Profilin 1 Negatively Regulates Osteoclast Migration in Postnatal Skeletal Growth, Remodeling, and Homeostasis in Mice

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ABSTRACT
Profilin 1 (Pfn1), a regulator of actin polymerization, controls cell movement in a context-dependent manner. Pfn1 supports the locomotion of most adherent cells by assisting actin-filament elongation, as has been shown in skeletal progenitor cells in our previous study. However, because Pfn1 has also been known to inhibit migration of certain cells, including T cells, by suppressing branched-end elongation of actin filaments, we hypothesized that its roles in osteoclasts may be different from that of osteoblasts. By investigating the osteoclasts in culture, we first verified that Pfn1-knockdown (KD) enhances bone resorption in preosteoclastic RAW264.7 cells, despite having a comparable number and size of osteoclasts. Pfn1-KD in bone marrow cells showed similar results. Mechanistically, Pfn1-KD osteoclasts appeared more mobile than in controls. In vivo, the osteoclast-specific conditional Pfn1-deficient mice (Pfn1-cKO) by CathepsinK-Cre driver demonstrated postnatal skeletal phenotype, including dwarfism, craniofacial deformities, and increased bone-resorbing activity. Our study, for the first time, demonstrated that Pfn1 has critical roles in inhibiting osteoclast motility and bone resorption, thereby contributing to essential roles in postnatal skeletal homeostasis. Our study also provides novel insight into understanding skeletal homeostasis in human disorders. © 2018 American Society for Bone and Mineral Research.

KEY WORDS: OSTEOCLAST; GENETIC ANIMAL MODELS; DEVELOPMENTAL MODELING; BONE HISTOMORPHOMETRY; DISEASES AND DISORDERS OF/RELATED TO BONE

Introduction
Skeletal homeostasis is maintained by various factors, including hormones, cytokines, growth factors, and coupling factors.1,2 Those factors influence cell–cell communication, signaling, gene expression, and behaviors including division, proliferation, vesicular transportation, and migration. By focusing on skeletal cell movement in growing mice,3–6 we previously demonstrated that profilin1 (Pfn1), one of the major modulators of actin cytoskeleton, plays important roles during skeletogenesis and bone homeostasis.5–7 The conditional Pfn1-deficient mice in limb mesenchyme had a failure in
chest-wall closure and endochondral ossification in long bones.\(^5\) Conditional Pfn1 deficiency in osteocytes resulted in impaired osteocytic dendrite formation and bone mass maintenance,\(^6\) consistent with the idea that Pfn1 enhances the movement of most mesenchymal cells by supporting the elongation of straight actin filaments assisted by formin.

However, pfn1 functions in modulating actin-filament elongation are known to be controversial, depending on the cell-type or cell-context difference.\(^6\) In epithelial and cancer cells, Pfn1 is known to inhibit their movement,\(^9\) possibly through inhibiting branched-end actin filaments\(^{10-12}\) that are necessary for structuring lamellipodia, podosomes, and invadopodias. Invadopodia is a podosome-like process in cancer cells; thus, Pfn1-deficient breast cancer cells are more invasive.\(^9\) Similarly, Pfn1-deficient cytotoxic T cells are more active in movement and cancer-killing functions than are Pfn1-positive cells.\(^{13}\)

Osteoclasts are critical players in bone remodeling and belong to the macrophage lineage cells. Their differentiation and activation requires cell–cell fusion, vesicle trafficking, transcytosis, and sealing zone formation, etc.—all of which require cytoskeletal reorganization.\(^{14,15}\) Characteristically, the osteoclasts utilize a cellular process, named podosome, with specific arrangements for their movement or attachment, depending on the differentiation/activation status.\(^{16}\) Most characteristically, the activated osteoclasts at the terminal differentiation stage create the structure, the podosome belt, which is required for active bone resorption. Because the core structures of podosome and lamellipodia are postulated to be negatively regulated by Pfn1, it is of interest to test if the inhibition of Pfn1 in osteoclasts leads to increased cellular movement and related behavior based on cytoskeletal changes.

Therefore, in this study, we investigated the Pfn1 function in osteoclasts by utilizing the preosteoclastic cell line and mice deficient for Pfn1, specifically in osteoclasts. By analyzing the phenotypic manifestations of mutant mice, we revealed the critical functions of Pfn1 in suppressing osteoclast movement and bone resorptive activity. In addition, we propose that observed skeletal phenotypes in these model mice might be related to the pathogenesis of certain osteolytic disorders that are possibly influenced by the modulation of the actin cytoskeleton.

### Materials and Methods

#### Animals

Pfn1-floxed (Pfn1\(^{fl/fl}\)) mice were generated previously.\(^7\) To obtain conditional mutant mice lacking Pfn1 in mature osteoclasts, Pfn1\(^{fl/fl}\) mice were crossed with Cathepsin K (CatK)-Cre knocked-in mice.\(^{17}\) Progenies were genotyped by genomic PCR. The neonatal, adolescent, and adult littermates were analyzed for the skeletal phenotypes at postnatal day 0 (p0), 4 weeks, and 8 weeks. Mice were housed under controlled conditions at 24°C on a 12-hour light and 12-hour dark cycle. All the experiments were approved by the animal welfare committee of Tokyo Medical and Dental University (#0170041A).

#### Radiological analysis

A skeletal soft X-ray radiogram of each animal was captured under anesthesia at 40 kV, 12 mA for 5 s, using Softex apparatus (Softex Japan Co., Kobe, Japan). Three-dimensional μCT images were obtained using the Scan-Xmate-ED90 (Comscan Tecnco, Sagamihara, Japan). The morphometric trabecular bone parameters such as bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular spacing (Tb.Spac), and trabecular separation (Tb.Sp) were analyzed on distal femurs, using Tri/3D-Bon software (Ratoc System Engineering, Tokyo, Japan). Cortical bone volume, cortical thickness, and center-line length were also analyzed as previously described.\(^{18,19}\) According to the guideline established by the ASBMR\(^{19}\) to evaluate the topological differences of the cross-sections, we calculated the ratios of the length on the short axis (SA) versus the long axis (LA) at the diaphysis.

#### Skeletal preparation

Neonatal skeletons stained with Alcian Blue and Alizarin Red, and stored in 80% glycerol in 0.2% KOH solution were photographed essentially according to previously described methods.\(^{20}\)

#### Histological and histomorphometric analysis

Paraffin-embedded decalcified sections were stained with hematoxylin and eosin or toluidine blue-O (pH 4.5). To visualize osteoclasts, sections were stained with substrate for tartrate-resistant acid phosphatase (TRAP). Bone resorption parameters including osteoclast number/bone surface (Oc.N/BS) were analyzed based on the TRAP-positive multinucleated cells on distal femoral sections.\(^{18}\) For some samples, nondecalcified femurs and skulls embedded in glycol methacrylate (GMA) were sectioned and stained. The analysis was based on the ASBMR guideline.\(^{21}\)

#### Immunoblot analysis

Cultured cells were solubilized at each time point with SDS sample buffer containing 62.5mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2.25% SDS. The total cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to an Amersham Hybond-P PVDF membrane (GE Healthcare, Piscataway, NJ, USA), which were then treated with blocking One P (Nacalai, San Diego, CA, USA) in Tris-buffered saline containing Tween 20 for 60 min. The membranes were incubated with antibodies against Pfn1 (GeneTex, Irvine, CA, USA), or GAPDH (14C10; Cell Signaling Technology, Beverly, MA, USA), washed, and incubated with horseradish peroxidase-labeled anti-rabbit IgG (GE Healthcare). Band intensities were measured and analyzed using a LAS4000 imager with ImageQuant TL software (GE Healthcare).

#### Gene-expression analysis

Total RNA was isolated from bone marrow cells and RAW264.7 cells using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Reverse transcription (RT) was carried out using 1-μg total RNA. Quantitative real-time PCR (qPCR) was carried out using StepOne equipment (Applied Biosystems; Thermo Fisher Scientific). The aliquots of 10-μL reagent mixture containing 2 μL of cDNA samples, 0.2 μM forward and reverse primers, and 5 μL of FAST SYBR Green Master Mix (Applied Biosystems) were applied and analyzed. The primer sequences for the Pfn1 were as follows: Pfn1-forward, 5'-CCCCCACCGTTCCCTTTGGC-3'; Pfn1-reverse, 5'-GCCCGCAGCCATGGTTT-3'.

#### Cell culture

RAW264.7 cells were maintained in culture with Dulbecco’s modified Eagle’s medium or alpha-MEM supplemented with
10% FBS by subconfluent stage. By replating the cells onto individual plates or wells, the osteoclastogenesis assay was conducted using RANKL (100 ng/mL; Oriental Yeast Co., Tokyo, Japan) as described previously.16,22 The siRNA transfection was conducted using lipofectamine RNAiMax reagent (Life Technologies; Thermo Fisher Scientific), according to the manufacturer’s standard protocol. The siRNA for the mouse Pfn1 (Ambion Silencer Select; #4390771-s171526, Thermo Fisher Scientific; Sense CAUUGGAUCUUGUAACCAAT, Antisense UUGUAU- GAAGAUCCAUUGTA) and control siRNA (Ambion Silencer Negative Control No.1 siRNA) was purchased from the vendor (Life Technologies; Thermo Fisher Scientific).

Bone marrow cells obtained from WT and Pfn1-cKO mouse femurs and tibias were plated on 100-mm plastic dishes at around 4 \times 10^5 cells/cm² in alpha-MEM supplemented with 10% FBS and penicillin/streptomycin. After 4 hours of incubation at 37 °C, 5% CO₂, nonadherent bone marrow cells were replated on the assay plates at 4 \times 10^5 cells/cm². For osteoclastogenesis assay, bone marrow cells were cultured 48 hours in the presence of M-CSF (10 ng/mL; R&D Systems, MN, USA); then the medium was changed to that containing both RANKL (100 ng/mL) and M-CSF. The osteoclast differentiation was monitored usually for 5 days, by periodically changing the medium. The osteoclast induction was also conducted by an optimized method.23,24 Bone marrow cells were cultured on a 60-mm plastic dish for 2 to 3 hours in alpha-MEM containing 10% FBS. Nonadherent cells were reseeded in multwell dishes at 2.6 \times 10^5 cells/cell² and cultured for 24 hours in alpha-MEM with 10% FBS and 1:50 CMG14-12 culture supernatant containing M-CSF. These cells were cultured for 72 hours with M-CSF and RANKL (36 ng/mL). The cells were fixed and stained for TRAP activity. To quantify the TRAP-positive multinucleated cells, microscopic images of entire wells were captured in tiled array by semi-automated microscope BX-2 (Keyence, Osaka, Japan).

Immunofluorescence analysis of cultured osteoclasts

To analyze the morphological features of the wild-type and Pfn1-cKO osteoclasts, localization of the Pfn1 was detected by immunofluorescence, using rabbit anti-Pfn1 antibodies (11680-1-AP; Proteintech-Japan, Tokyo, and GTX102072; GeneTex, with control siRNA (Ambion Silencer Negative Control No.1 siRNA) was purchased from the vendor (Life Technologies; Thermo Fisher Scientific). Bone marrow-derived macrophages plated on 6-well plates at 5 \times 10^3 cells/well were transfected with Pfn1- or control-siRNA (Ambion; Thermo Fisher Scientific) using Lipofectamine RNAiMax reagent and cultured for 6 hours. Then, siRNA was washed out, and the cells were incubated with RANKL for 72 hours. Similarly, nonadherent bone-marrow-derived cells (5 \times 10^5 cells/well) plated on a 24-well plate were subjected to the osteoclastogenesis assay as described above. Live imaging was conducted from 48 to 72 hours after adding RANKL, using BioStation CT (Nikon, Melville, NY, USA). The images were captured every 13 min for RAW264.7 cells and every 11 min for bone marrow cells. The average distances and the velocity at each interval were quantified using the manual tracking and chemotaxis tool of the ImageJ software.

For live-cell imaging of the modified wound-healing assay, RAW264.7 cells were plated on glass-bottom dishes (Matsunami, Bellingham, WA, USA) at 1 \times 10^5 cells/cm² in the presence of RANKL (100 ng/mL). To create a spotty wound on the bottom, a cloning cylinder was placed on the bottom. After 72 hours, the cells were transfected with Pfn1- or control-siRNA. At 4 to 5 days, the cloning cylinders were removed to start live imaging using the laser-scanning confocal microscope Fv-10i (Olympus, Tokyo, Japan). Similarly, nonadherent bone-marrow derived cells (1 \times 10^6 cells/cm²) plated on the glass-bottom dishes with cloning cylinders were subjected to osteoclastogenesis using M-CSF (10 ng/mL) and RANKL (100 ng/mL). The cloning cylinders were removed at approximately 2 days to start capturing the images every 15 min for 24 hours.

Statistical analysis

Statistical analysis was performed on the data obtained by biological, histological, radiological, and physical measurements. Normal distribution was tested by the Kolmogorov-Smirnov (K-S) test, when applicable; the data were basically represented by mean ± SD. The Student’s t test or the Mann-Whitney test was applied to compare the two experimental groups.

Results

Pfn1-KD affects osteoclast migration and matrix resorption

Pfn1 was expressed in osteoclasts, and its intracellular localization was detected by immunofluorescence (Supplemental Fig. 1A). To examine its cellular functions, we knocked down the Pfn1 mRNA in preosteoclastic RAW264.7 cells by siRNA transfection: The KD efficiency at 24 hours was 47.3 ± 2.52 % by qPCR analysis. In osteoclastogenesis by standard protocol, we found the number of TRAP-positive cells was comparable between the Pfn1-KD cells and control cells, whereas their size seemed to be slightly larger (Fig. 1A, B). However, detailed analysis of their size distribution using fluorescent images did not indicate significant differences between them (Supplemental Fig. 2B). We did detect increased locomotion of Pfn1-KD cells compared with that of controls by live-cell imaging analysis.
Similar results were obtained by modified scratch-wound healing assay with live-cell imaging (Supplemental Fig. 2C–E, Supplemental Movie 2). In addition, pit formation assay revealed that Pfn1-KD cells tended to resorb more mineralized matrix by larger pits with comparable numbers per area (Fig. 1C, D; 48 hours after transfection). The results suggest that the increased mobility of each osteoclast may amplify matrix-resorbing activity. This is consistent with a recent report showing that chemokine pathways that activate RhoA and ROCK increase bone resorption in culture without increasing the osteoclast number. To test if the Pfn1-KD osteoclasts are indeed more potent in bone resorption, RAW264.7 cells transfected with siRNA as negative control (siNT) or against Pfn1 (siPfn1) were subjected to the osteoclastogenesis using RANKL. (A) Multinucleated (n ≥ 3) TRAP-positive cells (Oc. N) were counted in all wells. Six wells per experimental group were evaluated. (B) Pit formation assay was conducted on artificial mineralized matrix by RAW264.7-derived osteoclasts. After 5 days of culture (48 hours after transfection), cells were lysed and the microscopic images were obtained (upper panels). The traced images (bottom panels) were analyzed by ImageJ. N.S. = not significant. Scale bars: 100 μm. (C) Average pit size in area was analyzed based on the traced images (C, bottom). (D) Live imaging analysis was performed for 24 hours on the osteoclastogenesis assay using siPfn1 transfected RAW264.7 cells. Representative images at 24 hours after reseeding are shown with the trails of multinucleated cells. Bottom panels are the magnified images for the boxed areas in the upper panels. (E) The average migration velocity, measured every 13 min per cell, was compared between the control and Pfn1-KD cells. Scale bars: 100 μm. Error bars: SDs. Student’s t test was applied. *p < 0.05, **p < 0.01.
Fig. 2. Continued.
resorption, we knocked down the Pfn1 on bone marrow cells. By evaluating the pit formation after 6 days of osteoclast induction, we found that the Pfn1-KD osteoclasts resorb more mineralized matrix than those transfected with control siRNA (Supplemental Fig. 18–E).

Growth failure arises postneonatally in Pfn1-cKO mice

To investigate the roles of Pfn1 in osteoclasts in vivo, conditional Pfn1-knockout (Pfn1-cKO) mice were generated by crossing the Pfn1fl/fl and CatK-Cre knock-in mice (CatK+/+). When the Pfn1fl/fl females were crossed with the CatK+/+; Pfn1fl/fl males or vice versa, the prevalence of the Pfn1-cKO mice (Pfn1fl/fl; CatK+/+) was approximately 25%. This natural segregation following the Mendelian law indicated that osteoclastic Pfn1 is dispensable for mouse embryogenesis. Neonatal skeletal preparation by Alizarin Red and Alcian Blue staining indicated no apparent difference between the Pfn1-cKO and control mice (Pfn1+/+; CatK+/+ mice: hereafter, described as WT; Supplemental Fig. 3).

However, at weaning, the mutant mice tended to be smaller in size, with a steeper nose than WT mice. The plain radiograms and 3D μCT images at 4 weeks revealed deformed limb long bones (Fig. 2A–E) and craniofacial bones (Fig. 2J). Morphometric analysis on trabecular and cortical femurs did not indicate significant differences, except for the shape of the diaphyseal cross-sections at this age (Fig. 2F–I).

At 8 weeks, the skeletal phenotypes became more remarkable with shortened body and femur length (Fig. 3A, E, H), as well as skull and long bone deformities in Pfn1-cKO mice (Fig. 3B–H). The shortened nose, maxilla, and distorted zygomatic arches (Fig. 3B) could be the elements contributing to the altered facial appearance with a steep nose-to-head curvature on their profile (Fig. 3B).

Anterior cranial and facial bones were hypoplastic in Pfn1-cKO mice as found by 3D μCT

To analyze the skull deformities more quantitatively, we utilized the reconstructed 3D μCT images (Fig. 4A). The quantification demonstrated a significant reduction of the midsagittal length of the nasal bone in Pfn1-cKO mice compared with that of WT mice (Fig. 4B; approximately 83% in male mice and 76% in female mice). The frontal bone was also slightly shorter in the nasal bone in Pfn1-cKO mice (Fig. 4C, to approximately 91%), but temporal bones were similar. By looking at the cranial bottom-side, the premaxilla were remarkably hypoplastic (Fig. 4E), whereas the sphenoid and occipital bones were only moderately hypoplastic (Fig. 4A, F, see also Supplemental Fig. 4D, E).

Interestingly, despite the longitudinal shortening of the frontals bones, the lateral width was significantly broader in the Pfn1-cKO mice (Supplemental Fig. 4A, B; approximately 108% in male mice and 104% in female mice). Similarly, the zygomatic arches were shortened and broadened (Fig. 4D and Supplemental Fig. 4C). We assumed the findings could indicate a mechanical weakness and imbalance among the cranial bones. This assumption was also consistent with the findings on lateral view of the reconstructed skull images: The calvarial bone curvature was flattened anteriorly and convex posteriorly, the facial bones, including the mandibula, were smaller in Pfn1-cKO mice (Fig. 4G and Supplemental Fig. 4F, G).

Long bones in extremities represent the Erlenmeyer-flask deformity with osteolytic appearance

Because the site-specific impairment of the cranial bone growth tended to be limited in the structures developmentally requiring chondrogenesis, we tested if there was a failure in cartilaginous bone growth in Pfn1-cKO mice. However, despite the shortening of the entire bone (Figs. 2 and 3), no obvious change was detected in femoral and tibial growth-plate chondrocytes and columnar structures in histological sections at 4 weeks (Supplemental Fig. 5) and 8 weeks (Fig. 5A, B). Instead, we noticed that the curves of the growth plate tended to be flattened in the Pfn1-cKO mice (this was confirmed by measurement; data not shown), possibly because of the expansion of bone marrow space. In parallel, they showed expanded distal femur metaphysis representing the Erlenmeyer-flask deformity at complete penetration at each stage, with the findings of trabecular bone loss and expanded bone marrow cavity (Figs. 2D–I and 5). These observations suggested that the Pfn1-cKO skeletal deformity may be causally related to the increased resorption of the bone nearby growing cartilaginous structures. Indeed, the Erlenmeyer-flask deformity is usually associated with osteolytic conditions in children, though osteopetrotic conditions would also contribute to the changes.26 Thus, we analyzed if bone structures of Pfn1-cKO mice at distal and midshaft femurs were consistent with this notion, by using 2D projection and 3D reconstruction of μCT images (Fig. 5C, D). The altered cross-sectional contour of the metaphysis (Fig. 5C) and diaphyseal cortex (Fig. 5D) were evident, where the Pfn1-cKO femurs represented a circular shape, in contrast with the elliptical shape in WT femurs (Fig. 5D). Significant differences were detected in the ratios of the SA versus LA length (Fig. 5E; 0.79 ± 0.015 versus 0.69 ± 0.027 in males, 0.80 ± 0.026 versus 0.69 ± 0.025 in females). In addition,
the trabecular bone parameters indicated the osteopenic conditions by lower BV/TV and Tb.th (Fig. 5C, E, F). The paradoxical stability of the Tb.N, Tb.Sp, and Tb.Spac (Supplemental Table 1) could be because of the expanded trabecular bone area in the mutant bones as indicated by cross-sectional images (Fig. 5C). The cortical bone thickness at the diaphysis was also significantly smaller in Pfn1-cKO femurs than in WT femurs (Fig. 5G). Bone-forming activity of the osteoblast seemed not to be so affected because the histomorphometric bone formation parameters in preliminary samples did not show any clear differences (data not shown). Therefore, the long-bone deformity observed in the Pfn1-cKO mice must be based on the osteolytic conditions in the metaphysis and diaphysis.

We then examined if the osteoclastic bone resorption was indeed increased in the histological sections of Pfn1-cKO mice (Fig. 6A). TRAP staining indicated that the number of...
multinucleated osteoclasts was increased at the primary and adjacent secondary trabecular surface of the Pfn1-cKO femurs (Fig. 6B, D). More importantly, prominent numbers of multinucleated TRAP-positive cells were detected in Pfn1-cKO mice at the endosteal regions of the metaphysis to the diaphysis, whereas the osteoclast distribution is usually rare in WT mice (Fig. 6C, E). In contrast, a few number of periosteal osteoclasts found in the WT metaphysis was absent in the Pfn1-cKO femurs, possibly indicating the compensational protection against periosteal remodeling. We assumed such an imbalance led to the expanded metaphyseal deformity in the Pfn1-cKO mice.

The osteolytic changes were also detected by skull μCT, especially the frontal and nasal bones with opened suture (Fig. 6F). In histology, the suture in mutant mice was filled with thick fibrocartilaginous tissues, whereas the suture in wild-type mice was almost closed. Correspondingly, the internal surface of the adjacent bones was strongly stained with residual TRAP activity when overstained (Fig. 6F; bottom panel), and the multinucleated TRAP-positive cells were increased (Fig. 6G; plastic section). We assumed the excessive bone resorption may have competed against the growth of ossifying fibrous suture in this region.
Pfn1-deficiency enhances osteoclast migration with slightly increased size

The increased osteoclast number in the metaphyseal endostium of Pfn1-cKO femurs may suggest an increased differentiation potential of preosteoclasts in bone marrow. However, the osteoclastogenesis assay using Pfn1-cKO and WT bone marrow cells indicated that the number of TRAP-positive osteoclasts was identical in Pfn1-cKO and WT cultures (Fig. 7A, B). Instead, we noticed the mutant osteoclasts were larger. A size-
distribution analysis of multinucleated osteoclasts using the Mann-Whitney test indicated that the osteoclast size, in addition to the total podosome-belt length, was significantly larger in Pfn1-deficient cells (Fig. 7G–I). The slightly increased osteoclast size may be related to the increased preosteoclast movement increasing the chance of fusion. To verify the increased movement of the Pfn1-cKO osteoclasts, the locomotion of bone-marrow-derived Pfn1-cKO osteoclasts was analyzed by live imaging (Fig. 7C). Although the number of multinucleated cells was comparable between the WT and Pfn1-cKO cells, the locomotive trails of the osteoclasts were significantly increased in the Pfn1-cKO cells under this condition (Fig. 8 and Supplemental Movie 3). Pit formation assay indicated the increased matrix resorption by Pfn1-cKO cells (Fig. 7D–F). Therefore, these results show, for the first time, that Pfn1 has an essential role in the maintenance of osteoclast movement, and hence for proper skeletal development and homeostasis.
Discussion

This study demonstrated that Pfn1 functions negatively in osteoclast movement and bone-resorbing activity. A novel aspect of Pfn1 function was also demonstrated by postnatal growth failure in craniofacial and limb bones associated with an osteolytic expansion in osteoclast-specific Pfn1-deficient mice. Of note, the long bone deformity at metaphysis resembled the Erlenmeyer-flask deformity that is known to be associated with several human disorders that result from dysregulated bone

Fig. 7. Continued.
The increased osteoclast movement observed in our Pfn1-cKO mice is consistent with a recent theory that Pfn1 functions negatively in the assembly of branched actin networks required for the formation of lamellipodia and podosomes.\(^{(11–13)}\) Our findings are also consistent with previous reports suggesting a suppressive role for profilins on lamellipodia and invadopodia in cancer cells, contributing to their invasive features.\(^{(14,15,27)}\) The molecular mechanisms of how the profilins work in both enhancing and inhibiting cell movement still remain unclear.\(^{(11–13)}\) The classical understanding of the profilins is that profilins support the formin-bound barbed-end elongation of the actin filaments by efficiently adding the ATP-bound actin monomers.\(^{(28,29)}\) This molecular feature assists elongation of the nonbranched filopodial filaments contributing to mesenchymal cell movement. Mesenchymal cell movement is basically supported by Pfn1 in most situations. However, the different processes, namely the lamellipodia and podosomes, are structured mainly with branched filamentous networks.\(^{(11–13,30,31)}\) For these structures, the elongation relies on different molecules, including Ena/Vasp and Arp2/3, which have been shown to be inhibited by Pfn1.\(^{(11–13)}\) Thus, our observation provides another example of this inhibition by Pfn1. Osteoclast movements have been suggested to rely on this suppression by Pfn1. Interestingly, a recent study has found that cofilin, an actin-binding protein that interacts with cortactin, is required for Pfn1 movements.\(^{(32)}\) Thus, our observation could be explained by the increased endostal bone resorption based on longer survival of more-potent osteoclasts (Fig. 8C). Accelerated endosteal osteolysis can be the cause of an expanded thinner bone collar. Periosteal expansion, which contributes to the characteristic Erlenmeyer-flask deformity, could be explained by peristomal osteoblast compensation against mechanical impairment caused by endosteal bone resorption: The increased periosteal bone formation has been masked by the peristomal osteoclasts in Pfn1-cKO femurs that are usually found in WT femurs. Increased endosteal bone resorption would also explain the abnormal lateral broadening and impaired longitudinal growth of the facial and cranial bones in Pfn1-cKO mice (Figs. 3I, 4A, and 6D–F). The Erlenmeyer-flask deformity, a term describing a specific abnormality of the distal femur, is a clinical sign of the dysregulated long-bone remodeling during longitudinal growth.\(^{(26)}\) Although various skeletal disorders, including osteopetrosis, could be associated with this abnormality, its predominant cause is increased bone resorption.\(^{(26)}\) The typical group of associated syndromes includes osteopetetic disorders such as Engelmann disease and Melnick-Needles syndrome; the unclassified group includes Gaucher disease and Nasu-Hakola disease, whose characteristics include bone marrow expansion and infiltration. Thus, the phenotypic feature of the Pfn1-cKO mice could be regarded as typical Erlenmeyer-flask deformity based on the increased bone resorption at metaphysis.\(^{(26)}\) A recent study has found that macrophages utilize the MT1-MMP for the turnover of podosomes as well as invadopodia.\(^{(35)}\) The MT1-MMP KO mice, like Mmp2-deficient mice,\(^{(37)}\) showed osteolytic skeletal deformities resembling our Pfn1-cKO mice. Because these mutant mice recapitulate the human disorder, multicentric osteolysis nodulosus arthropathy (MONA)\(^{(38,39)}\), it might be of interest to investigate if the increased migration of MT1-MMP-deficient osteoclasts contributes to the osteolytic phenotype. Our Pfn1-cKO mice could be used to investigate the pathogenesis of these related disorders. Our results also suggest the importance of the osteoclastic cytoskeleton in the disorders associated with Erlenmeyer-flask deformity.

Lastly, the causative mechanisms of the short stature of the Pfn1-cKO mice were not defined in the present study. Despite an
apparent growth failure in proximal limb bones and the facial bones, the only detectable histological findings were the expanded distribution of the osteoclasts. In a previous study, Yang et al reported that the Catk-Cre knock-in mice that we utilized hereto delete the Pfn1-flox allele in osteoclasts had aberrant periosteal Cre expression in the chondrogenic progenitor cells, specifically at the groove of Ranvier.\textsuperscript{40} However, despite displaying altered growth plate curvature, the chondrocytes in this region did not show any alteration in the columnar alignment that we observed histologically in chondrocyte-specific Pfn1-cKO mice in a previous study.\textsuperscript{47} The possible involvement of osteoblastic precursor cells and osteocytes in this region was not thoroughly investigated, but the absence of alterations in growth plate chondrocytes and osteocytes in dwarf mice is not surprising because this is often the case in the dysplastic mutant mice and human disorders associated with impaired bone turnover and short stature.\textsuperscript{41,42} Short stature in osteopetrosis patients is a recurrent symptom, with no apparent pathological changes in growth plates. Similarly, most patients with osteodysplastic disorders—especially associated with Erlenmeyer-flask deformity—are short in stature with no specific growth-plate abnormalities. Melnick-Needles syndrome is caused by the recurrent point mutations clustered at exon 22 of the filamin A gene (FLNA), one of the actin filament-binding proteins.\textsuperscript{43} Therefore, whether the alteration of cytoskeletal protein functions in osteoclasts leads to growth failure in various mutant alleles in the conditional gene KO mice would be an interesting line of investigation.

In summary, we investigated the osteoclast-specific functions of Pfn1 in mice and in cultured osteoclasts, and revealed its inhibitory function on osteoclast movement. The increased migration of osteoclasts because of Pfn1 deficiency resulted in an expansion of endosteal bone resorption at the metaphysis and diaphysis, leading to Erlenmeyer-flask deformity. Our study explored a novel aspect of the molecular mechanisms that contribute to the pathogenesis of various skeletal disorders, which may lead to a better understanding and treatment of such disorders.

**Disclosures**

All authors declare that they have no conflict of interest.

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