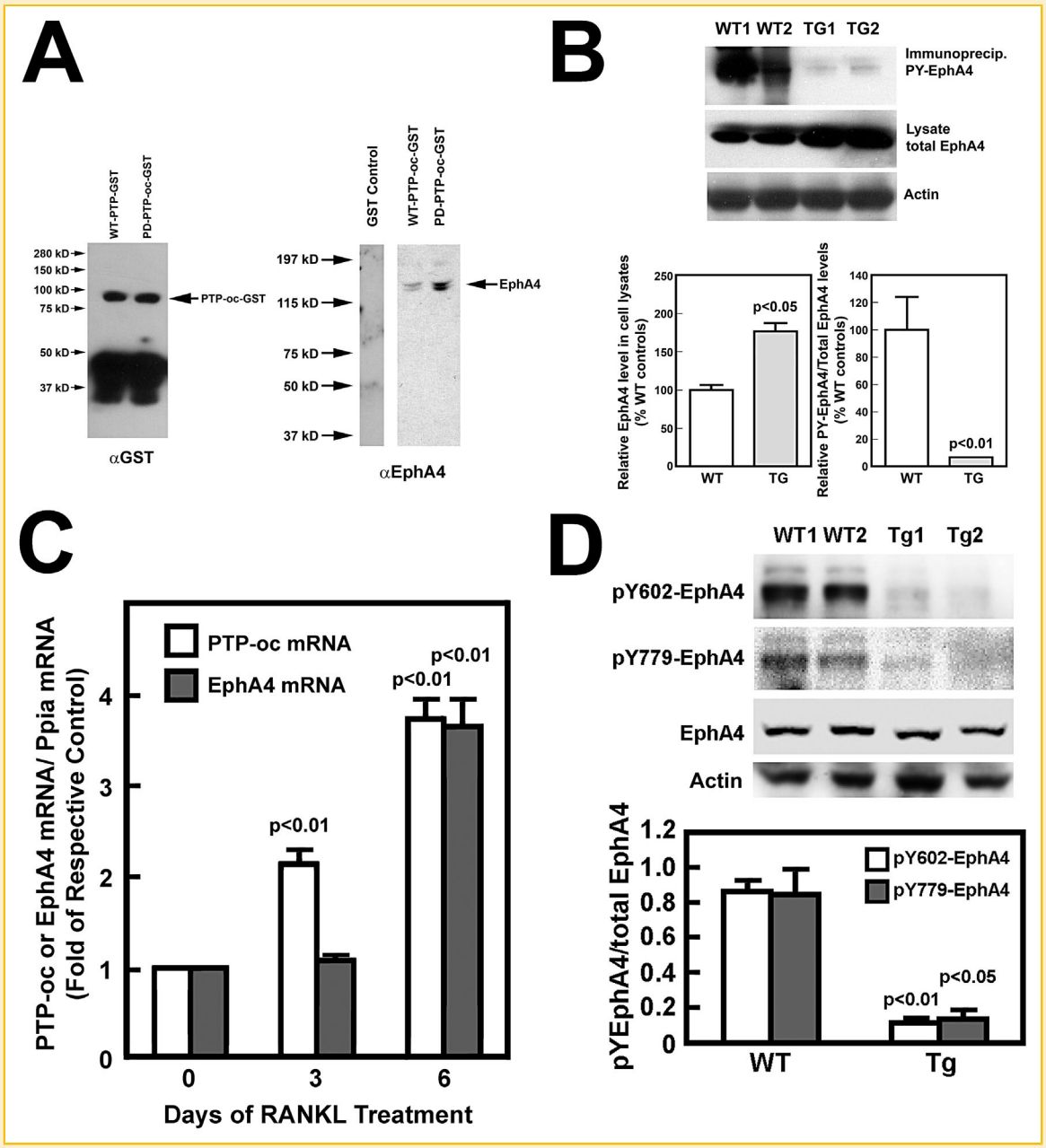

Fig. 1. EphA4 is a potential cellular substrate of PTP-oc. Panel A shows the GST pull-down analyses of "trapped" substrate of PTP-oc in RAW/C4 cells. The GST pull-down assay was performed in RAW/C4 cells. The pull-down cellular pY proteins were analyzed by Western blots using a specific anti-EphA4 antibody. The 120-kD pY protein band was shown to be EphA4. Panel B shows the effect of PTP-oc overexpression on PY levels of EphA4 in mouse osteoclasts. In order to detect total pY-EphA4, cell lysates (650 μg protein) of PTP-oc transgenic (Tg) and those of WT osteoclasts were immunoprecipitated with anti-EphA4 antibody (4 μg). The co-immunoprecipitated proteins were then subjected to Western blot and blotted with an anti-pY (4G10) antibody. To adjust for the loading amounts of total EphA4 protein, each cell lysate (40 μg) was analyzed by Western blot using the anti-EphA4 antibody. Top shows the Western blot, and bottom shows the relative amounts of total EphA4 (left) and pY-EphA4 per total EphA4 (right) in PTP-oc TG and WT osteoclasts (mean ± SEM, n = 4). Panel C shows the PTP-oc expression or *Epha4* expression during the RANKL-induced osteoclast differentiation. Marrow-derived osteoclast precursors were treated with RANKL and mCSF for 0, 3, or 6 days. PTP-oc and *Epha4* mRNAs (each normalized against *Ppia* mRNA) were reported as % WT controls. Panel D shows the effects of PTP-oc overexpression on pY602- and pY779-EphA4 levels in marrow-derived osteoclasts. Cell lysate (40 μg) of two preparations each of PTP-oc transgenic (Tg) and WT osteoclasts was analyzed by Western blot using specific antibody against pY602, pY779, or total EphA4. Top shows the Western blot, and bottom shows the relative amounts of pY602-and pY779-EphA4, normalized against total EphA4 (mean ± SEM, n = 4 each).

protein band pulled down with the PD-PTP-oc trapping mutant was the pY-EphA4. The pull-down EphA4 remained tyrosine-phosphorylated even after a 24-h pretreatment with PP2, prior to the GST pull down assay (not shown), indicating that the increased tyrosine phosphorylation levels of EphA4 was not due to the increased Src PTK activity in the pull-down complex. No significant amounts of 120-kD pY-EphA4 or other pY-containing proteins were pulled down from lysates of the empty GST plasmid-transfected cells, indicating that the pull-down of pY-EphA4 was specific for PTP-oc. To further test the hypothesis that the pY-EphA4 is a cellular substrate of PTP-oc in osteoclasts, we compared the tyrosine phosphorylation level of EphA4 in cell lysates of PTP-oc Tg osteoclasts with that in lysates of corresponding WT osteoclasts. The relative level of immunoprecipitated pY-EphA4 protein (assessed by Western blot using an anti-pY [4G10] antibody) in PTP-oc Tg osteoclasts, normalized against total amounts of EphA4 protein, was reduced to <5% of that in WT osteoclasts. Interestingly, the total level of EphA4 protein in PTP-oc Tg osteoclasts was twice as much as that in WT osteoclasts (Fig. 1B). Figure 1C shows that the time-dependent increase in cellular PTP-oc mRNA levels in osteoclasts during the RANKL treatment was accompanied with a similar time-dependent increase in the *Epha4* mRNA level. This raises an interesting possibility that overexpression of PTP-oc in mature osteoclasts could lead to an up-regulation of EphA4 expression. In this regard, it is conceivable that the increased EphA4 protein level in PTP-oc Tg osteoclasts was the result of a feedback up-regulation of EphA4 expression in response to the PTP-oc-mediated inactivation of the EphA4 forward signaling.

There are four functionally important phosphorylated tyrosine residues (pY596, pY602, pY779, and pY928) in EphA4 [Zisch et al., 2000; Wybenga-Groot et al., 2001]. Phosphorylation of Y596 and Y602 at the juxtamembrane domain opens up the closed inactive configuration of EphA4 for activation [Wybenga-Groot et al., 2001]. Phosphorylation of Y779 at the activation loop enhances its PTK activity. Phosphorylation of Y928 at the C-terminal Sterile Alpha Motif does not affect its PTK activity but it allows binding of SH2-containing signaling proteins [Zisch et al., 2000]. We focused on pY602 and pY779, because they are essential for the PTK activity of EphA4 and because specific antibodies against these two pY residues were commercially available. We did not evaluate pY928, because phosphorylation of this residue did not affect its PTK activity. Figure 1D shows that PTP-oc Tg osteoclasts had very low levels of pY602 and pY779 (i.e., <5% of that in control osteoclasts for each), supporting the premise that these pY residues are potential substrates of PTP-oc in osteoclasts.

Functional role of the phosphorylation of key tyrosine residues of EphA4 in the regulation of osteoclast activity. Before we assessed the importance of the phosphorylation level of key tyrosine residues of EphA4 in the regulation of osteoclast activity, we sought to confirm that the enhanced bone resorption activity in *Epha4* null osteoclasts was indeed the consequence of deficient *Epha4* expression by performing a "rescue" experiment, in which the *Epha4* gene was re-introduced back into *Epha4* null precursors with lenti-WT-*Epha4* transduction. To assess the transduction efficiency, we measured the relative cellular level of EphA4 of marrow-derived WT osteoclast progenitors (normalized against cellular actin level) 2 days after transduction with Lenti-*Epha4* or Lenti-*gfp* vector. The Lenti-*Epha4*-transduced osteoclast precursors had ~2.5-fold more EphA4 protein than the Lenti-*gfp*-transduced control precursors (left panel of Fig. 2A), indicating that the lentiviral vector was effective in transducing osteoclast precursors. The relative level of EphA4 protein in Lenti-*gfp*-transduced control osteoclast precursors was very similar to that in non-transduced WT osteoclast precursors (~30% of the respective cellular actin level) (right panel of Fig. 2A), suggesting that the lentiviral transduction did not have significant effects on cellular EphA4 levels in osteoclast precursors. The right panel of Fig. 2A also confirms the *Epha4* mRNA findings in Fig. 1C that mature osteoclasts expressed 2- to 3-fold more cellular EphA4 protein than untreated precursor cells.

To evaluate whether re-introduction of *Epha4* expression in *Epha4* null osteoclasts could "reverse" the observed enhanced bone resorption activity in *Epha4* null osteoclasts [Stiffel et al., 2014], we transduced *Epha4* null osteoclast precursors with lenti-WT-*Epha4* or lenti-*gfp* control vector. The lenti-*Epha4*- and the lenti-*gfp*-treated marrow-derived osteoclast precursor cells, after plated on dentine slices, were treated with RANKL/mCSF to induce osteoclast differentiation. The average pit area per resorption pit created by these differentiated osteoclasts was measured after 5 days. The average resorption pit size created by lenti-WT-*Epha4*-transduced osteoclasts was reduced to ~60% ($P < 0.01$) of the average size of the pits created by the lenti-*gfp*-transduced or untreated osteoclasts (Fig. 2B). The average pit size per pit created by lenti-WT-*Epha4*-transduced osteoclasts was similar to that created by WT osteoclasts (~0.3 mm^2 per pit) [Lau et al., 2012], suggesting that reintroduction of *Epha4* in *Epha4* KO osteoclasts appeared to "restore" the phenotype of *Epha4* null osteoclasts back to the original phenotype of lower bone resorption activity.

Vav3 is an essential mediator of osteoclast resorption [Faccio et al., 2005], and phosphorylation of its pY173 residue is required for Vav3 activation [Lopez-Lago et al., 2000]. Increased pY173-Vav3 phosphorylation levels in osteoclasts were associated with the PTP-oc-induced [Sheng et al., 2009], or the EphA4 deficiency-related [Stiffel et al., 2014], up-regulation of bone resorption activity. Accordingly, we determined the effect of re-introduction of WT-*Epha4* into *Epha4* null osteoclasts on the pY173-Vav3 levels (Fig. 3A). As expected, re-introduction of the WT-*Epha4* into *Epha4* null osteoclasts significantly ($P < 0.05$) reduced the pY173-Vav3 level to ~60% of that in *Epha4* null osteoclasts.

To determine the importance of the phosphorylation status of Y602 and Y779 of EphA4 in regulation of osteoclast activity, we assessed the effects of overexpression of Y602F-*Epha4* or Y779F-*Epha4* dominant negative mutants in WT osteoclasts on the pY173-Vav3 level. Figure 3B shows that overexpression of the Y779F-*Epha4* mutant in Lenti-Y779F-*Epha4*-transduced WT osteoclasts significantly increased (~4-fold, $P < 0.01$) the pY173-Vav3 level (normalized against respective total Vav3). In contrast, over-expression of the Y602F-*Epha4* mutant only resulted in a small and statistically insignificant increase (Fig. 3C), suggesting that the phosphorylation of Y779 may have greater regulatory effects than that on Y602 with respect to regulation of Vav3 activation. We next sought to confirm the effects of transgenic overexpression of WT-, Y602F-, and Y779F-*Epha4* in WT osteoclasts on in vitro bone

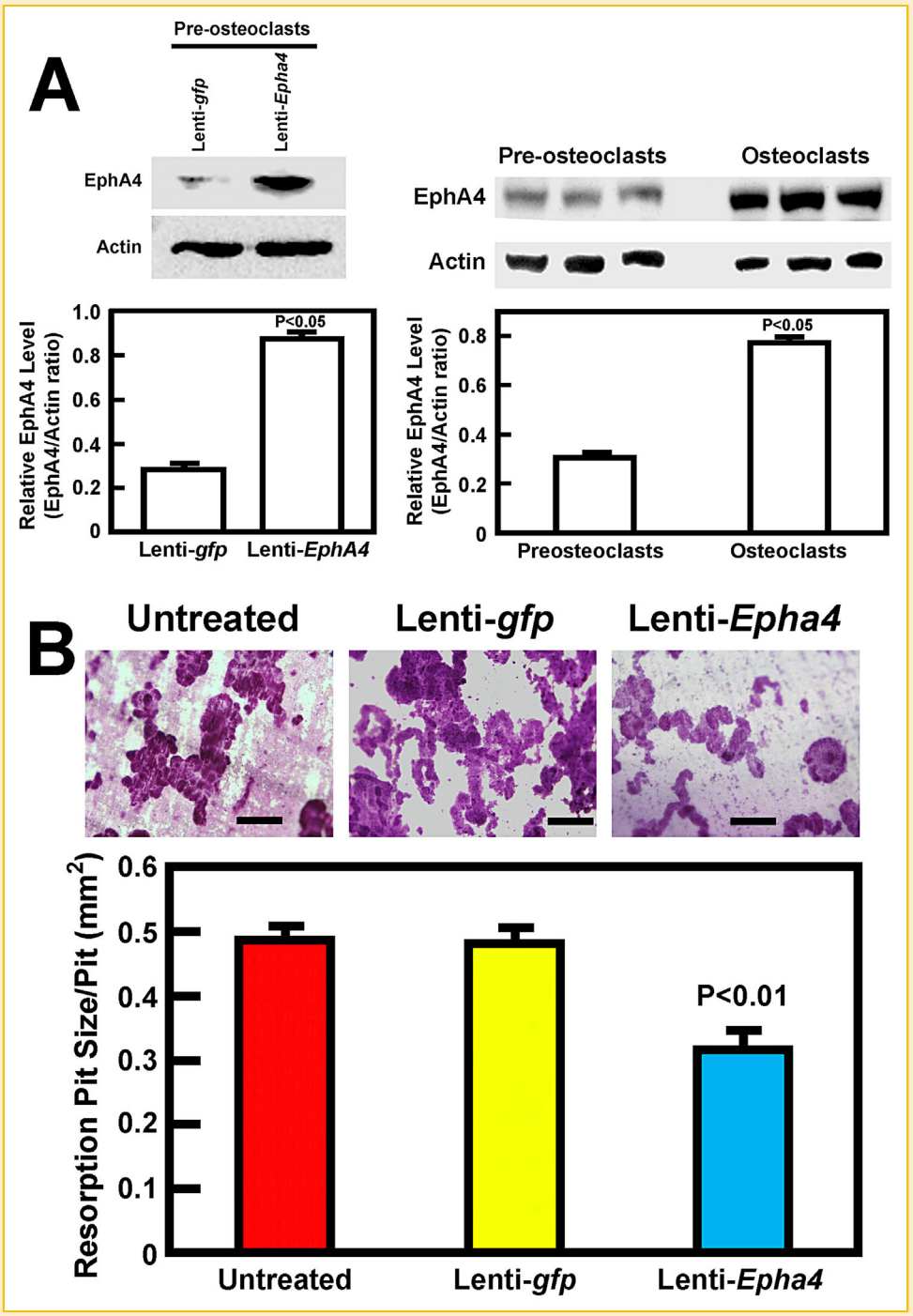

Fig. 2. Lentiviral-based vector reintroduction of *Epha4* back into *Epha4* null osteoclastic cells effectively reversed the phenotype of an enhanced bone resorption activity in vitro. Panel A shows that lenti-viral based vectors can effectively transduce primary mouse osteoclast precursors. Freshly isolated non-attached marrow-derived osteoclast precursors were transduced with either Lenti-*gfp* or Lenti-WT-*Epha4* vector with an MOI of 10. Cell lysate of each transduced cells was prepared 24-h later, and Western immunoblot against EphA4 was performed. Cellular level of actin was also measured to reflect equal extract loading. Panel B shows that lentiviral-based reintroduction of EphA4 in the brain extract of a normal mouse. Lentiviral-based reintroduction of *Epha4* back into *Epha4* null osteoclasts reduced the average size of the resorption pit. Marrow-derived osteoclast precursors on thin dentine slices were treated with lenti-*gfp* or lenti-*Epha4*, or untreated for 24 h. After the viral transduction, cells were treated with RANKL/mCSF to induce osteoclast differentiation. Resorption pits created by these cells formed after 6 days were measured with the Osteometric system as previously reported. Top shows representative photomicrograph of resorption pits created by each treated cells. Bottom summarizes the average resorption pit size per pit (mean $\pm$ S.E.M., n = 4 each).

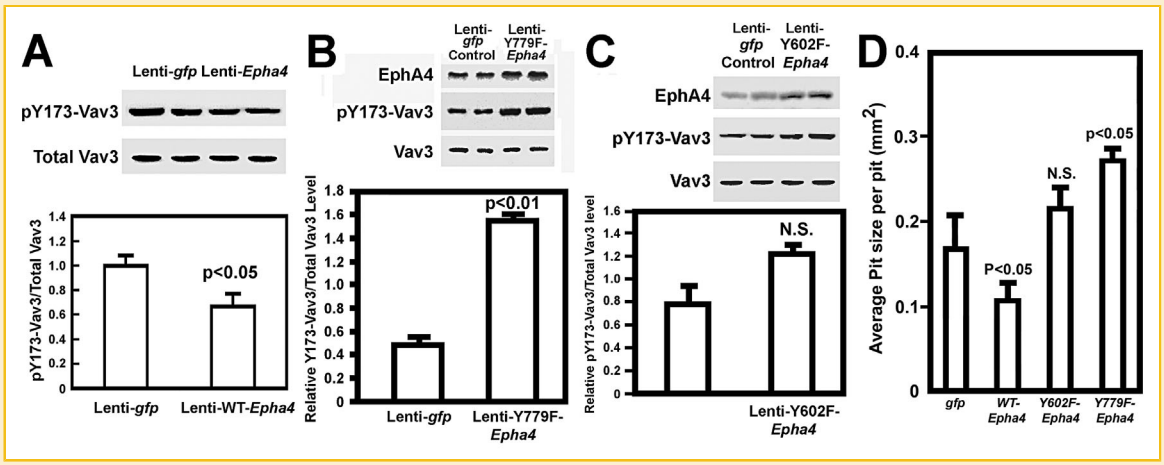

Fig. 3. Effect of reintroduction of WT-*Epha4* in *Epha4* null osteoclasts (A), and that of overexpression of Y779F-*Epha4* mutant (B) or Y602F-*Epha4* mutant (C) in WT osteoclasts on the cellular pY173-Vav3 level and bone resorption activity (D). The lentiviral transduction of *Epha4* null or WT osteoclasts was performed as described in the Methods. Cell lysate (40 μg) of each transduced osteoclasts were then analyzed by Western blots. The EphA4, pY173-Vav3, and total Vav3 protein levels were detected with each respective specific antibody and the relative levels of each protein were analyzed by laser densitometry. In each panel, top shows the respective Western blot, and bottom shows the relative amounts of pY173-Vav3 per total Vav3 level (mean ± S.E.M., n = 4 for each). In D, the bone resorption activity, which was assessed by the resorption pit formation assay, was indicated by the average resorption pit size per pit. Results are shown as (mean ± S.E.M., n = 2–3 each).

resorption activity (Fig. 3D). As expected, overexpression of WT-*Epha4* significantly reduced the average resorption pit size per resorption pit by ~40%. Consistent with the results on pY173–Vav3, overexpression of Y779F-*Epha4* dominant negative mutant significantly enhanced the bone resorption activity of WT osteoclasts. Conversely, transgenic overexpression of Y602F-*Epha4* mutant only slightly, but not significantly, increased the average resorption pit size per pit.

PTP-oc exerts contrasting effects on the activation status of Rho-GTPase versus Rac-GTPase in mature osteoclasts. There are three major classes of the Rho family of small GTPases: Rho, Rac, and Cdc42, which are central components in governing cytoskeleton reorganization and subsequent cell migration and motility [Bar-Sagi and Hall, 2000]. Vav3 is a Rac guanine nucleotide exchange factor (RacGEF), which activates Rac-GTPases by promoting GDP to GTP exchange on Rac. In this regard, overexpression of PTP-oc in osteoclasts significantly increased the GTP binding to Rac [Sheng et al., 2009], supporting the interpretation that the PTP-oc-mediated activation of Vav3 would translate into an activation of Rac-GTPase. It has been reported that EphA4 has opposing effects on Rac-GTPase compared to Rho/Cdc42 GTPases in the neurons [Sahin et al., 2005]. Therefore, we also compared the effect of overexpression of PTP-oc on the relative amount of GTP-bound Rac versus that of GTP-bound Rho (Fig. 4). We found that transgenic overexpression of PTP-oc significantly increased (~3-fold) the level of GTP-bound Rac when compared to WT osteoclasts. Conversely, PTP-oc overexpression significantly reduced the level of GTP-bound Rho (by ~50%) compared to WT osteoclasts (Fig. 4C), indicating that the PTP-oc signaling also exerts opposing effects on the activation status of Rho-GTPase versus Rac-GTPase. Because the total Rho and Rac protein levels in the cell lysates of PTP-oc Tg osteoclasts were not different from those in cell lysates of WT osteoclasts, it would appear that PTP-oc modulates the activation status, but not the synthesis, of Rho versus Rac proteins in osteoclasts.

Members of Rho family small GTPase proteins are activated by their own guanine nucleotide exchange factors (GEFs) and inactivated by their own GTPase Activating Proteins (GAPs). While Vav3 is a RacGEF, Ephexin functions as a RhoGEF in neurons. In this regard, EphA4 has been shown in neurons to phosphorylate (at Y87) of Ephexin that enhances its RhoGEF activity, which then activates Rho-GTPase [Shamah et al., 2001; Sahin et al., 2005]. If our hypothesis that PTP-oc dephosphorylates and inactivates the EphA4 signaling in osteoclasts is correct, it follows that overexpression of PTP-oc should also reduce pY87-Ephexin and inhibits its RhoGEF activity. Consistent with our hypothesis, Figure 5A shows that the pY87-Ephexin level in PTP-oc Tg osteoclasts was reduced to <20% of that in WT osteoclasts. Thus, the reduced pY87-Ephexin level in PTP-oc Tg osteoclasts was consistent with the observed decrease in the activation status of Rho-GTPase (Fig. 4C). Similarly, the pY87-Ephexin level in *Epha4* null osteoclasts was significantly reduced compared to that in WT osteoclasts (Fig. 5B). This reduction corresponded to the decrease in Rho-GTPase activation in *Epha4* null osteoclasts (i.e., reduced to ~60% of the Rho-GTPase activation in WT osteoclasts [not shown]). Consequently, the differential modulation of activation status of Rac-GTPase versus Rho-GTPase seen in PTP-oc-overexpressing or in *Epha4* deficient osteoclasts could be the combined consequence of the PTP-oc/EphA4-induced activation of Vav3 and the PTP-oc/EphA4-mediated inactivation of Ephexin.

DISCUSSION

The present studies have offered some important mechanistic insights about the molecular mechanism by which PTP-oc up-regulates the bone resorption activity of mature osteoclasts. According, while our previous studies have gathered substantial amounts of compelling evidence that PTP-oc enhanced the

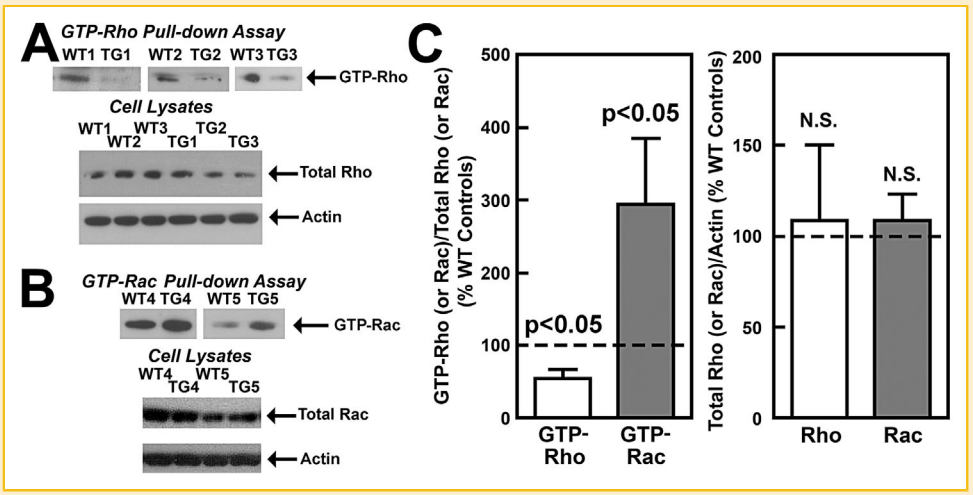

Fig. 4. Overexpression of PTP-oc in osteoclasts results in upregulation of Rac activation along with a corresponding downregulation of Rho GTPase. The activation status of Rac and Rho was determined by relative amounts of GTP-bound Rac or Rho to total Rac or Rho, respectively) in osteoclasts of three pairs of PTP-oc transgenic (TG) mice and WT littermates. GTP-bound Rho or Rac were assayed with respective commercial kits. Total Rac or Rho levels were determined by Western blots. Panels A and B show the Western of GTP-bound and total Rho and Rac, respectively. Left side of Panel C summarizes the relative ratio of GTP-Rho (or Rac) to total Rho (or Rac) in the PTP-oc Tg osteoclasts as the relative percentage of WT osteoclast control. Right side of Panel C summarizes the relative amounts of Rho (or Rac) in transgenic osteoclasts as relative percentage of WT osteoclasts. Results are shown as mean ± S.E.M. (n = 3 for each). The dashed lines are 100% of respective WT osteoclasts.

functional activity of osteoclasts in part through up-regulation of several Src PTK-dependent signaling pathways [Amoui et al., 2007; Lau et al., 2012], we also have strong, albeit circumstantial, evidence that Src-independent pathways are also involved. The findings of the current study have led us to advance an interesting concept, in which PTP-oc not only acts through up-regulation of multiple stimulatory Src PTK-dependent mechanisms to stimulate bone resorption activity of mature osteoclasts, but it can also act through suppression of an inhibitory signaling pathway, namely the EphA4 signaling, in a Src-independent manner in osteoclasts. In this regard, we have

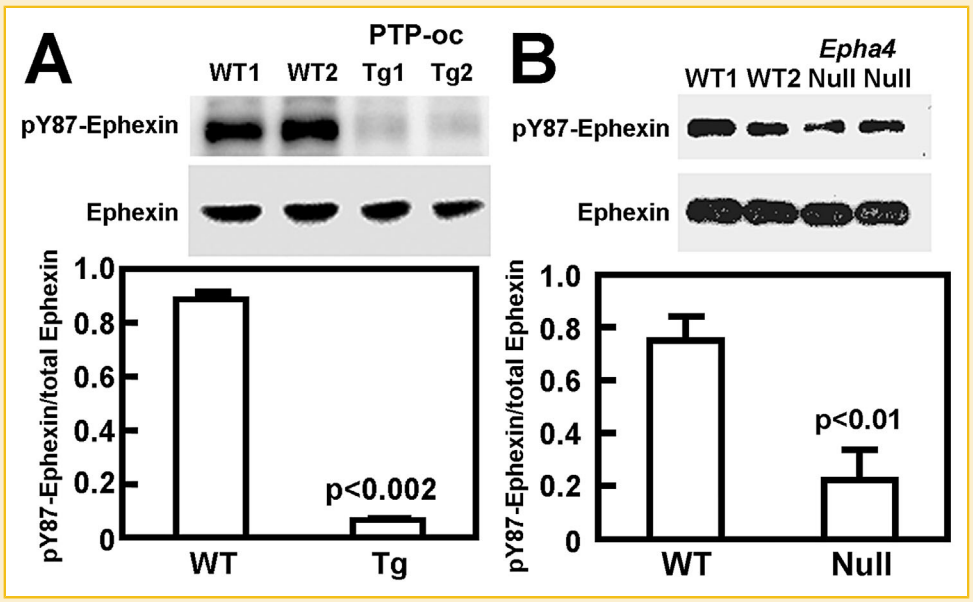

Fig. 5. Overexpression of PTP-oc (A) or deficient *Epha4* expression (B) in osteoclasts results in reduced pY87 phosphorylation levels of ephexin in osteoclasts. The pY87-Ephexin and total Ephexin levels in marrow-derived osteoclasts from 10-week-old male PTP-oc transgenic (Tg) and WT mice were analyzed by Western blot using specific anti-pY87- and anti-Ephexin, respectively. In each panel, top shows the respective Western blot, and bottom summarizes the results, which are shown as the pY87-Ephexin/total Ephexin ratio (mean ± S.E.M., n = 2 for each).

recently demonstrated that the EphA4 signaling is a potent suppressive mechanism for regulation of the bone resorption activity of osteoclasts [Stiffel et al., 2014]. Incidentally, the EphA4 signaling is activated by the phosphorylation of several key tyrosine residues. Dephosphorylation of these pY residues would inhibit the EphA4 signaling [Zisch et al., 2000; Wybenga-Groot et al., 2001]. Our concept contends that EphA4 is a cellular substrate of PTP-oc in mature osteoclasts, and that the PTP-oc-mediated dephosphorylation of EphA4 would inactivate the EphA4 signaling. Since the EphA4 functions as a potent negative regulatory pathway of the functional activity, the PTP-oc-mediated inactivation of the EphA4 in mature osteoclasts would therefore lead to an enhancement in their bone resorption activity. Thus, it appears that PTP-oc can simultaneously act through multiple stimulatory and inhibitory mechanisms to enhance bone resorption activity of mature osteoclasts.

This study has offered several lines of strong evidence that EphA4 is a cellular substrate of PTP-oc in osteoclasts. The first piece of suggestive evidence came from the PTP-oc-GST fusion protein pull-down assay. Accordingly, we found that pY-EphA4 was pulled down with PD-PTP-oc substrate trapping mutant, but not with WT-PTP-oc. Our previous studies have suggested that the increased phosphorylation level of some of the pulled down pY-proteins (i.e., Syk and β_3-integrin) was due to the enhanced PTK activity of the co-pulled down Src PTK in the complex [Lau et al., 2012]. However, because the 24-h pretreatment with the Src PTK inhibitor, PP2, did not affect the pY-EphA4 levels in the pull-down assay, we conclude that the increased pY phosphorylation level in the pull-down EphA4 was unlikely related to, or dependent of, the PTP-oc-mediated activation of the Src PTK. The second piece of supporting evidence was derived from our findings that overexpression of PTP-oc in osteoclasts decreased the total pY levels as well as the pY levels of pY602 and pY779 residues of EphA4 each to $<5\%$ of that in the WT osteoclasts. Although we did not measure the pY levels of the other functionally important pY residues (e.g., pY596 and pY928), the fact that overexpression of PTP-oc reduced the total pY levels of EphA4 to $<5\%$ is entirely compatible with our conclusion that all pY residues in EphA4 could be effectively dephosphorylated by PTP-oc. On the other hand, we cannot entirely rule out the unlikely possibility that it may involve other mechanisms than direct PTP-oc-mediated dephosphorylation of EphA4 that could indirectly result in reduction in the overall phosphorylation status of EphA4. Nevertheless, our premise that PTP-oc is a key regulator of EphA4 by direct dephosphorylation of its key phosphotyrosine residues is consistent with a previous report, which showed that a closely related PTP, PTPRO, was capable of dephosphorylating EphA4 in vitro and modulating its sensitivity to Efn ligands in retinal ganglion cells [Shintani et al., 2006].

The concept that dephosphorylation of EphA4 is a downstream event of the PTP-oc-mediated up-regulation of osteoclast activity is supported by several in vivo and in vitro observations. Firstly, transgenic mice with targeted overexpression of PTP-oc in cells of osteoclastic lineage (PTP-oc Tg mice) [Sheng et al., 2009] and *Epha4* null mice [Stiffel et al., 2014] each exhibited the same low bone size phenotype characterized by low bone mass and density that were associated with an increase in osteoclastic resorption but without an increase in osteoclast number on the bone surface. Secondly, osteoclasts derived from PTP-oc Tg mice or from *Epha4* null mice both shared very similar characteristics of increased bone resorption activity and enhanced cell spreading/migration [Sheng et al., 2009; Stiffel et al., 2014]. Thirdly, overexpression of PTP-oc or deficient *Epha4* expression in osteoclasts both led to up-regulation of the β_3-integrin signaling in part through upregulation of the Vav3-Rac signaling [Sheng et al., 2009; Stiffel et al., 2014]. Consequently, these similarities in the overall effect of overexpression of PTP-oc and that of *Epha4* deficiency in osteoclasts support the likely possibility that PTP-oc may regulate osteoclast activity in part through reduction of the suppressive action of the EphA4 forward signaling on the osteoclast activity.

The molecular mechanism by which the EphA4 forward signaling acts to negatively modulate the bone resorption activity (including cell migration and spreading) of osteoclasts has not been fully understood. Two intriguing findings of this study may provide relevant mechanistic insights. First, transgenic overexpression of the Y779F-EphA4 dominant negative mutant, but not the Y602F-EphA4 mutant, in osteoclasts increased the pY175-Vav3 level. This may imply that the pY779 residue is more essential than the pY602 residue for its inhibitory action on the Vav3 activation and perhaps also on the subsequent activation of β_3-integrin signaling that stimulated bone resorption activity of osteoclasts. On the other hand, it should be noted that while Y779 phosphorylation is required for enhancing its PTK activity, which is required for the forward signaling of EphA4 [Zisch et al., 2000; Kullander and Klein, 2002], the Y602 phosphorylation is also important in its PTK activation process [Zisch et al., 2000; Kullander and Klein, 2002]. Thus, it is surprising that overexpression of the Y602F mutant was much less effective in "reversing" its suppressive action on the Vav3/Rac signaling. It is possible that while pY602 phosphorylation may be required for opening up the closed inactive configuration of the EphA4, its importance on the overall activation of its PTK activity is less than that of the pY779. On the other hand, because the phosphorylation of Y779 in EphA4 is mediated by FGF receptor-mediated transphosphorylation upon Efn-induced activation [Yokote et al., 2005], and the phosphorylation of Y602, Y596, and Y928 are mediated primarily by autophosphorylation [Zisch et al., 2000; Wybenga-Groot et al., 2001], it is also conceivable that the reduced pY596, pY602, or pY928 levels in PTP-oc overexpressing osteoclasts were the consequences of an inactivation of its PTK activity due to the PTP-oc-mediated dephosphorylation of the pY779 residue. Alternatively, it is possible that mutation of Y602 alone is not sufficient to prevent EphA4 from acquiring the open, active conformation, as double Y596E/Y602E mutations are needed to alter its PTK activity [Shamah et al., 2001]. Much additional work is needed to clarify the significance of this intriguing observation.

The second and perhaps more intriguing finding is that despite both Rho and Rac belong to the same Rho family of small GTPases, overexpression of PTP-oc or deficient *Epha4* expression in osteoclasts each had similar opposing effects on the activation status of Rho-GTPase versus Rac-GTPase; both leading to the condition that favors Rac-GTPase activation. These findings could be extrapolated to suggest that activation of the EphA4 signaling would lead to activation of Rho-GTPase at the expense of

suppression of Rac-GTPase. Consistent with this extrapolation, other investigators have reported that activation of the EphA4 signaling in neuron indeed induced differential activation of Rho GTPase over Rac GTPase [Sahin et al., 2005]. The significance of the differential actions of PTP-oc or EphA4 on Rho-GTPase versus Rac-GTPase activation in osteoclasts is not clear. However, activation of Rho-GTPase is essential for cell adhesion, whereas activation of Rac-GTPase is involved in cell spreading and migration [Arthur et al., 2002]. Since cell adhesion needs to be reduced in order for cell to spread and migrate, it makes physiological sense that the Rho and the Rac signaling are to be coordinately and reciprocally regulated. Accordingly, we speculate that PTP-oc enhances, while EphA4 inhibits, osteoclast spreading and migration by promoting the Rac signaling and at the same time reducing cell adhesion through suppression of the Rho signaling. Consistent with this speculation, activation of the integrin and the cadherin signaling has each been reported to suppress RhoA activity but up-regulate Cdc42 and Rac1 in other cell types [Arthur et al., 2002]. The fact that there is a strong similarity between PTP-oc overexpression and *Epha4* deficiency on the differential regulation of Rho versus Rac signaling supports our contention that PTP-oc may up-regulate spreading/migration of osteoclasts in part through the relief of the inhibitory action of EphA4 on the Vav3/Rac signaling, which is a key component of the β_3-integrin signaling required for osteoclast resorption [Ross and Teitelbaum, 2005].

Each member of the Rho family of small GTPases is activated by their respective GEFs [Rossman et al., 2005]. We now have compelling evidence that overexpression of PTP-oc [Lau et al., 2012] or knocking out *Epha4* expression [Stiffel et al., 2014] in osteoclasts each led to increased phosphorylation and activation of Vav3 (a RacGEF), and simultaneously resulted in dephosphorylation and inactivation of Ephexin (a RhoGEF). The changes in Vav3 and Ephexin corresponded well to the observed up-regulation of the Rac signaling and the corresponding down-regulation of Rho signaling in PTP-oc overexpressing or *Epha4* null osteoclasts. Therefore, the mechanism contributing to the differential regulatory actions of PTP-oc or EphA4 on the Rho versus Rac signaling could likely be the consequence of similar opposite actions of PTP-oc or EphA4 on Ephexin versus Vav3, respectively.

The mechanistic explanation contributing to the opposing actions of PTP-oc or EphA4 on the activation status of Vav3 versus Ephexin remains speculative. It has been demonstrated that Vav3 [Fang et al., 2008] and Ephexin [25] each binds to EphA4, through their plekstrin-homology (PH) domain [Bustelo, 2002; Ogita et al., 2003], for their phosphorylation and activation by the EphA4 PTK catalytic domain. There is also suggestive evidence that Vav3 and Ephexin both bind to the same structural domain of EphA4 that includes the Y596/Y602 residues [Wegmeyer et al., 2007; Takeuchi et al., 2009]. The binding of Vav3 to EphA4 [Fang et al., 2008], but not that of Ephexin to EphA4 [Shamah et al., 2001], requires the phosphorylation of these two juxamemberane tyrosines (i.e., pY596/pY602). Accordingly, a possible contributing factor that determines which of these two GEFs are phosphorylated (and activated) by the EphA4 PTK will depend on the binding affinity and recruitment of each GEF to the EphA4. In this regard, we have advanced a novel mechanistic model that could account for the opposite effects of PTP-oc or EphA4 on the Ephexin/Rho versus Vav3/Rac signaling (Fig. 6). In this model, both Vav3 and Ephexin bind to the same site of EphA4 that involves the Y596/Y602 residues. There is a competition between Vav3 and Ephexin for the same binding site on EphA4. We postulate that the binding affinity for Ephexin as opposed to Vav3 is determined by the phosphorylation status of pY596/pY602 residues of the EphA4. When the PTK activity of the EphA4 receptor is activated and the Y596/Y602 residues are autophosphorylated, the

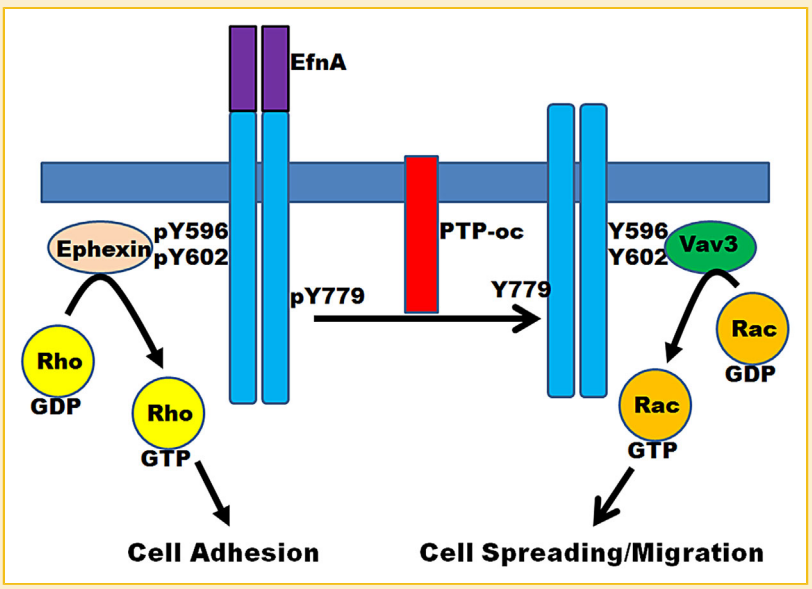

Fig. 6. A proposed molecular mechanism of EphA4-mediated differential regulation of Rho signaling versus Rac signaling. Please see the text for detailed description of the proposed mechanism.

EphA4 protein would assume the conformation that favors the binding of Ephexin over Vav. Conversely, when Y596/Y602 are dephosphorylated or incompletely phosphorylated, the enzyme would bind Vav3 more favorably than Ephexin. We further postulate that this conversion in binding affinity from one that favors Ephexin binding to one that prefers Vav3 binding is triggered by the PTP-oc-mediated dephosphorylation of pY779-EphA4 and the subsequent dephosphorylation of pY596/pY602. We termed this mechanism the "EphA4 phosphorylation switch," since this mechanism is reminiscent of a previously proposed regulatory mechanism for the activation of the integrin signaling known as the "integrin phosphorylation switch" [Oxley et al., 2008]. In that model, the integrin phosphorylation switch model postulates that the binding to the cytoplasmic tail of β_3-integrin of talin (a positive mediator of the integrin signaling) [Garcia-Alvarez et al., 2003; Tadokoro et al., 2003], versus Dok1 (a negative regulator of the integrin signaling) [Kawamata et al., 2011] would depend on the pY747-β_3-integrin phosphorylation status. This proposed "EphA4 phosphorylation switch" mechanism is intriguing, and therefore merits further investigations. Much additional work will be needed to confirm this putative but novel "EphA4 phosphorylation switch" regulatory mechanism. Regardless of how PTP-oc or EphA4 acts to differentially regulates Rac-GTPase versus Rho-GTPase, it is our premise that the PTP-oc-mediated dephosphorylation of EphA4 would activate Vav3 and subsequently the Rac-GTPase signaling. Because both Vav3 [Faccio et al., 2005] and Rac-GTPase [Croke et al., 2011], the enhanced Vav3/Rac-GTPase activation induced by PTP-oc overexpression or *Epha4* deficiency PTP-oc would subsequently lead to an overall increase in bone resorption activity of the osteoclast.

In conclusion, this study provides compelling evidence that EphA4 is a cellular substrate for PTP-oc in osteoclasts and that the molecular mechanism contributing to the PTP-oc-induced up-regulation of the osteoclast activity in part involves the PTP-oc-mediated dephosphorylation and inactivation of the EphA4 signaling. Accordingly, our studies together bring forth a novel concept that PTP-oc not only acts to promote several positive regulatory signaling of the osteoclast activity (such as the Src, the JNK, the β_3-integrin, and the Syk/ITAM signaling) through the PTP-oc-mediated activation of Src PTK, but also acts to suppress a key negative regulatory signaling of the osteoclast activity, namely the EphA4 forward signaling, through the PTP-oc-mediated dephosphorylation and inactivation of the EphA4 in osteoclasts in a Src-PTK-independent manner. Understanding of how PTP-oc regulates the EphA4 forward signaling and how the EphA4 forward signaling exerts its negative effects on osteoclast activity not only could disclose important insights into the potential pathophysiological roles of EphA4 or PTP-oc in various bone-wasting diseases but may also provide novel gene targets for the development of new and effective anti-resorptive therapeutics.

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