Journal of Oral Biosciences 61 (2019) 135–140

Contents lists available at ScienceDirect

Journal of Oral Biosciences

journal homepage: www.elsevier.com/locate/job

Review

Regulation of osteoclast function via Rho-Pkn3-*c*-Src pathways

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ARTICLE INFO

Article history:
Received 4 June 2019
Received in revised form
3 July 2019
Accepted 8 July 2019
Available online 7 August 2019

Keywords:
Actin cytoskeleton
Bone resorption
Osteoclast
Protein-serine-threonine kinases
Wnt5a protein

ABSTRACT

Background: Wnt signaling pathways are largely divided into the β-catenin-dependent canonical pathway and β-catenin-independent non-canonical pathways. The roles of Wnt signaling in bone metabolism have been extensively investigated.

We previously attempted to clarify the roles of Wnt-non-canonical signaling in bone resorption and demonstrated that Wnt5a-receptor tyrosine kinase-like orphan receptor 2 (Ror2) signaling promoted osteoclast differentiation by enhancing RANK expression in osteoclast precursor cells. However, the roles of Wnt5a-Ror2 signaling in osteoclast function remain unclear.

Highlight: Trabecular bone mass was significantly greater in osteoclast-specific Ror2-deficient ($Ror2^{\Delta OCL/\Delta OCL}$) mice than in control mice due to the decreased bone-resorbing activity of osteoclasts. Wnt5a-Ror2 signaling activated Rho in osteoclasts via dishevelled-associated activator of morphogenesis 2 (Daam2). The expression of protein kinase N3 (Pkn3), a Rho effector, increased during osteoclast differentiation. Trabecular bone mass was significantly greater in Pkn3-deficient mice than in wild-type mice due to the decreased bone-resorbing activity of osteoclasts. Pkn3 bound to c-Src and Pyk2 in a Wnt5a-Ror2 signaling-dependent manner, thereby enhancing the kinase activity of c-Src in osteoclasts. The binding of Pkn3 to c-Src was essential for the bone-resorbing activity of osteoclasts.

Conclusion: Wnt5a-Ror2 signaling promotes the bone-resorbing activity of osteoclasts by activating the Daam2-Rho-Pkn3-*c*-Src pathways. Pkn3 inhibitors, therefore, have potential as therapeutic agents for osteoporosis and bone destruction in inflammatory diseases.

Contents

Abbreviations: CA, constitutively active; CaMK2, Ca^{2+}/calmodulin-dependent protein kinase 2; cKO, conditional knock-out; Clc-7, chloride channel 7; CTX-1, type I collagen C-terminal telopeptide; Daam2, dishevelled-associated activator of morphogenesis; GSK3β, glucose synthase kinase 3β; HUVEC, human umbilical vein endothelial cell; JNK, c-Jun N-terminal kinase; LEF, lymphocyte enhancer factor; LPA, lysophosphatidic acid; Lrp, low density lipoprotein receptor-related protein; M-csf, macrophage colony-stimulating factor; MEF, mouse embryonic fibroblast; MMP9, matrix metalloproteinase-9; Opg, osteoprotegerin; PCP, planar cell polarity; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; Pkn3, protein kinase N3; Prk, PKC-related kinase; PRR, proline-rich region; Pyk2, proline-rich tyrosine kinase 2; Rankl, receptor activator of nuclear factor kappa B; Ror2, receptor tyrosine kinase-like orphan receptor 2; shRNA, short hairpin RNA; TCF, T-cell factor; V-ATPase, vacuolar-type H^+-ATPase.

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https://doi.org/10.1016/j.job.2019.07.002
1349-0079/

1. Introduction

Osteoclasts differentiate from monocyte/macrophage-lineage osteoclast precursor cells and this process is regulated by two cytokines, receptor activator of nuclear factor kappa B (Rankl) and macrophage colony-stimulating factor (M-csf) [1–3]. These cytokines are secreted by osteoblast-lineage cells, such as osteoblasts and osteocytes [1–5]. Osteoblast-lineage cells also secrete a decoy receptor of Rankl, osteoprotegerin (Opg), to inhibit osteoclast differentiation.

Osteoclasts migrate to the bone surface and resorb bone [6–8]. Such bone-resorbing osteoclasts form two types of resorption lacunae: a trench type formed by motile osteoclasts and a pit type formed by the non-motile osteoclasts [6,7]. Osteoclasts are polarized to resorb bone matrices [3,9,10]. They make contact with bone through sealing zones and the area surrounded by the sealing zone is then isolated. Additionally, podosome clusters form in non-resorbing osteoclasts, dynamically changing their actin cytoskeleton during migration and the formation of sealing zones on the bone surface.

Sealing zones are formed by rearrangement of the actin-rich protein complex, podosomes, and are also referred to as actin rings [3,9,10]. A podosome contains many signaling molecules that regulate the actin cytoskeleton, such as integrins, the tyrosine kinases c-Src and proline-rich tyrosine kinase 2 (Pyk2), and small G-proteins [10]. These molecules play important roles in the formation of sealing zones in osteoclasts.

The cell membrane surrounded by the sealing zone of osteoclasts exhibits a wavy structure called the ruffled border [3,9,10]. Proteases, such as matrix metalloproteinase (MMP) −9 and cathepsin K, are secreted from the ruffled border by the fusion of lysosome-like vesicles. Protons and chloride ions are also secreted via vacuolar-type H^+-ATPase (V-ATPase) and chloride channel (Clc)-7, respectively, in the ruffled border. Bone matrices, such as collagen and hydroxyapatite, are then degraded. These bone degradation products are taken up into the osteoclasts via endocytosis, transported through the cytosol, and secreted from the functional secretory domain, which is opposite to the sealing zone. This series of processes is called transcytosis [11,12].

In this review, we provide an overview of Wnt signaling along with the recently elucidated mechanisms responsible for enhancements in the bone-resorbing activity of osteoclasts by Wnt non-canonical pathways.

2. Wnt signaling

Wnt signaling pathways are largely divided into canonical and non-canonical pathways (Fig. 1A and B) [13–15]. In the canonical pathway, the key molecule is β-catenin [13,14]. In the absence of Wnt ligands, β-catenin forms a complex with glucose synthase kinase 3β (GSK3β) and Axin2 and is phosphorylated by GSK3β. Phosphorylated β-catenin is then degraded by the proteasome. The binding of Wnt ligands to receptors causes the accumulation of β-catenin in the cytoplasm due to its suppressed degradation, and it subsequently translocates into the nucleus. Nuclear β-catenin regulates the expression of target genes together with transcription factors such as T-cell factor/lymphoid enhancer factor.

Non-canonical pathways are signal transduction pathways that are independent of β-catenin [13,16]. They include the calcium pathway, through which intracellular calcium concentrations are increased, and the planar cell polarity (PCP) pathway with the activation of small G proteins, such as Rac, Rho, and cdc42. In the PCP pathway, the activation of Rho requires an adapter protein, the

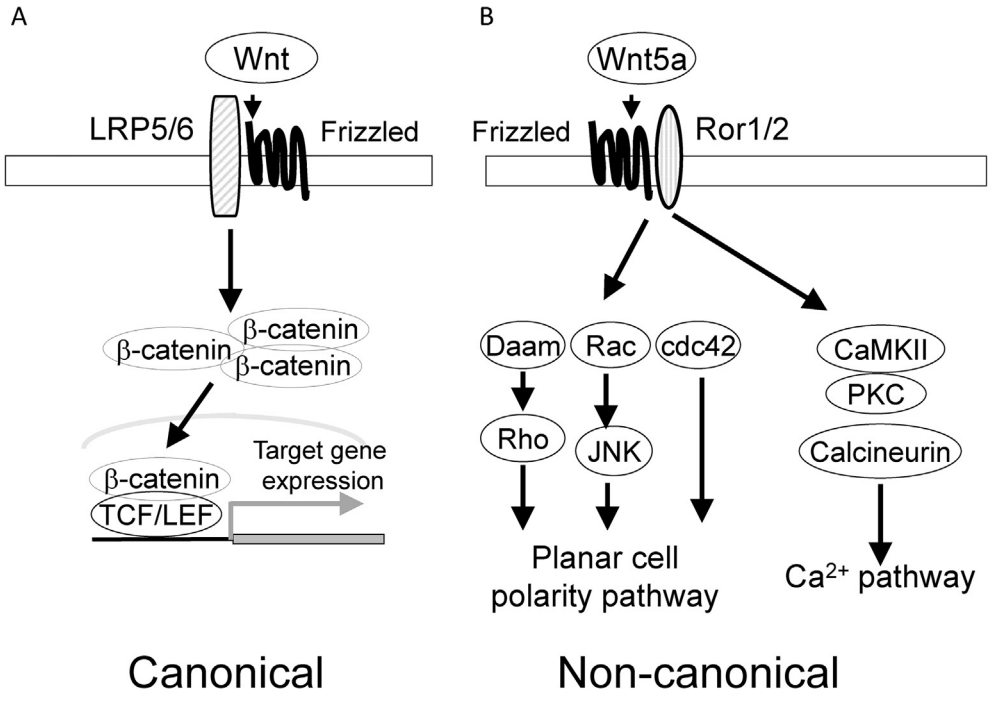

Fig. 1. Schematic representation of Wnt signaling pathways. **A**: Wnt canonical pathways. The binding of Wnt ligands to Frizzleds and LRP5/6 leads to the inhibition of β-catenin degradation. Increased β-catenin translocates into the nucleus, which, in turn, regulates the expression of target genes together with transcription factors, such as TCF/LEF. LRP, low density lipoprotein receptor-related protein; TCF, T-cell factor; LEF, lymphocyte enhancer factor. **B:** Wnt non-canonical pathways. The binding of Wnt ligands, particularly Wnt5a, to Frizzleds and Ror1/2 activates Wnt non-canonical pathways. Wnt non-canonical pathways contain planar cell polarity (PCP) pathways and calcium pathways. Small G proteins, such as Rho, Rac, and cdc42, are activated in PCP pathways. In calcium pathways, intracellular calcium concentrations are increased, which, in turn, activates CaMK2. Ror, receptor tyrosine kinase-like orphan receptor; CaMK2, Ca^{2+}/calmodulin-dependent protein kinase 2.

dishevelled-associated activator of morphogenesis (Daam) [17]. Nineteen types of Wnt ligands and ten types of Frizzled receptors have been reported in mammals [13,14]. Furthermore, the co-receptor low density lipoprotein receptor-related protein (Lrp) 5/6 and receptor tyrosine kinase-like orphan receptor (Ror) 1/2 play important roles in Wnt signaling. The combination of these ligands with the receptors and co-receptors influences which signaling pathways are activated.

We previously reported the roles of Wnt non-canonical pathways in osteoclast differentiation [18]. Osteoclast precursor cells expressed co-receptor Ror2, which activates the Wnt non-canonical pathways. In contrast, Wnt5a, the ligand for Wnt non-canonical pathways, was strongly expressed in osteoblasts. Osteoblast-specific *Wnt5a*-deficient mice were generated by crossing *Osterix*-Cre mice and *Wnt5a*-floxed mice, and osteoclast precursor cell-specific *Ror2*-deficient mice by crossing *Rank*-Cre mice and *Ror2*-floxed mice, and their bone phenotypes were then analyzed. Osteoclast formation was suppressed in both conditional knock-out (cKO) mice. We investigated the underlying molecular mechanisms in detail and clarified that Wnt5a-Ror2 signaling increased RANK expression by activating c-Jun N-terminal kinase (JNK) in osteoclast precursor cells, thereby promoting osteoclast differentiation. We recently reported that Wnt5a-Ror2 signaling plays important roles not only in osteoclast differentiation, but also in regulating osteoclast function through the Rho-Pkn3-*c*-Src pathways [19]. This issue will be described in more detail in Chapter 4.

3. Protein kinase N (Pkn) family

Pkn1 (Pknα or protein kinase C-related kinase (Prk) 1) is a serine/threonine kinase that was cloned in 1994 [20,21]. Pkn2 (also called Pknγ or Prk2) and Pkn3 (also called Pknβ or Prk3) were identified in 1995 and 1999, respectively [20,22,23]. In 1996, two groups independently reported that Pkn1 was activated by RhoA [24,25]. Pkn2 and Pkn3 were then also shown to be activated by Rho [23,26].

In a previous study on the cloning of Pkn3 in 1999, a Northern blot analysis revealed that *Pkn3* was expressed in tumorigenic cells [23]. Therefore, the roles of Pkn3 in tumor cells were investigated. In 2004, Pkn3 was reported to be expressed in the prostate cancer cell line PC-3 [27]. In PC-3 cells, phosphoinositide 3-kinase (PI3K) not only increased the expression of Pkn3, but also enhanced its activity. PI3K-Pkn3 signaling enhanced the proliferation of PC-3 cells. Furthermore, the small interference (si) RNA-mediated knockdown of Pkn3 in PC-3 cells suppressed the metastasis of PC-3 cells transplanted into nude mice to the lymph nodes. These findings suggest that Pkn3 plays an important role in the control of cell growth, thereby contributing to tumor progression and metastasis. Aleku et al. developed a liposome incorporating an siRNA against Pkn3 (Atu027) and reported its administration to mice in 2008 [28]. The administration of Atu027 to mice implanted with PC-3 cells suppressed the size of the primary tumor as well as the metastasis of PC-3 cells to the lymph nodes. Atu027 is currently being tested as a cancer treatment in clinical trials [29,30].

Pkn3 is also expressed in human umbilical vein endothelial cells (HUVECs) [28,31]. The knockdown of Pkn3 did not affect the proliferation of HUVECs [28]. However, the formation of tube-like structures of HUVECs cultured on matrigel was suppressed by the knockdown of Pkn3 [31]. In 2012, Möpert et al. reported that migration was suppressed and that the formation of TNF-α-induced stress fibers and focal adhesions was inhibited in HUVECs in which Pkn3 was knocked down [31]. These findings suggest that Pkn3 plays an important role in the regulation of the actin cytoskeleton in HUVECs.

In 2016, Mukai et al. reported the phenotypes of *Pkn3*-deficient (Pkn3 KO) mice; using real-time PCR, Pkn3 was shown to be ubiquitously expressed in normal tissues [32]. However, the expression levels of Pkn3 in major organs were lower than those of Pkn1 and Pkn2. Pkn3 KO mice were born according to Mendel's law, and their appearance and fertility were normal. These findings suggest that Pkn3 is not essential for development or growth, and that the functions of Pkn1 and Pkn2 are compensatory. However, the mobility of mouse embryonic fibroblasts (MEFs) derived from Pkn3 KO mice was lower than those from wild-type (WT) mice [32]. There was also a delay in the repair of injured blood vessels. The infusion of melanoma BL16B6 cells from the tail vein resulted in tumor formation in the lungs. The sizes of these tumors were smaller in Pkn3 KO mice than in WT mice. These findings are consistent with those obtained with siRNAs against Pkn3.

The phospholipid lysophosphatidic acid (LPA) activates the Rho-Pkn1 pathway via the LPA receptor in fibroblastic Swiss 3T3 cells [24,33]. LPA receptors are G protein-coupled receptors, and six isoforms ($LPA_{1\text{-}6}$) have been reported to date [34]. The expression of the LPA_1 receptor reportedly increases during osteoclast differentiation and LPA promotes the differentiation and fusion of osteoclasts [35,36]. Furthermore, the LPA_1 antagonist Ki16425 suppresses actin ring formation in osteoclasts [35]. Therefore, LPA signals, as well as Wnt5a, may regulate osteoclast function via Pkn3, and further studies are necessary to clarify the relationship between LPA signals and Ror2 signals.

4. Roles of the Pkn family in the promotion of osteoclast function

Ror2 was expressed not only in osteoclast precursor cells, but also in osteoclasts [18,19]. During osteoclast differentiation, the expression of Wnt5a was markedly increased [19]. In addition, osteoclasts formed from precursor cells isolated from the E14.5 fetal liver of Wnt5a-deficient mice exhibited impaired bone-resorbing activity. These findings suggest that Wnt5a secreted from osteoclasts promotes their bone-resorbing activity in an autocrine manner.

In order to clarify the importance of Ror2 signaling in osteoclast function *in vivo*, osteoclast-specific *Ror2*-deficient mice ($Ror2^{\Delta OCL/\Delta OCL}$) were generated by crossing *Ror2*-floxed mice and *Ctsk*-Cre mice [19]. The trabecular bone mass of the femur was significantly greater in $Ror2^{\Delta OCL/\Delta OCL}$ mice than in control mice. Bone histomorphometry revealed that the number of osteoclasts did not significantly differ between $Ror2^{\Delta OCL/\Delta OCL}$ mice and control mice; however, the depth of the resorption lacunae of osteoclasts was significantly shallower in $Ror2^{\Delta OCL/\Delta OCL}$ mice. Consistent with these findings, serum type I collagen C-terminal telopeptide (CTX-1), a marker of bone resorption, was also significantly lower in $Ror2^{\Delta OCL/\Delta OCL}$ mice. No significant difference was observed in the parameters of bone formation, such as osteoblast numbers and the bone formation rate, between $Ror2^{\Delta OCL/\Delta OCL}$ mice and control mice. Consistent with these findings, serum alkaline phosphatase activity, a bone formation marker, was normal in $Ror2^{\Delta OCL/\Delta OCL}$ mice. These findings suggest that Ror2 signaling in osteoclasts promotes bone resorption *in vivo*. In accordance with these *in vivo* findings, osteoclasts derived from $Ror2^{\Delta OCL/\Delta OCL}$ mice exhibited impaired bone-resorbing activity due to the failed formation of actin rings. Furthermore, Wnt5a activated Rho and Rac in a Ror2 signaling-dependent manner in osteoclasts. In order to clarify which small GTPase is important for actin ring formation downstream of Ror2 signaling, constitutively active (CA)-RhoA or CA-Rac1 was overexpressed in $Ror2^{\Delta OCL/\Delta OCL}$ osteoclasts using an adenovirus. The expression of CA-RhoA, but not CA-Rac1, rescued actin ring formation as well as pit-forming activity in $Ror2^{\Delta OCL/\Delta OCL}$ osteoclasts.

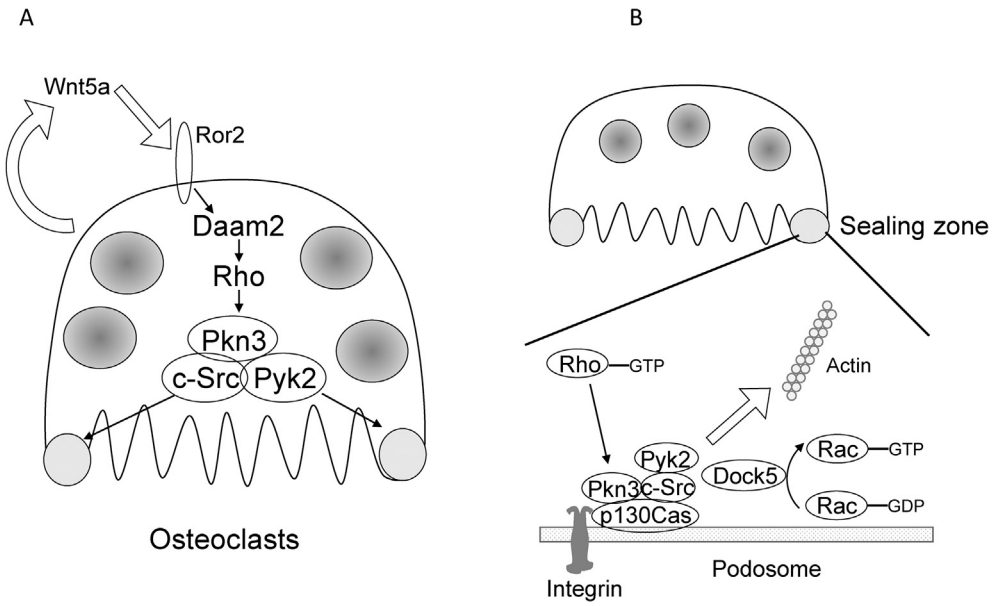

Fig. 2. Regulatory mechanisms of the bone-resorbing activity of osteoclasts via Pkn3. **A:** The roles of Wnt non-canonical signaling in osteoclast function. Wnt5a-Ror2 signaling activates Rho via Daam2. Pkn3 is activated downstream of Rho, which enhances the tyrosine kinase activity of c-Src. These signaling pathways play an important role in the bone-resorbing activity of osteoclasts. **B:** Schematic representation of hypothesized signaling pathways in the podosomes of osteoclasts. Activation of integrin signaling activates c-Src. c-Src phosphorylates p130Cas, which is followed by the activation of Rac via Dock5 [43]. Pkn3 may interact with p130Cas and get involved in the phosphorylation of p130Cas [42] in osteoclasts. Integrated signaling regulates the actin cytoskeleton in the podosomes of osteoclasts.

These findings suggest that Wnt5a-Ror2 signaling enhances the bone-resorbing activity of osteoclasts through the activation of Rho. We then investigated whether Daams are involved in the activation of Rho by Wnt5a-Ror2 signaling. Osteoclasts were seen to express Daam2, but not Daam1. The knockdown of Daam2 by short hairpin (sh) RNA suppressed Wnt5a-induced Rho activation and inhibited the bone-resorbing activity of osteoclasts. These findings suggest that Wnt5a-Ror2 signals activate Rho via Daam2 in osteoclasts.

In order to clarify the molecular mechanisms that promote the bone-resorbing activity of osteoclasts downstream of Rho, the expression of Rho effectors in osteoclasts was examined by a real-time PCR analysis. Among the 13 Rho effectors identified to date [37], the expression of Pkn3 was significantly increased during osteoclast differentiation [19]. Although Pkn1, 2, and 3 were knocked down in osteoclasts using specific shRNA, their bone-resorbing activity was significantly reduced only by the knockdown of Pkn3. However, the knockdown of mDia2 by shRNA or the addition of Y27632, a Rock inhibitor, did not affect the bone-resorbing activity of osteoclasts, although mDia2 and Rock are reportedly involved in the bone-resorbing activity of osteoclasts [19,38,39]. These findings suggest that among Rho effectors, Pkn3 plays an important role in regulating osteoclastic bone resorption. To clarify the importance of Pkn3 in bone resorption *in vivo*, bone phenotypes in Pkn3 KO mice were examined using a μCT analysis. The bone mass of femurs was significantly greater in Pkn3 KO mice than in WT mice. Bone histomorphometry revealed that the number of osteoclasts was not significantly different between Pkn3 KO mice and WT mice; however, the depth of the resorption lacunae of osteoclasts was significantly shallower in Pkn3 KO mice. Consistent with these findings, serum CTX-1 was also significantly lower in Pkn3 KO mice. No significant differences were observed in bone formation parameters, such as osteoblast numbers and bone formation rates, between Pkn3 KO mice and WT mice. In accordance with these findings, serum alkaline phosphatase activity was normal in Pkn3 KO mice. These results suggest that Pkn3 promotes bone resorption *in vivo*.

Pkn2 has been reported to bind to the tyrosine kinase Fyn, a member of the Src family of tyrosine kinases [40]. C-Src is a tyrosine kinase that is essential for the formation of actin rings in osteoclasts [41]. Therefore, we hypothesized that Pkn3 may bind to c-Src in osteoclasts. An immunoprecipitation analysis revealed that Pkn3 binds to c-Src and Pyk2 in a Ror2 signaling-dependent manner [19]. Furthermore, the tyrosine kinase activity of c-Src was lower in $Ror2^{\Delta OCL/\Delta OCL}$ osteoclasts and Pkn3 KO osteoclasts. These findings suggest that Wnt5a-Ror2 signaling promotes osteoclastic bone resorption by enhancing the activity of c-Src via Pkn3 (Fig. 2A). Mutants lacking the proline-rich region (PRR) of Pkn3 failed to bind to c-Src and Pyk2. However, Pkn3 mutants deleted the kinase domain bound to c-Src and Pyk2. The enforced expression of WT Pkn3 in Pkn3 KO osteoclasts rescued its bone-resorbing activity, whereas the expression of Pkn3 lacking the PRR or kinase domain did not. These findings suggest that not only the binding of Pkn3 to c-Src and Pyk2, but also the kinase activity of Pkn3, is important for osteoclastic bone resorption. Further studies are needed to clarify what the Pkn3 substrate is and how Pkn3 activates c-Src.

Pkn3 was recently shown to bind to p130Cas in order to promote cell proliferation in MEFs [42]. Podosome rearrangement results in the formation of invadopodia in MEFs that express CA-Src (Y527F). Pkn3, actin, and p130Cas co-localized in invadopodia in MEFs expressing CA-Src (Y527F). This suggests that Pkn3 also plays an important role in the invadopodia of tumor cells. If Pkn3 interacts with p130Cas in osteoclasts, there may be crosstalk between the signaling pathways of Rho-Pkn3 and p130Cas-Dock5-Rac in podosomes (Fig. 2B) [43]. In the future, the roles of Rho and Rac in the regulation of the bone-resorbing activity of osteoclasts will have to be completely clarified.

5. Conclusions

Our studies revealed the molecular mechanisms by which Wnt5a-Ror2 signaling regulates osteoclast function. The long-term administration of anti-resorptive drugs such as bisphosphonates

and anti-RANKL antibodies suppressed bone remodeling, thereby increasing the risk of atypical femoral fractures [44]. We also demonstrated that Pkn3 KO mice showed normal bone formation without any changes in osteoclast numbers, even though the bone-resorbing activity of osteoclasts was markedly suppressed. Therefore, the inhibition of Pkn3 may effectively increase bone mass without bone remodeling.

Ethical approval

Ethical approval is not required because this is a review article.

Conflicts of interest

The authors declare that they have no conflict of interest.

Role of funding sources

Shunsuke Uehara was supported by the Japanese Association for Oral Biology Grant-in-Aid for Young Scientists and awarded by the Japanese Association for Oral Biology as Rising Members Award. However, this entity is not involved in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit this manuscript for publication.

Acknowledgements

This work was supported by The Japan Society for the Promotion of Science KAKENHI Grant Numbers JP16K11494, JP19K10050 (SU), JP16H05508 (NU), and JP16H02691 (YK).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.job.2019.07.002.

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