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A supra-cellular model for coupling of bone resorption to formation during remodeling: lessons from two bone resorption inhibitors affecting bone formation differently

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ABSTRACT

The bone matrix is maintained functional through the combined action of bone resorbing osteoclasts and bone forming osteoblasts, in so-called bone remodeling units. The coupling of these two activities is critical for securing bone replenishment and involves osteogenic factors released by the osteoclasts. However, the osteoclasts are separated from the mature bone forming osteoblasts in time and space. Therefore the target cell of these osteoclastic factors has remained unknown. Recent explorations of the physical microenvironment of osteoclasts revealed a cell layer lining the bone marrow and forming a canopy over the whole remodeling surface, spanning from the osteoclasts to the bone forming osteoblasts. Several observations show that these canopy cells are a source of osteoblast progenitors, and we hypothesized therefore that they are the likely cells targeted by the osteogenic factors of the osteoclasts. Here we provide evidence supporting this hypothesis, by comparing the osteoclast-canopy interface in response to two types of bone resorption inhibitors in rabbit lumbar vertebrae. The bisphosphonate alendronate, an inhibitor leading to low bone formation levels, reduces the extent of canopy coverage above osteoclasts. This effect is in accordance with its toxic action on periosteoclastic cells. In contrast, odanacatib, an inhibitor preserving bone formation, increases the extent of the osteoclast-canopy interface. Interestingly, these distinct effects correlate with how fast bone formation follows resorption during these respective treatments. Furthermore, canopy cells exhibit uPARAP/Endo180, a receptor able to bind the collagen made available by osteoclasts, and reported to mediate osteoblast recruitment. Overall these observations support a mechanism where the recruitment of bone forming osteoblasts from the canopy is induced by osteoclastic factors, thereby favoring initiation of bone formation. They lead to a model where the osteoclast-canopy interface is the physical site where coupling of bone resorption to bone formation occurs.

1. Introduction

Bone remodeling consists of bone resorption by osteoclasts followed by bone formation by osteoblasts. The mechanism ensuring the restoration of resorbed bone is gaining increasing attention because malfunction of this mechanism contributes to bone loss and fractures. An important concept is that osteoclasts are part of this mechanism, probably through the release of pro-osteoblastic factors [1–3]. The basis of this concept is that general inhibitors of osteoclasts, such as alendronate (ALN), a bisphosphonate, lead to decreased bone formation, whereas inhibition restricted to their resorptive activity sustains or even increases bone formation [2,4,5] while increasing the number of non-resorbing osteoclasts [6–9]. An example of the latter inhibitors is odanacatib (ODN), a selective inhibitor of cathepsin K, the main proteinase degrading collagen during bone resorption. This compound is presently in a phase III clinical trial for the treatment of osteoporosis.

Importantly, osteoclasts and bone forming osteoblasts are separated in time and space during the remodeling cycle, and it is still not understood how the osteoclast-derived factors make osteoblasts restitute locally the bone matrix [1]. We previously

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proposed that osteoclast osteogenic products should target cells in the immediate osteoclast surroundings, thereby promoting the reversal phase of the remodeling cycle, which is critical for recruitment of osteoprogenitors and initiation of bone formation [10,11]. In line with this hypothesis, we found that ODN induces a shorter reversal phase, higher osteoblast recruitment, and an increase in osteoclast surface in ovariectomized rabbits, whereas ALN did not show these responses [7]. Accordingly, a previous analysis of the same rabbits, showed that ODN had a positive effect on bone formations rate, whereas ALN decreased bone formation rates [5]. Amongst the cells which may be targeted by osteoclasts are reversal cells on the bone surface [7,10,11], canopy cells at the interface of the bone marrow and the bone remodeling site [12–14], and vasculature-associated cells at the canopy-marrow interface [12,15]. All belong to the osteoblast lineage and may serve as progenitors of bone forming osteoblasts [11]. Particular attention on the likely participation of canopy cells in this process is drawn by decreased bone formation in disease situations of canopy deficiency [12,14,16]. Here we hypothesized that canopy cells might be the unidentified partners of the osteoclasts, allowing them to exert their anabolic role. We tested this hypothesis by extending the analyses of our earlier study performed in ovariectomized rabbits, where the extent of osteoclasts surfaces, the reversal phase, and bone formation were promoted by ODN, but not by ALN [5,7]. The question we asked was whether the distinct effects of ODN and ALN we reported on these parameters would coincide with distinct effects of ODN and ALN on the osteoclast-canopy interface, thereby suggesting a causal relationship.

2. Materials and methods

2.1. Immunohistochemistry, histomorphometry and electron microscopy

The present study is a follow-up of our recent study reporting the effects of ALN and ODN on post-osteoclastic events in ovariectomized rabbits [7]. We used the same lumbar vertebrae from the four experimental groups: sham-operated, ovariectomized treated with vehicle, ALN, or ODN [7]. For immunohistochemical staining, paraffin sections (3.5 μm thick) from the second lumbar vertebrae were processed as described [7]. Immunostaining for the endocytic collagen receptor urokinase plasminogen activator receptor-associated protein (uPARAP/Endo 180), was performed using a monoclonal mouse antibody, 2h9F12, [17] which was detected with a polymeric alkaline phosphatase conjugated system (Bright Vision, Immunologic, Duiven, Holland) and visualized by liquid permanent red (DakoDenmark A/S, Glostrup, Denmark). Negative controls were performed by using an isotype-matched mouse control immunoglobulin (IgG1, MOPC-21, Ab18443, Abcam). Sections were counterstained with Mayer's haematoxylin and mounted.

Histomorphometric parameters were assessed in the trabecular bone of Masson-Goldner trichrome-stained sections (6 μm thick) prepared from the plastic-embedded fourth lumbar vertebrae as described by Jensen et al. [7]. The parameters included the proportion of bone surface covered by osteoclast (Oc.S), reversal (Rv.S), and osteoid surface (OS), where each parameter was determined in relation to the presence or absence of a canopy [12,14–16]. Reversal surfaces were defined as eroded surfaces without osteoclasts. Eroded surfaces were identified through visualization of broken lamellae in polarized light. Canopies were defined as a continuous layer of elongated cells lining the bone marrow and separated from the bone matrix by osteoclasts, reversal cells, or osteoblasts, and sometimes by a lumen [12,13] (Fig. 1A, yellow arrowheads). For every single hit on reversal perimeters, the presence of both an osteoclast and osteoid in the vicinity was recorded. Vicinity was defined as being within the same 2D remodeling unit as the reversal surface itself [7]. All measurements were done blinded with respect to sham, OVX, ALN, and ODN treatment.

Samples for electron microscopy were prepared and analyzed as previously described [15].

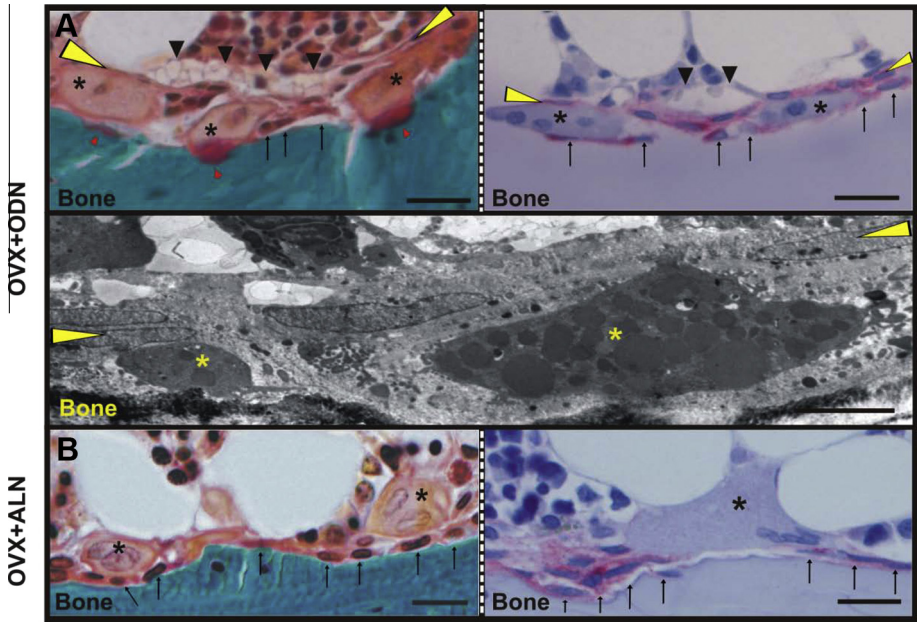

Fig. 1. Proximity of osteoclasts and canopy cells as it appears in histological sections. (A) Association between osteoclasts (*asterisk*) and canopy cells (*yellow arrowheads*) in the vertebral trabecular bone of ODN-treated OVX rabbits as it appears by using Masson-Goldner trichrome (*upper left*), uPARAP immunohistochemical staining (red) (*upper right*), and electron microscopy (*lower*). Note the close proximity of vascular structures (*black arrowheads*) and canopies (*upper panels*). (B) The association between osteoclasts (*asterisk*), and canopy cells is frequently lost in ALN-treated OVX rabbits as illustrated by Masson-Goldner trichrome (*left*) and uPARAP immunohistochemical staining (*right*). Arrows in (A) and (B) indicate reversal cells. Scale bar: 20 μm, except for *A, lower panel*: 2 μm.

2.2. Statistical analysis

All parameters for each group were tested for normality using the D'Argostino–Pearson omnibus test. Overall comparisons of the groups were performed using one-way analysis of variance (ANOVA) when data allowed for parametric statistics, whereas the Kruskal–Wallis test was applied when non-parametric statistics were needed. The post-test for individual comparison of groups was the Tukey–Kramer test for parametric analyses and Dunn's multiple comparison test for non-parametric analyses. The relationship between two parameters was analyzed by a two-sided Chi-square test. p values lower than 0.05 were considered statistically significant. The specific analysis used for the individual measurements are indicated in the respective figure legend. All statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Differences in proximity of osteoclasts to canopies in ODN- and ALN-treated rabbits

The analysis of bone remodeling sites in vertebrae of OVX rabbits treated with ODN revealed the typical features emphasized in our previous report [7]: osteoclasts frequently associated with demineralized collagen in the resorption zone, and in close proximity with the reversal cells (Fig. 1). The latter cells could be clearly visualized both in Masson staining and through immunoreactivity of uPARAP/Endo 180, the endocytic collagen receptor present in osteoblast lineage cells and involved in collagen uptake and bone formation [18,19]. In addition, the present analysis identified a layer of elongated cells between the osteoclasts and the bone marrow, which appeared identical with the so-called canopy, recently identified at remodeling sites of human cancellous bone [12–14,16] (Fig. 1A). These canopy cells were visible through Masson staining (Fig. 1A, upper left panel) and were clearly revealed through uPARAP/Endo 180 immunoreactivity (Fig. 1A, upper right panel), as well as by using electron microscopy (Fig. 1A, lower panel). Vascular spaces containing erythrocytes were also identified at the bone marrow side of these canopies, as typically reported in human cancellous bone [12,15]. The same analysis performed on remodeling sites of ALN-treated bones showed also osteoclasts and reversal cells as previously described [7], but canopy cells were most often not detected, whether through Masson or uPARAP staining (Fig. 1B). The qualitative histological analysis thus suggests a difference of effect of ODN and ALN on canopy coverage.

3.2. Quantifications of the osteoclast-canopy interface in ODN- and ALN-treated rabbits

Next, we systematically quantified the degree of coverage of osteoclasts by canopy cells in ODN and ALN-treated rabbits, compared to control conditions. Fig. 2A shows that treatment of OVX rabbits with ALN resulted in a significant reduction in the proportion of osteoclasts covered by canopy cells, whereas ODN did not do so. In contrast, the degree of coverage of reversal and osteoid surfaces by canopies was similar and high after ALN and ODN treatment. This indicates that the effect of ALN on reducing canopy coverage is osteoclast-specific. The specificity of this effect is in line with the observation of Coxon et al. [20] that osteoclasts release ALN from the bone matrix, and after transcytosis, allow its

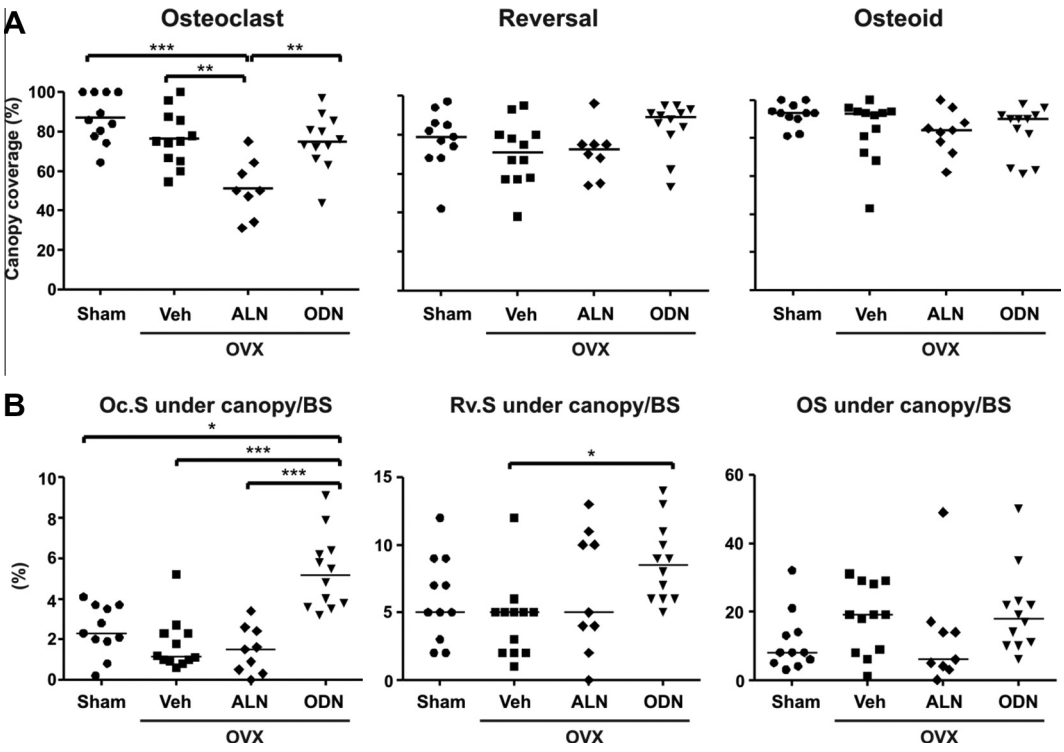

Fig. 2. Effect of ODN and ALN treatment on the canopy-bone surface interface. The trabecular bone surfaces (BS) were sorted according to the presence of osteoclasts (Oc.S, *left graphs*), reversal surfaces (Rv.S, *middle graphs*), and osteoid (OS, *right graphs*). (A) The proportion of each of these surfaces covered by a canopy was assessed in sham and OVX rabbits treated with vehicle (Veh), alendronate (ALN), or odanacatib (ODN). (B) The total length of the interface between canopies and each of these bone surfaces is shown for each group as percentage of total bone surface. In (A) and (B), each symbol represents the measurement obtained in one rabbit. The horizontal bars indicate the median except for *A*, *left*, where the mean values are indicated. Overall differences were analyzed by the Kruskal–Wallis test (*A*, *middle and right*: ns; *B*, *left*: $p < 0.0001$; *B*, *middle and right*: ns) or ANOVA (*A*, *left*: $p < 0.0001$). Post-test for pairwise comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

delivery specifically to cells positioned in the immediate vicinity of the osteoclast. In this respect, it is interesting to note that canopy cells are positioned along the basolateral secretory domain of osteoclasts [21–23], and appear thus particularly exposed to ALN release [20]. If the canopy is a source of osteoprogenitors, it makes sense that ALN-induced damage of the canopy cells would harm osteoblast recruitment, delay initiation of matrix deposition, and slow down bone formation as reported in the previous studies performed on the same rabbits [5,7]. This is consistent with the view that osteoclasts appear involved to some extent in the negative effect of bisphosphonates on bone formation [24]. The same scenario has been shown in a series of other situations of canopy deficiency, such as multiple myeloma, Cushing's syndrome, and post-menopausal osteoporosis [12,14,16].

Conversely, in situations where the canopy is preserved, the position of the canopy cells is ideal for exposure to the osteogenic products released through the basolateral secretory domain of the osteoclasts. This vesicular release was proposed to play a role in coupling bone resorption and formation [25]. One may therefore expect that the total length of the osteoclast-canopy interface may influence the efficiency of osteoblast recruitment during the reversal phase. Interestingly in this respect, Fig. 2B shows that ODN treatment resulted in a fourfold increase in the total length of osteoclast-canopy interfaces, whereas ALN had no effect on this parameter. Also when comparing the effect of ODN and ALN on the length of the canopy-bone surface interface at various phases of the remodeling cycle, we notice that it is above the osteoclasts that they show the biggest differences, and that this is the only place where these differences are statistically significant (Fig. 2B). Thus we found that ODN and ALN, which in the previous studies on the same rabbits were reported to affect differently osteoblast densities, bone formation, and bone density [5,7], also affect differently the interface between osteoclasts and canopies, but not the interaction of the canopy with other features, suggesting therefore a critical partnership between osteoclasts and canopies.

3.3. Relation between the extent of the osteoclast-canopy interface and the length of the reversal phase in ODN- and ALN-treated rabbits

Next we reasoned that the way the extent of osteoclast-canopy interface may affect bone formation would be reflected by the length of the reversal phase, which is connecting osteoclastic resorption with osteoblastic formation [7,10]. As an indicator of the length of the reversal phase, the proximity of reversal surfaces to both osteoclast and osteoid was recorded. Fig. 3A shows that reversal surfaces were twice as frequently in the vicinity of both osteoclasts and osteoid surfaces in ODN versus ALN treatment, thus indicating that compared to ALN, ODN treatment leads to a faster initiation of osteoid deposition after the departure of the osteoclast. Furthermore, plotting the proximity of reversal surfaces to both osteoclast and osteoid surfaces against the total length of osteoclast-canopy interface clearly shows that the shorter osteoclast-canopy interfaces measured in the ALN group correspond with a slower initiation of bone formation, whereas longer osteoclast-canopy interfaces of the ODN group correspond to a faster initiation of bone formation (Fig. 3B). The overall pattern of the plot suggests a positive correlation between length of osteoclast-canopy interface and rate of initiation of bone formation up to a plateau, which is reached upon treatment with ODN.

3.4. The osteoclast-canopy partnership coupling bone resorption to bone formation

There is compelling evidence for an involvement of osteoclasts in coupling of bone resorption to formation. This evidence includes observations on low bone formation under osteoclast-poor conditions [26,27], as well as preserved/increased bone formation in osteoclast-rich conditions, as seen with ODN treatment. Other examples of the latter are suppression of the function of c-Src or the chloride channel-7 (ClC-7), which all coincide with well performing bone formation [2,6,8,9]. Intensive search to identify the mechanism behind the coupling activity of the osteoclast has led to the identification of a number of anabolic factors released by the osteoclast, as reviewed by Henriksen et al. [1]. These factors include for example TGF-beta, IGF, S1P, TRAcP, as well as EphrinB2, and are considered to be the actual factors coupling bone resorption and formation. However, an important information which has been missing for understanding the mechanism behind these coupling signals is the identity of the target cell of these osteoclast products [1].

There is also another line of research showing evidence for an involvement of bone remodeling compartment canopies in coupling bone resorpion to formation. First, situations of canopy deficiency, such as multiple myeloma, Cushings syndrome, or post-menopausal osteoporosis show decreased osteoblast recruitment and bone formation [12,14,16], and second, proliferation and osteoblast differentiation markers support that the canopy is an important reservoir of osteoblast progenitors during remodel-

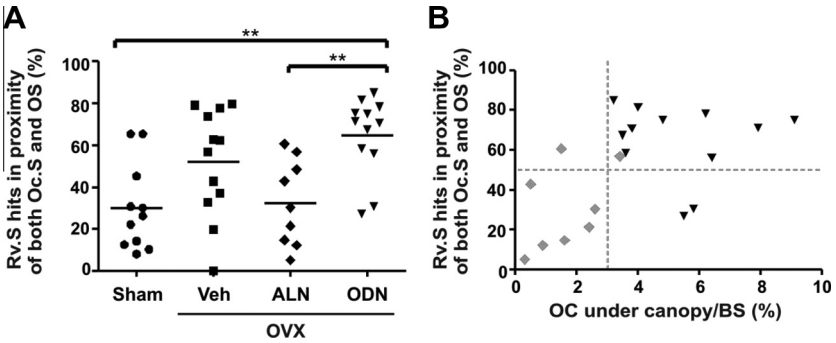

Fig. 3. Relation between the extent of osteoclast-canopy interface and the length of the reversal phase in ODN- and ALN-treated rabbits. (A) The proximity of reversal surfaces (Rv.S) to both osteoclasts (Oc.S) and osteoid surfaces (OS) was assessed in sham and OVX rabbits treated with vehicle (Veh), alendronate (ALN), or odanacatib (ODN). Data are expressed as the percentage of reversal surface hits with both Oc.S and OS in the vicinity as defined in the methods. Horizontal bars indicate mean values. Overall differences were analyzed by ANOVA: $p = 0.0011$. Post-test for pairwise comparisons: $^{**}p < 0.01$. (B) For each OVX rabbit treated with ALN (*grey diamonds*) or ODN (*black triangles*) the proximity of reversal surfaces to both osteoclasts and osteoid surfaces shown in (A) was plotted against the proportion of bone surface covered by osteoclasts under canopies shown in Fig. 2. Based on the distribution pattern of the measurements we categorized the rabbits according to the presence of a high or low extent of BS covered by OC under canopy (limit put at 3%) and a high or low extent of Rv.S in proximity of Oc.S and OS (limit put at 50%). The limits are indicated by the dotted lines. The relationship between the two variables was analyzed by a Chi-square test: $p = 0.0022$. In (A) and (B), each symbol represents the measurement done in one rabbit.

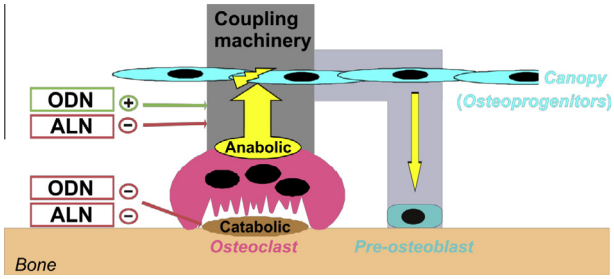

Fig. 4. Model proposing the osteoclast-canopy interface as the physical site coupling bone resorption to bone formation. According to this model, the osteoclast has two polarized functional domains: (i) the apical one (corresponding to the ruffled border) is catabolic and directed against the bone matrix to be solubilized; (ii) the basolateral one (corresponding to the secretory domain which releases the osteoclast products transported through transcytosis [22,23,25]) is anabolic and oriented towards the canopy cells described in [12]. The canopy cells are a reservoir of osteoprogenitors differentiating into mature bone forming osteoblasts during the bone remodeling cycle [11]. Note the strategic position of the canopy cells, which makes them the ideal target cells for the factors released by the osteoclast. The present data show that ODN increases the interface between osteoclasts and canopy cells, which is expected to favor the exposure of the canopy cells to the anabolic factors of the osteoclast. In contrast, ALN released by the osteoclast [20] poisons the canopy cells, and, as shown here, leads to a reduction in the extent of canopy coverage above osteoclasts – which is expected to reduce their exposure to the anabolic factors of the osteoclast. For further explanations, see text.

ing of human bone [11]. However, the external trigger acting on canopy cells to induce osteoblastogenesis has not been identified.

The interest of the present findings is to integrate the knowledge provided by these two lines of research: our findings indicate (1) that the osteoclasts may be the previously unrecognized triggers inducing osteoblastogenesis in the canopy cells, and (2) that the canopy cells might well be the previously unrecognized target for the osteoclastic osteogenic factors. In this way osteoclasts and canopy cells appear as partners in the coupling mechanism, and the osteoclast-canopy interface appears the central physical place where coupling occurs. This concept is illustrated in Fig. 4. Further studies are however necessary to investigate the presence of receptors for osteoclastic anabolic factors in the canopy cells. In this respect, it is of interest that we identified uPARAP/Endo 180 both in the canopy cells and in the cells recruited on the eroded bone surfaces. This collagen receptor was found to play a role in bone formation [18] and in cell migration [28], and to participate in the chemotactic response of osteoblast lineage cells to collagen [29]. Immunostainings performed on human bone also showed TRAcP in canopy cells (unpublished), supporting the hypothesis that they take up osteoclast products.

The present findings also help understanding the intriguing question why ODN and ALN, which inhibit equally well bone resorption, affect bone formation differently. Our earlier analysis suggested that this difference results from distinct effects of ODN and ALN on the reversal phase [7]. Here we propose that within the reversal phase, it is specifically the osteoclast-canopy interface that is differently affected by ALN and ODN, thereby affecting the osteoblast recruitment, which is critical for bone formation.

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Disclosures

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