MUSCULOSKELETAL PATHOLOGY

Osteoblast Recruitment Routes in Human Cancellous Bone Remodeling

Helene B. Kristensen,* Thomas L. Andersen,* Niels Marcussen,[†] Lars Rolighed,[‡] and Jean-Marie Delaisse*

From the Department of Clinical Cell Biology,* Lillebaelt/Vejle Hospital, Institute of Regional Health Science, University of Southern Denmark, Vejle; the Department of Clinical Pathology,[†] Odense University Hospital, Odense; and the Department of Surgery P,[‡] Aarhus University Hospital, Aarhus, Denmark

It is commonly proposed that bone forming osteoblasts recruited during bone remodeling originate from bone marrow perivascular cells, bone remodeling compartment canopy cells, or bone lining cells. However, an assessment of osteoblast recruitment during adult human cancellous bone remodeling is lacking. We addressed this question by quantifying cell densities, cell proliferation, osteoblast differentiation markers, and capillaries in human iliac crest biopsy specimens. We found that recruitment occurs on both reversal and bone-forming surfaces, as shown by the cell density and osterix levels on these respective surfaces, and that bone formation occurs only above a given cell density. Canopies appeared an important source of osteoprogenitors, because (i) canopy cells proved to be more pro-liferative and less differentiated than bone surface cells, as shown by the inverse levels of Ki-67 and procollagen-3 N-terminal peptide versus osterix, and (ii) canopy cell densities, found to decline with age, and canopy-capillary contacts above eroded surfaces correlated positively with osteoblast density on bone-forming surfaces. Furthermore, we showed that bone remodeling compartment canopies arise from a mesenchymal envelope surrounding the red bone marrow, which is lifted and hypertrophied on initiation of bone resorption. This study, together with earlier reports, led to a model in which canopies and nearby capillaries are critical for reaching the osteoblast density required for bone formation. *(Am J Pathol 2014, 184: 778—789; http://dx.doi.org/10.1016/j.ajpath.2013.11.022)*

Human bone is remodeled throughout life, a process that is critical to maintain healthy and functional bone. This remodeling process consists of bone resorption by osteo-clasts, followed by bone formation by osteoblasts (OBs).[1] It is widely accepted that bone formation involves recruitment of OBs to the resorption site, and lack of OB recruitment leads to bone diseases characterized by bone loss and increased fracture risk, such as osteoporosis.[1] Hence, un-derstanding the mechanisms that lead to the generation of OBs has become an increasingly important issue in bone research. However, most research is focused on OB differ-entiation for itself.[2] Examples are the identification of mo-lecular pathways that drive the differentiation process,[3,4] the study of effects of systemic endocrine factors or osteoclastic, monocytic, and endothelial cell products on this process,[5–9] and elegant investigations tracing OB recruitment in model systems such as bone development, fracture healing, and intermittent parathyroid hormone treatment.[10–13] However, it is still unknown when and from where OBs are recruited

during remodeling of adult human cancellous bone, and which physiological cell-cell interactions coordinate the recruitment process throughout the remodeling cycle. Thus, there is an unmet need to identify the origin of OB pro-genitors in the biological context in which diseases such as osteoporosis originate.

Bone marrow is well-known to be rich in osteoprogeni-tors and is, therefore, often regarded as a likely source.[14,15] These bone marrow osteoprogenitors are believed to be associated with the vasculature,[12,15–17] thereby fitting many diverse observations showing that vascularization is critical for osteogenesis.[5] However, OB progenitor sources posi-tioned closer to the actual bone remodeling sites probably also deserve attention. Cells lining quiescent bone surfaces have osteogenic potential.[11,18–20] These bone-lining cells

(BLCs) surround the resorbing osteoclasts and are, thus, ideally positioned to colonize the eroded surface where bone formation has to occur. These post-resorptive/preformative surfaces, called reversal surfaces,[21] are poorly investigated,[22-24] but the so-called reversal cells lining these surfaces[21] have been reported to show OB-lineage characteristics.[22,23] Another frequently proposed osteoprogenitor source close to bone-forming surfaces is the cell layer situated at the periphery of the bone marrow immediately next to the bone-forming OBs. The basis of this proposal is mainly morphological,[25-27] although cell proliferation was mentioned in this region.[25,28,29] Interestingly, this cell layer covering the mature bone-forming OBs appears as a part of the canopies recently reported to cover most bone remodeling sites.[30] These canopies express a range of osteoblastic factors, show abundant contacts with capillaries, and their absence coincides with lack of bone formation.[30-33] Thus, canopies may well represent a reserve of osteoprogenitors available for recruitment to both the neighboring bone-forming and reversal surfaces. Finally, it is worth nothing that these canopies are actually the cell layer of the outer surface of the bone marrow and, therefore, appear to coincide with the mesenchymal cell layer that, in a few studies, is claimed to envelop the entire bone marrow.[26,34-37] When isolated and cultured, these envelope cells showed increased expression of OB-lineage markers, including alkaline phosphatase, thereby showing that they represent a possible OB reserve proximal to the endosteal surfaces.[35] Overall, these observations suggest that the OBs required for bone remodeling may originate from the bone surface, the canopies covering bone remodeling sites, or more remote sites in the bone marrow.

In the present study, we evaluate the relative contribution of these three sources to the generation of OBs during remodeling of adult human cancellous bone, paying special attention to bone remodeling compartment (BRC) canopies. In addition, we determine at which step of the remodeling cycle OB progenitors are recruited on the bone surface. Therefore, we quantified the following: i) cell densities on the quiescent, eroded, and bone-forming surfaces, as well as in BRC canopies; ii) cell proliferation and OB differentiation markers in both the cells on the bone surfaces undergoing remodeling and canopy cells; and iii) the frequency of canopy-capillary contacts. Age effects were also considered. Furthermore, because the canopies are likely to originate from a pre-existing mesenchymal bone marrow envelope, which is poorly known, we document the presence of such an envelope at the level of quiescent bone surfaces.

Materials and Methods

Patients and Biopsy Specimens

For histochemistry and immunohistochemistry (IHC), we included 14 paraffin-embedded iliac crest biopsy specimens with no apparent pathological feature in either bone or bone marrow (Department of Medicine/Hematology, Lillebaelt Hospital, Vejle, Denmark; Danish National Committee on Biomedical Research Ethics; journal number S-20070121). None were used in our earlier studies. Eight biopsy specimens were from men, and six were from women. The mean age was 43.5 (range, 20 to 72) years. The biopsy specimens were fixated, decalcified, and dehydrated, as previously described.[33]

For electron microscopy (EM), we included EPON-embedded transiliac bone biopsy specimens from another two control patients (one was provided by INSERM 1033, Lyon University, Lyon, France, and the other was obtained from the Department of Endocrinology, Odense University Hospital, Odense, Denmark; Danish National Committee on Biomedical Research Ethics, journal number S-20110112) and from three patients with primary hyperparathyroidism (PHPT) (Department of Surgery P, Aarhus University Hospital, Aarhus, Denmark; Danish National Committee on Biomedical Research Ethics, journal number S-20070121).

Histochemistry and IHC Procedure

Sixteen adjacent sections (3.5 μm thick) were obtained from each of the 14 iliac crest biopsy specimens. They were processed for either staining with modified Masson's trichrome, as previously described,[31] or IHC. The IHC stained sections underwent deparaffinization, blocking of endogenous peroxidase activity, rehydration, epitope retrieval, and blocking of unspecific adhesion. Either single or double immunostainings were used for quantifications. For single immunostainings, the sections were incubated with either a rabbit polyclonal antibody against procollagen-3 N-terminal peptide (P3NP) (a gift from Prof. Juha Risteli, Department of Clinical Chemistry, University of Oulu, Oulu, Finland), osterix (Abcam, Cambridge, UK), or a monoclonal mouse IgG1 antibody against Ki-67 (clone MIB-1; Dako, Glostrup, Denmark). The P3NP and osterix antibodies were detected with alkaline-phosphatase (AP)−conjugated goat anti-rabbit IgG (Brightvision; Immunologic, Duiven, The Netherlands), and visualized with liquid permanent red (LPR) (Dako). Detection of Ki-67 was performed by using the same secondary antibody, as previously described,[33] and visualization was performed with 3,3′ diaminobenzidine (DAB$^+$) (Dako). After visualization with LPR or DAB$^+$, the P3NP-, osterix-, and Ki-67−stained sections were counterstained with Mayer's hematoxylin and mounted. For double immunostainings, the sections were first incubated with a monoclonal mouse IgG1 antibody against CD56 (clone 56C04; LabVision, Kalamazoo, MI), detected by using the same secondary antibody, as previously described,[33] followed by a first visualization with DAB$^+$. Then, the sections were blocked with mouse serum, and incubated with either the monoclonal mouse IgG1 anti-CD34 class II fluorescein isothiocyanate−labeled antibody (clone QBend10; Dako), detected with the AP-conjugated Fab anti-fluorescein isothiocyanate (Roche, Basel, Switzerland), or the monoclonal mouse IgG2b anti−tartrate-resistant acid

phosphatase (TRAcP; clone ZY-205; Zymed, San Francisco, CA), detected with the AP-conjugated goat anti-mouse IgG2b (Jackson ImmunoResearch, West Grove, PA). Finally, the sections underwent a second visualization with LPR, and they were counterstained with Mayer's hematoxylin and mounted.

A triple-immunostained section with P3NP/CD34/TRAcP, detected sequentially,[31] and a silver nitrate—stained section[38] were included for illustration only.

Negative controls were performed by omitting the primary antibody and by using irrelevant antibodies of the same species and class as for the positive antibodies. Positive controls for Ki-67, CD34, and CD56 were performed as previously described.[33] Positive controls for TRAcP, P3NP, and osterix were provided by the respective staining of the cytoplasm of multinucleated cells situated on the bone surface, the adventitia of large arteries, and the nuclei of mature OBs.

Electron Microscopy, Microscopy, and Image Analysis

These procedures were performed as previously described.[33]

Histomorphometry

Of the 16 sections obtained from each of the 14 biopsy specimens, 8 were stained as outlined in Table 1. The Masson's trichrome—stained sections 3 and 15 were used to sort the cancellous bone surfaces into quiescent, osteoclastic, reversal, and bone-forming osteoid surfaces, each defined according to the standard bone histomorphometry nomenclature.[21] The endocortical bone was excluded. Reversal surfaces were distinguished from quiescent surfaces through the visualization of broken lamellae in polarized light.[22] BLCs, osteoclasts, reversal cells, and OBs are the actual bone surface cells in direct contact with the bone matrix or osteoid. TRAcP- and CD56-stained sections 6 and 13 were used to help identify osteoclasts and BRC canopies, respectively. Canopies were defined as a continuous layer of elongated cells lining the bone marrow, separated from the bone matrix by osteoclasts, reversal cells, or OBs, and sometimes by a lumen containing erythocytes.[30,31] The distribution of canopies over the bone surfaces was systematically recorded. All this information was established in agreement between two observers (H.B.K. and T.L.A.) and collected in handwriting on printed images of sections 3 and 15, taken at ×2.5 magnification. These records were used as a map allowing us to associate specific events of the remodeling cycle with activities immunodetected on the adjacent sections. The quantifications of the histomorphometric parameters and of other features or activities were performed on the respective sections, as indicated in Table 1.

Three different methods were used for histomorphometric quantifications: i) bone surface distribution, capillary contacts, and P3NP were quantified with a cycloid grid[39] and extrapolated orthogonal lines at the intersection points[33]; ii) the density of nuclear profiles on the bone surface and in the canopy was quantified using the Osteomeasure system (Osteometrics, Decatur, GA); and iii) Ki-67— and osterix-positive nuclei were quantified by analyzing all nuclei on the bone surface or in the canopy.

In two biopsy specimens, a low number of osteoclastic hits rendered a reasonable estimate of capillary-canopy contacts too uncertain, leading to the exclusion of those biopsy specimens.

Statistical Analysis

The quiescent, osteoclast, reversal, and osteoid surfaces from each patient were considered as related events that are separated in space. A statistical test was chosen, as indicated in the figure legend, depending on i) whether data were divided into two groups or into three or more groups, and ii) whether data allowed for parametric or nonparametric statistics, according to either the D'Agostino and Pearson omnibus normality test or normality tests performed in Stata 11 (StataCorp LP, College Station, TX). The posttest for three or more groups was Bonferroni's multiple comparison test in the case of a parametric analysis, and Dunn's multiple comparison test in the case of a nonparametric analysis. All statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

Results

Distribution of Bone Surface Types, Canopies, and Their Contacts with Capillaries

The proportion of quiescent, osteoclastic, reversal, and osteoid surface relative to total bone surface showed typical values for normal individuals (Figure 1A). In accordance with

Table 1 Respective Staining and Analyses Performed on Adjacent Sections

	Section no.*							
	2	3	6	7	13	14	15	16
Staining	Ki-67	Masson's trichrome	CD56/TRAcP	CD56/CD34	CD56/TRAcP	P3NP	Masson's trichrome	Osterix
Purpose	Data on density and proliferation	Mapping and data on extent of bone surface and capillary-canopy contacts	Mapping	Data on extent of bone surface and capillary-canopy contacts	Mapping	Data on P3NP distribution	Mapping	Data on osterix distribution

*Lacking numbers in the series of 16 corresponds to sections that were withdrawn for technical reasons.

A Bone surface distribution   **B** Extent of canopy coverage   **C** Capillaries in contact with the bone surface/the canopy
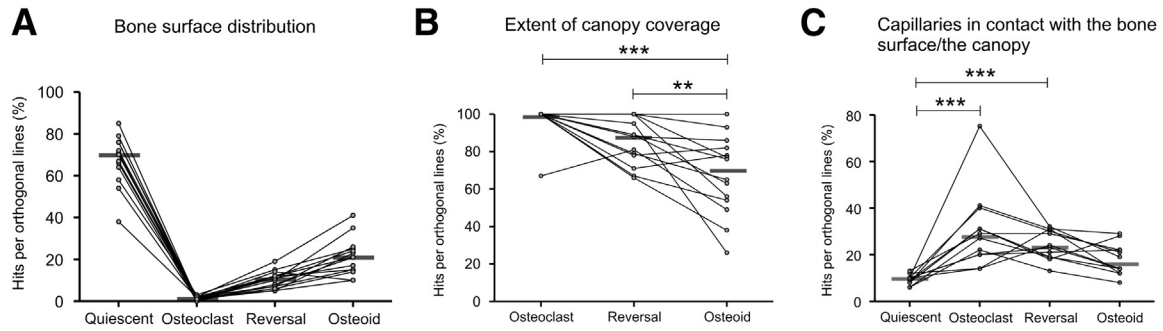
Figure 1 Relative distribution of the four different types of bone surface (**A**), extent of canopy coverage over bone surfaces undergoing remodeling (**B**), and presence of capillary contacts with quiescent bone surfaces or with canopies (**C**). Each dot represents the proportion of intersections between a cycloid grid and a bone surface (**A**), the percentage of orthogonal lines extrapolated from the cycloid grid and hitting a canopy (**B**), or a contact point between a capillary and either a quiescent bone surface or a canopy (**C**) (Table 1, serial sections 3 and 7). Measurements performed on the same biopsy specimen are connected with a line. The horizontal bars depict the median (**A** and **C**) or the mean (**B**). $N = 14$ (**A** and **B**), $N = 12$ (**C**). Overall comparisons were analyzed by using the Friedman test ($P < 0.0001$; **A** and **C**) or the repeated-measures analysis of variance ($P < 0.0001$; **B**). Posttest for individual comparisons: **$P < 0.01$, ***$P < 0.001$.

observations on other cohorts, almost all osteoclast surfaces were covered with a canopy, and the canopy presence steadily decreased over reversal and osteoid surfaces (Figure 1B).[30–33] We have previously demonstrated an increased presence of capillaries next to remodeling sites, and stressed the light microscopically assessed canopy-capillary contacts.[33] In the present cohort, we show, for the first time, that the percentage of canopy-capillary contacts peaks over osteoclast surfaces with approximately threefold more hits compared with quiescent surfaces (Figure 1C).

Cell Density and Proliferation on the Bone Surface and in the Canopy

It has been hypothesized that mature bone-forming OBs originate from OB-lineage cells already present on the bone surface (ie, BLCs and reversal cells).[18–21,40] A qualitative assessment of cancellous bone surfaces shows that cell densities increase during remodeling. To investigate at which step of the remodeling cycle this increase occurs, we quantified cell densities on the different types of bone surfaces. We found that the reversal cell density was 45% higher compared with the BLC density, and that OB density on osteoid was 26% higher compared with the reversal cell density (Figure 2A). Thus, the cell density increased the most during

the reversal phase, whereas it peaked on osteoid surfaces. Importantly, osteoid was only seen when cell densities were well above the densities exhibited by quiescent surfaces, which suggests that this increase is a prerequisite for matrix deposition (Figure 2A). Moreover, because the canopies may constitute a pool of OB progenitors close to the bone surfaces undergoing remodeling,[30–33,37,41] cell densities were recorded at the level of the canopy and were found to be in the range of those on reversal surfaces (Figure 2B).

Next, we investigated whether osteoblastic cells might be generated through proliferation on the bone surface and in the canopy. We detected the proliferation marker, Ki-67, in the canopy, on the reversal surfaces, and on osteoid surfaces (Figure 3A). Most often, the Ki-67–positive cells in the canopy and on the bone surface were positioned individually, whereas groups of Ki-67–positive cells were seen more rarely (Figure 3A). In the cells along the bone surfaces, the overall proliferation index was approximately 5%, showing that only a subset of cells proliferates. This subset might belong to a pool of more immature cells, because the mature bone-forming OBs are believed to be non-proliferative, whereas the more immature OB-lineage cells are known to be able to divide.[42,43] In accordance with this view, we could not find mature osteonectin-positive bone-forming OBs also positive for Ki-67 (data not shown). Interestingly, the
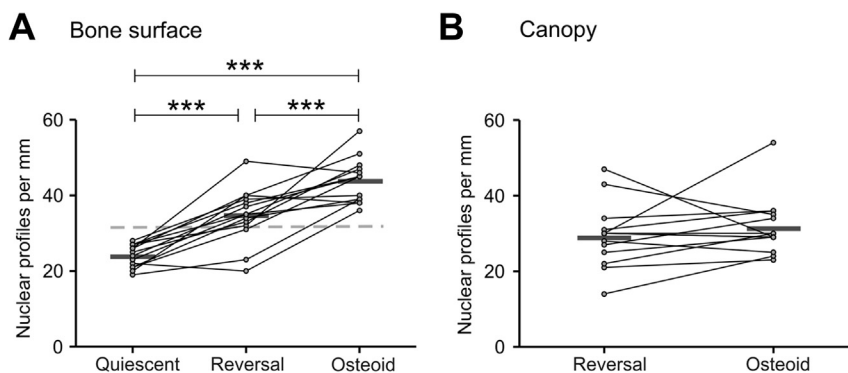
A Bone surface   **B** Canopy

Figure 2 Density of the cells in direct contact with the bone surface (**A**) and in the canopy (**B**) at successive stages of the remodeling cycle (Table 1, serial section 2). Each dot represents the number of nuclear profiles per millimeter bone surface at these respective locations. Measurements performed on the same biopsy specimen are connected with a line. The range of cell densities on quiescent and osteoid surfaces does not overlap (dashed gray line; **A**). The horizontal bars depict the mean. $N = 14$. Three-group comparisons were analyzed by using the repeated-measures analysis of variance ($P < 0.0001$; **A**). Posttest for individual comparisons: ***$P < 0.001$. Two-group comparisons (**B**) were made by using the paired t-test: $P = 0.3890$.
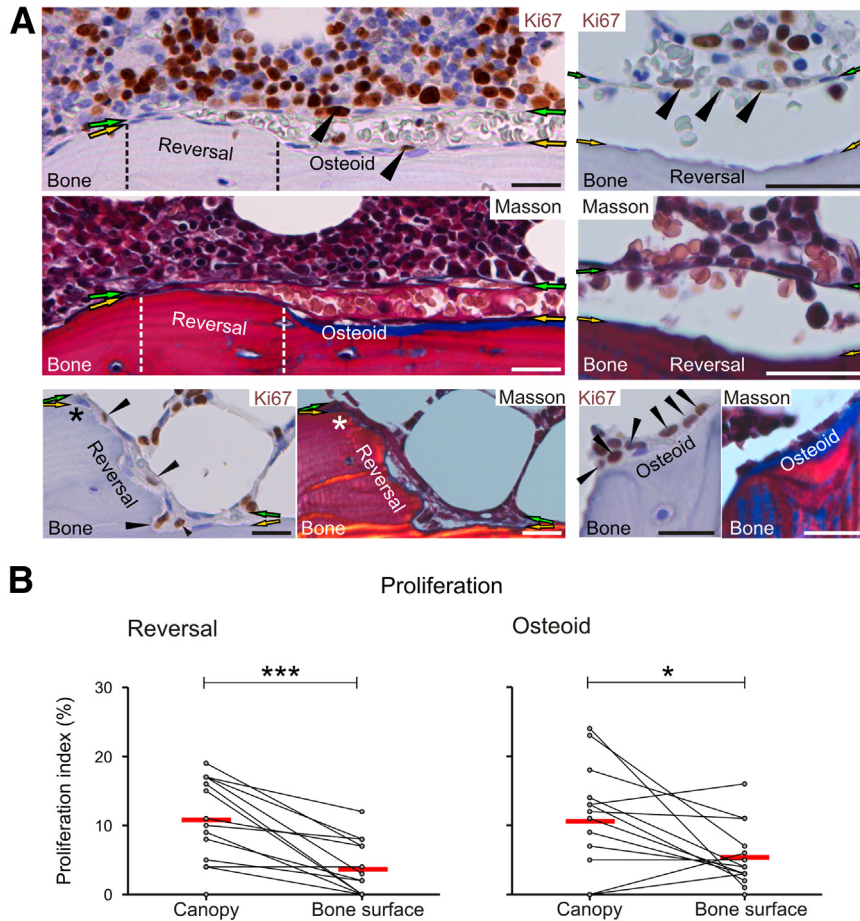
Figure 3 Proliferation of cells in direct contact with the bone surface and in the canopy at successive stages of the remodeling cycle. **A:** Histological appearance of the staining for Ki-67 (Table 1, serial section 2), as illustrated in four pairs of adjacent Ki-67— and Masson's trichrome—stained sections. **Arrowheads** indicate Ki-67—positive nuclei. When the canopy is present, **green arrows** indicate the canopy, and **yellow arrows** indicate the bone surface; Ki-67 labeling is visible in the canopy (**top panels**), on a reversal surface (**bottom left panels**), and on osteoid surfaces (blue in Masson's trichrome; **top left panels** and **bottom right panels**). An osteoclast is indicated (**asterisks**). Scale bar = 25 μm. **B:** Quantification of the Ki-67 immunoreactivity in the canopy and in the bone surface cells at reversal and formation sites. Each dot represents the percentage of Ki-67 —positive nuclear profiles at these respective locations. Measurements performed on the same biopsy specimen are connected with a line. The horizontal bars depict the mean. $N = 14$. Statistical analysis was performed using the paired t-test. $*P < 0.05$, $***P < 0.001$.

proliferation index in the canopy was twofold to threefold higher than on the bone surface (Figure 3B). This proliferation index did not change significantly in relation to the bone remodeling step, whether at the level of the canopy or along the bone surface (Figure 3B).

Distribution of Osterix-Positive Cells at Bone Remodeling Sites

Osteoblastogenesis requires not only cell proliferation, but also differentiation. Osterix is one of the main transcription factors required for OB differentiation.[3] We show that osterix nuclear immunoreactivity can be assessed according to a semi-quantitative scale, as illustrated (Figure 4A). On reversal surfaces, 40% of the cells were weakly positive for osterix, whereas 10% were strongly positive. In contrast, on osteoid surfaces, approximately 40% of the cells were strongly positive, whereas 20% were weakly positive (Figure 4B). This shows that OB differentiation is a process that starts on reversal surfaces, and appears to still occur on bone-forming surfaces. Furthermore, we found that 20% of the canopy cells were weakly positive for osterix and that nearly none were strongly positive (Figure 4C). On the contrary to osterix immunoreactivity on bone surfaces, the osterix immunoreactivity in the canopy was uniform and seemingly independent of which remodeling surface the canopy was covering (Figure 4C).

When comparing osterix immunoreactivity in the canopy and on the bone surface, we found, on average, two times fewer cells with osterix-positive nuclei in the canopies, whatever the remodeling step (Figure 4D). In conclusion, bone remodeling sites show OB differentiation along two different axes: one from the canopy to the bone surface and one along the bone surface, from the reversal bone surface to the bone-forming surface.

Distribution of P3NP Immunoreactivity at Bone Remodeling Sites

Collagen type 3 or its propeptide, P3NP, is not found in mineralized bone matrix, but well in vessel walls, bone marrow reticular fibers, mesenchymal stromal cells,[44] and OB-lineage cells on bone surfaces, where the levels are highest in the more immature cells.[45,46] Figure 5A illustrates P3NP immunoreactivity associated with reticular fibers in close connection to vascular walls and with canopies, whereas a lower level of immunoreactivity or no immunoreactivity was associated with bone surface OB-lineage cells and with the mineralized bone matrix. Comparative quantifications in the canopy and on the bone surface showed a twofold higher level in the canopies, independently of which bone remodeling surface the canopy covered (Figure 5B). In conclusion, P3NP levels decrease from the canopy to the bone surface, contrary to osterix.
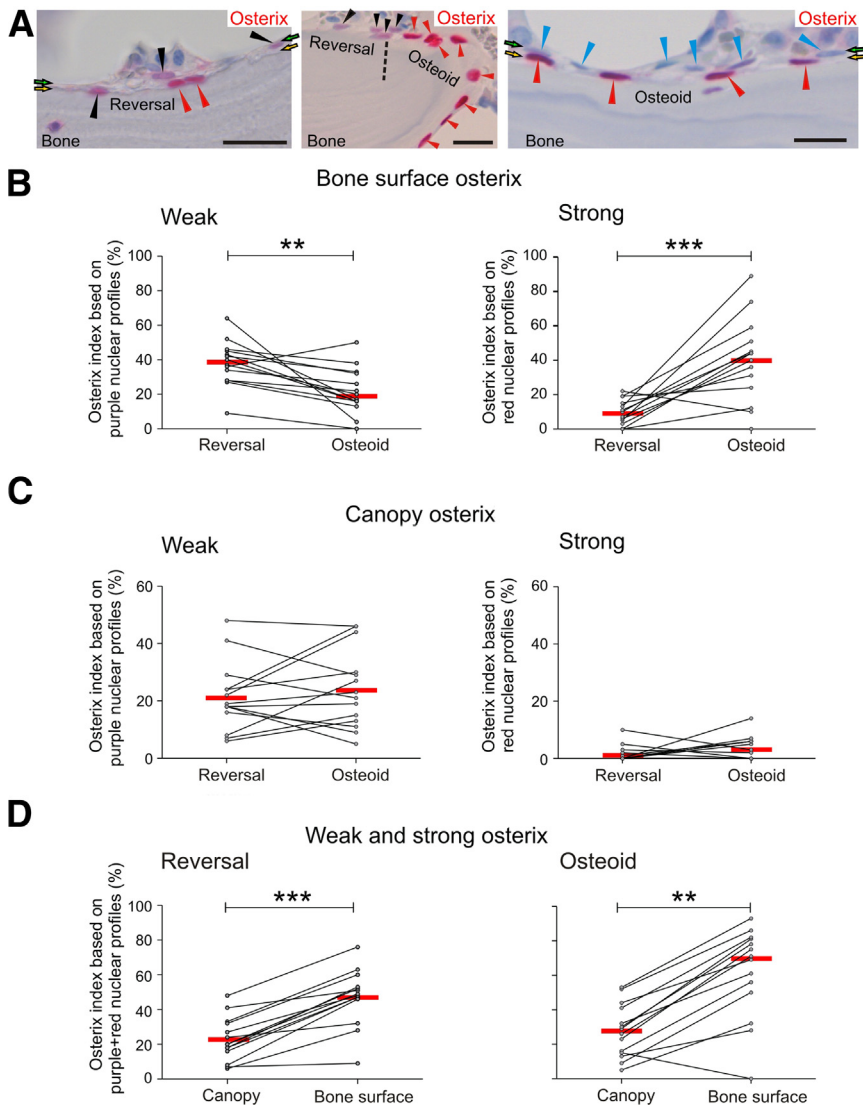
Figure 4 Osterix immunoreactivity in cells directly in contact with the bone surface and in the canopy at successive stages of the remodeling cycle. **A:** Histological appearance of the osterix immunoreactivity and illustration of the semiquantitative scale are used for its quantification (Table 1, serial section 16). When the canopy is present, **green arrows** indicate the canopy, and **yellow arrows** indicate the bone surface. Scale bar = 25 μm. Nuclei with high (**red arrowheads**), low (**black arrowheads**), or no (**blue arrowheads**) immunoreactivity are indicated, based on their respective red, purple, and blue color. **Left panel:** A low level of osterix in a canopy cell situated above two highly osterix-positive cells on the reversal surface. **Middle panel:** The level of osterix is low on the reversal surface and high on the osteoid surface. **Right panel:** Close apposition of the osteoid surface and the canopy. The flattened OBs are highly positive for osterix, whereas most of the canopy cells are negative. **B–D:** Quantifications of osterix immunoreactivity on the bone surface (**B**), in the canopy (**C**), and in both (**D**), at reversal and bone-formation sites. Each dot represents the percentage of purple nuclear profiles weakly expressing osterix (**B**, **left panel**, and **C**, **left panel**) at these respective positions, or red ones strongly expressing osterix (**B**, **right panel**, and **C**, **right panel**), or purple plus red ones representing the sum of those expressing osterix weakly and strongly (**D**). The horizontal bars depict the mean (**B**, **right panel**, and **C** and **D**, **left panel**) or median (**B**, **left panel**, and **D**, **right panel**). $N = 14$. Statistical analysis was performed using either the paired t-test (**B**, **right panel**, and **C** and **D**, **left panel**) or the Wilcoxon signed rank test (**B**, **left panel**, and **D**, **right panel**). ****$P < 0.01$, ***$P < 0.001$, $P = 0.3887$ (**C**, **left panel**), $P = 0.1950$ (**C**, **right panel**).

Evidence Compatible with Cell Translocation between the Canopy and the Bone Surface

OB recruitment from the canopy requires cell migration to the bone surface. This is in line with the presence of smooth muscle actin (SMA), a motility factor, in the canopy cells[33] and is here supported by the presence of osterix-positive cells in the space between the canopy and the bone surface, as well as by EM evidence for cell translocation from the canopy cell layer to the adjacent bone surface cell layer (Figure 6A).

Evidence Supporting the Existence of a Mesenchymal Bone Marrow Envelope over Quiescent Bone Surfaces

An important question is the origin of the OB-lineage cell canopy covering the bone remodeling sites. We hypothesized that this canopy may represent a local specialization of the mesenchymal cell layer claimed to envelop the bone marrow in several species, including human.[26,34–37] Therefore, we investigated whether such an envelope could be detected at the level of quiescent bone surfaces in the present bone biopsy specimens. Figure 6B shows light microscopy evidence supporting that the cells covering quiescent bone surfaces can be resolved into two separate layers: one along the bone surface and the other along the bone marrow. This double layer is seen whether the separation is achieved on initiation of bone resorption or artefactually (Figure 6B). EM further emphasizes the existence of an extra cell layer above the BLCs, and illustrates the morphological characteristics of the cells with flattened nuclei and very elongated overlapping cell extensions (Figure 6C), as reported around bone marrow plugs[34,36] and as is typical for bone marrow mesenchymal cells.[14,45] Both controls and patients with PHPT showed the existence of this bone marrow envelope on EM analysis of quiescent bone surfaces.
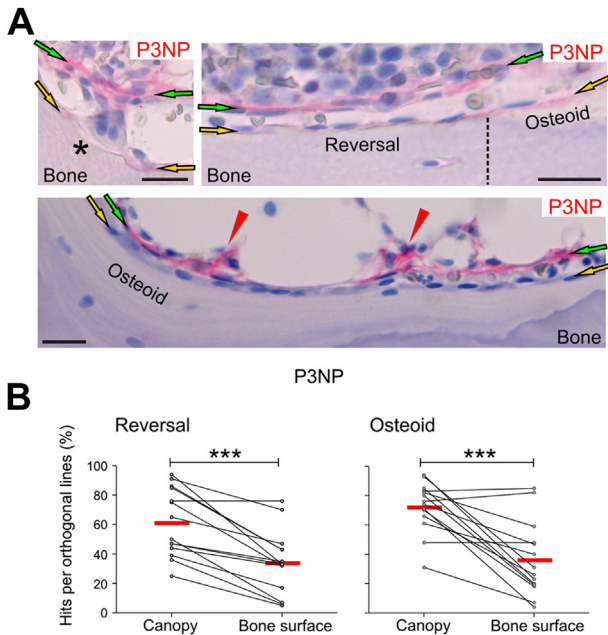
A

B

Figure 5 P3NP immunoreactivity on the bone surface and in the canopy at successive stages of the remodeling cycle. **A:** Histological appearance of the P3NP immunoreactivity (Table 1, serial section 14). When the canopy is present, **green arrows** indicate the canopy, and **yellow arrows** indicate the bone surface. The P3NP-stained meshwork of bone marrow collagen is indicated (**red arrowheads**). An osteoclast is indicated (**asterisk**). Scale bar = 25 μm. P3NP was occasionally present both in the canopy and on the eroded bone surface (**top panel**); however, most often, it was present only in the canopy (**bottom panel**). **B:** Quantifications of P3NP immunoreactivity at reversal and osteoid surfaces. Each dot represents the percentage of P3NP signals that were hit by the intersections between a cycloid grid and the bone surface (quantifications on the bone surface), or by the orthogonal lines extrapolated from these intersections (quantifications in the canopy). Measurements performed on the same biopsy specimen are connected with a line. The horizontal bars depict the mean. $N = 14$. Statistical analysis was performed using the paired t-test. ***$P < 0.001$.

Relation between OB Recruitment, Canopies, Capillary-Canopy Contacts, and Age

The hypothesis that canopies might be a reservoir of OB progenitors[30–33,37,41] is also supported by a series of correlations. First, we found a positive correlation between density of canopy cells and bone-forming OBs (Figure 7A). Second, the prevalence of capillary-canopy contacts tightly correlates with the density of bone-forming OBs (Figure 7A). This is of interest because it has been speculated that interaction between capillaries and canopies may promote OB recruitment.[32] Third, age is known to compromise OB recruitment,[1,42] and we show that age significantly affects the degree of canopy coverage over remodeling surfaces and canopy cell density above reversal surfaces (Figure 7B).

Discussion

Considerable knowledge on OB recruitment has recently been obtained in experimental models. However, the mechanism of OB recruitment during remodeling of adult human cancellous bone remains poorly known. The present study evaluated the generation of OBs during remodeling in this particular bone type, taking into account cell numbers and cell differentiation during the successive steps of the remodeling cycle. It leads to a model in which OB recruitment may proceed along three concurrent routes (Figure 8): route 1 originates from BLCs and proceeds along the bone surface; route 2 originates from the bone marrow envelope cells, which specialize into the canopies covering the bone remodeling site and supply OB progenitors to the reversal and bone-forming surfaces; moreover, our data are compatible with the existence of a route 3, which probably originates from perivascular cells, reaching canopies along capillaries.

Recruitment Route 1

Right before initiation of bone remodeling, bone surfaces are covered with OB-lineage cells, called BLCs. BLCs are considered as osteoblastic cells at a post–bone-forming differentiation stage.[40] However, there are indications that they may revert to bone-forming OBs on hormonal[11,19] or mechanical[18] stimulation. When bone remodeling is initiated, BLCs retract to allow osteoclasts to attach and resorb the bone matrix.[47,48] It makes sense to believe that once the osteoclasts have left, BLCs will re-occupy the space they had freed and will colonize the eroded surfaces, then called reversal surfaces, shown as route 1 in Figure 8.[23,49–51] Supporting this view, close to 100% of the cells on reversal surfaces, called reversal cells, are osteoblastic,[22] and here we show that half of them are positive for osterix, a factor that is mandatory for differentiation into mature bone-forming OBs.[3] A differentiation gradient along reversal surfaces is clearly seen, because osterix is more frequently present and at higher levels in reversal cells next to osteoid compared with those next to osteoclasts.[22] Furthermore, cell numbers are already significantly higher on reversal surfaces compared with quiescent surfaces. Importantly, our study is the first to show that osteoid is detected only above a critical level of cell density (Figure 2A), suggesting that this gain in cell number on reversal surfaces is a prerequisite for initiation of bone formation (ie, for coupling resorption to formation). This observation is in accordance with our recent report showing that the remodeling cycle aborts after osteoclastic resorption, if the bone surface cell density remains low.[22] One may speculate that some of the extra cells gained on reversal surfaces come from osteocytes released from their lacunae through osteoclastic resorption,[51] but this hypothesis remains to be investigated. One could also envision a gain through cell proliferation on the bone surface, but we found a proliferation index of only 4% to 5% on the bone surface. Thus, cell proliferation at the bone surface appears to only slightly contribute to this gain in cell density.

Recruitment Route 2

Herein, we propose that many of these newly generated cells may originate from the canopies covering bone remodeling
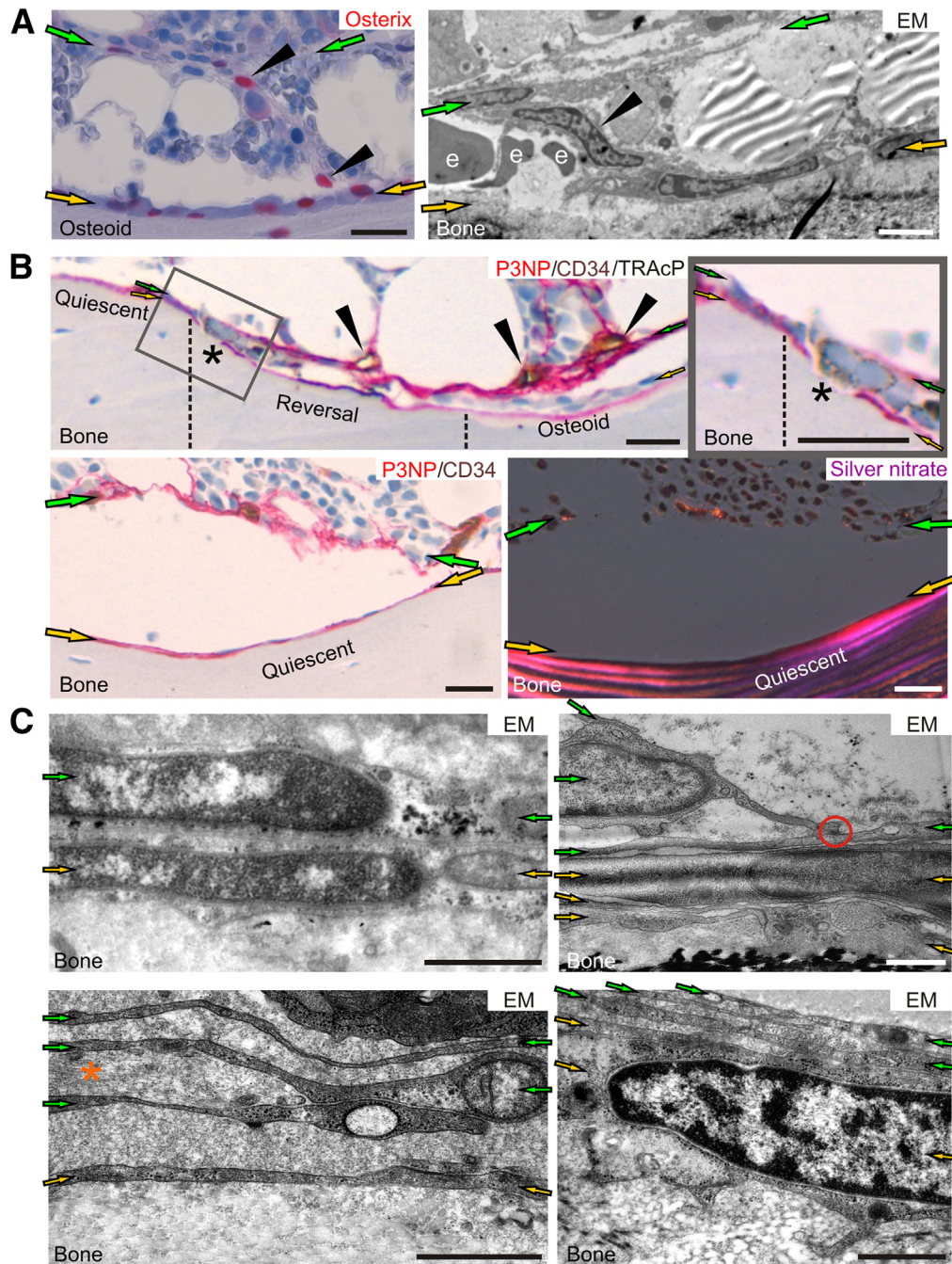
Figure 6 Illustrations of the mesenchymal bone marrow envelope and of possible cell translocations from the canopy to the bone surface (Table 1, serial sections 3 and 7). The **yellow arrows** indicate the bone surface, whereas the **green arrows** indicate the canopy (**A**) or the mesenchymal bone marrow envelope (**B** and **C**). Scale bars: 25 μm (**A**, **left panel**, and **B**); 2.5 μm (**A**, **right panel**); 1 μm (**C**). **A:** Images supporting cell translocation between the canopy and the bone surface. **A**, **left panel:** Two ovoid-shaped highly osterix-positive cells (**arrowheads**) are detected in the space between a canopy and an osteoid surface. e, erythrocytes. **A**, **right panel:** EM shows a cell (**arrowhead**) appearing to translocate from the canopy to the bone surface. **B:** Separation of the cells covering quiescent surfaces into two distinct layers, either by initiation of bone resorption (**top panel**) or artefactually (**bottom panels**). **B**, **top panel:** A triple-stained section for P3NP (red), CD34 (capillaries, brown; **arrowheads**), and a TRAcP (osteoclast, silver; **asterisk**) shows a sequence of three types of bone surfaces: quiescent, resorption initiated by an osteoclast (**asterisk**), and formation as detected by osteoid. The transition from quiescence to resorption is framed and shown at the right. Note that P3NP staining over quiescent surface splits into two distinct layers at the level of the osteoclast, one along the bone and the other along the bone marrow, thereby forming a canopy over the surface undergoing remodeling. Note the former is disrupted at the osteoclast-bone interface. **B**, **bottom panels:** Adjacent sections stained for P3NP/CD34 (**left panel**) and silver nitrate (polarized light; **right panel**), respectively. **B**, **bottom left:** Because of an artifact, the bone marrow happened to be separated from the quiescent bone surface. This artifact gives the opportunity to distinguish two cell layers both stained with P3NP: one along the bone surface and the other along the bone marrow. **B**, **bottom right:** The unbroken lamellae running parallel to the surface demonstrate that this surface is quiescent. **C:** EM analysis indicating the existence of a mesenchymal bone marrow envelope above quiescent surfaces. **C**, **top panels:** Two layers of elongated nuclei from a patient with PHPT (**left panel**) and a control (**right panel**). A cell junction is encircled. **C**, **bottom panels:** One or several layers of overlapping cellular extensions, both on the quiescent bone surface and in the mesenchymal bone marrow envelope of a control. Collagen is noted (**asterisk**).
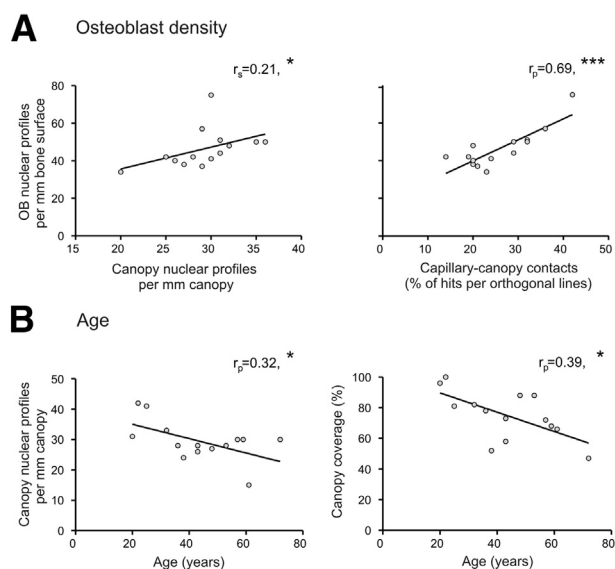
Figure 7 Correlation between canopy parameters and density of mature bone-forming OBs (**A**) or age (**B**). Each dot represents OB density plotted against canopy cell density (**A**, **left panel**) or the percentage of orthogonal lines hitting capillary-canopy contacts above the eroded surface (**A**, **right panel**), or age plotted either against cell density in canopies covering the eroded surface (**B**, **left panel**) or extent of canopy coverage (**B**, **right panel**). N = 14. Statistical analysis was performed with either the Spearman rank correlation test (**A**, **left panel**) or the Pearson rank correlation test (**A**, **right panel**, and **B**). The straight lines represent the best-fitted linear relationship between the parameters. *P < 0.05, ***P < 0.001.

sites, shown as route 2 in Figure 8. Canopies consist of OB-lineage cells,[30–33] and we demonstrate that canopy cells are two to three times more proliferative than bone surface cells at all remodeling steps. More important, this proliferation does not result in increasing canopy cell densities during the remodeling cycle, which indicates that the cells generated through proliferation are leaving the canopy. In accordance with this, we provide images compatible with cell translocation from the canopy to the bone surface. Furthermore, canopy cells are at an early OB differentiation stage, as previously suggested by their high expression of CD56 and SMA, and now also of P3NP, the propeptide of collagen type 3. Type 3 collagen has been reported in early OB-lineage cells in several situations, including cell culture,[46] bone development, and fracture healing,[45,52] and their fibroblast-like appearance is reminiscent of the morphological characteristics of the canopy cells. Moreover, a low level of osterix in canopy cells indicates that they are OB-lineage cells at an early differentiation stage. Interestingly, while canopy cells show high proliferation and P3NP, but low osterix, levels, bone surface cells show low proliferation and P3NP, but high osterix, levels at all remodeling steps. Thus, the bone surface appears the main site for OB differentiation, shown as route 1 in Figure 8, whereas the canopies appear to be more specialized in proliferation, shown as route 1 in Figure 8. Furthermore, it should be noted that, most often, the canopy covers the entire bone remodeling site,[30–33] and may deliver osteoprogenitors to

the reversal surface and to the osteoid surface, in contrast to previous reports that only propose the cell layer above the mature bone-forming OBs as OB progenitors.[26–29,34,53]

An effective role of canopies in OB recruitment was already supported by earlier observations in multiple myeloma and Cushing syndrome, in which canopy deficiency coincides with deficient bone formation.[31,32] Herein, we further show a direct association between canopy cell density and OB recruitment. Moreover, we document that aging coincides with a decline in canopy coverage and canopy cell density, which suggests the involvement of the canopy in the mechanism of age-induced bone loss.[1,54] Thus, the role of the canopy as an osteoprogenitor source is supported not only by expression of markers and cell numbers but also by decreased OB recruitment or bone formation in diverse situations of canopy deficiency.

Origin of the BRC Canopies: The Bone Marrow Envelope

Because the canopies appear to be a significant source of osteoprogenitors, an intriguing question is their origin. An attractive hypothesis is that canopies represent the phenotypic appearance taken by the mesenchymal bone marrow envelope above bone remodeling sites, shown as route 2 in Figure 8. So far, this envelope was mainly analyzed in marrow plugs. It consists of a cell layer claimed to surround the entire red bone marrow in different animal species, and is often called a marrow sac.[26,34–37,53] Our proposal that canopies coincide with this envelope is based on several observations. First, canopies consist of the outer surface cell layer of the bone marrow.[26,34–37] Second, cells isolated from this envelope differentiate into OBs,[35] just as canopy cells appear to do in the present study. Third, EM of marrow plugs shows that bone marrow envelope cells are <0.1 μm thick and have long cell extensions,[36] as described for the putative OB progenitors deeper in the bone marrow.[14,45] These envelope cells may well be the endosteal representatives of the bone marrow mesenchymal osteoprogenitors that originate from the invagination of cells of the primitive periosteum into the primitive marrow cavity during bone development.[45] Thus, the role of this endosteal envelope in bone remodeling may be similar to the one the periosteum plays in fracture healing.[10] Fourth, herein, we show the presence of a mesenchymal bone marrow envelope at the level of quiescent surfaces—a presence that we are the first to document. Fifth, we repeatedly observed that the bone marrow envelope dissociates from the BLCs at the periphery of the bone remodeling sites. It is, thus, likely that canopies result from envelopes that are lifted and become hypertrophied on initiation of bone resorption and, therefore, become visible at the light microscopic level. This lifting may arise from the increased osmotic pressure generated by the osteoclast resorption products. Moreover, we show that the place where the envelope develops into a canopy is systematically associated with osteoclasts and
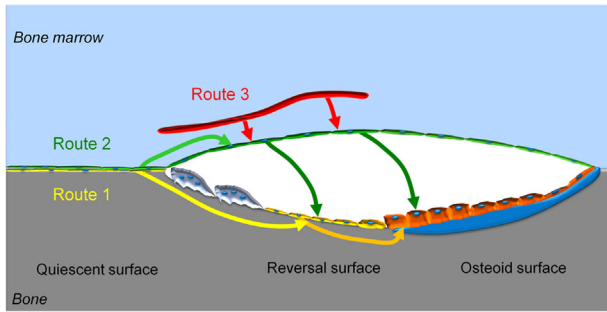
Figure 8 Model proposing the existence of three distinct routes for recruitment of mature bone-forming OBs during human cancellous bone remodeling. Route 1 originates from the BLCs situated at quiescent bone surfaces (yellow). When an osteoclast (blue) resorbs bone, surrounding BLCs are at a privileged position to colonize the resorbed surfaces after departure of the osteoclast, thereby contributing to reversal cells (pale orange). Reversal cells may then be triggered to differentiate into mature bone-forming OBs (dark orange). Route 2 originates from the mesenchymal bone marrow envelope (pale green), which lifts from the bone surface and differentiates into a canopy (dark green) at remodeling sites. Canopy cells appear then triggered for osteoblastogenesis and are dispatched to reversal and osteoid surfaces, thus joining route 1. Route 3 originates from osteoprogenitors associated with capillaries (red). The capillaries are in contact with canopies, thereby favoring delivery of osteoprogenitors to the bone-remodeling sites. Thus, route 3 joins route 2. Note the strategic position of osteoclasts and capillaries along these routes, allowing them to trigger OB recruitment. It is anticipated that the relative importance of these three routes may depend on the pathophysiological situation. See the Discussion for details.

coincides with the highest frequency of capillary-canopy contacts. Thus, on initiation of bone remodeling, the canopies are optimally exposed to the well-known stimulatory effects that osteoclasts[8] and endothelial cells[5] exert on osteoblastogenesis. Accordingly, our study shows a tight correlation between capillary-canopy contacts and OB density (Figure 7). Another observation in accordance with an effective function of the envelope in bone formation is that yellow bone marrow, where bone formation is known to be depressed,[55] does not show envelope cells,[56] probably as a result of their transformation into adipocytes.[45]

Balance of the Recruitment Routes and Route 3

This discussion naturally leads to an attempt to analyze the balance between the gain in osteoblastic cells on the bone surface during bone remodeling and the potential of the bone surface and the canopy to generate new cells (Table 2). Of the 44 cells per millimeter during the bone formation phase, 19 appear to represent new cells generated in equal amount during the reversal and the formation phase. Our evaluations show that proliferation, mainly in the canopy, has the potential to provide more than half of these new cells. However, our data provide only a snapshot of the proliferation status, and we do not know how the proliferation pattern develops over time. Therefore, we cannot evaluate the real contribution of this proliferation to the gain in cell density, and whether it meets the demands of new cell generation. It is of interest in this respect that a frequently proposed recruitment source of OB progenitors consists of vasculature-associated osteoprogenitors in the bone marrow, such as pericytes.[12,15,17] These may, thus, represent a third source of osteoprogenitors, shown as route 3 in Figure 8. This third source is supported by the increased presence of capillaries at bone remodeling sites, together with their association with proliferative cells, SMA-positive pericytes, and SMA-positive canopies,[33] as well as by the presence of SMA in reversal cells.[22,33] Furthermore, our results suggest that canopies mediate the delivery of perivascular cells to the bone surface, and indicate a close correlation between capillary-canopy contacts and the density of bone-forming OBs.

It is remarkable that, despite the great interbiopsy heterogeneity in the case of cell density, proliferation, and differentiation, all biopsy specimens show the same changes along the recruitment routes, thereby strengthening the likelihood of our model (Figure 8). Also, it should be noted that our quantifications, summarized in Table 2, may have underestimated the number of OBs that have to be generated, as follows: i) we may have overestimated BLC density, because we have possibly included bone marrow envelope cells that

Table 2 Overview of Cell Densities and Proliferation Indexes on the Bone Surface and in the Canopy at the Different Stages of the Remodeling Cycle

Variable	Quiescent	Reversal	Osteoid
Bone surface route 1			
Cell density (cells/mm)	25	35	44
Increase in cell number*		10 (35−25)	9 (44−35)
Proliferation index (%)		4.1	5.5
Generation potential[†]		1.4 (35 × 0.041)	2.4 (44 × 0.055)
Marrow surface (ie, canopy) route 2			
Cell density (cells/mm)		30	31
Proliferation index (%)		12	10.7
Generation potential[†]		3.6 (30 × 0.012)	3.3 (31 × 0.107)
Total generation potential through cell proliferation (cells/mm)[‡]		5 (1.4 + 3.6)	5.7 (2.4 + 3.3)

Data from Figures 2 and 3.

*Mean increase in cells/mm on reversal surfaces determined by subtracting number on quiescent surfaces, and on osteoid surfaces determined by subtracting number on reversal surfaces.

[†]Generation potential in cells/mm calculated using the proliferation index and cell density.

[‡]Total generation potential calculated by adding generation potential of routes 1 and 2.

are difficult to distinguish from BLCs through light microscopy; ii) possible apoptosis of bone surface cells[57] was not considered; iii) in two dimensions, a larger nucleus has a greater possibility of being sampled[58] and, consequently, compared with the density of OB nuclei, the density of the large BLC nuclei could have been overestimated, whereas the density of the small reversal cell nuclei could have been slightly underestimated; and iv) our proliferation index may overestimate cell division, because it is based on Ki-67 that stains cells that are ready to, but do not necessarily, divide.[59] Furthermore, more work is needed to interpret the position of osteoprogenitors between the canopy and the bone surface as a migration from the canopy toward the bone surface.

Conclusion

In conclusion, recruitment of OB progenitors during remodeling of adult human cancellous bone occurs both on reversal and bone-forming surfaces. OB progenitors may originate from the three concurrent sources shown in Figure 8. Our study especially highlights the involvement of the canopies covering the remodeling sites in this recruitment process. Canopies are a privileged site of cell proliferation and should also serve for transfer of perivascular cells to the bone surface,[33] which is the main site for final OB differentiation. This canopy-mediated supply of osteoprogenitors appears critical to reach the OB density required for bone formation — in line with earlier reports of a link between canopies and bone formation.[31,32] These canopies originate from the mesenchymal bone marrow envelope, which is lifted on initiation of bone resorption. The presence of osteoclasts and capillaries in the envelope-canopy transition area fits the current knowledge on the molecular mechanism of osteogenesis.

Acknowledgments

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