Osteoclast-derived IGF1 induces RANKL production in osteocytes and contributes to Pagetic lesion formation

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Graphical abstract

The role of osteoclast-derived IGF1 on Pagetic lesion formation (PLF) in Paget's disease (PD). Our results with "tumor"-IGF1 and "non-tumor"-IGF1 mice support a model in which osteoclast-IGF1 induces the generation of osteoclasts and promotes bone loss. A small reduction of IGF1 expression reduces bone growth and leads to hyperosteoabsorptive balance. Hyperosteoabsorptive balance increases Ca^2+ release in osteoclasts and can increase osteoblast bone formation as the expression of the coupling factors in osteoblasts. IGF1 further induces local bone formation and development of PLF in bones with low Ca^2+. The data suggest that IGF1 play a role of PLF formation.

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Osteoclast-derived IGF1 induces RANKL production in osteocytes and contributes to Pagetic lesion formation

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Key words: Paget’s disease, measles virus nucleocapsid protein, osteoclast, IGF1, osteocyte
Abstract

We previously reported that measles virus nucleocapsid protein (MVNP) expression in osteoclasts (OCLs) of Paget’s disease (PD) patients or targeted to the OCL lineage in transgenic (T-MVNP) mice increases IGF1 production in osteoclasts (OCL-IGF1) and develop PD-OCLs and pagetic bone lesions (PDLs). Conditional deletion of Igf1 in OCLs of T-MVNP mice fully blocked development of pagetic bone lesions (PDLs). In this manuscript we examined if osteocytes (OCys), key regulators of normal bone remodeling, contribute to PD. OCys in PDLs of patients and of T-MVNP mice expressed less sclerostin, and had increased RANKL expression compared to OCys in bones from WT mice or normal patients. To test if increased OCL-IGF1 is sufficient to induce PDLs and PD-phenotypes, we generated TRAP-Igf1 (T-Igf1) transgenic mice to characterize if increased IGF1 expression in the absence of MVNP in OCLs is sufficient to induce pagetic lesions and pagetic OCLs. We found that T-Igf1 mice at 16 months of age developed PD-OCLs, PDLs, and OCys with decreased sclerostin and increased RANKL similar to T-MVNP mice. Thus, pagetic phenotypes could be induced by OCLs expressing increased IGF1. OCL-IGF1 in turn increased RANKL production in OCys to induce PD-OCL and PDLs.
Introduction

Paget’s Disease (PD) of bone usually occurs in patients >50 years of age and represents the most exaggerated example of coupled bone remodeling (1). PD patients have characteristic pagetic lesions (PDLs) that are highly localized areas in bone, where both osteoclast (OCL) and osteoblast (OB) activities are markedly increased. This results in local overproduction of poor-quality bone that can cause significant clinical problems (1). Interestingly, PD patients rarely develop new focal lesions over the course of their disease. The primary cellular abnormality in PD resides in the osteoclast (2), because highly effective PD treatments, such as zoledronic acid, that target OCL activity also normalize the rapid bone formation (3).

We previously reported that OCLs from 70% of PD patients we tested express measles virus nucleocapsid protein (MVNP) (4), and transgenic mice >12-month-old with MVNP targeted to OCLs (MVNP-mice) develop PDLs and abnormal OCLs characteristic of PD (5, 6). We found that OCLs from PD patients, but not normal donors express elevated levels of IL-6 and IGF1. Similarly, MVNP expression in OCLs of PD patients also induces high levels of IL-6 in PD-OCLs (7), which in turn increases IGF1 expression in OCLs in an autocrine manner. The OCL-derived IGF1 (OCL-IGF1) then upregulates expression of the coupling factors ephrinB2/EphB4 on OCLs and OBs, respectively (8, 9). Importantly, conditional deletion of Igf1 in OCLs of MVNP (MVNP/Igf1-cKO) mice totally blocked development of PDLs and the abnormal OCLs and bone remodeling characteristic of PD (9). These results suggest that increased expression of IGF1 in OCLs is needed for the development of PD-OCLs and PDLs in vivo.
Although much is known about abnormal OCL and OB activity in PD, little is known about the mechanisms responsible for the focality and persistence of bone lesions in PD. The persistence of solitary lesions in patients with PD suggests cellular imprinting for which local modification of resident cells, such as osteocytes (OCys) within the pre-pagetic niche, provides an attractive mechanism. OCys are the most abundant and long-lived cells in bone, derived from late OB lineage cells that become imbedded within mineralized matrix and intercommunicate via neurite-like extensions between cells within bone canaliculi. OCys are critical regulators of local bone remodeling, the primary source of RANKL in adult bone, and producers of and responders to IGF1 (10). However, little is known about their contributions to PD. Here we report that OCys are abnormal in PD. We generated TRAP-\textit{lgf1} (T-\textit{lgf1}) mice to test if high levels of OCL-IGF1 alone are sufficient to induce PDLs. T-\textit{lgf1} mice developed PDLs and pagetic OCLs and OCys at 16 months of age. Further, OCys from T-MVPN and T-\textit{IGF1} mice secreted high amounts of RANKL and low sclerostin in response to OCL-IGF1, suggesting a mechanism by which high IGF1 produced by PD-OCLs promotes a positive feedback loop between OCLs and OCys, leading to development of pagetic lesions.
Results

**OCys in MVNP-mice and PD patients are abnormal.** To characterize the effects of increased OCL-derived IGF1 on OCys in PD, we analyzed sclerostin expression in bone sections of femurs from 20 months old WT, *Igf1*-cKO, MVNP and MVNP/*Igf1*-cKO mice.

OCys in *MVNP*-mice showed lower sclerostin staining compared to the other genotypes (Figure 1A), and the numbers of sclerostin-expressing OCys per bone area were also significantly reduced compared to the other genotypes (Figure 1B). However, circulating serum sclerostin concentrations were similar in all genotypes (Figure 1C). OCys in *MVNP* mice were morphologically abnormal and had very short dendritic processes compared to the well-developed dendritic processes of OCys in WT, *Igf1*-cKO and *MVNP/*Igf1*-cKO mice (Figure 1A, arrow). Canalicular length of OCys in *MVNP* mice was also significantly shorter than in the other genotypes (Figure 1D, p<0.001) but did not appear to be sexually dimorphic. Importantly, OCys in a bone biopsy from a Paget's patient were similarly abnormal and showed reduced sclerostin expression and shorter dendritic processes compared to OCys in a bone biopsy of a normal patient (Figure 1E, arrow).

**OCy morphology and sclerostin expression in PDLs.** We and others previously found that OCLs are increased numbers and hyper-multinucleated in PDLs (1-3). Therefore, we examined OCys in PDLs that were characterized by such OCLs. As shown in Figure 1, in bone areas in mice without PDLs, sclerostin expression and dendritic processes of OCys in *MVNP*-mice were significantly reduced compared to WT mice. Sclerostin expression and dendritic processes of OCys were further reduced in *MVNP*-mice at sites of PDLs
(Figure 2A), which also had lower numbers of sclerostin-expressing OCys per bone area and decreased canalicular length compared to MVNP-mice without PDLs or WT mice (Figure 2B and C). These results suggest that OCys may contribute to the increased pagetic OCL formation and to formation of PDLs.

**Characteristics of primary OCys from bones of WT and MVNP-mice.** Since sclerostin expression and dendrite formation are characteristics of mature OCys (11), and OCys produce IGF1 (12), we analyzed Sost and Igf1 mRNAs in primary OCys isolated by collagenase digestion of long bones from 20-month-old WT and MVNP mice. Sost gene expression in OCys of MVNP-mice was reduced by 30% compared to WT mice, while Igf1 gene expression in OCys from WT and MVNP-mice was unchanged (Figure 3A). Fluorescent immunostaining of primary OCys from MVNP-mice showed decreased average intensity of staining for the OCy maturation markers DMP1 and sclerostin compared to OCys in WT mice (Figure 3B and C). These results suggest that OCys from MVNP-mice exhibit impaired maturation compared to WT OCys.

Since only a limited numbers of OCys can be obtained by collagenase digestion, we isolated OBs and OCys derived from bone outgrowth cells of WT and MVNP mice. Differentiated OBs can be detected in outgrowth cells in bones from MVNP and WT mice at day 15 of culture, and when cultured for an additional 15 days, they differentiate into OCy-like cells. Outgrowth cells from bone reflect the phenotype of OCys in bone (9) and demonstrate that OCy maturation appears decreased in OCys-like cells from MVNP mice (Supplemental Figure 1A-C). Further, OCy-like cells from bones of MVNP mice produced
and secreted less sclerostin than OCy-like cells derived from the other three genotypes (Supplemental Figure 1D).

**OCys from MVNP-mice express increased RANKL compared to WT, Igf1-cKO, and MVNP/Igf1-cKO mice.** Since OCy-derived RANKL plays a key role in OCL formation (10-15), we examined RANKL expression by OCys in bone sections of 20-month-old mice. We found that OCys of MVNP-mice expressed increased RANKL compared to OCys of WT and MVNP/Igf1-cKO mice (Figure 4A). Interestingly, the numbers of RANKL-expressing OCys per bone area in MVNP-mice were higher than in the other genotypes (Figure 4B). RANKL released into conditioned media of OCys from MVNP mice was also significantly higher than in OCy conditioned media (CM) of the other genotypes ($p<0.01$) (Figure 4D).

We then determined if RANKL mRNA in primary OCys isolated by collagenase digestion was also increased in MVNP mice compared with WT mice and found this to be the case ($p<0.01$) (Supplemental Figure 2 A and B). Thus, RANKL production by the 30-day outgrowth cells from bone reflected differences in RANKL production by primary OCys in WT and MVNP mice bones, with increased RANKL production by MVNP OCy-like cells compared to WT OCy-like cells. Further, RANKL was also increased in OCy-like cells from MVNP-mice at the protein level (31-kDa band), which is the membrane-bound form of RANKL (Supplemental Figure 2C-E).

**OCL-IGF1 increases RANKL production by OCys and induces formation of PD_OCLs.** We then asked if the large amounts of OCL-IGF1 secreted by MVNP-mice
could increase RANKL production by OCys and contribute to pagetic OCL formation in cocultures of OCL-precursors and OCys from either WT or MVNP mice. When MVNP OCL-precursors were cocultured with MVNP OCy-like cells, OCL formation and RANKL in culture media was increased 2.5-fold compared to cocultures of WT OCL-precursors with WT OCy-like cells, with intermediate levels of OCL formation and RANKL production seen in either WT/MVNP mixed cocultures (Figure 5 A, B and D). As shown by the arrows in Figure 5C, pagetic-like hyper-multinucleated OCLs only formed in cocultures of MVNP OCL-precursors with MVNP OCy-like cells. IGF1 levels were only increased in media of cocultures containing OCL-precursors from MVNP-mice, regardless of whether OCys of WT or MVNP-mice were in the coculture (Figure 5E). Thus, increased IGF1 is secreted by OCL-precursors and OCLs from MVNP-mice rather than by OCys in the cocultures.

Since IGF1 from OCLs could increase OCL formation via autocrine actions on OCLs (9), we examined the contribution of OCL-IGF1 on OCL formation in cultures of purified OCL-precursors treated with anti-IGF1 or anti-IGF1Receptor (IGF1R) in the absence of OCys. Treatment with anti–IGF1 or anti–IGF1R inhibited OCL formation by 25% in WT OCL-precursor cultures and 40-60% in MVNP-OCL precursor cultures (Supplemental Figure 3A). We then assessed RANKL produced by OBs and OCys from MVNP and WT mice in CM of OBs and OCy-like cells cultured for 72 hours. Although both OBs and OCy-like cells secreted RANKL, OCy-like cells secreted more RANKL than OB (p<0.001), and RANKL secretion was significantly higher in CM from MVNP-OCys compared to CM of WT-OCys (Supplemental Figure 3B). Thus, OCL-IGF1 from MVNP mice increases OCL-formation in part via autocrine mechanisms, and via MVNP-OCy-
like cells, which express higher RANKL, and further increase OCL numbers and pagetic-like OCL formation (Figure 5 A-C).

**OCL formation in cocultures of OCL-precursors and OCy-like cells is decreased by transducing OCys with Igf1 receptor siRNA or treatment with anti-IGF1 or anti-IGF1R.** We then asked if OCL-IGF1 was directly increasing RANKL production in OCys. When MVNP-OCys transduced with Igf1-Receptor siRNA (MVNP-IGF1R siRNA-OCy) or control siRNA were cocultured with MVNP-OCL-precursors, OCL formation and RANKL in CM of cocultures containing MVNP-IGF1R siRNA-OCys were significantly lower (by 65%) compared with MVNP-control siRNA-OCys cocultures (Figure 6A and B). The Igf1R siRNA-transduced OCy-like cells from WT and MVNP mice still had suppressed IGF1R expression after 48 hours (Supplemental Figure 4).

Further, addition of anti-IGF1 or anti-IGF1R to OCL/OCy cocultures inhibited OCL formation and decreased RANKL levels in CM of MVNP-OCL-precursors cocultured with MVNP-OCys (by 80%) compared with vehicle or control IgG cocultures (Figure 6C and D). In addition, development of pagetic-like OCLs by MVNP-OCL-precursors was also decreased in these cocultures (data not shown). Similar inhibition of OCL formation was observed in coculture of WT-OCL precursors and WT-OCy (70%), but RANKL levels in CM were below the detection range of the RANKL ELISA. We also found that OCys in co-culture adopted a spindle shape when the action of IGF1 was blocked.

**Bone resorption capacity of OCLs cocultured with OCy-like cells.** To test the bone resorption capacity of OCLs formed in cocultures of OCL-precursors with OCys, the same
numbers of OCLs and OCy-like cells were cocultured without RANKL on bone slices for 72 hours. Coculture of MVNP-OCLs with MVNP-OCy-like cells formed numerous large resorption pits, while coculture of WT-OCLs with WT-OCy-like cells formed low numbers of small pits (Figure 7A). Anti-IGF1 receptor treatment of MVNP-OCL-precursors cocultured with MVNP-OCys on bone slices decreased the bone resorption rate by 80% compared with bone resorption in control-IgG-treated cocultures (Figure 7B). In contrast, anti-IGF1R decreased bone resorption rates in cocultures WT-OCL with WT-OCy-like cells by 30%. WT-OCys-MVNP-OCLs and MVNP-OCys-WT-OCLs co-cultures were not evaluated in the bone resorption pit analysis, because the number of OCLs formed and the concentration of RANKL in the co-culture medium (Figure 5 and 6) were too low to demonstrate bone resorption.

**TRAP-Igf1 mice expressed PDLs like those seen in TRAP-MVNP mice.** To test if high levels of OCL-IGF1 alone are sufficient to induce PDLs and pagetic OCLs, we generated a TRAP-Igf1 (T-Igf1) mouse that overexpresses IGF1 in OCLs. To confirm the role of OCL-IGF1 obtained in MVNP mice, we selected T-Igf1 mice as founders with the same level of OCL-IGF1 as in MVNP mice (mice expressing twice as much IGF1 as in WT mice). T-Igf1 mice were aged to assess if they develop PDLs and pagetic OCLs at 16 months of age for comparison to similar ages of MVNP and WT mice. OCLs from bone marrow culture of these T-Igf1 mice express IGF1 at the same level as MVNP mice and express twice as much IGF1 as WT, as shown in Supplemental Figure 7A. Interestingly, the expression of IL-6 in OCLs of T-Igf1 mice was at the same level as in WT mice and lower than in MVNP mice. Furthermore, OCL-IGF1 levels in the bones of T-Igf1 and MVNP
mice were detected at similar levels and more strongly stained with anti-IGF1 than in WT, as shown in Figure 8A. There was no difference in plasma IGF1 levels between WT and T-Igf1 mice (Supplemental Figure 7B).

T-Igf1 mice developed the same PD phenotype as MNVP mice (Figure 8B). Mice of this age were examined because we most consistently find PDLs in MVNP mice at 12 months of age or older (9). PDLs were detectable in 50% of T-Igf1 mice (6 of 12) by μCT and histological analysis (Table 1) and found in vertebrae, femurs, and tibiae. The number of PDLs formed ranged from 1 to 5 in each bone. The results of μCT analysis (Figure 8C) and histological analysis (Figure 8E) in T-Igf1 mice were similar to MVNP mice. Very large OCLs also appeared in T-Igf1 mice (Figure 8D). These results suggest that IGF1 is the major OCL product driving PDL formation in the MVNP model. Even in the absence of MVNP and IL-6 in OCLs, IGF1 alone facilitates PDL formation.

Further, OCys in T-Igf1-mice showed lower sclerostin staining compared to WT mice (Figure 8F), and the numbers of sclerostin-expressing OCys per bone area were also significantly reduced compared to WT mice (Figure 8G). Canalicular length of OCys in T-Igf1 mice was also shorter in MVNP-mice (Figure 8G). DMP1 expression was also lower in OCys of TRAP-Igf1 mice than in WT (data not shown). These results are similar to the observations in MVNP mice shown in Figure 1A and B.

Detection of phospho-Akt, RANKL and p16INK4A in WT, MVNP, and T-Igf1 mice. Since IGF1 stimulates phosphorylation of PI3K/ Akt via IGF1R (9), we examined phospho-Akt expression in bones from these mice. Interestingly, more phospho-Akt staining was observed in T-Igf1 and MVNP than in WT mice (Figure 9A). OCys and OCLs from MVNP
and T-\textit{Igf1} mice stained positively for p-Akt compared with OCys and OCLs from WT mice. These results suggest that OCL-IGF1 not only transduces signals to OBL and OCys via IGF1R, but also has autocrine actions on OCL, as shown Supplemental Figure 3A.

Next, we assessed IGF1 effects on OCys by histomorphology. We stained with TRACP and RANKL by IHC of bones from T-\textit{Igf1} and WT mice. OCys near OCLs expressed more RANKL in T-\textit{Igf1} mice than in WT mice (Figure 9B). These results are similar to RANKL production in OCys of \textit{MVNP} mice, as shown Figure 4.

Farr et al., recently reported that the removal of senescent OCy reduced RANKL production by OCys and restored osteogenesis (16). Therefore, we examined the production of p16\textsuperscript{INK4a} and RANKL in \textit{MVNP}, T-\textit{Igf1} and WT mice. p16\textsuperscript{INK4a} and RANKL were visualized by immunofluorescent (IF) staining in single cells. The expression of p16\textsuperscript{INK4a} and RANKL in \textit{MVNP} and T-\textit{Igf1} mice was stronger than in WT (Figure 9C). p16\textsuperscript{INK4a} - and RANKL-positive OCys were counted within a 250-\textmu m\textsuperscript{2} areas at 500 \textmu m below the growth plate. The ratio of p16\textsuperscript{INK4a} - and RANKL-positive OCys of \textit{MVNP} and T-\textit{Igf1} mice was increased 2.5 to 3.5-fold compared with WT. Moreover, p16\textsuperscript{INK4a} /RANKL double-positive OCys accounted for 25\% of total OCy in \textit{MVNP} and T-\textit{Igf1} mice, which was 3-fold higher than WT (Figure. 9D). The results confirm previous observations that RANKL production is part of senescence-associated secretory phenotype (SASP) of OCys.
Discussion

In the current study we found that OCys in PD were both functionally and morphologically abnormal. As shown in Figure 1, OCys adjacent to PDLs of a PD patient and from MVNP-mice have shorter dendritic processes compared with OCys from a normal patient and WT mice respectively. MVNP-OCys also expressed lower levels of genes associated with OCy maturation, such as sclerostin, DMP1 and FGF23, and higher levels of collagen type1 and BSP compared to Igf1-cKO, MVNP/Igf1-cKO and WT mice (Supplemental Figure 1). These results suggest that OCy maturation may be impaired or delayed in PD. In support of this notion are our similar findings for expression of OCy maturation markers in primary osteocytic cells isolated from MVNP and WT mouse bones (Figure 3).

We recently reported that pagetic OCLs and OCLs expressing MVNP produce high levels of IGF1 that are required for development of pagetic OCLs and PDLs in MVNP-mice (8, 9). Our current results suggest that these changes in OCy marker gene expression may reflect effects of OCL-IGF1 on OCy differentiation. PDLs were also seen in IGF1 transgenic mice created to study the contribution of OCL-IGF1 to OCy (Figures 8 and 9). This seems likely since OCys from MVNP/Igf1-cKO mice have more normal-appearing OCys. Further, IGF1 treatment of WT or MVNP-OCys decreased sclerostin expression (Supplemental Figure 5). The shorter dendritic processes of OCys in the bone biopsy of a PD patient and MVNP-mice could reflect a block in transition of osteoid OCys to mature OCys or decreased expression of genes involved in dendrite formation, such as or E11 or kalirin (17).
Interestingly, OCys in MVNP-mice with PDLs had significantly decreased canalicular length and sclerostin expression per bone area compared to OCys in MVNP mice lacking PDLs (Figure 2). This difference may be due to the increased OCL numbers/bone area in bones with a PDL, which could result in increased local OCL-IGF1 levels compared to bones from MVNP-mice without PDLs. Our finding that serum levels of IGF1 (9) and sclerostin in the 4 genotypes were similar (Figure 1D), in combination with the presence of normal appearing OCys in MVNP/igf1-cKO mice support the importance of high local IGF1 in PDL formation.

The basis for impaired differentiation of OCys in MVNP-mice is unclear. Possibly, this may result from increased expression of Runx2 in late OBs induced by OCL-IGF1. Komori et al. reported that forced expression of Runx2 in late-stage OBs suppresses their differentiation into OCys, using a mouse model in which Runx2 was forcibly expressed in late-stage OBs driven by an osteocalcin promoter (18, 19). We previously found that IGF1 increases the expression of EphB4 and Runx2 in primary OBs from MVNP-mice (8). As shown in supplemental Figure 6A, the expression of Runx2 was only increased in MVNP-OCys derived from day 30 cultures. Further, when IGF1 was added to cultures of late OBs (day 15 of culture). Runx2 expression in OBs in MVNP-mice was increased 6.9-fold compared to WT mice treated with vehicle (Supplemental Figure 6B). These results suggested that OCL-derived IGF1 induces the expression of Runx2 in OBs and impedes their differentiation into OCys.

In support of this notion, primary OCys isolated from MVNP and WT mouse bones showed similar changes in OCy gene expression as OCy-like cells present in 30-day outgrowth cells from bones of MVNP and WT mice. Sost mRNA in primary OCys from
MVNP mice were 30% lower than those in primary OCys from WT mice. Sclerostin expression by immunohistochemical analysis of primary OCys showed a similar pattern of results (Figure 3).

OCys present in the canalicular lacunae of MVNP-mice showed increased RANKL expression and secretion compared to MVNP/Igf1-cKO, Igf1-cKO and WT mice (Figure 4A). The numbers and percentages of RANKL-expressing OCys were significantly increased in MVNP-mice compared to the other genotypes (Figure 4B), although serum RANKL was similar in all genotypes. Further, RANKL in CM of OCys from MVNP-mice was significantly higher than in CM of the other genotypes (Figure 4C and D). These results suggest that RANKL production by OCys is increased in PD patients that have prolonged exposure to high local OCL-IGF1. These high local levels of RANKL may induce formation of large numbers of PD-OCLs, that secrete high levels of IGF1. Since PD-OCL precursors are hyper-responsive to RANKL (20, 21), IGF1 may then induce OCy RANKL production that further increases PD-OCL formation eventually resulting in a PDL. Consistent with this notion, coculture of MVNP-OCL-precursors and MVNP-OCys showed increased pagetic OCL formation, and the CM of these cocultures contained higher RANKL compared to cocultures of WT-OCL precursors with OCys from WT and MVNP-mice (Figure 5). Importantly, when MVNP-OCys transduced with Igf1-receptor siRNA (MVNP-IGF1R siRNA-OCy) or control siRNA were cocultured with MVNP-OCL-precursors, OCL formation and RANKL levels in CM of MVNP-OCL-precursors with MVNP-Igf1receptor siRNA-OCys were significantly lower (65%) vs. MVNP-control siRNA-OCys cocultures. Further, addition of anti-IGF1 or anti-IGF1receptor to these cocultures similarly suppressed expression of a pagetic phenotype in MVNP-OCLs. In addition, anti-
IGF1 receptor treatment of MVNP-OCL-precursors cocultured with MVNP-OCys on bone slices, decreased the bone resorption rate by 80% compared with control-IgG-treated cocultures. In addition, the observation of phospho-Akt in OCys of bone sections from MVNP and T-igf1 mice suggests that IGF1 activates signaling in OCys (Figure 9A). These results demonstrate that PD-OCL-IGF1 induces RANKL and decreases sclerostin expression in OCys via the IGF1 receptor on OCys.

Pagetic OCLs in MVNP mice express elevated IGF1, and OCL-cKO of igf1 blocks local PDL formation in this model, while further increases in IGF1 secretion by OCLs may increase PDLs. We showed previously that IL6, while insufficient to initiate pagetic lesions, could enhance the actions of IGF1 (8). To evaluate if elevated levels of OCL-IGF1 alone are sufficient to induce PDL and pagetic OCLs, we generated a T-igf1 mouse that overexpresses IGF1 in OCLs. As shown in Figure 8, PDLs were induced by overexpression of IGF1 in OCL. At 16 months of age, PDLs were found in 54% of T-igf1 mice, allowing us to distinguish the contribution of IGF1 effects on OCys in PD and PDLs. Increased OCL-IGF1 in the absence of MVNP expression was sufficient to induce PD.

Finally, 25% of OCys at sites of PDLs showed senescence, and RANKL secretion was observed in these OCys, suggesting a relationship between senescence and PDLs (Fig. 9D). Most recently, Farr et al. reported that deletion of p16 from OCys reduced RANKL from OCys (16). Insulin/IGF-1 signaling induces intracellular oxidative burden and associated oxidative damage (22). IGF1 induces specific p53 acetylation via inhibition of SIRT1, leading to premature senescence.

Taken together, our results with TRAP-igf1 and MVNP/igf1-cKO mice support a model in which OCL-IGF1 induces PD-OCL formation and PDLs. A small collection of
PD-OCLs secrete high local levels of IGF1 that induce local OCy-RANKL production, suppress OCy-sclerostin expression, and enhance formation of PD-OCLs from OCL-precursors that are hyperresponsive to RANKL. This increases local bone destruction. OCy-derived RANKL could in turn recruit additional PD-OCL precursors to this specific site in bone, analogous to OCL recruitment by apoptotic OCys. This could result in multiple cycles of PD-OCL formation that increases local bone destruction and can induce local rapid bone formation via the expression of the coupling factors Ephrin B2 on OCLs and EphB4 on OBs (8). IGF1 further stimulates local bone formation and development of PDLs (8, 9) in a bone site with low sclerostin levels. Thus, our results suggest that OCys play a key role for PDLs formation in PD.
Materials and Methods

Chemicals. Rabbit IgG (AB-105-C), anti-IGF1 (AF791), anti-IGF1R (MBA391-100), were purchased from R&D (Minneapolis, MI). αMEM was from Thermo Fisher and FBS from Sigma-Aldrich. An anti-p-Akt antibody (#9271) was purchased from Cell Signaling Technology (Danvers, MA). An anti-p16INK4A antibody (#03119) was purchased from GeneTex (Irvine, CA).

Animal studies. Animals were housed at VCU in individually ventilated cages in a barrier vivarium, which excludes all known mouse viruses and parasites and most bacteria (including Helicobacter). The mice were fed standard mouse chow (irradiated Teklad LM-485 diet) and autoclaved water. Mice of both sexes and multiple ages were euthanized under isoflurane anesthesia, followed by cervical dislocation, for collection of bone tissues, which were shipped overnight to Indiana University School of Medicine in DMEM plus 10% FBS with penicillin/streptomycin at 20°C.

Generation of mice with cKO of IGF1 in OCLs. Mice with cKO of IGF1 in OCLs were generated by breeding IGF1fl/fl mice that carry loxP sequences flanking exon 4 of the gene (24) (Jackson Laboratory 016831) with transgenic mice expressing Cre recombinase under the control of a 2.3-kb murine TRACP promoter (25) to generate TRACP-Cre (+)/IGF1fl/fl mice. These mice were further bred to WT or TRACP-MVNP mice (5), to generate mice of the following 4 genotypes: (a) WT, (b) IGF1-cKO, (c) MVNP, and (d) MVNP/IGF1-cKO, as we previously reported and characterized (9). All mice were on a C57BL/6J background. At each generation, only 1 parent carried the TRACP-MVNP transgene and only 1 carried the TRACP-Cre transgene, so offspring carrying either were heterozygous for the transgene. Since our previous studies showed that 40% of MVNP-
mice at 16-22 month of ages (average 20 months old) had a detectable pagetic lesion (PDL), we used mice in this age range for this study. All experiments were performed using mice of both sexes. In previous studies, μCT and histomorphometry showed no statistically significant differences in MVNP-induced bone changes between male and female mice (9).

**Generation of mice with IGF1 in OCLs**

We expressed the murine IGF-1 isoform containing the E peptide (isoform 4), which increases local bioavailability of IGF-1 while minimizing release into the systemic circulation (26). The founder mice were generated and bred to WT mice to obtain germline transmission. OCLs were isolated from one transgenic offspring and one WT control from founder mice under the control of a 2.3-kb murine TRACP promoter (22). IGF1 expression was assessed by Western blot analysis. The mice are on a C57BL/6J background. We identified the line whose OCL-IGF1 expression level most closely matches that from MVNP mice, using the same approach that examined if overexpression of IL-6 in OCLs was sufficient to induce PD/PDLs (27). The mice were aged to assess if they develop PDLs and pagetic OCLs and OCys at 16 months of age with comparison to 12- and 18-month-old MVNP and WT mice.

**PD patient and healthy donor** Deidentified resin-embedded bone sections from a transiliac crest bone biopsy of a 58-year-old female patient with PD and from a 30-year-old female patient as a healthy control were generously provided by Dr. Brendan Boyce (University of Rochester, Rochester, NY). Both subjects had been treated with calcein and tetracycline before biopsy. The samples were embedded without decalcification in methyl methacrylate. Sections were stained with an anti-sclerostin antibody (Abcam
Histological examination of bone sections from this PD patient and healthy donor were previously reported (9).

**Immunohistochemical analysis.** The femurs from 20 months old mice were fixed in 10% buffered formalin and decalcified in 10% EDTA for 2 weeks at 4°C and embedded in paraffin. Longitudinal sections (5μm) were cut and mounted on glass slides. Deparaffinized sections were treated with 1% horse serum for 1 hour, followed by addition of primary antibodies against sclerostin (Abcam ab63097), RANKL (Santa Cruz sc-59982) or control rabbit IgG (Santa Cruz sc-2027). The sections were incubated overnight and then stained with anti–rabbit IgG conjugated to peroxidase (Vector Laboratories). Scoring of staining intensity was performed by a blinded observer, using a scale of 1-4+ to grade the staining intensity.

For immunofluorescence, 4x10³ isolated OCys or OCy-like cells were cultured with 10% FCS in αMEM overnight on 4-well chamber slides (Falcon, #354104) overnight and fixed with 4% paraformaldehyde for 30 minutes at room temperature. Cells were permeabilized in 0.1% Triton-100 for 5 min before staining. Goat anti-DMP1 (Abcam ab81985) or rabbit anti-sclerostin (Abcam ab63097) were added for 12 hours, with goat or rabbit IgGs (R&D Systems) as staining control. Anti-goat Alexa 594 conjugate (Life Technologies A11058) or anti-rabbit Alexa 488 conjugate (Invitrogen 11008) were then added for 2 hours. Sections were examined by confocal (Zeiss 710) and fluorescence microscopy (Olympus IX73).

Cell morphological studies employed fluorescent staining of F-actin filaments with Alexa Fluor 488 phalloidin conjugate to visualize OCy dendritic processes (Thermo Fisher A12379) and were examined by fluorescence microscopy.
Measurements of canalicular lengths and staining for sclerostin and RANKL. Lumbar vertebrae were decalcified in 10% EDTA at 4°C for 2 weeks and embedded in paraffin. The decalcified sections were immunostained for sclerostin, and canalicular lengths were measured using ImageJ software. The count of positive OCys for each antibody was performed in a square at 500 μm below the growth plate.

Isolation of primary OCys from mouse long bones. Primary osteocytes were isolated from femurs and tibiae of WT and MVNP-mice, according to a method previously described by Miyagawa, et al. with modifications (28). Briefly, mouse tibiae and femurs were minced into 0.5-mm pieces and digested with 1.25 mg/ml collagenase (Wako, Osaka, Japan) in Ca²⁺-, Mg²⁺-free Hanks’ Balanced Salt Solution (HBSS) at 37°C. Cells released after the first and second (15 min. each) and third to fifth (20 min. each) digestion were collected through a 100-μm nylon cell strainer as Fractions 1 to 5, respectively. Residual bone pieces were treated with 4 mM EGTA in Ca²⁺-, Mg²⁺-free HBSS for 15 min. and then with 1.25 mg/ml collagenase for 20 min. at 37°C to collect cells for osteocyte-rich fractions (Fractions 6 to 9).

Isolation of OBs and OCy-like cells. After flushing the bone marrow from tibiae and femurs of WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice, the bones were cultured in αMEM + 10% FCS for 15 days. The original bone was removed, and the outgrowth cells from the bone were treated with 0.25% trypsin and 0.05% EDTA for 10 minutes at 37°C. These cells were used as primary OBs without further passage (8, 9). Cells were either stained for alkaline phosphatase or with alizarin red, or cell lysates were collected and analyzed for protein expression. Similarly, cells separated by trypsin from the outgrowth cells of bones that had been cultured for 30 days were used as OCys-like cells without further
passage. As shown Supplemental Figure 1A, cells derived day15 outgrowth cells of bones expressed OB makers. Cells from day 30 culture expressed the OCy markers sclerostin, DMP1, and ORP150 by Western blotting and were used to determine RANKL production by OCys and for coculture with OCL-precursors for assessing OCL formation.

**OCL formation from purified OCL precursors.** Nonadherent bone marrow cells were harvested and enriched for CD11b(+) mononuclear cells using CD11b microbeads (MACS, 120-000-300) and a Miltenyi Biotec MACS magnetic cell-sorting system. These cells were cultured with 10ng/mL M-CSF (R&D Systems) in αMEM containing 10%FCS for 3 days. This stage of purified OCL precursor expresses RANK receptor and TRACP (8) and forms OCLs in the presence of 50ng/ml RANKL for 2-4 days. The cells were stained for TRACP (Sigma-Aldrich), and TRACP+ multinucleated cells (≥3 nuclei/cell) were scored as OCLs.

**Coculture of purified OCL-precursors and OCy-like cells.** OCL-precursors (5x10⁴/well) and OCy-like cells (5x10³/well) isolated by the methods described above were cocultured in αMEM with 10%FCS for 72 hours in 96 well-plates.

**Transfection of Igf1R siRNA into OCy-like cells.** OCy-like cells (5x10³) were plated in 96-well plates 12 hour before transfection with 100nM siRNAs (Cell Signaling. Control siRNA (#6568) or mouse-specific Igf1 receptor siRNA (# 12482) were transfected into OCy-like cells as described (29).

**Isolation of mature OCLs from bone marrow (BM) cultures.** BM cells flushed from long bones of WT or MVNP-mice were cultured (2.5 x 10⁷ cells/10-cm dish) with 10ng/ml M-CSF for 3 days, followed by 50ng/ml RANKL for 4 days as described (8). At the end of
culture, trypsin-EDTA (Corning) was added for 3 minutes to remove non-osteoclastic cells. OCLs were released from the plates by gently scaping with a rubber policeman.

**Coculture of OCLs and OCy-like cells for bone resorption.** OCLs (2×10^3 /well) and OCy-like cells (5×10^3 /well) were cocultured on bovine bone slices (Immunodiagnostic Systems DT-1BON1000-96) in 96-well plates with αMEM + 10% FCS ± anti-IGF1R (0.5µg/mL) for 72 hours. The cells were then removed, the bone slices stained with acid hematoxylin, and the areas of bone resorbed determined as previously described (30).

**RNA Extraction and Real-time Polymerase Chain Reaction (PCR) Analysis.** Total RNA was extracted using TRlzlol (Invitrogen), treated with DNase (Qiagen), and reverse transcribed with random hexamers (Promega) and SuperScript II (Invitrogen). cDNA was analyze using TaqMan with Real Time PCR (Applied Biosystems). To generate a standard curve for real-time PCR, amplicons of interest were first cloned into a pT7-blue vector (Novagen), and serial 10-fold dilutions of the plasmid included in the assay. The copy number of the target cDNA in each sample was estimated by referring to the standard curve, which was standardized to that of Gapdh in each sample. Specific primers were; Sost forward, 5’-TCC TGA GAA CAA CCA GAC CA-3’, reverse, 5’- GCA GCT GTA CTC GGA CAC ATC-3; Igf1 forward 5’-ACC GAG GGG CTT TTA CTT CA-3’, reverse, 5’-TGG CTC ACC TTT CCT TCT CC-3’, Tnfsf11, 5’-AGC CAT TTG CAC ACC TCA C-3’ and 5’-CGT GGT ACC AAG AGG ACA GAG T-3’. Gapdh forward, 5’-GTG TTC CTA CCC CCA ATG TG-3’, reverse 5’-ATA GGG CCT CTC TTG CTC AG-3.

**Sclerostin, RANKL and IGF1 ELISA assays.** Collected mouse sera were stored at -80°C until tested. Sclerostin and IGF1 were measured using ELISA kits for murine Sost (Abcam, ab213889) and murine IGF1 (Abcam ab100695), and RANKL was measured using an
ELISA kit for murine/rat RANKL (R&D Systems MTR00), according to the manufacturer’s instructions.

**μCT and histomorphometry.** Femora, tibiae, and vertebrae from WT, TRAP-**Igf1** and TRAP-**MVNP** at 16 months of age were fixed in 10% buffered formalin at 4°C. Bone microstructure analyses were performed using a μCT scanning system (viva CT 40, Scanco Medical) with an isotropic voxel size of 10.5 μm and the scanner settings of 55 kVp, 25 μA, and 350 ms integration time. Structural parameters were analyzed in reconstructed 3-dimensional images using evaluation software (μCT v1.6, Scanco Medical) according to the recommended guideline (31). The regions of interest were defined using previously described methods (32, 33). The cancellous bone and marrow compartments of the L5 vertebral body were examined between the cranial and caudal growth plates. The cortical bones parameters were analyzed in 100 slices at the tibial midshaft, starting 5.5 mm from the proximal metaphysis. The μCT data were then exported as a sequence of 8-bit DICOM grayscale images, and simultaneous multiplanar reconstructed (MPR) images were viewed using ImageJ software (NIH).

The lumbar vertebrae were decalcified in 10% EDTA at 4°C and embedded in paraffin. OCLs containing active TRACP were stained red as described by Liu et al. (34). OCL perimeter (Oc.S/BS) was defined as the length of bone surface covered with TRACP+ multinuclear cells. OB perimeter (Ob. S/BS) was also measured in the same field.

**Statistics.** Significance was evaluated using a 1-way ANOVA with a Tukey test. Differences with \( p < 0.05 \) were considered significant.
**Blinding.** To avoid bias, all data were collected in a blinded fashion, with the observer unaware of the experimental group. Key studies were performed by more than 1 individual to confirm observational consistency.

**Study approvals:** All animal studies were performed as described in approved IACUC protocols from VCU and IU and an ACURO protocol from the Department of Defense, in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals. Human patient samples used deidentified archival material not collected for this study and were IRB-exempt.
 Author contributions

GDR, JMC and NK designed the study, interpreted the data, and wrote the manuscript. KM, HT, PLM, JDC, and NK performed the experiments. MAS and JJW designed and generated the transgenic mice.
Acknowledgments

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References

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Figure 1. Osteocyte morphology and sclerostin expression in WT, Igf1-cKO, MVNP and MVNP/Igf1-cKO mice at 20 months of age and a PD and normal patient. (A) Sclerostin expression was determined by immunostaining as described in Methods. Arrows indicate canalicular tubes. Scale bars=10 µm. (B) Ratio of sclerostin-positive OCys/total OCys per area (0.5mm²). Results are expressed as the mean ± SEM from 3 male (blue) and female (red) mice of the 4 genotypes, analyzed using a 1-way ANOVA with Tukey test. (C) Serum sclerostin from mice in (B) measured by ELISA, expressed as mean ± SEM. (D) Canalicular length per 0.5 mm² was measured using ImageJ software from the same sections shown in (A) and used for (B). Results are expressed as the mean ± SEM from one average value per mouse (randomly selected 30 measurements from each mouse) of 3 male (blue) and female (red) mice of the 4 genotypes, analyzed using a 1-way ANOVA with Tukey test. The same mice were used in A-D. No statistical differences were found for results between males and females. (E) OCy phenotype and sclerostin expression in bone specimens from a Paget's patient (PD) and a normal donor. Transiliac crest bone biopsies were taken from a 58-year-old female with PD (center panel) and a 30-year-old healthy female control (left) and immunostained for sclerostin. PD patient sample was also stained with control IgG (right). Scale bars=10 µm.
Figure 2. Changes in osteocyte morphology and sclerostin expression within pagetic lesions of MVNP mice. (A) OCy phenotype (upper panels, sclerostin immunostaining as in Figure 1A, scale bar=20µm) and Alexa Fluor 488-phalloidin fluorescent staining for F-actin of sections adjacent to those above to show osteocyte dendritic processes (lower panels, scale bar=5µm) in bone specimens from 20-month-old WT and MVNP mice ± pagetic lesions (PDL). (B) Canalicular length per 0.5mm² measured as in Figure 1D. (C) Numbers of sclerostin-positive OCys per 100 randomly counted OCys in three biological replicates from WT and MVNP mice, shown as mean ± SEM, analyzed by 1-way ANOVA with Tukey test.
Figure 3. Characterization of primary OCys from WT and MVNP mice. (A) Sost and Igf1 mRNAs: Primary OCys were isolated from long bones from 20-month-old WT and MVNP mice by sequential digestion with collagenase as described in Methods. Fractions 6-9 were used. RNA was extracted from $2 \times 10^6$ cells from 4 individual WT or MVNP mice and analyzed by TaqMan PCR. Data for Sost and Igf1 are the mean ± SEM (3 technical replicates from the mice) analyzed using the Mann-Whitney U-test in A and C. (B) Sclerostin and DMP1 protein: OCys were fixed, stained with anti-sclerostin or DMP1 antibody and fluorescent secondary antibody conjugates, and examined by immunofluorescence. Scale bar=10µm. (C) The pixel intensity of positive cells was measured using a laser confocal microscope. Results shown relative pixel intensity of cells in 30 random cells from 3 wells, mean ± SEM from one average value per sample and analyzed using a 1-way ANOVA with Tukey test. The experiment was performed three times using different biological replicates with similar results.
Figure 4. RANKL-positive OCys in 20-month-old WT, Igf1-cKO, MVNP and MVNP/Igf1-cKO mice. (A). RANKL in OCys in decalcified femoral sections was immunostained as described in Methods. Scale bars=50 μm. (B) The ratio of RANKL-positive cells/0.5 mm² square of area was determined in bones from 10 WT, 6 Igf1-cKO, 10 MVNP and 6 MVNP/Igf1-cKO mice. Blue dots are data from males and red from females, expressed as the mean ± SEM, analyzed using a 1-way ANOVA with Tukey test. (C) Serum RANKL was measured by mouse ELISA kit and expressed as mean ± SEM for 6 WT, 6 Igf1-cKO, 5 MVNP and 5 MVNP/Igf1-cKO mice. No statistical difference was found between results from male (blue) and female (red) samples. (D) RANKL media conditioned by OCy-like cells (1 × 10⁵ cells/ml) from 30-day bone outgrowth cultures grown for 72 hours was assayed by ELISA. Results shown are mean ± SEM analyzed by a 1-way ANOVA with Tukey test.
Figure 5. OCL formation by co-culture with OCy-like cells from MVNP and WT mice. (A). OCL-precursors from 16-month-old male WT and MVNP mice were co-cultured with OCy-like cells from 15-month-old male WT and MVNP mice for 72 hours and stained for TRACP. Results are mean ± SEM (n=4) Data for A-E analyzed using a 1-way ANOVA with Tukey test. (B) Nuclear numbers per OCL, expressed as the mean ± SEM of 30 randomly counted OCLs from each coculture. (C) OCL morphology from photomicrographs. Scale bars=100μm. Arrows point to large, polynucleated OCLs. (D) Media were collected at the end of the cultures shown in Figure 5A, and RANKL protein was measured by ELISA and shown as mean ± SEM (n=4). Biological replicates from female mice gave similar results (not shown). (E) The conditioned media in D were also assessed by IGF1 ELISA. Similar results were found in 2 biological replicates and with cells from female mice (not shown).
Role of IGF1 and receptor in OCL formation in cocultures from MVNP and WT mice. OCL precursors from 17-month-old mice and OCy-like cells from 15-month-old ones were cocultured for 72 hours as in Figure 5. (A) Numbers of TRACP-stained OCLs when OCy-like cells were transduced with IGF1R or control siRNAs prior to coculture. (B) RANKL protein in coculture conditioned media (CM) measured by ELISA. Results are with cells from male mice. Female mice gave similar results (not shown). (C) OCL formation in coculture treated with control rabbit IgG (20ng/ml), anti-IGF1 (10ng/ml) or anti-IGF1R (0.5 µg/ml). Cells were stained for TRACP. (D) RANKL protein in the CM from (C) measured by ELISA. (C) and (D) were repeated and performed with cells from female mice, with similar results (not shown).
Figure 7. Bone resorption by OCLs formed in cocultures with OCy-like cells and role of IGF1. Mature OCLs (from 16-month-old female WT and MVNP mice) and OCy-like cells (from 19-month-old female WT and MVNP mice) were cocultured on bone slices with rabbit IgG (20 ng/ml), or anti-IGF1R (0.5 µg/ml) for 72 hours in 96-well plates. (A). Bone slices were stained with hematoxylin after removal of cells. Scale bars=200μm. Photomicrograph images are representative of 2 independent experiments using 2 biological replicates. (B). Bone resorption was assayed, and independent replicate values in each experimental group of bone resorption were plotted on the graph. The results expressed as mean ± SEM, analyzed using a 1-way ANOVA with Tukey test. Each stained bone slice was divided into quadrants. Under the microscope, each quadrant was scored as + or – for pits in 500 squares using a 20x25 grid. Results were expressed as % of the 500 squares per slice scored positive for resorption. Similar results were seen for 2 independent biological replicates.
Figure 8. The detection of IGF1, bone structure and histomorphometry analysis in WT and T-Igf1 mice.

(A) Detection of IGF1. Femur sections were stained with anti-IGF1. Arrows point to OCLs. Scale bars: 10 μm. Results are representative of 3 biological replicates. (B) Representative μCT images of fifth lumbar vertebrae. Scale bars: 1.0 mm. (C) Bone volume and structural parameters of trabecular bone in vertebrae. Results are mean ± SEM for WT (5 male (blue), 6 female (red), 16 ± 3 months), MVNP with PDL (3 female (red), 19 ± 1 months), and T-Igf1 with PDL (1 male (blue), 5 female (red), 15 ± 1 months). The data were analyzed using a 1-way ANOVA with Tukey test. (D) OCL morphology. OCLs in vertebral sections were stained with TRACP. Scale bars: 50 μm. Staining results are representative of 3 biological replicates. (E) Bone morphometric analysis. Results are expressed as the mean ± SEM for WT (3 male (blue), 2 female (red), 16 ± 3 months), MVNP with PDL (1 male (blue), 2 female (red), 21 ± 2 months), and T-Igf1 with PDL (1 male (blue), 3 female (red), 15 ± 1 months). The data were analyzed using a 1-way ANOVA with Tukey test. (F) Sclerostin in OCys. Femur sections from these mice were stained with anti-sclerostin antibody as described in Methods. Scale bars: 10 μm. Staining results are representative of 3 biological replicates. (G) Ratio of sclerostin-positive OCys/total OCys per area and canalicular length (0.25 mm square). Results are expressed as the mean ± SEM from WT (each 3 male (blue) and 4 female (red), 15 ± 1 months) and T-Igf1 with PDL (1 male (blue), 5 female (red), 15 ± 1 months) from the same sections shown in (F) as described in Methods analyzed using a 1-way ANOVA with Tukey test.
Figure 9. Detection of phospho-Akt, RANKL and p16INK4A in WT, MVNP, and T-Igf1 mice. (A) Detection of phospho-Akt. Femur sections were stained with anti-pAkt. Scale bars: 10 µm. Staining results are representative of 3 biological replicates. (B) RANKL in OCys. Femur sections were stained with anti-RANKL antibody and TRACP as described in Methods. Scale bars: 50 µm. Staining results are representative of 3 biological replicates. (C) RANKL and p16INK4A in OCys. Sections stained with anti-p16INK4A and anti-RANKL. (D) The ratio of RANKL- and p16INK4A-positive cells/250 µm² at 500 µm below the growth plate was determined in bones of WT (4 male, 5 female, 16 ± 3 months), MVNP (3 female, 19 ± 1 months) and T-Igf1 (1 male, 5 female, 15 ± 1 months). Blue circles are data from males and red from females, expressed as the mean ± SEM, analyzed using a 1-way ANOVA with Tukey test.
Table 1. The number of mice and PDLs in TRAP-igf1 or WT mice

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PDLs were screened by μCT and IHC as described in Methods. Markedly abnormal structure was seen 6 of the 12 T-igf1 mice (50 %) at 16 months of age.