Non-canonical Wnt signals regulate cytoskeletal remodeling in osteoclasts

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Abstract
Osteoclasts are multinucleated cells responsible for bone resorption. Osteoclasts adhere to the bone surface through integrins and polarize to form actin rings, which are formed by the assembly of podosomes. The area contained within actin rings (also called sealing zones) has an acidic pH, which causes dissolution of bone minerals including hydroxyapatite and the degradation of matrix proteins including type I collagen by the protease cathepsin K. Osteoclasts resorb bone matrices while moving on bone surfaces. Osteoclasts change their cell shapes and exhibit three modes for bone resorption: motile resorbing mode for digging trenches, static resorbing mode for digging pits, and motile non-resorbing mode. Therefore, the actin cytoskeleton is actively remodeled in osteoclasts. Recent studies have revealed that many molecules, such as Rac, Cdc42, Rho, and small GTPase regulators and effectors, are involved in actin cytoskeletal remodeling during the formation of actin rings and resorption cavities on bone slices. In this review, we introduce how these molecules and non-canonical Wnt signaling regulate the bone-resorbing activity of osteoclasts.

Keywords Actin · Bone resorption · Osteoclast · Rho effectors · Wnt non-canonical pathway

Introduction
Bone is continually remodeled by bone-forming osteoblasts and bone-resorbing osteoclasts [1–4]. The balance between bone formation and resorption maintains bone mass. An imbalance between bone resorption and formation leads to bone metabolic diseases, including osteoporosis [1–4]. Osteoporosis occurs when bone resorption is higher than bone formation. Similarly, when bone formation is higher than bone resorption, bone mass increases. Estrogen deficiency enhances bone resorption, which, in turn, reduces bone mass in postmenopausal osteoporosis [3]. Inflammatory cytokines, such as TNF-α, IL-6, and IL-17, induces bone destruction by osteoclasts in inflammatory diseases such as rheumatoid arthritis and periodontal disease [4, 5]. Antiresorptive agents have two pharmacological actions, inhibition of osteoclast formation and inhibition of the bone-resorbing activity of osteoclasts [6–8]. An anti-receptor activator of NF-κB ligand antibodies (denosumab) inhibits osteoclast differentiation [6, 7]. Bisphosphonates suppress the bone-resorbing activity of osteoclasts [6, 8]. A deeper understanding of the mechanisms by which osteoclasts resorb bone may lead to the development of new antiresorptive drugs.

Osteoclasts are one of the cells, in which actin cytoskeleton is highly organized. Recent studies have established roles of actin cytoskeletons in cell functions such as cell migration, adhesion, mitosis, and sensing the external environment [9–11]. Polymerization and depolymerization of globular-actin reorganize the cytoskeleton and are required for cell movement through the formation of lamellipodia and filopodia [9, 10, 12, 13]. When cells contact the extracellular matrix (ECM), focal adhesions and podosomes are formed at the adhesion site, and the cytoskeleton becomes organized [10, 14, 15]. Podosomes are unique structures in osteoclasts, invasive cancer cells, vascular smooth muscle cells, endothelial cells, and myeloid cells such as macrophages and dendritic cells [14–18]. Podosomes are also formed in fibroblasts expressing a constitutively active form of the non-receptor tyrosine kinase c-Src or v-Src, indicating that critical roles of active c-Src in podosome formation...
Osteoclasts strongly express c-Src, and c-Src-deficient osteoclasts exhibited impaired bone-resorbing activity with defects of the formation of actin rings, ring-like structures of podosomes [21]. Thus, an understanding of signals that activate c-Src may lead to the discovery of new therapeutic targets for bone diseases such as osteoporosis and periodontal disease.

In this review, we introduce important findings on how actin rings are regulated by signals from integrins and small GTPases to exert bone-resorbing activity of osteoclasts and discuss how non-canonical Wnt signals regulate bone-resorbing activity of osteoclasts through small GTPases.

**Osteoclasts**

Osteoclasts are multinucleated cells responsible for bone resorption (Fig. 1a) and differentiate from monocyte–macrophage-lineage progenitor cells [22–24]. Two cytokines, macrophage colony-stimulating factor (M-CSF, also called CSF-1) and receptor activator of nuclear factor-κB (RANK) ligand (RANKL), are essential for osteoclast differentiation [22–24]. Osteoclast precursors express RANK and the M-CSF receptor c-Fms. When M-CSF and RANKL bind to c-Fms and RANK, respectively, osteoclast precursors differentiate into osteoclasts.

Osteoclasts change their cell shapes and exhibit the following states: motile resorbing osteoclasts [25, 26], static resorbing osteoclasts, and motile non-resorbing osteoclasts.
Podosomes are actin-rich, adhesive structures observed in several kinds of cells including osteoclasts, and are important for rigidity sensing, bone resorption, antigen sampling, and formation of invadopodia [14, 15, 17, 28, 34–36]. A better understanding of podosome structures helps to clarify how podosomes are remodeled in osteoclasts stimulated by various signals including Wnt signals. Here, phenotypes of osteoclasts formed from mice lacking the genes encoding proteins constituting podosomes are introduced.

The state of podosomes is dependent on both the activity of osteoclasts and ECM. Bone-resorbing osteoclasts have actin rings. In contrast, non-resorbing osteoclasts without actin rings have podosome clusters in their cytoplasm (Fig. 1b, left). Osteoclasts cultured on plastic plates have a belt-like structure of podosomes called the podosome belt at the periphery of cells (Fig. 1b, middle), indicating that ECM affects the distribution of podosomes in osteoclasts. Osteoclasts cultured on plastic plates can secrete acid comparable to osteoclasts on bone [37]. Therefore, there may not be a large functional difference between actin rings and podosome belts.

Osteoclasts have been considered to stop arbitrarily and form actin rings to resorb bone. Two phases of mature osteoclasts were observed using intravital multiphoton microscopy: static resorbing osteoclasts and motile non-resorbing osteoclasts [27]. However, it has recently been reported that osteoclasts move laterally on the bone surface and generate long trench without disassembling and reconstructing podosomes [25, 26]. Thus, osteoclasts resorb bone under either two states such as pit or trench resorption modes (hereafter, resorption pits and trenches are simply called resorption cavities unless, otherwise, distinguished). It should be clarified how these modes are switched in osteoclasts in future.

Podosomes are composed of a large number of proteins and can be divided into an actin-rich protrusive core and an adhesive ring around the core ([29], Fig. 1b, c). The adhesive ring includes the adhesion molecule integrin, adaptor proteins, the tyrosine kinase c-Src, and proline-rich tyrosine kinase 2 (Pyk2). The adapter proteins paxillin, talin, and vinculin connect integrin and actin. The podosome core contains Wiskott–Aldrich syndrome protein (WASP), neural-WASP (N-WASP), and the Arp2/3 complex, and is the site where F-actin elongates [38–41]. Cortactin is also in the podosome core and stabilizes F-actin [42].

Osteoclasts deficient in c-Src, Pyk2, Wasp, Cortactin, Talin, or Vinculin have impaired bone-resorbing activity in vitro [43–48]. c-Src-deficient mice have osteopetrosis from impaired bone resorption [43]. Formation of actin rings and resorption cavities is markedly impaired in c-Src-deficient osteoclasts [21, 44]. Pyk2-deficient mice also have increased bone mass from the suppression of bone-resorbing activity of osteoclasts [45]. Pyk2-deficient osteoclasts do not form actin rings because of the reduced stability of microtubules from excessive Rho activation [45], suggesting that excess activation of Rho inhibits the podosome formation.

The bone mass in c-Src-deficient mice exhibited higher than that in Pyk2-deficient mice, even though c-Src forms a complex with Pyk2 for the podosome formation [46]. Furthermore, mice with either osteoclast-specific Talin1 deficiency or an osteoclast precursor-specific Vinculin deficiency showed increased bone mass, but failed to show osteopetrosis as shown.
in c-Src-deficient mice [47, 48]. These findings suggest that c-Src has other important roles in osteoclast function in addition to its role in podosomes.

Outside-in signals from integrins activate c-Src to form actin rings in osteoclasts. To explore the roles of integrins in bone resorption, β3 integrin-deficient mice have been generated and studied, because osteoclasts strongly express αvβ3 integrins [49, 50]. β3 integrin-deficient mice developed osteopetrotic phenotype due to defects in bone-resorbing activity of osteoclasts [51]. Unlike c-Src-deficient mice, the osteopetrotic phenotype was developed 3–6 months after birth. This finding also suggests that c-Src is involved in osteoclast functions other than podosome formation and that other integrins complement the functions of β3 integrin in osteoclasts during developmental stages.

Integrin signals reportedly crosstalk with growth factor signals. When osteoclasts are stimulated with growth factors, such as M-CSF and hepatocyte growth factor, small G proteins such as Rac, Rho, and Cdc42, are activated [51]. Activation of these small G proteins is not observed in β3 integrin-deficient osteoclasts or in wild-type osteoclasts cultured in suspension. These results indicate that adhesion-induced activation of integrin signaling promotes growth factor signaling. Furthermore, the mechanisms by which integrin signaling and c-Src promote osteoclast activity have been studied [52]. The tyrosine kinase Syk forms a complex with αvβ3 integrin, c-Src, and immunoreceptor tyrosine-based activation motif proteins such as DNAK activation protein of 12 kDa and FcRγ [53]. This complex activates the small G protein Rac to induce the bone-resorbing activity of osteoclasts.

Dynamin, a large GTPase involved in endocytosis, also plays roles in actin-ring formation in osteoclasts to bind a complex of c-Src, c-Cbl, and Pyk2 ([54], Fig. 1c). In fact, dynamin reportedly regulates the actin cytoskeleton in several cell types [55] and co-localizes with actin in osteoclasts [56, 57]. Furthermore, osteoclasts overexpressing dynamin K44A, a mutant form deficient in GTP binding, have decreased bone-resorbing activity [56]. Treatment of osteoclasts with dynasore, a GTPase-activating protein (GAP) promotes inactivation by increasing the GTPase activity of small G proteins [59]. The role of Rac GEFs in osteoclastic bone resorption has been reported [60–63]. Mice lacking the Rac GEF Vav3 have increased bone mass [60]. Osteoclasts derived from Vav3-deficient mice have suppressed activation of Rac by RANKL, M-CSF, and adhesion. Therefore, the formation of actin rings and resorption cavities are also impaired in Vav3-deficient osteoclasts. Although osteoclasts deficient in Dock5, another Rac GEF, fail to form actin rings and resorption cavities in in vitro experiments, bone mass is mildly increased in Dock5-deficient mice (approximately 20% increased from control litters) compared with Vav3-deficient mice (approximately 300% increase from control litters) [60, 62]. This finding suggests that Vav3, but not Dock5 is mainly involved in bone-resorbing activity of osteoclasts.

Crk-associated substrate (p130Cas), an adapter protein, is phosphorylated by c-Src [64]. Osteoclast-specific p130Cas-deficient mice have high bone mass from impaired bone-resorbing activity [63]. Mechanistically, osteoclasts lacking p130Cas have impaired interactions among c-Src, Pyk2, and Dock5, which decreases Rac activity and impairs the formation of actin rings and resorption cavities. FERM, ARH/RhoGEF, and pleckstrin domain protein (FARP) 2, a Rac1-specific GEF, is also expressed in osteoclasts [61]. Osteoclasts that express FARP2 lacking a GEF domain (a dominant negative form) have impaired the formation of actin rings and resorption cavities, suggesting that FARP2 is also required for bone-resorbing activity of osteoclasts. In contrast to Vav3, FARP2 regulates the localization of active Rac1, but not the activation of Rac1 in osteoclasts. FARP2 negatively regulates phosphorylation of β3 integrin to reduce the adhesion activity [61]. Thus, FARP2 is involved in podosome rearrangement in osteoclasts and enhances bone-resorbing activity of osteoclasts.

Croke et al. [65] generated osteoclast-specific Rac1; Rac2−/− double knockout mice (Rac DKO mice) by crossing Rac1Δfl/fl; Rac2−/− mice with LysM Cre mice or with Cathespin K Cre mice. Both Rac DKO mice using LysM Cre (LysM Rac DKO) and using Cathepsin K Cre (Csk Rac DKO) have an osteopetrotic phenotype. Osteoclasts formed from LysM Rac DKO fail to form actin rings and resorption cavities, but osteoclasts formed from Csk Rac DKO have normal bone-resorbing activity in vitro. Wang et al. [66] also generated Rac DKO mice by crossing Rac2−/− and Rac1Δfl/fl with LysM Cre mice to analyze the bone phenotype. In contrast to Croke’s report, the mice have a mild increase in bone mass and impaired bone-resorbing activity in osteoclasts. Furthermore, RANKL-induced osteoclast formation was impaired in cultures of osteoclast precursors derived from Rac DKO mice. These studies reveal the importance of Rac in the bone-resorbing activity of osteoclasts.

**Rac, Cdc42, and Rho**

Small G proteins are converted from the GDP-bound (inactive) form to the GTP-bound (active) form by a guanine nucleotide exchange factor (GEF) (Fig. 2a). Activated small G proteins become inactive by their own GTPase activity. GT-Pase-activating protein (GAP) promotes inactivation by increasing the GTPase activity of small G proteins [59].
Osteoclast-specific Cdc42-deficient mice also have increased bone mass because of the decreased bone-resorbing activity of osteoclasts [67]. Actin-ring formation is slower in osteoclasts derived from Cdc42-deficient mice. Elongation and branching of F-actin occur via WASP, N-WASP, and the Arp2/3 complex, which are downstream of Cdc42 activity [29]. These findings suggest that Cdc42 plays critical roles in the formation of podosome cores in osteoclasts.

In contrast to Rac and Cdc42, roles of Rho in bone-resorbing activity of osteoclasts have not been established. When C3 exoenzyme, an inhibitor of Rho, is added to osteoclast cultures, actin rings disappear [68], which suggests that Rho is necessary for actin-ring formation in osteoclasts. In contrast, overexpression of a constitutively active form of Rho reduces actin-ring formation in osteoclasts [29]. Furthermore, when Pleckstrin homology (PH) domain-containing family G 5 (Plekhs5), a Rho GEF, is knocked down in osteoclasts, bone-resorbing activity is impaired [70]. These studies suggest that the activation of Rho may be tightly regulated in a narrow optimal window to promote the bone-resorbing activity of osteoclasts. Myosin IXB is a GAP for Rho in osteoclasts [71]. When myosin IXB is knocked down, the activity of Rho but not Rac slightly increases in osteoclasts. siRNA-mediated knockdown of Myosin IXB inhibits the formation of podosome belts and increases actin rings in osteoclasts cultured on glass. Interestingly, osteoclasts knocked down for Myosin IXB cultured on dentin slices which have impaired bone-resorbing activity, even though actin rings are formed normally. These osteoclasts have decreased phosphorylation of tyrosine residues necessary for c-Src activity and abnormal localization of c-Src, which suggests that Rho regulates the activity and localization of c-Src.

Because various effector molecules are activated downstream of Rho, regulation of Rho may be required for the bone-resorbing activity of osteoclasts. There are 13 Rho effectors that bind active Rho ([72], Fig. 3b), and they are classified into three groups: Group 1 contains Rho-associated, coiled-coil containing protein kinase (Rock) 1, Rock2,
and citron-K, which are serine/threonine kinases. Group 2 contains mammalian homolog of Drosophila Diaphanous (mDia) 1–3, which have formin homology (FH) 1, 2 domains. mDia1, mDia2, and mDia3 are involved in actin elongation. Group 3 contains protein kinase N (Pkn) 1–3, which are serine/threonine kinases. Rhophilins and Rhotekins are involved in protein–protein interactions, which have a Rho-binding domain (RBD) and PSD95, Disks large, ZO-1 (PDZ) or PH domains, but no kinase domain. Rock1 and Rock2 positively control bone resorption by recruiting CD44, an osteopontin receptor, to the plasma membrane of osteoclasts [73]. On the other hand, mDia2 negatively regulates bone resorption by promoting deacetylation of tubulin through histone deacetylase (HDAC) 6 [74]. The expression of Pkn3 increases during osteoclast differentiation and Pkn3-deficient mice have high bone mass because of decreased bone resorption [75]. Multiple effector signals may cooperate or counteract each other downstream of Rho. Taken together, Rho may regulate osteoclast activity, but further studies are needed to clarify the role of Rho in osteoclast function.

Non-canonical Wnt signaling pathways

Wnt proteins are involved in the development and homeostasis of various organs through β-catenin-dependent and β-catenin-independent signaling [76–81]. There are 19 ligands involved in Wnt signaling in human and mouse. The ligands bind to frizzled receptors and the co-receptor low-density lipoprotein receptor-related protein (LRP) 5/6, and then, β-catenin-dependent canonical signaling pathways are activated [76, 79]. This signaling induces cytosolic accumulation and nuclear translocation of β-catenin. Nuclear β-catenin and T cell factor/lymphoid enhancer factor induce the transcription of target genes.

On the other hand, Wnt5a, a typical non-canonical Wnt ligand, binds to receptor tyrosine kinase-like orphan receptor (Ror) 1/2 and activates β-catenin-independent signaling pathways such as the planar cell polarity (PCP) pathway and calcium pathway [77, 78]. In the PCP pathway, Rho, Rac, and Cdc42 are activated. Frizzled is associated with disheveled and disheveled-associated activator of morphogenesis (Dama) to activate Rho, Rac, and Cdc42. In the calcium pathway, intracellular calcium increases through receptor-coupled G proteins and phospholipase C.

Wnt5a is secreted from osteoblasts and binds to the Ror2 receptor in osteoclast precursor cells, which promotes RANKL-induced osteoclast differentiation [80]. Mechanistically, Wnt5a activates c-Jun N-terminal kinase (JNK) in osteoclast precursor cells through Ror2-mediated signaling, which enhances the expression of RANK (Fig. 3a). Expression of Wnt5a and Ror2 markedly increases during osteoclast differentiation [75], which suggests that Wnt5a regulates the function of mature osteoclasts through Ror2 receptors. Osteoclast precursor cells prepared from the fetal liver of Wnt5a-deficient mice with M-CSF and RANKL have impaired bone-resorbing activity due to defects in actin-ring formation. These results suggest that Wnt5a secreted from osteoclasts autonomously promotes bone-resorbing activity.

To clarify the role of Ror2 signaling in osteoclast function, bone from osteoclast-specific Ror2-deficient (Ror2ΔOCL/ΔOCL) mice (by crossing Ror2ΔΔ mice with Cathepsin K-Cre mice) was analyzed. Ror2ΔOCL/ΔOCL have high bone mass because of the impaired bone-resorbing activity of osteoclasts. Wnt5a activates both Rac and Rho, but overexpression of a constitutively active form of RhoA but not Rac1 rescues the impaired bone-resorbing activity of Ror2ΔΔ osteoclasts. These findings suggest that
Wnt5a-Ror2 signaling activates Rho, which promotes the bone-resorbing activity of osteoclasts. Daams 1 and 2 activate Rho downstream of frizzled and disheveled [75, 81], and shRNA-mediated knockdown of Daam2 suppresses the bone-resorbing activity of osteoclasts. These findings suggest that Wnt5a-Ror2 signaling activates Rho through Daam2 to promote the bone-resorbing activity of osteoclasts.

Expression of Pkn3 markedly increases in osteoclasts, and actin-ring formation and bone-resorbing activity are lower in osteoclasts derived from Pkn3-deficient mice. Similar to Ror2ΔOCL/ΔOCL mice, Pkn3-deficient mice have increased bone mass due to impaired bone resorption, but not increased bone formation. Pkn3 is associated with c-Src and Pyk2 in a Ror2- and Daam2-dependent manner. In addition, the kinase activity of c-Src decreases in Ror2ΔOCL/ΔOCL and Pkn3-deficient osteoclasts. The proline-rich region of Pkn3 is necessary for binding between Pkn3 and c-Src, and for the bone-resorbing activity of osteoclasts. Furthermore, Pkn3 lacking the kinase domain bound to c-Src but failed to rescue the impaired bone-resorbing activity of Pkn3-deficient osteoclasts. This finding suggests that the kinase domain is also required for the activation of c-Src by Pkn3. The mechanism by which Pkn3 activates c-Src in osteoclasts needs to be clarified in future. Taken together, Wnt5a-Ror2 signaling activates Rho via Daam2 in osteoclasts. Activation of Rho promotes the formation of Pkn3, c-Src, and Pyk2 complexes, and increases c-Src activity in osteoclasts, thereby promoting actin-ring formation and bone resorption (Fig. 3b). Thus, Pkn3 represents a therapeutic target for osteoporosis and inflammatory bone diseases such as periodontal disease and rheumatoid arthritis.

Conclusion

Many molecules and signaling pathways involved in the formation of actin rings and bone-resorbing activity of osteoclasts have been identified. Bone resorption by osteoclasts is a tightly regulated multistep process. Mouse genetic approaches have revealed the role of Rac in osteoclast function, and several Rac GEFs, such as Vav3, Dock5, and FARP2, are involved. However, it is still not clear why so many molecules are necessary to activate Rac during bone resorption. Rho is also activated by many signals, and further studies are needed to clarify how Rac cooperates with Rho to organize podosomes in osteoclasts. These studies may lead to the development of new antiresorptive drugs and will clarify when and where molecules are activated during cytoskeletal remodeling in osteoclasts. The development of multiphoton fluorescence microscopes and new probes, such as LifeAct-GFP [82], will enable real-time analysis of actin dynamics and other molecules.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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