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A Novel *miR17*/Protein Tyrosine Phosphatase-oc/EphA4 Regulatory Axis of Osteoclast Activity

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Abstract

Information about the molecular mechanisms leading to the activation of the osteoclast is relatively limited. While there is compelling evidence that the signaling mechanisms of Src and integrin β_3 are essential for osteoclast activation, the regulation of these two signaling mechanisms is not fully understood. In this review, evidence supporting a novel regulatory axis of osteoclast activation that plays an upstream regulatory role in both the Src and integrin β_3 signaling during osteoclast activation is discussed. This regulatory axis contains three unique components: a structurally unique transmembrane protein-tyrosine phosphatase, PTP-oc, EphA4, and *miR17*. In the first component, PTP-oc activates the Src signaling through dephosphorylation of the inhibitory tyr-527 of Src. This in turn activates the integrin β_3 signaling, enhances the JNK2/NFκB signaling, promotes the ITAM/Syk signaling, and suppresses the ITIM/Shp1 signaling; the consequence of which is activation of the osteoclast. In the second component, EphA4 inhibits osteoclast activity by suppressing the integrin β_3 signaling. PTP-oc relieves the suppressive actions of EphA4 by directly dephosphorylating EphA4. In the third component, PTP-oc expression is negatively regulated by *miR17*. Accordingly, suppression of *miR17* during osteoclast activation upregulates the PTP-oc signaling and suppresses the EphA4 signaling, resulting in the activation of the osteoclast. This regulatory axis is unique, in that each of the three components acts to exert suppressive action on their respective immediate downstream inhibitory step. Because the final downstream event is the EphA4-mediated inhibition of osteoclast activation, the overall effect of this mechanism is the stimulation of osteoclast activity.

Keywords

Osteoclasts; Protein-tyrosine Phosphatase; EphA4; Integrin β_3; Src; *miR17~92*; Bone Resorption

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Introduction

Bone resorption is essential for all aspects of bone physiology. The most prevalent bone disease associated with an abnormality in bone resorption is osteoporosis, and the pathology of most subtypes is an excessive bone resorption. Bone resorption is determined by the number, activity, and life-span of mature osteoclasts. In this regard, there are many subtypes of osteoporosis (1). While most subtypes are caused by an increase in the number of active osteoclasts, several subtypes are due to “hyperactive” osteoclasts (1,2). Accordingly, information about the underlying molecular mechanism(s) regulating not only osteoclast formation but also osteoclast activity is essential for the understanding of the physiology of bone resorption as well as the pathology of the various subtypes of osteoporosis. Mechanistic information about these processes may also yield novel drug targets for development of effective therapies for the various subtypes of osteoporosis and other bone-wasting diseases.

Mechanistic studies during the past two and a half decades have led to enormous advances in our understanding of the regulation of osteoclastogenesis at the molecular level. It is now evident that osteoclastogenesis is regulated largely through two local protein factors – macrophage colony-stimulating factor (mCSF) and receptor activator of NFκB ligand (RANKL) (3–5). Their respective signaling pathways have been well established (4,6–11). Conversely, mechanistic information about the regulation of osteoclast activity (or activation) is comparatively scarce, but yet there is compelling evidence that the signaling of Src and also that of integrin β_3 are two key mediators of osteoclast activation (12–17). These two factors work concertedly with each other and with other pathways, e.g., the immunoreceptor tyrosine-based activation motif (ITAM) pathway and the immunoreceptor tyrosine-based inhibition motif (ITIM) pathway, to modulate osteoclast activity (14,18). Accordingly, the molecular mechanisms regulating osteoclast activation appear complex and likely involve multiple interacting pathways.

Our initial investigation into the regulatory mechanisms of the Src signaling in osteoclasts has discovered a novel regulatory mechanism for osteoclast activation that involves the activation of both the Src and integrin β_3 signaling by a structurally unique osteoclastic non-receptor-type transmembrane protein-tyrosine phosphatase (PTP) (19–24), which is referred to as PTP-oc (25). Intriguingly, PTP-oc also acts directly on EphA4 to suppress its forward signaling (26), which is a negative regulator of the osteoclast activity through inhibition of the integrin β_3 signaling (27), resulting in further enhancement in the osteoclast activity. The PTP-oc expression in osteoclasts is determined by post-transcriptional regulation in part through *miR17* (28). Consequently, it appears that the *miR17*, the PTP-oc, and the EphA4 represent three novel upstream regulators of the Src and integrin β_3 signaling in osteoclasts. This novel mechanism of osteoclast activity is referred to as the *miR17*/PTP-oc/EphA4 regulatory axis of osteoclast activation.

This review summarizes the current evidence that supports the *miR17*/PTP-oc/EphA4 regulatory axis in the context of osteoclast activation. Because the *miR17*/PTP-oc/EphA4 regulatory axis is upstream to the regulation of the integrin β_3 and Src signaling in the

context of osteoclast activity, the current understanding of the integrin β_3 and Src signaling is also discussed.

Roles of integrin $\alpha_v\beta_3$ in the activation of osteoclasts

Integrins are a family of cell-surface glycoproteins, which function as receptors for extracellular matrix proteins and which are involved in cell-cell, cell-matrix adhesion, cell proliferation, and differentiation. Integrins are heterodimers containing an α subunit and a β subunit. There are > 20 integrins consisting of > 16 α subunits and > 8 β subunits in vertebrates. The extracellular domains of both α and β subunits contribute to the ligand binding, and the α/β pairings specify the ligand-binding specificity. The osteoclast attaches to the bone surface predominantly through the vitronectin receptor, integrin $\alpha_v\beta_3$, which recognizes the RGD sequence of bone matrix proteins (29). The requirement for $\alpha_v\beta_3$, however, can be compensated by $\alpha_2\beta_1$ overexpression (30,31). The cytoplasmic domains are crucial for signal transduction. Upon activation by the ligand binding, the cytoplasmic tails of α and β subunits separate (32), which in turn triggers the cytoplasmic regions of the heterodimer to initiate intracellular signal transduction (33). The ligand-induced mechanism is referred to as the outside-in signaling. Integrins can also be activated through an indirect, inside-out signaling mechanism, in which other receptors, typically those of growth factors, transmit signals to the integrin's cytoplasmic regions (33), leading to separation of the intracellular domains of α and β subunits and the activation of downstream signaling events (33).

Integrin $\alpha_v\beta_3$ is an integral component of the osteoclast cytoskeleton. Interaction of integrin $\alpha_v\beta_3$ with bone matrix ligands induces polarization of the cytoskeleton of the osteoclast to mobilize its resorptive machinery toward the bone-cell surface, where it creates an isolated resorptive compartment consisting of a sealing zone of an actin ring that surrounds ruffled borders. Deficient in integrin β_3 in osteoclasts compromises the re-organization of osteoclast cytoskeleton and abridges osteoclastic resorption, as mice lacking integrin β_3 develop an osteosclerotic phenotype due to the dysfunctional osteoclasts without reduction in the number of osteoclasts (34).

The cytoplasmic tail of integrin β_3 is constitutively associated with Src. Binding of an extracellular matrix ligand to integrin β_3 leads to autophosphorylation of tyrosine-416 (Y-416) of the Src (14), which activates its PTK activity. This in turn phosphorylates several key tyrosine residues at the cytoplasmic C-terminal tail of integrin β_3. The phosphorylation of integrin β_3 promotes recruitment of downstream cytoskeleton-organizing and signaling molecules (such as Pyk2, Cbl, paxillin, vinculin, $p130^{Cas}$, Erk1/2) for the Src PTK-mediated phosphorylation and activation (16,17,35,36). The recruitment of Pyk2 to integrin β_3 promotes autophosphorylation of pY-402 of Pyk2 (37), providing the binding site for Cbl to form the Pyk2-Src-Cbl complex. This tri-molecular signaling complex is essential for the initiation of signal transduction mechanism needed for osteoclast adhesion, migration, and bone resorption (38). Additionally, recruitment of $p130^{Cas}$ (Crk-associated substrate) to the Pyk2-Src-Cbl complex further increases the PTK activity of Src by displacing the Src intra-molecular interactions, resulting in the Src-dependent phosphorylation of the associated co-

localized cytoskeletal proteins (such as F-actin, vinculin, paxillin, and PI3K) at the sealing zone of an actively resorbing osteoclast (39,40).

Activated integrin β_3 also recruits Syk to the complex for the Src-mediated phosphorylation (41). Syk is a key mediator of the ITAM signaling initiated by Fc receptor γ-chain (FcRγ) or DNAX-activating protein of 12kDa (Dap12) (14). The SH2 domain of activated Syk interacts with key pY residues of Dap12 and recruits the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), which is an adaptor for the guanine nucleotide exchange factor (GEF), Vav3. Syk then phosphorylates and activates Vav3 (42), which converts Rac (a member of Rho GTPases family) from the inactive GDP-bound conformation to the active GTP-bound conformation (43). The Vav3-mediated activation of Rac is central to the reorganization of the actin cytoskeleton to form actin rings via the Arp2/3 complex, which plays major roles in podosome formation, osteoclast motility, and osteoclastic resorption (44). That integrin β_3-deficient osteoclasts lack matrix-induced Rac activation (45) indicates that the Vav3-dependent Rac activation is an important downstream component of the integrin β_3 signaling.

Roles of Src in the activation of osteoclasts

Active osteoclasts expressed high levels of Src on the ruffled border membranes and podosomes of mature osteoclasts (46,47). The most compelling evidence for an essential role of Src in osteoclast activation is that disruption of the Src gene led to a form of osteopetrosis characterized by a slight increase in the number of, but totally inactive, osteoclasts (48–50). Src-deficient osteoclasts are unable to form ruffled borders, display abnormal cytoskeletal structure, retard cell migration, and lack bone resorption activity (50,51).

Src is a member of a family of nine structurally similar, non-receptor, cytoplasmic membrane-associated PTKs. This family of non-receptor PTK shares similar structural organization (52). The N-terminal region (known as SH4 domain) contains a myristolyation site and membrane-localization signal sequence; both of which are essential for attachment to the cytoplasmic membrane. The SH4 domain is followed sequentially in the peptide chain by the proline-rich motif-binding SH3 domain (53), the phosphotyrosine (pY) motif-binding SH2 domain (54), the kinase domain (also known as SH1 domain), and a short C-terminal sequence containing a negative regulatory pY-527 residue (52). Because SH3 domain binds signaling proteins with proline-rich motifs and SH2 domain binds pY-containing signaling proteins, Src also functions as an adapter protein.

The PTK activity of Src is regulated by the phosphorylation status of the inhibitory pY-527 in the C-terminal tail and the stimulatory pY-426 in the activation loop of the PTK domain. When in the inactive state, Y-527 is phosphorylated by Csk (C-terminus Src kinase). The pY-527 forms an intramolecular association with its own SH2 domain near the N-terminus, which stabilizes the closed, inactive conformation. Dephosphorylation of pY-527 disrupts this intramolecular interaction, permitting the protein to acquire an open, active conformation that allows unhindered access of substrates to the PTK domain for phosphorylation. This process also frees up SH2 and SH3 domains to bind downstream

effectors needed for the activation of the Src signaling. The Y-416 residue in the open, active conformation can undergo autophosphorylation, which further increases its PTK activity (52).

There is strong evidence that the Src PTK activity is required for the functional activity of osteoclasts, including osteoclast spreading, migration, and bone resorption activity (38,50,55,56), whereas inhibition of the Src PTK activity abolished bone resorption activity of osteoclasts (57–60). On the other hand, because transgenic expression of Y416F and K295R dominant-negative Src mutants with inactive PTK partially rescued the osteopetrotic phenotype in Src-deficient mice (61), and because bone-targeted Src SH2 inhibitors blocked Src cellular activity and osteoclast-mediated resorption (62), both the PTK and adaptor activities of Src are essential.

The first clue suggesting a commonality of intracellular signaling mechanism between integrin β_3 and Src in osteoclasts came from the observation that mutant mice lacking integrin $\alpha_v\beta_3$ or Src share very similar osteoclastic phenotype and develop severe osteopetrosis associated with an impaired bone resorption that is due to completely inactive osteoclasts despite slight increases in osteoclast number (34,50). Similar to integrin $\alpha_v\beta_3$-deficient osteoclasts, osteoclasts of Src knockout mutants have abnormal cytoskeletal organization, are unable to attach to bone surface, spread and migrate, and do not have ruffled borders (50). The integrin β_3 signaling-associated activation of the Src signaling in osteoclasts leads to 1) reduction in the strength of association between the integrin and cytoskeleton, 2) promotion of movement of the integrin $\alpha_v\beta_3$ to the migratory machinery of the osteoclast in the form of lamellipodia, 3) acceleration of the disassembly of podosomes through the Src PTK-mediated phosphorylation of cortactin (63), and 4) formation of actin ring, sealing zone, and ruffled borders (12).

In addition to its indispensable role in cytoskeleton reorganization and polarization, the Src signaling also has a regulatory role in osteoclast survival (48,50). One of the mechanisms by which the Src signaling suppresses osteoclast apoptosis is mediated through the Src-dependent activation of the JNK2/NFκB signaling (20). Additionally, Src can be translocated into the mitochondria of osteoclasts to phosphorylate and activate cytochrome c oxidase to modulate mitochondrial respiration. The Src-dependent activation of cytochrome c oxidase is also essential for actin ring formation and bone resorption activity (64). The foregoing provides strong supports for an essential role of the Src signaling in the activation of the osteoclast. However, there is still a significant gap in knowledge of how the Src signaling is regulated in the context of osteoclast activation.

Osteoclastic protein-tyrosine phosphatase, PTP-oc

Activation of the Src PTK activity is mediated predominantly by the dephosphorylation of pY-527 of Src, which is mediated by a cellular protetin-tyrosine phosphatase (PTP). Thus, it is important to know the identity and regulation of the PTP responsible for the dephosphorylation of pY-527 of Src in osteoclasts. Several cellular PTPs have been reported to have modulatory actions on osteoclast formation and/or activity, including SH2 domain-containing PTP-1 (Shp1), dual-specificity phosphatase MAPK phosphatase-1 (MKP1), dual-

specificity tyrosine phosphorylation-regulated kinase 2 (Drk2), SH2 domain-containing PTP-2 (Shp2), PTP-PEST (also known as PTPN12), cytosolic PTP-ε, and an osteoclastic protein-tyrosine (PTP-oc). The role of these PTPs in the regulation of osteoclast functions was reviewed elsewhere (65–67). However, this review focuses only on PTP-oc.

PTP-oc is a structurally unique transmembrane PTP, which was originally cloned from a rabbit osteoclast cDNA library (25). PTP-oc is not a receptor-associated PTP. It has a very short extracellular domain and only a single PTP catalytic domain. It has no SH2, SH3, or other typical domains found in adaptor proteins. Other than a short insert at the cytosolic juxtamembrane region, PTP-oc shares complete sequence identity with the transmembrane and cytosolic domains of a receptor-associated PTP, termed PTPRO [also known as Glepp1, PTP-Φ, CRYP2, PTPBK]. Although PTP-oc and PTPRO are derived from the same gene structure, PTP-oc is not a splicing variant of PTPRO. Instead, its expression is driven by an alternative, cell-type-specific intronic promoter (68–70). PTP-oc is expressed predominantly in cells of monocyte-macrophage lineage, osteoclasts, and B-lymphocytes.

Role in PTP-oc in regulation of Src and integrin β_3 signaling mechanisms in osteoclasts

There is a large body of evidence that PTP-oc is a positive regulator of osteoclast activity (19–24). The most compelling evidence is that transgenic mice with targeted overexpression of PTP-oc in cells of osteoclastic lineage had a large increase in bone resorption without an effect on bone formation, leading to a marked reduction in bone mass (19). Because a relatively weak TRAP exon-1c promoter (71) was used to drive PTP-oc expression, the level of PTP-oc overexpression in osteoclasts of the transgenic mice was only ~2-fold of that in osteoclasts of wild-type littermates, such that the observed effects were not due to a non-physiologically high overexpression level of PTP-oc in osteoclasts (19). Overexpression of PTP-oc in osteoclasts increased not only their bone resorption activity, but also their cell size, spreading/migration. The PTP-oc increases osteoclast activity in a large part through an interplay between the PTP-oc-induced Src signaling and the β_3-integrin signaling in osteoclasts. Accordingly, PTP-oc dephosphorylates the inhibitory pY527 of Src, thereby activating its PTK activity (20–23). This in turns: promotes Src PTK-dependent activation of the JNK2/NFκB pathway to enhance osteoclast survival (20), phosphorylates integrin β_3 and activates the integrin β_3 signaling (24), and phosphorylated pY525/526 of Syk to enhance the ITAM signaling (24). The ITAM/Syk signaling then acts in concert with the β_3-integrin signaling to promote osteoclast adhesion, spreading, and cytoskeleton re-organization (14,16,24). PTP-oc also suppresses the ITIM signaling, which is a potent negative regulatory mechanism on osteoclast differentiation and bone resorption (72,73), through the Src-mediated phosphorylation of and inactivation of Shp1 (24). Accordingly, the inactivation of the Shp1-mediated ITIM signaling in osteoclasts further promotes osteoclast activation. Consequently, PTP-oc plays a central upstream regulatory role in the coordinated regulation of the JNK2/NFκB, integrin β_3, ITAM/Syk, and ITIM/Shp1 pathways through the PTP-oc-mediated activation of Src (Fig. 1).

Role of EphA4 forward signaling in the regulation of osteoclast activity

In addition to the direct activation of the Src PTK activity, PTP-oc also inhibits the forward signaling of EphA4 by dephosphorylating key pY residues of EphA4 in osteoclasts (26). EphA4 belongs to the largest family of receptor PTKs, Eph receptors. Eph receptors are classified into A (A1-A10) and B (B1-B6) families. Eph receptors are activated by membrane-associated ligands, ephrins (Efns), located on the cell surface of neighboring cells (74). There are also two Efn ligand families: EfnA (A1-A5) and EfnB (B1-B3). The binding of Efn to Eph induces conformational change in the cytoplasmic domain of the Eph receptor (75), which induces autophosphorylation of two juxtamembrane tyrosine residues that relieves the inhibitory action on its PTK domain and also provides binding sites for SH2-containing signaling proteins (76). These effects together trigger the downstream signaling events. Both families of EfnA and EfnB ligands are capable of receptor-like signaling (77). Interaction of a ligand with EphB receptors induces protein-tyrosine phosphorylation of EfnB ligands, which then recruits and activates SH2- or PDZ-containing proteins to transmit the signal. Activation of EfnA receptors triggers clustering and recruitment of regulatory proteins to the lipid raft to initiate the signal. The Eph receptor-mediated signal is known as the forward signaling and the Efn ligand-mediated signal is called the reverse signaling.

The Efn-Eph bidirectional signaling is an important regulatory mechanism in various organs and cells (78), including osteoblasts (79–82) and osteoclasts (83–85), and plays an essential role in various physiological and pathological processes, such as insulin secretion, bone homeostasis, immune function, blood clotting, pathological forms of angiogenesis, and cancer (86). In osteoclasts, the reverse signal of EfnB2 (83) and that of EfnB1 (84) each exerts an inhibitory action on osteoclastogenesis via its suppressive action on the Fos-Nfatc1 cascade. EphA2-mediated activation of the reverse signaling of EfnA2 in osteoclast precursors enhanced osteoclast differentiation and bone resorption (85). Because this effect was not blocked by an EphA2 dominant negative mutant, the reverse signaling of EfnA2, and not the forward signaling of EphA2, is responsible for the enhanced osteoclast differentiation.

Of all Eph receptors, mature osteoclasts express predominantly EphA4 (27,83,85). Deletion of *Epha4* in mice leads to an osteopenia phenotype due to an increased bone resorption but without an effect on bone formation (27). The increased bone resorption is the consequence of an increase in the osteoclast activity and not an increase in the number of osteoclasts. Deficient *Epha4* expression (by siRNA) or signaling (with EphA4-fc chimeric protein) in osteoclasts *in vitro*, not only increased the bone resorption activity of osteoclasts, but also enlarged their cell size, increased the number of nuclei per cell, and increased *Ctsk* expression. Conversely, activation of the EphA4 forward signaling (with EfnA4-fc chimeric protein) yielded opposite effects. Transgenic reintroduction of EphA4 in *Epha4* knockout osteoclasts “restored” the original phenotype of lower bone resorption activity (26). Thus, the forward signaling of EphA4 in osteoclasts seems to play a potent negative regulatory role in the osteoclast activity.

The molecular mechanism by which the forward signaling of EphA4 negatively modulates osteoclast activity is not fully understood, but it may be mediated in part through

suppression of the integrin β_3 signaling (26). In this regard, the integrin β_3 signaling is regulated by a mechanism known as the "integrin phosphorylation switch" (87), in that phosphorylation of Y-747 of integrin β_3 shifts the binding affinity of the cytoplasmic tail of integrin β_3 from one that favors talin [which mediates the critical final step of integrin β_3 activation (88)] to one that prefers Dok1 [a potent negative regulator of the integrin signaling (89)]. The increased binding of Dok1 to integrin β_3 in turn suppresses activation of Vav3 that leads to reduced osteoclast spreading/migration and bone resorption activity (29). Activation of the EphA4 forward signaling increases the phosphorylation level of pY-747-integrin β_3 (26). Thus, it is possible that EphA4 suppresses osteoclast activation by inhibiting the integrin β_3 signaling through this unique "integrin phosphorylation switch" mechanism (Fig. 2).

Role of PTP-oc in the regulation of EphA4 signaling

The forward signaling of EphA4 is inhibited by the dephosphorylation of key tyrosine residues (75,76). There are several pieces of circumstantial evidence that PTP-oc regulates the forward signaling of EphA4 by direct dephosphorylation of EphA4 (Fig. 2). First, overexpression of PTP-oc in osteoclasts drastically reduced the total pY as well as pY602 and pY779 residues of EphA4 (26). Second, in a PTP-oc-GST fusion protein pull-down experiment, the pY-EphA4 was pulled down by the phosphatase-dead (PD)-PTP-oc substrate trapping mutant, but not by the active WT-PTP-oc (26). Because the pull-down assay was not affected by pretreatment with PP2, a Src PTK inhibitor (26), the regulation of EphA4 signaling by PTP-oc is independent of the Src PTK. Consistent with the notion that EphA4 is a cellular substrate of PTP-oc, PTPRO is also capable of dephosphorylating EphA4 in retinal ganglion cells (90).

The concept that the dephosphorylation of EphA4 is a key downstream event of the PTP-oc in the regulation of osteoclast activity is supported by several observations: First, *Epha4* knockout mice (27) and transgenic mice with targeted overexpression of PTP-oc in osteoclasts (19) showed similar trabecular bone loss and hyperactive osteoclast phenotypes, which suggests significant mechanistic similarities between PTP-oc overexpression and *Epha4* deficiency. Second, osteoclasts derived from mice with overexpression of PTP-oc and those from *Epha4* knockout mice shared similar characteristics of increased bone resorption and cell spreading/migration activities (19,27). Third, osteoclasts with PTP-oc overexpression and those with deficient *Epha4* expression upregulated the integrin β_3 signaling via the Vav3-Rac signaling (19,27). Consequently, PTP-oc imposes its stimulatory effects on osteoclast activity not only by enhancing the stimulatory pathways (integrin β_3- and ITAM/Syk signaling) but also by suppressing the inhibitory pathways (ITIM/Shp1 and EphA4 signaling) in both Src PTK-dependent and –independent manners.

Regulation of PTP-oc expression in osteoclasts

Information about how PTP-oc is regulated is critically important not only with respect to the understanding of pathophysiological roles of this osteoclastic enzyme in the regulation of osteoclast activity, but it could also provide potential novel targets for PTP-oc-based anti-resorptive therapies. In this regard, the expression of PTP-oc in rabbit osteoclasts were

significantly upregulated by certain resorption stimulators [i.e., PTH, $1,25(OH)_2D_3$, PGE_2, and IL-1α but not IL-6, RANKL, or TNFα], and reduced by certain resorption inhibitors [i.e., alendronate but not calcitonin] (21,22). However, none of these resorptive cytokines altered the promoter activity of the 2-kb mouse proximal PTP-oc promoter in osteoclastic cells *in vitro* (68). Thus, PTP-oc expression in osteoclasts may be regulated primarily by post-transcriptional mechanisms.

Regulatory roles of miR17~92 in the PTP-oc-mediated osteoclastic resorption

The miRNAs, an abundant class of highly evolutionarily conserved, single-stranded, non-coding RNAs of 19-25 nucleotides, are one of the key post-transcriptional regulators of gene expression (91). They bind to the complementary sequence in the 3′UTR of target mRNAs to induce post-transcriptional suppression of target gene expression (92). They are key regulators of numerous pathways and cellular processes. In bone, a number of miRNAs have been reported to be regulators of osteoblast differentiation [*miR29a*, *29b*, *141*, *200a*, *210*, *206*, *133*, *135*, *125b*, *26a*, *2861*, and *196a* (93,94)] and chondrocyte differentiation [*miR18a*, *199a*, *146a*, *222*, *140*, and *27b* (94)].

The first evidence that miRNAs are key regulators of osteoclastogenesis came from the findings that overexpression of pre-*miR223* blocked the RANKL-induced osteoclastic differentiation (95) by modulating the expression of nuclear factor I-A and m-CSF receptor (96). That conditional disruption of Dicer in osteoclasts drastically reduced the number of mature osteoclasts and developed osteopetrosis in mice further indicates an important regulatory role for miRNAs in osteoclastogenesis (96,97). In addition, the interferon-β treatment induced *miR155* expression, which in turn inhibited osteoclastogenesis (98), and *miR21* enhances osteoclastogenesis through downregulation of PDCD4 (99). Conversely, relatively little is known about the regulatory role of mRNAs in the activation process of the osteoclast.

The potential role of *miR17* in the regulation of PTP-oc in osteoclastic cells was recently investigated (28), because the expression of a structurally-related PTP (PTPRO) was shown to be negatively regulated by *miR17* (100). The *miR17* is expressed as one of the six cluster genes of *miR17~92* (i.e., *miR17*, *miR18*, *miR19a*, *miR19b*, *miR20*, and *miR92*). The *in vivo* evidence that *miR17~92* is a negative regulator of osteoclast functions came from the characterization of the *miR17~92* osteoclast conditional knockout mice (28), in that the *miR17~92* conditional knockout mutants had highly active osteoclasts and lost substantial trabecular bone associated with marked increase in bone resorption, but without an effect on bone formation parameters.

There are several strong pieces of *in vitro* evidence supporting the premise that the PTP-oc expression in osteoclasts is regulated in part by *miR17* (28): First, an online search with the Targetscan database for potential miRNA target sequences has identified a highly conserved target site for *miR17* on the 3′UTR of the PTP-oc mRNA, and the presence of the *miR17* target site was confirmed by a miRNA target reporter assay (28). Second, the RANKL-induced osteoclast differentiation upregulated PTP-oc expression with a corresponding time-

dependent downregulation of *miR17*, and there was an inverse correlation between the levels of *miR17* and the levels of PTP-oc mRNA in osteoclasts (28). Third, the suppression of *miR17* activity with an anti-*miR17-5p* locked nucleic acid (LNA) oligonucleotide, but not the suppression of *miR19a* activity with an anti-*miR19a-5p* LNA oligonucleotide, increased the cell size and the number of nuclei of osteoclasts, which are indices of activated osteoclasts (28). Fourth, the same resorption activators [i.e., IL-1 and $1,25(OH)_2D_3$ but not IL-6 and TNFα] that increased cellular PTP-oc mRNA and protein levels (21,22) also reduced the cellular *miR17* level in osteoclasts (28). Lastly, transgenic overexpression of pre-*miR17~92* in mouse osteoclasts drastically suppressed the expression of PTP-oc (28). Together, these observations indicate that *miR17*, but not *miR19a* (another member of the *miR17~92* cluster genes), is a negative regulator of PTP-oc expression in osteoclasts. However, because the 3′UTR of PTP-oc mRNA also contains potential target sites for *miR20* and *miR92* (28), the possibility that these two members of the *miR17~92* cluster genes could also yield similar negative regulation of PTP-oc in osteoclasts has not been excluded.

Potential mechanism by which miR17 regulates osteoclast activity

Characterization of the osteoclasts derived *miR17~92* conditional knockout mutants have disclosed information that is relevant to the mechanism by which *miR17* acts to regulate osteoclast activity. Specifically, deletion of *miR17~92* in osteoclast precursors significantly increased fusion of osteoclast precursors (28). Intriguingly, the expression levels of three osteoclastic genes known to be involved in osteoclast fusion, i.e., osteoclast-stimulatory transmembrane protein (*OC-Stamp*), d2 isoform of vacuolar (H^+) ATPase V0 domain (*ATP6v0d2*), and osteoclast-associated receptor (*Oscar*), in *miR17~92* conditional knockout osteoclasts were not different from those in wild-type osteoclasts (28). This suggests that *miR17~92* modulates osteoclast fusion through mechanisms that are either independent of or downstream to these osteoclast fusion genes. On the other hand, deletion of *miR17~92* in osteoclasts increased the expression of integrin β_3 and also activated Vav3 functional activity (28). Vav3 is a key downstream component of the integrin β_3 signaling (29,44) and is an upstream activator of Rac1/2. Activation of Rac1 and/or Rac2 in osteoclasts appears to play an essential role in osteoclast fusion (101,102). Thus, the possibility that *miR17* may regulate osteoclast fusion through an alternative mechanism that involves suppression of the integrin β_3-dependent Vav3-mediated activation of Rac1/Rac2 should also be considered. In addition, deficient expression of *miR17~92* in osteoclasts increased expression of several degradative enzymes, such as Mmp3 and Mmp9. It is conceivable that *miR17* suppresses osteoclast activity is also mediated through suppression of the release of degradative enzymes that are required for osteoclastic resorption.

An inter-regulatory relationship among miR17, PTP-oc, and EphA4 in osteoclast activation

Regardless of how *miR17* acts to negatively regulate osteoclast activity, the foregoing provides strong, albeit circumstantial, evidence that *miR17* forms a close regulatory partnership with PTP-oc and EphA4 in modulating the osteoclast activity via regulation of

the integrin β_3 signaling. Consistent with an inter-regulation among *miR17*, PTP-oc, and EphA4, the *miR17~92* osteoclast conditional knockout mutants (28), the PTP-oc overexpression mutants (19), and the *Epha4* knockout mutants (27), each show very similar bone and osteoclast phenotypes with each other. However, it is interesting to note that, unlike *miR17~92* osteoclast conditional knockout and *Epha4* knockout mutant mice, which showed osteopenic and activated osteoclast phenotypes in both male and female mutant mice, transgenic overexpression of PTP-oc in osteoclastic cells yielded significant osteopenic phenotype only in male mutant mice. One of the potential explanations for the apparent sex-related differences is that the relatively low (~ 2-fold increase) overexpression of PTP-oc in osteoclasts of the PTP-oc transgenic mice [due to the use of a relatively weak TRAP exon-1c promoter to drive transgene expression (71)], along with an apparent negative interaction between the estrogen receptor signaling and the PTP-oc/Src signaling (19), may yield only a subtle osteoclast phenotype in female mutant mice. The potential mechanistic reason has merits and needs to be further investigated.

A proposed novel miR17/PTP-oc/EphA4 regulatory axis of osteoclast activation

A molecular mechanism that could account for the enhancing effect of PTP-oc on the osteoclast activity is proposed (Fig. 3). In this mechanism, PTP-oc, in Src PTK-dependent manner, a) enhances osteoclast survival via activation of the JNK2/NFκB signaling, b) promotes cell spreading, migration, cytoskeletal reorganization (to form sealing zone and ruffled borders to resorb bone) through activation of the integrin β_3 signaling, and c) activates the ITAM/Syk signaling to further synergistically enhance the integrin β_3 signaling (as shown in Fig. 1). The forward signaling of EphA4 inhibits osteoclast activity, primarily through its negative regulatory actions on the integrin β_3 signaling (step 1a) and on the production of degradative enzymes, such as Mmp3, Mmp9, and Ctsk (step 1b). PTP-oc also directly dephosphorylates several key pY residues of EphA4, in Src PTK-independent manner, leading to an inhibition of the forward signaling of EphA4. Because the forward signaling of EphA4 is a negative regulator of the integrin β_3 signaling (see Fig. 2). The PTP-oc-mediated inhibition of EphA4 forward signaling will further activate the osteoclast activity. Additionally, cellular PTP-oc expression in osteoclasts is suppressed by *miR17* (step 2). Certain resorption activators [e.g., IL1, PTH, and $1,25(OH2)_2D$] increase cellular PTP-oc expression in osteoclasts by suppressing *miR17* expression (step 3). Accordingly, *miR17* acts to inhibit osteoclast activation through the suppression of PTP-oc expression.

There are three novel Src-independent components in this mechanism: the first component relates to the concept that PTP-oc reduces the inhibitory action of the EphA4 signaling by dephosphorylating its key pY residues (step 1a) that decreases the inhibitory action of the EphA4 signaling on the β_3-integrin signaling and the osteoclastic release of degradative enzymes (Mmp3, Mmp9, and Ctsk) (step 1b). The second component deals with the inhibitory action of *miR17* on PTP-oc mRNA expression (step 2). The third component is the suppressive role of certain resorption activators on *miR17* expression (step 3). This novel regulatory circuitry of osteoclast activation is referred herein to as the "*miR17*/PTP-oc/EphA4 regulatory axis of the osteoclast activity". This regulatory axis is unique, in that each

of the three key components acts to exert suppressive action on their respective immediate downstream inhibitory step. Because the final downstream event is the EphA4-mediated inhibition of the osteoclast activation, the overall effect of this mechanism is the stimulation of osteoclast activity.

Concluding remarks

An excessive bone resorption is often a significant pathology for osteoporosis and many degenerative bone disorders, such as osteoarthritis and osteolytic metastasis. A viable way to treat patients with an excessive resorption is an anti-resorptive therapy. However, the bone resorption process is highly complex and involves a large number of genes and pathways. As a result, the pathology of osteoporosis is very heterogeneous, and there are a number of subtypes of osteoporosis. Accordingly, a single anti-resorptive therapy is unlikely to be effective for all subtypes, as it has been exemplified by the large variable response to Fosmax in individual patients. Therefore, although several FDA approved anti-resorptive therapies are already available, there is still a great need for additional and different types of anti-resorptive therapies.

The foregoing describes a novel *miR17*/PTP-oc/EphA4 regulatory axis of osteoclast activation. The mechanistic investigations into this *miR17*/PTP-oc/EphA4 regulatory axis have identified two novel potential drug targets for anti-resorptive therapy: 1) EphA4, and 2) *miR17*. In this regard, EphA4 is an attractive drug target for an anti-resorptive therapy for the following reasons: a) mature osteoclasts express primarily the EphA4 (27,83,85); b) the forward signaling of EphA4 can effectively be activated by soluble EphA4-binding Efn-fc chimeric proteins (27); c) several Efn fragment-based EphA4 ligands (103) and EphA4-interacting small-molecules (104) have been identified; and d) direct injection of EfnA5-fc (an EphA4 ligand) into the spinal cord of mice with spinal cord injury promoted EphA4-induced axonal regeneration and functional recovery (105,106). The *miR17* is also an appealing drug target, as *miR17* modulators or mimics could be used to develop novel and effective anti-resorptive therapies. It is recognized that the current miRNA-based therapeutic technology has numerous and significant challenges that limit its therapeutic utility. However, because numerous miRNA-based therapeutic approaches have shown great promises in various preclinical models (107–109), the miRNA-based technology has received a great deal of attention from the biotech community and a great deal of efforts and resources are being put forth in addressing the various limitations of the technology. In this regard, several pre-clinical and phase I miRNA mimic-based anti-cancer clinical trials are on-going with encouraging results. It is hopeful that the challenges of the miRNA-based therapeutic technology can be overcome and that the *miR17*-based therapies will become a reality in the near future. Even if it turns out that the *miR17*-based therapies are untenable, further studies on this *miR17*/PTP-oc/EphA4 regulatory axis, especially the regulatory mechanism of *miR17* in PTP-oc-mediated resorption, could disclose additional novel drug targets.

Finally, current anti-resorptive therapies suppress osteoclast differentiation but also reduce bone formation, as bone resorption is usually tightly coupled to bone formation (110). This relationship is known as the “bone coupling”. In this regard, transgenic mice with targeted

overexpression of PTP-oc in osteoclasts (19) as well as *miR17~92*-(28) or *Epha4*-deficient (27) mice exhibited elevated bone resorption but without corresponding increases in bone formation, suggesting that alteration of the *miR17*/PTP-oc/EphA4 regulatory axis of osteoclast activity could allow the preservation of the bone coupling process. Accordingly, further investigation into the mechanism by which the regulatory axis of osteoclast activity regulates bone resorption without affecting bone formation may disclose potential therapeutic targets for development of novel therapies that could preserve the bone coupling process. This can be clinically relevant since therapies that preserve the bone coupling process are highly desirable for treatment of osteoporosis and related bone-wasting diseases.

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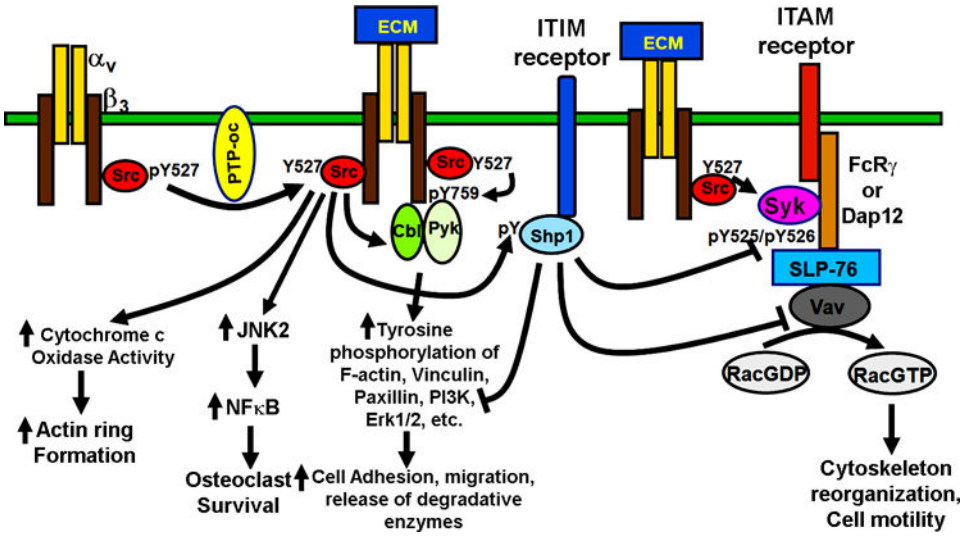

Figure 1. The molecular mechanism by which PTP-oc enhances osteoclast activity in part by activating the PTK activity of Src in osteoclasts

In this mechanism, PTP-oc dephosphorylates the pY527 residue of Src, resulting in the activation of its PTK activity, which in turn phosphorylates and activates various downstream signaling proteins, including JNK2, Cytochrome c oxidase, vinculin, paxillin, PI3K, Erk1/2, etc., to reduce osteoclast apoptosis, promote actin ring formation, cell adhesion, migration, release of degradative enzymes. The activated Src PTK also phosphorylates and activates Syk, leading to the activation of the ITAM signaling and the integrin β_3 signaling that in turn promote cytoskeleton reorganization and cell motility. In addition, the activated Src PTK also phosphorylates and inhibits Shp1, which results in the relief of the suppressive action of the ITIM/Shp1 signaling. The overall effect of these actions is an increase in the osteoclast activity.

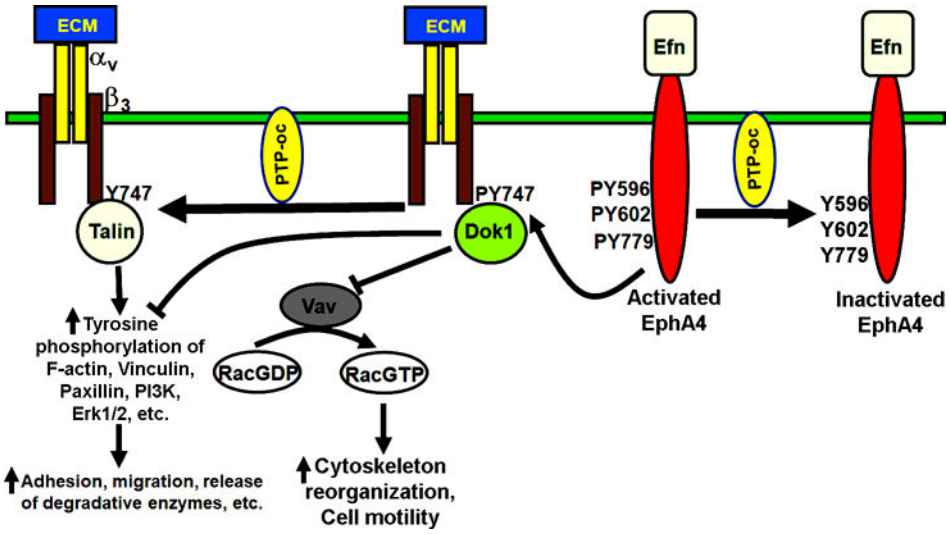

Figure 2. A proposed molecular mechanism by which the forward signaling of EphA4 acts to suppress osteoclast activity by reducing the integrin β_3 signaling in osteoclasts

In this mechanism, binding of an appropriate Efn ligand to EphA4 results in the phosphorylation of several key tyrosine residues (i.e., Y596, Y602, Y779, etc.) that leads to activation of its PTK activity and the forward signaling. The activated EphA4 then phosphorylates the Y747 residue of integrin β_3. The phosphorylation of pY747 changes the binding affinity of the C-terminal tail of integrin β_3 from one that favors the stimulatory Talin to that prefers the inhibitory Dok1. The binding of Talin is required for the activation of the integrin β3 signaling that lead to the activation of osteoclasts; whereas the binding of Dok1 prevents the subsequent activation of the integrin β_3 signaling. Accordingly, EphA4 suppresses the integrin β_3 signaling and thereby osteoclast activation through the phosphorylation of pY747 and the increased binding of Dok1 over Talin to the C-terminal tail of integrin β_3. Conversely, PTP-oc enhances osteoclast activation by direct dephosphorylating and suppressing the signaling of EphA4.

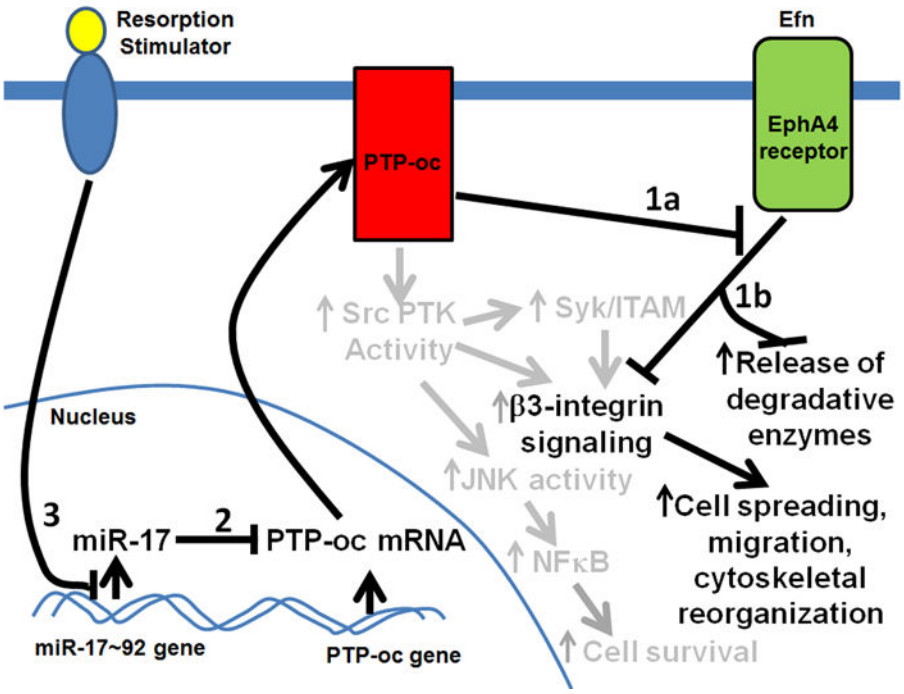

Figure 3. The proposed miR17/PTP-oc/EphA4 regulatory axis of osteoclast activity

Please see the text for the description of the proposed *miR17*/PTP-oc/EphA4 regulatory axis of osteoclast activation.