Coordination of fusion and trafficking of pre-osteoclasts at the marrow-bone interface

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Abstract

Fusion is the final osteoclast differentiation step leading to bone resorption. In healthy trabecular bone, osteoclast fusion is restricted to bone surfaces undergoing resorption, and necessarily requires site-specific recruitment of mononucleated pre-osteoclasts originating from the bone marrow. However, the spatiotemporal mechanism coordinating recruitment and fusion is poorly investigated. Herein we identify a collagen/vascular network as a likely structure supporting this mechanism. We therefore used multiplex immunohistochemistry and electron microscopy on human iliac crest bone samples, in combination with functional assays performed in vitro with osteoclasts generated from healthy blood donors. First, we found that putative pre-osteoclasts are in close vicinity of a network of collagen fibers associated with vessels and bone remodeling compartment canopies. Based on 3D-reconstructions of serial sections, we propose that this network may serve as roads leading pre-osteoclasts to resorption sites, as reported for cell migration in other tissues. Importantly, almost all these bone marrow pre-osteoclasts, but only some osteoclasts, express the collagen receptor OSCAR, which is reported to induce fusion-competence. Furthermore, differentiating osteoclasts cultured on collagen compared to mineral show higher fusion rates, higher expression of fusogenic cytokines, and a CD47 plasma membrane distribution pattern reported to be typical of a pre-fusion state—thus collectively supporting collagen-induced fusion competence. Finally, these in vitro assays show that collagen induces high cell mobility. The present data lead to a model where collagen fibers/vasculature support the coordination between traffic and fusion of pre-osteoclasts, by serving as a physical road and inducing fusion competence as well as cell mobility.
Introduction

Osteoclasts (OCs) are multinucleated cells that have the unique ability to resorb bone[1]. One of the mechanisms ensuring that this destructive activity is kept under control results from the highly regulated generation of OCs and/or nucleus renewal/supplementation[1–4]. OCs are generated on the bone surface by fusion of mononuclear tartrate-resistant acid phosphatase positive (TRAcP+) pre-osteoclasts (preOCs) that differentiate from hematopoietic myeloid lineage cells in the bone marrow[5]. Obviously, selective transfer of preOCs to bone surfaces about to be resorbed is a prerequisite for fusion as well as bone resorption. However, there is only little knowledge about this mechanism. It has been proposed that opposed effects of chemokines, sphingosine-1-phosphate (S1P) and stromal derived factor-1 (SDF-1) respectively, can drive the mobility of “monocytes with OC progenitor potential” from the bone marrow to the circulation and vice-versa[6]. This view explains how preOCs can access resorption sites deep inside the cortical bone matrix, but not how relevant this mechanism is in trabecular bone. It clarifies how preOCs can move in and out the blood vessels, but does not tell whether they are delivered close to or far from the resorption sites. The mechanism relating site-specific fusion and preOC traffic remains thus to be understood.

When trying to answer the above questions, it is of interest to take into account intrinsic properties of the OC fusion mechanism. Recent in vitro studies showed that fusion partnership is not random, but is highly selective, depending for example on whether cells are donors or acceptors of nuclei, or whether they have so-called founder or follower characteristics[7–11]. Furthermore, time-lapse observations showed that two thirds of the OCs gain nuclei by addition of one nucleus at a time, and that this nucleus is most often delivered by a moving mononucleated cell to an immobile multinucleated cell[7]. This finding is especially intriguing in the context of the in vivo situation where mononucleated TRAcP+ preOCs originating from the bone marrow deliver their nucleus to an OC on the bone surface[4, 12].
Still, direct in vivo observations of the spatial framework supporting this unique fusion scenario are lacking[13]. What renders preOCs mobile? What renders them fusion-competent? What are the roads guiding them through the complex landscape of the bone marrow and specifically towards the bone surface undergoing remodeling? Physical structures defining roads for cell migration are known from other tissues. For example, cancer cells follow axons and blood vessels in the brain, and collagen fibers in the breast[14]. T-cells are guided by a reticular/collagen network in the lymph nodes[15, 16], and also by collagen fibers in the dermis[17]. In the bone marrow, a similar network of reticulin and collagen type I and III has been reported [18, 19]. Furthermore, the marrow cavity contains a vascular network, stretching from the inner marrow cavity to the bone marrow envelope next to the bone surface[20, 21]. The contacts of vessels with the bone marrow envelope are especially abundant over the remodeling sites where this envelope is lifted thereby forming a canopy covering the whole remodeling site[21, 22]. Thus, one may speculate that a network of vessels and collagen/reticulin fibers supports the migration of bone marrow cells in a similar manner as observed in the above examples.

These observations prompted us to identify more precisely the in vivo-relevant features that might support traffic of preOCs and site-specific fusion into multinucleated OCs. We therefore used a combined in vivo and in vitro approach. First, we performed multiplex immunohistochemistry to assess the proximity of preOCs, OCs, collagen fibers, vasculature, bone surface, and bone remodeling compartment canopies in human iliac crest biopsy specimens. In parallel, we addressed the functional relevance of this proximity by generating preOCs from human blood-derived monocytes and comparing the impact of collagen and mineral on their mobility, fusion, and cytokine expression.

**Materials and methods**

*Patients and biopsy specimens*

The histological analysis was conducted on decalcified paraffin-embedded iliac bone biopsy specimens (3 mm diameter) from 10 controls (6 men and 4 women, age 69±12 years). These control biopsy specimens
were selected from patients under investigation for a hematological malignancy, as previous described\cite{21}. The selected patients had neither any apparent pathology detected in their bone biopsy specimen according to the prior pathological examination, nor been subjected to any treatment known to affect their bone. The transmission electron microscopic (TEM) assessments were performed on EPON-embedded fragments of trans-iliac bone biopsy specimens from five patients with primary hyperparathyroidism (PHPT) undergoing surgical treatment for PHPT. The reason for choosing biopsy specimens from PHPT patients to conduct the TEM analysis is their increased activation frequency of bone remodeling, thus facilitating the detection of remodeling sites at the TEM level.

*Immunohistochemistry and histochemical procedures*

In order to evaluate the co-localization and proximity of the different cells and structures, we combined the immunostainings of OC and preOC markers, type-5 TRAcP and OSCAR, with each other and with an endothelial cell marker (CD34), and collagen type I or III. Immunoreactivity for OSCAR was detected with goat anti-OSCAR antibodies [sc-34230 (n-terminal domain) and sc-34233 (c-terminal domain), Santa Cruz, Dallas, TX], which were labeled with rabbit anti-goat antibodies (Jackson ImmunoResearch, West Grove, PA) and then with a polymer of alkaline phosphatase conjugated goat anti-rabbit IgGs (PowerVision, Immunovision, Springdale, AZ). Immunoreactivity for TRAcP was detected with mouse IgG2b anti-TRAcP antibodies (clone ZY-9C5, Invitrogen, Life Technology, Nærum, Denmark), which were labeled with biotin-conjugated IgG2b subtype-specific secondary goat antibodies (Jackson ImmunoResearch), and then with gold-conjugated anti-biotin tertiary goat antibodies (Aurion, Wageningen, The Netherlands). The gold particles were silver-enhanced (12 mM silver acetate and 45 mM hydroquinone in citrate buffer pH 3.8) until visible at the light microscopic level. Immunoreactivity for CD34 was detected with FITC-conjugated mouse IgG1 anti-CD34 antibodies (clone QBEnd10, DAKO, Glostrup, Denmark), which were labeled with horseradish peroxidase conjugated sheep anti-FITC Fab fragments (Roche, Basel, Switzerland)). Immunoreactivity for collagen type I was detected with rabbit anti-collagen type I antibodies (ab34710 and
ab292, Abcam, Cambridge, UK) that were labeled with a polymer of alkaline phosphatase or horseradish peroxidase conjugated goat anti-rabbit IgGs (PowerVision, ImmunoVision), while Immunoreactivity for collagen type III was detected with mouse IgG1 anti-collagen type III antibodies (clone FH-7a, Abcam) that were labeled with alkaline phosphatase conjugated IgG1 subtype-specific secondary goat antibodies (Jackson ImmunoResearch). The horseradish peroxidase and alkaline phosphatase were visualized with DAB+ (DAKO) and Liquid Permanent Red (LPR, DAKO), and counterstained with Mayer’s Haematoxylin. The reticular fibers were stained using the Gordon and Sweet’s silver impregnation procedure[23].

**Immunofluorescence labelling**

The immunofluorescence detection with mouse IgG2b anti-TRAcP antibodies (clone ZY-9C5, Invitrogen) were labeled with Alexa-488-labelled goat anti-mouse-IgG2b antibodies (Life Technology, Nærum, Denmark), and combined with the immunofluorescence detection with mouse IgG2a anti-Cathepsin K antibodies (ab66237, Abcam) and rabbit anti-collagen type I antibodies (ab34710, Abcam), or mouse IgG1 anti-TRAcP 5a antibodies (clone 220, gift from Anthony J. Janckila, University of Louisville, KY, USA), which were labeled with Cy3-labelled goat anti-mouse-IgG2a antibodies (Jackson ImmunoResearch) and Alexa-568-labeled goat anti-rabbit (Molecular probes/Thermofischer Scientific), or Alexa-594-labelled goat anti-mouse-IgG1 antibodies (Jackson ImmunoResearch). The sections were mounted with Prolong Gold+ DAPI (Molecular probes/Thermofisher Scientific) and sealed with nail polish.

**In situ hybridization**

In order to investigate the mRNA expression collagen type I and III in canopy cells, sections were in situ hybridized using a modified version of the RNAscope 2.5 high-definition procedure (310035, Advanced Cell Diagnstics [ACD]). Following pretreatment, the sections were hybridized with probes for collagen type I (Probe-Hs-Col1A1, 401891, ACD) or Collagen type III (probe-Hs-Col3A1, 549431, ACD) and amplified according to the instructions provided by the manufacturer. In addition, the signal was amplified using
digoxigenin-labeled tyramide signal amplification (NEL748001KT, PerkinElmer, Skovlunde, Denmark), followed by a detection with alkaline phosphatase-conjugated sheep anti-digoxigenin FAB fragments (11093274910, Roche, Basel, Switzerland) and visualization with liquid permanent red (DAKO). Finally, the sections were counter stained with Mayer’s hematoxylin.

Microscopic analysis and micrographs

The estimated co-localization between osteoclastic markers of putative preOCs and OCs, and the estimated proximity between putative preOC and the collagen and vascular network was performed on double- and triple-immunostained sections from controls. Random bone marrow cells were selected in systematic random regions of interest using a point-grid. The identification of the vasculature was not only based on their CD34 immunoreactivity, but also their morphological appearance, as CD34 is also present in the morphological distinguishable hematopoietic stem and progenitor cells. The microscopic analysis was conducted on an upright DM2500 microscope (Leica, Wetzlar, Germany) and micrographs were obtained with a DP71 digital camera (Olympus, Center Valley, PA, USA). The illustrations and graphs were assembled using CorelDraw x5 (Corel corporation, Ottawa, Canada).

3D-reconstruction

The 3D-reconstructions were based on micrographs obtained from 40 consecutive TRAcP immunostained sections and from 30 consecutive TRAcP/CD34/Collagen type 1 immunostained sections from paraffin-embedded iliac crest biopsy specimens from controls. In each Z-stack of micrographs, the bone, vasculature, collagen, OCs and preOCs were marked and converted into 3D-reconstructions using the Amira software v4.0 (Mercury Computer Systems, Merignac, France), as previously described[20].

Electron microscopy

This procedure was performed as previously described[21].
Cell culture

CD14+ monocytes were isolated from blood from healthy human blood donors. Blood from a total of 34 blood donors was used for all the cell culture experiments. Cells were isolated as described elsewhere[24] and seeded into culture flasks and incubated for two days at 37°C and 5% CO₂ in a humidified atmosphere in αMEM (ThermoFisher Scientific, Nærum, Denmark) with 10% FCS (Biological Industries, Kibbutz Beit-Haemek, Israel), 1% Penicillin-Streptomycin (Sigma-Aldrich, Copenhagen, Denmark) and 25 ng/ml M-CSF (R&D Systems/Bio-Techne, Abingdon, UK). Subsequently, media was refreshed and 25 ng/ml M-CSF and 25 ng/ml RANKL was added and cells were incubated for another three to four days. After this period the cells had reached an early maturation and fusion stage (verified by microscopy prior to initiation of experiments). Cells at this stage were used for all the experiments described below.

Generation of different substrates

Bone slices (IDS Nordic, Herlev, Denmark and Boneslices.com, Jelling, Denmark) were rendered inorganic as described previously[25] by incubation with 5-7% hypochlorite for 30 min followed by extensive washing. This treatment resulted in the substrate hereafter referred to as “mineral”. Removal of the inorganic matrix was done by incubating individual bone slices with 0.4 M sterile EDTA pH 8.0 (ThermoFisher Scientific), while gently shaking at room temperature for seven days with renewal of the solution five times. At the end of incubation the discs were fully flexible. Discs were washed four times 15 min in αMEM and were stored at 4°C for up to 3 days before use. This treatment resulted in the substrate hereafter referred to as “collagen”.

OC fusion on different substrates

Cells were detached by the use of accutase (Fisher Scientific, Roskilde, Denmark) and 25 000, 50 000, or 75 000 cells were seeded on the appropriate surface discs (mineral, collagen or bone) in αMEM, 10% FCS and 25 ng/ml M-CSF and RANKL with four to five discs per condition. Cells were incubated for 48h, fixed for 30
min with 3.7% formaldehyde and washed with PBS. Cells were permeabilized with PBS, 0.5% BSA and 0.05% saponin for 30 min. Subsequently, they were stained with phalloidin AF568 (Molecular Probes/ThermoFisher Scientific) for 20 min, washed in PBS, individually mounted on glass slides with ProLong Gold+DAPI (Molecular Probes/ThermoFisher Scientific) and were sealed with a coverslip and clear nail polish. When cells were immunofluorescently labelled for CD47 this was done according to the previously published procedure[8] using primary antibody mouse anti-human CD47 (clone B6H12; BD, Franklin Lakes, NJ, USA) and Alexa Fluor 568 conjugated goat anti-mouse (Invitrogen, Carlsbad, CA, USA) in combination with phalloidin AF488 (Molecular Probes/ThermoFisher Scientific). Around 20 systematic random images per disc were made using an Axio Imager Z1 microscope (Carl Zeiss, Oberkochen, Germany) with a 20x objective and the Isis software version 5.3.1 (Metasystems, Altlussheim, Germany). These digital images were used for manually quantifying the number of OCs (2 nuclei or more) per image, the number of nuclei for each OC (with 2 nuclei or more), the number of mononucleated cells in the image and the morphological appearance of each cell suggestive of active migration. Migrating cells were identified by the presence of lamellopodia, pseudopodia, and/or podosomes in polarized cells with a clear leading edge[26] and based on our experience from our published studies on imaging moving OCs by time-lapse[7, 11, 27]. See figure legends for further details. Of note, we would have liked to verify the differences in mobility by performing time-lapse recordings as we have done previously, but the strong auto-fluorescence and the very light and rubber-like nature of the EDTA-treated bone slices rendered this technically impossible.

**Gene expression level on different substrates**

Cells were detached by the use of accutase (Fisher Scientific, Roskilde, Denmark) and 120 000 to 130 000 cells were seeded on the appropriate surface discs (mineral, collagen or bone) in αMEM, 10% FCS and 25 ng/ml MCSF and RANKL with 3x 10 discs per condition. After 1.5h the cells on the discs were lyzed in three separate tubes per condition using Trizol lysis buffer containing 143 mM 2-mercaptoethanol (ThermoFisher Scientific). RNA purification (Trizol, ThermoFisher Scientific), cDNA synthesis (iScript, BioRad, Copenhagen,
Denmark) and TaqMan Q-RT-PCR (ThermoFisher Scientific) were performed and normalized to two reference genes, Abl and GUS, as previously described[25]. Primer/probes used: GUS, Hs99999908_m1; Abl, Hs00245443_m1; IL1, Hs01555410_m1; TNFα, Hs00174128_m1; IL8, Hs00174103_m1 (ThermoFisher Scientific).

**Statistical analysis**

Statistical analyses: Fig. 1c: Paired student’s t-test; Fig. 4c: one-way ANOVA followed by a Bonferroni’s multiple comparison test. The Gaussian distribution of the differences was assessed using D’Agostino & Pearson Omnibus normality test; Fig. 5a: Wilcoxon matched-pairs signed rank test; Fig. 5b: paired student’s t-test. The Gaussian distribution of the differences was assessed using D’Agostino & Pearson Omnibus normality test; Fig. 6a,b,d: Chi-square with Yates’ correction; Fig. 6a, b, c: Kruskal-Wallis test. p<0.05 was defined as statistically significant. All statistical analyses and graphical illustrations were performed using GraphPad Prism v6 (GraphPad software Inc., La Jolla, CA, USA).

**Study approval**

All methods were carried out in accordance with relevant guidelines and regulations. The use of human bone biopsy specimens was approved by The Scientific Ethical Committee for the Region of Southern Denmark with approval number S-20070121. The use of human blood donors was approved by The Scientific Ethical Committee for the Region of Southern Denmark with approval number S-20070019. All subjects were 18 years or older and informed written consent was obtained from the PHPT patients and blood donors. Formal written consent was not required for the retrospective study of bone biopsy specimens from controls, which had been under investigation for a hematological disorder.

**Data availability**
The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

**Results**

_The identification and distribution of preOCs in the marrow cavity_

OCs (multinucleated) and putative preOCs (mononucleated) were identified in serial sections of human iliac crest bone biopsy specimens by using TRAcP immunostaining, as previously reported[20]. 3D reconstructions of these stained serial sections highlight that the TRAcP+ OCs were exclusively present on the bone surface, while the putative TRAcP+ preOCs (TRAcP+ mononucleated cells herein termed T’mncs) were distributed throughout the bone marrow cavity (Fig. 1A and online resource 1). Of note, some of these putative preOCs in the marrow cavity were closely positioned in small clusters of two to five cells without any evidence for cell fusion (Fig. 1a and movie in online resource 1). This observation supports the notion that the condition for preOC fusion into multinucleated OCs is exclusively present at the bone surface under physiological conditions[12]. To further characterize the putative TRAcP+ preOCs in the marrow cavity, their immunoreactivity for other OC markers was tested. Interestingly, immunoreactivity for the collagen receptor, osteoclast-associated receptor (OSCAR), was detected in 94% of the T’mncs but only in 30% of the OCs (Fig. 1b, c). Similar results were obtained whether using an antibody directed against the C-terminus (Fig. 1b, c) or the N-terminus of OSCAR (online resource 2). In contrast, immunofluorescence labelling of the osteoclastic marker, cathepsin K, was only detected in some of the T’mncs, but as expected, in all the mature OCs on the bone surface (Fig. 1d). It is worth noting that the TRAcP antibody (clone ZY-9CS) used for the immunostaining shown in Fig. 1a-c, recognizes two isoforms of TRAcP: TRAcP5a, which is common in inflammatory macrophages, and TRAcP5b, which is a truncated from of TRAcP5a and is a hallmark of OCs[28]. We had thus to verify whether the TRAcP+ staining generated by this antibody indeed identifies putative preOCs and OCs. We therefore used a second antibody (clone 220) specific for TRAcP5a and performed a co-staining. This co-staining showed that the immunoreactivity of intact TRAcP5a
was only observed in the perinuclear region of both OCs and putative preOCs (Fig. 1e). In contrast, the antibody recognizing TRAcP5a+b had a broader cytoplasmic distribution. Thus, the anti-TRAcP (clone ZY-9C5) antibody routinely used in the present study truly identifies osteoclastic cells.

**Collagen, reticulin and vasculature form a connective mesh in the bone marrow cavity**

Immunostainings of collagen type I and III reveal that the bone marrow cavity contains a dense collagen network, also enwrapping the bone marrow vasculature, adipocytes and other cells (Fig. 2a-d). Part of this collagen network appears to be composed of reticular fibers (Fig 2e, f) in line with previous studies reporting that reticular fibers are composed of collagen type I and III fibrils embedded in a matrix of glycoproteins and glycosaminoglycans[29]. Close to the bone surface, collagen type I and III immunoreactivity and reticulin staining label the canopies covering remodeling sites (Fig. 2c, d, f, Fig. 1d). Canopy cells appear to contribute themselves to collagen production since they show type I and III collagen mRNA – although at a weak level (online resource 3). At the electron microscopy level, bundles of collagen fibers were observed between the canopy cells (Fig. 3). Important, the 3D reconstruction of Fig 4d allows to appreciate the continuity of the collagen/vascular network from the bone marrow to the canopies covering the remodeling sites. Collectively, these observations show that a network of collagen and reticular fibers forms a very dense mesh including vasculature and BRC canopies. This mesh provides a frame for cell-positioning and migration, and deserves attention when trying to understand how the bone surface and the bone marrow are functionally connected.

**Putative preOCs are in close contact with the vascular-collagen network in the marrow cavity**

In accordance with the density of the collagen network, the TRAcP⁺ or OSCAR⁺ mononucleated cells were most often observed adjacent to collagen in the bone marrow in double-immunostained histological sections (Fig. 4a, b) and in a 3D-reconstruction (Fig. 4d). In order to get a quantitative evaluation, we analyzed the frequency of different degrees of proximity to collagen type I using sections double-immunostained for type I collagen and either TRAcP or OSCAR (Fig. 4c). This quantification revealed that
93% of these TRAcP$^+$ or OSCAR$^+$ mononucleated cells (a total of 667 cells) were no more than one cell layer away from collagen type I in the marrow cavity. A similar analysis on 1152 random bone marrow cells revealed that 73% of these cells were no more than one cell layer away from collagen type I in the bone marrow (Fig. 4c). These quantifications stress that although close proximity to type I collagen is a widespread characteristic of bone marrow cells, it is even more so for putative preOCs (Fig. 4c). Furthermore, collagen fibers in close proximity of T$^+$mncs were often fibers enwrapping vasculature, as shown both in triple-immunostained 2D-sections (Fig. 4e) and in a 3D-reconstruction (Fig. 4d). In fact, nearly half of T$^+$mncs were no more than one cell layer away from collagen type I enwrapping vasculature (Fig. 4f).

**Enhanced fusion of preOCs on collagen compared to mineral surfaces**

Our *in situ* observations show that the mononucleated preOCs are in very close contact with collagen fibers while the multinucleated OCs, to which the preOCs deliver their nuclei[7], are on bone. We hypothesized that collagen compared to bone may favor preOCs to come in a fusion-competent state. To test this hypothesis, we compared the specific effects of collagen and mineral on OC fusion. We generated mononucleated TRAcP$^+$ cells from CD14$^+$ monocytes, reseeded them on either mineral (hypochlorite-treated bone slices) or collagen (EDTA treated bone slices) surfaces, and cultured them for 48h. Systematic immunofluorescent images of the resulting cells labelled with phalloidin and DAPI were counted for the number of cells and their respective number of nuclei. The analysis of 10 independent experiments showed that OCs (≥2 nuclei) formed on collagen had significantly more nuclei than those formed on mineral surfaces, despite the variation in nuclearity amongst the experiments (Fig. 5a). Culturing the cells on bone led to an intermediate situation (online resource 4). Furthermore, the frequency of OCs was significantly higher on collagen compared to mineral in the same 10 experiments (Fig. 5b).

**Enhanced fusion of OCs on collagen surfaces compared to mineral is favored by enhanced migration**
What may be the reason for the enhanced fusion of OCs when seeded on collagen compared to mineral? We know that migration is critical to allow fusion partners to meet as previously demonstrated by time-lapse[7]. We thus compared fusion rates on collagen and mineral in response to echistatin, an inhibitor of beta-integrins, and known to inhibit migration of preOCs and OCs[30, 31]. In Fig. 6a it can be seen that the fusion rate of OCs on both mineral and collagen drops to the same level (approximately 5%) in the presence of echistatin, but from different starting points (10% and 20%, respectively). This suggests that approximately 3-fold more of the fusion events on collagen involved migration compared to on mineral. In agreement with this observation, systematic quantifications of the cellular morphology show that approximately 10% of all cells seeded on mineral were found to have a phenotype coherent with migration in contrast to about 30% when seeded on collagen (Fig. 6b). Thus, high fusion rates on collagen may relate with the ability of collagen to favor migration. Of note, we would have liked to verify the differences in mobility by performing time-lapse recordings as we have done previously [7, 11], but the strong auto-fluorescence and the physical nature of the EDTA-treated bone slices rendered this technically impossible.

*The distribution of CD47 on the cell membrane of preOCs and OCs is much more frequently in clusters on collagen compared with mineral surfaces.*

We and others have shown that CD47 is involved in OC fusion[8, 11, 32] and that its localization is often clustered on the cell membrane of putative fusion partners[8]. In this respect, it is interesting that the distribution of CD47 on the cell membranes of preOCs and OCs is very different depending on whether cultures were on mineral or on collagen (Fig. 6c). On mineral only 7% of the cells were found to have CD47 located in distinct clusters on the cell membrane whereas it was 77% on collagen (Fig. 6d). These results may suggest an enhanced fusion potential of preOCs and OCs in contact with collagen compared to mineral.

*The expression of autocrine fusogenic cytokines in preOCs and OCs is elevated on collagen compared to mineral surfaces.*
It is well known that the expression and release of autocrine factors can boost osteoclastogenesis and fusion. In this regard, interleukin 1 β (IL1β), tumor necrosis factor α (TNFα), and IL8[33–36] are mentioned as factors that can facilitate osteoclastogenesis in an autocrine fashion. Q-RT-PCR revealed that the expression of all three genes is strongly induced upon contact with mineral and collagen as compared to plastic (Fig. 7a, b, c). But it is also clear that the expression level of these genes is higher upon contact with collagen as compared to mineral. More specifically IL1β was on average induced 3.4-fold (Fig. 7a), TNFα 8-fold (Fig. 7b) and IL8 5-fold (Fig. 7c) more on collagen than on mineral. Again, these data suggest an enhanced fusion potential on collagen compared to mineral.

**Discussion**

The originality of the present study is to address OC fusion in the context of bone tissue. One of the peculiarities of OC fusion in healthy trabecular bone, is its site-specificity[12] - which obviously demands a strict coordination between traffic of mononucleated preOCs to the marrow-bone interface and their fusion. We herein propose a model where such coordination is supported by a collagen and vascular network (i) that serves as a road leading the preOCs from the marrow cavity to resorption sites, (ii) that promotes a migratory phenotype, and (iii) that favors maturation and programming into a fusion competent state (Fig. 8). Interestingly, this model converges with recent in vitro observations showing that the intrinsic fusion propensity of osteoclasts depends on their nuclearity, mobility, and fusion competence[7–11], and with recent in vivo observations showing that their maintenance depends in the iterative fusion with mononucleated progenitors [4].

To begin with, the present 3D distribution of TRAcP immunostaining highlights unambiguously that preOCs are generated in the marrow of human bone. This conclusion is made strong by the combined use of two TRAcP antibodies: (i) an antibody recognizing both the 5a-isoform of inflammatory macrophages and the 5b-isoform specific of OCs, and (ii) an antibody recognizing only the 5a-isoform. Furthermore, 94% of the
mononucleated TRAcP+ cells of the bone marrow also show OC lineage factors such as OSCAR [37] and some show cathepsin K[38]. Next, this 3D analysis provides direct evidence for the concept that fusion occurs at the marrow-bone interface[12], since multinucleated OCs are not seen in the bone marrow, and conversely, TRAcP+ preOCs do not accumulate on the bone surface. The latter observation strongly suggests that fusion occurs as soon as preOCs are dispatched at the bone surface. How can this be?

First, the present immunostainings draw the attention on a dense network of collagen fibers in the bone marrow. This collagen network forms a 3D-continuum with the vasculature (shown to be enwrapped by collagen) and the canopy cell layer (shown to be penetrated by collagen) covering the remodeling sites. Important, the number of connections between vessels and the canopy at resorption sites is about three times higher than that between vessels and quiescent surfaces[21, 22]. Thus, this collagen network establishes a privileged physical connection between the sites of the marrow where the mononucleated preOCs are “differentiating” - i.e. nearly always next to collagen and most often next to vasculature - and the bone resorption sites where they are transferred to engender multi-nucleation. To the best of our knowledge, it is the first time that such a connection is visualized. This visualization was made possible because of the 3D images of Fig. 4d, since such a connection is a 3D structure and cannot be seen in a 2D section. This physical connection deserves attention with respect to cell trafficking, since a series of physiological and pathophysiological situations have been reported where cells use vessel-collagen scaffolds as a road guiding them to their operational sites[14, 16, 17, 39] (see also Introduction). Of note, the present quantifications of T+mncs in high magnification pictures show the proximity of these cells to the vascular collagen network (i.e. their presence on the putative roads), and 3D-analyses of resorption sites stress the striking position of vessels just next to OCs[20]. Overall, these observations support a site-specific delivery of preOCs, rather than a random delivery at bone surfaces, and help explaining preferential fusion at the “gathering site” of the preOCs. Bringing preOCs very close to their destination also helps explaining why fusion occurs as soon as preOCs are dispatched at the bone surface.
Second, in addition to serve as an access road, collagen appears to promote cell mobility – which is critical for fusion as clearly shown by using time-lapse[7]. Collagen is well-known to induce a mesenchymal/migratory phenotype in mature OCs[40, 41]. Herein, it seems that also the contact of preOCs with collagen compared to mineral favors a mobile phenotype, as based both on inhibition of migration by echistatin and morphological evaluation. Furthermore, the time-lapse experiments have shown that most mobile OCs are mononucleated, that cells with more nuclei tend to be less mobile and less prone to fuse with each other, and that the highest fusion rates are between mobile and immobile cells[7]. These data taken together, point to a model where collagen-induced mobility contributes to make the mononucleated preOCs nuclei-donors, whereas the less mobile multinucleated OCs on the bone surface would act as nuclei acceptors.

Third, in addition to being a road and inducing mobility, collagen provides also fusion competence, as supported by several of our observations. (i) Fusion rates are increased on collagen compared to mineral. (ii) Our quantifications of OSCAR immunoreactivity in TRAcP+ cells show that almost the whole population of mononucleated preOCs associated with the collagen network is positive, but only 30% of the multinucleated OCs on the bone surface. This is of interest because OSCAR is a collagen receptor that contributes to signaling through immunoreceptor tyrosine-based activation motifs (ITAMs)[37]. ITAMs are known to program macrophages into a fusion competent state[42], and also appear critical for regulation of OC multinucleation and migration[37, 43]. (iii) Our observations show that CD47 forms clusters on the plasma membrane of 77% of the cells in contact with collagen, but in only 7% of the cells in contact with mineral. This is of interest because the cluster configuration was reported to be typical of mononucleated cells that are about to fuse[8], and because CD47 was found to play a rate limiting role specifically in the fusion of mononucleated preOCs[11]. The present CD47 clusters thus indicate that collagen strongly induces a “ready-to-fuse” state. (iv) Our results show that the expression of autocrine factors such as IL1β,
TNFα and IL8 is upregulated in preOCs when in contact with collagen compared to mineral. Functional assays have demonstrated that these factors promote differentiation of multinucleated OCs [33–36, 44].

The present study thus leads to propose a model where the coordination between preOC fusion and preOC trafficking involves collagen fibers that serve as physical roads, induce movement along these roads, and secure that the cells have gained fusion competence when they reach their destination (Fig. 8). These properties can explain how preOCs have the ability to meet preferentially at critical points and then immediately fuse. This model represents a physical frame for orderly fusion, which matches recent in vivo observations indicating that functional maintenance of OCs is achieved by acquisition of new nuclei one at a time [4], and matches as well our recent in vitro finding of “non-random” fusion [7]: 1) the cells on the bone surface are likely to correspond to less mobile nuclei acceptor cells while 2) the mononucleated cells migrating along the vascular-collagen network of the neighboring bone marrow are likely to correspond to highly mobile nuclei donor cells. This interpretation is also supported by another study of Levaot and co-workers identifying OC fusion partners as founders or followers [9]. Placed in the present context, it could be speculated that preOCs initially arriving at the bone surface are founders, while those subsequently arriving at this site can be considered as followers fusing with the founder. However, at the present stage this model (Fig. 8) does not explain what determines the direction of the migration. The answer to this question is awaiting the analysis of the tissue distribution of factors known to exert chemotactic activity towards preOCs in vitro. Important ones to consider may be factors such as SDF-1, receptor activator of nuclear factor kappa-B ligand (RANKL), macrophage-colony stimulating factor (M-CSF), vascular endothelial growth factor (VEGF), IL8, macrophage inflammatory protein 1α (MIP1α) [45–47], as well as site-specific signals in the extracellular matrix. Knowledge on the convergence between physical and molecular guidance should provide full understanding of how OC fusion and preOC trafficking are integrated. Another issue that needs attention is that we may have missed preOCs with immediate fusion potential, since TRAcP-negative/RANK-positive preOCs have been reported in mice [13]. Whatever the answer to these
questions, the present data stress a mechanism whereby OCs, which require a large size for efficient bone resorption[48], are generated at their operational site by fusion of mononuclear units that can move more easily through a constrained 3D microenvironment.
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Conflict of interests

All authors (KS, TLA, MH, LR, NM and JMD) declare that they have no conflict of interests
References


**Fig. 1:** The distribution of TRAcP$^+$ mononuclear cells (T$^+$mnc) within the human bone marrow cavity and of OCs on the bone surface. a) 3D-reconstruction of two trabeculae (yellow) separated by the marrow cavity: note that TRAcP$^+$ OCs (red) are present only on the bone surface and numerous TRAcP$^+$mnc (blue) are located in the bone marrow. b) Examples of TRAcP$^+$ (black) mncs positioned in the deep bone marrow (BM) (right) or towards the bone surface (left): note all show C-terminal-OSCAR immunoreactivity (red).
Examples of TRAcP+ (black) OCs on the bone surface (dotted line) with (upper left picture) or without (lower left picture) C-terminal-OSCAR immunoreactivity. c) Prevalence of OSCAR immunoreactivity in TRAcP+ OCs and T+mncs. Each dot represents the mean percentage of TRAcP+ OCs or T+mncs that are OSCAR+ in a given biopsy specimen (n=10). The percentages of OCs and T+mncs from the same biopsy specimen are connected with a line. The red horizontal lines represent the means. Statistics: paired Student’s t-test: ***, p<0.001. d) Pictures of sections co-labeled for collagen type I (Coll1), cathepsin K (catK) and TRAcP+ at the marrow bone interface (left) and the deep bone marrow (right). Combined immunofluorescence for collagen type I (Coll1 - red) and TRAcP+ (green) (upper pictures) show an OC on the bone surface and covered by a collagen-containing canopy (weak red staining) (left), and a T+mncc in the deep bone marrow. Combined immunofluorescence for TRAcP and CatK (lower pictures) shows that both the OC and the T+mncc have CatK. e) Pictures of an OC on the bone surface (upper) and a T+mncc at the marrow-bone interface (lower) showing the restricted perinuclear immunoreactivity revealed by an antibody specific for TRAcP5a (red/yellow, clone 220), in contrast with the broad distribution of immunoreactivity revealed by an antibody recognizing both TRAcP5a and 5b (green, clone ZY-9C5). The insets show a magnification of the framed areas. Scale bars are 20 µm.
Fig. 2: The histological appearance of the entwined collagen-vascular-network in the human bone marrow (BM) (a, b, e) and towards the marrow-bone interface (c, d, f). Pictures showing the distribution of collagen type I (a, c) and collagen type III immunoreactivity (b, d) (red staining) and reticulin staining (g, h) (dark
blue/black). Note the spatial relationship between the collagen and CD34+ vascular spaces (green arrowheads) in the deep bone marrow and close to bone remodeling compartment (BRC) canopies (yellow arrowheads). The canopies cover characteristic BRCs with bone resorbing TRAcP+ OCs on eroded surfaces (ES) and osteoblasts (OB) on the bone forming surfaces. More intense red at the bone surface in (d) is due to heterogeneity in collagen immunoreactivity. The insets show a magnification of the framed areas. Scale bars are 20 µm.
**Fig. 3:** Transmission electron microscopy revealing collagen fibers (asterisk) between BRC canopy cells. The figure shows a light microscopy overview picture (a) to identify morphologically and locationally a lifted BRC canopy (yellow arrowheads) and a series of five transmission electron microscopy images (b-f), each at increasing magnifications (see boxed areas). The outlines of the canopy cell areas ("cc" in d-f) are delineated by yellow hatched lines (b-f). At the two higher magnifications (e, f), collagen bundles (asterisks) in between the canopy cells (CC) are visible. Scale bars are 20 µm (a) and 0.5 µm (b-f).
**Fig. 4:** Proximity of TRAcP⁺ (a) and OSCAR⁺ (b) mononucleated cells (mnc) and osteoclasts to collagen type I fibers. a) Pictures of double-immunostaining of TRAcP and collagen type I at the marrow-bone interface (left) and the deep bone marrow (right): note TRAcP⁺ OC (upper left) and mnc (T⁺mnc) (all other images)
next to collagen type I. b) Pictures of double-immunostaining of OSCAR and collagen type I in the deep bone marrow: OSCAR⁻ mncs (O⁻ mnc) are seen next to collagen type I. c) Prevalence of random bone marrow (BM) cells, TRAcP⁺ mncs and OSCAR⁺ mncs adjacent, close to (one cell layer distance) and away (over one cell layer distance) from collagen type I in the bone marrow. The bars show the mean percentages ± standard deviation of the respective cell type adjacent, close or away from collagen type I (n=10 patients). The statistical difference was calculated by one-way ANOVA followed by a Bonferroni’s multiple comparison test: ***, p<0.001. d) 3D-reconstruction of serial sections showing T⁺ mncs (blue) in the spatial framework provided by collagen fibers (green) and vasculature (light purple) at the level of a bone resorption site (red osteoclast). Points of interest are (i) the continuous nature of this network thereby physically connecting the canopy (black grid covering the osteoclast) and the deep marrow, and (ii) the proximity of T⁺ mncs (blue) to this network over this entire zone. e) Picture of CD34⁺ (brown) vascular spaces (green arrowheads) enwrapped with collagen type I (red). Note a T⁺ mnc (black) next to vasculature. f) Prevalence of T⁺ mncs adjacent and close to collagen (coll.) type I either associated or not to vasculature. The columns represent the mean ± standard deviation (n=9 patients). The insets in the images show a magnification of the framed areas (a, b, e). Scale bars are 20 µm.
Fig. 5: PreOCs fuse more readily when seeded on collagen compared to mineral. a) The number of nuclei per multinucleated OC on mineral or collagen from different experiments (n=10). Each connected pair of points represents the mean of four to five discs used in a given experiment after 48h incubation on the two different surfaces. Statistics: Wilcoxon matched-pairs signed rank test; **, p<0.01. b) The frequency in percent of multinucleated OCs per total cells on mineral or collagen from different experiments (n=10). Each connected pair of points represent the mean from a given experiment after 48h incubation on different surfaces. Statistics: Paired t-test; *, p<0.05.
Fig. 6: Contacts of preOCs with collagen as opposed to mineral favors migration as well as clustering of CD47. a) Comparative effect of echistatin on fusion levels assessed on mineral or collagen. The graph illustrates for each experimental condition, the number of nuclei found in OCs is shown in percentage of total number of nuclei. The bars represent the mean ± SD from different experiments (n=3). Statistics
shown were done by pooling the actual nuclei counts of the three data-sets. Chi-square with Yates' correction was used to relate the number of nuclei found in OCs with or without echistatin-exposure with the total number of nuclei in all cells for each surface (mineral: total count=16,432; collagen: total count=8360); ***, p<0.001. b) The graph illustrates the mean percentage ± SD of all preOCs/OCs showing a migratory phenotype on mineral or collagen surfaces from different experiments (n=3). Statistics shown were done by pooling the actual cell count (total count=1853) of the three data-sets. Chi-square with Yates' correction was used to compare the number of cells with or without a migration morphology on the different surfaces; ***, p<0.001. c) Immunofluorescent labeling of preOCs/OCs seeded on mineral or collagen surfaces using anti-CD47 antibody (red), phalloidin labeling f-actin (green), and DAPI labeling the nucleus (blue). White arrowheads point to examples of CD47+ clusters. This type of images were used for quantifications shown in d. d) The graph illustrates the mean percentage ± SD of preOCs/OCs showing either CD47 distributed in clusters or evenly distributed over the plasma membrane in different experiments (n=3). Statistics shown were done by pooling the actual cell count (total count=1465) of the three data-sets. Chi-square with Yates' correction was used to compare the number of cells with or without CD47+ clusters on the different surfaces; ***, p<0.001.
Fig. 7: Comparison of the expression levels of autocrine pro-differentiation cytokines by preOCs cultured on plastic, mineral and collagen surfaces. For each condition, gene expression levels of a) IL1β, b) TNFα and c) IL8 were normalized to that of ABL and GUS. The expression levels are shown relative to the expression level on plastic (n=3 experiments). Statistics: Kruskal-Wallis test; *, p<0.05; **, p<0.01.

Fig. 8: Model for preOC trafficking and cell-cell fusion at resorption sites. PreOCs are closely associated to the collagen and vascular network, which favors differentiation, makes them highly mobile, and may serve as a “road” from the bone marrow cavity to the resorptive sites on the bone surfaces. Once there, preOCs will eventually fuse into mature multinucleated OCs. This model provides the in vivo frame for the orderly fusion of preOCs/OCs with distinct nuclearity and mobility, which was recently discovered to determine nuclei delivery and acceptance in vitro [7, 9] and in vivo [4]. The black arrows illustrate the guidance of preOCs to the resorptive site.