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Does collagen trigger the recruitment of osteoblasts into vacated bone resorption lacunae during bone remodeling?

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ABSTRACT

Osteoblast recruitment during bone remodeling is obligatory to re-construct the bone resorbed by the osteoclast. This recruitment is believed to be triggered by osteoclast products and is therefore likely to start early during the remodeling cycle. Several osteoclast products with osteoblast recruitment potential are already known. Here we draw the attention on the osteoblast recruitment potential of the collagen that is freshly demineralized by the osteoclast. Our evidence is based on observations on adult human cancellous bone, combined with in vitro assays. First, freshly eroded surfaces where osteoblasts have to be recruited show the presence of non-degraded demineralized collagen and close cell–collagen interactions, as revealed by electron microscopy, while surface-bound collagen strongly attracts osteoblast lineage cells in a transmembrane migration assay. Compared with other extracellular matrix molecules, collagen's potency was superior and only equaled by fibronectin. Next, the majority of the newly recruited osteoblast lineage cells positioned immediately next to the osteoclasts exhibit uPARAP/Endo180, an endocytic collagen receptor reported to be involved in collagen internalization and cell migration in various cell types, and whose inactivation is reported to lead to lack of bone formation and skeletal deformities. In the present study, an antibody directed against this receptor inhibits collagen internalization in osteoblast lineage cells and decreases to some extent their migration to surface-bound collagen in the transmembrane migration assay. These complementary observations lead to a model where collagen demineralized by osteoclasts attracts surrounding osteoprogenitors onto eroded surfaces, and where the endocytic collagen receptor uPARAP/Endo180 contributes to this migration, probably together with other collagen receptors. This model fits recent knowledge on the position of osteoprogenitor cells immediately next to remodeling sites in adult human cancellous bone.

Introduction

Bone remodeling renews the bone matrix through a tightly regulated process starting with resorption of old bone by osteoclasts (OCs), then a "reversal phase", followed by reconstruction of new bone by osteoblasts (OBs) [1]. It has been known for a long time that reconstruction of new bone during remodeling demands OB recruitment [1]. A series of observations indicate that this recruitment is closely associated with osteoclast activity [2,3], and is therefore likely to already occur during the resorption and reversal phase. Accordingly, histomorphometry of adult human cancellous bone revealed that virtually all the cells on eroded surfaces next to the OC are OB-lineage cells that mature during the reversal phase [4]. Furthermore, a critical density level of these cells appears required for the actual initiation of bone matrix deposition [4,5].

Extensive research has addressed the origin of the osteoprogenitors as well as the nature of the signals recruiting them to the remodeling sites and inducing OB differentiation. The bone marrow is rich in mesenchymal stem cells (MSCs), and accordingly, osteoprogenitors are often proposed to originate from the bone marrow [6]. It has been proposed that the signals attracting the MSCs to the remodeling sites are molecules released by the OCs which then diffuse into the bone marrow. This proposal received support from cell migration assays showing that a series of soluble cytokines, chemokines, growth factors, or other OC products such as collagen fragments and sphingosine 1-phosphate exert chemotaxis on MSCs or on more mature OB-lineage cells [2,3,7,8].

However, there is also evidence for osteoprogenitors originating from sites close to bone surfaces undergoing remodeling. The existence

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of a layer of osteoprogenitors at the periphery of the bone marrow is clearly documented [5,9]. It was reported that this osteoprogenitor layer gets the appearance of a canopy above bone remodeling sites [5], and the absence of canopies coincides with lack of bone formation [10–12]. It was also proposed that bone lining cells (BLCs) are able to differentiate into bone forming OBs [13–16]. BLCs are believed to retract to give the OC access to the bone surface [17,18], and to recolonize the resorption site after the OC has left, like in a wound-repair process [19,20]. This colonization may be triggered by the erosion of the bone surface by the OC which creates new epitopes. Examples of cell migration induced by changes in the extracellular matrix (ECM) are shown in other tissues [21,22] and are called haptotaxis when the signal is ECM-bound, in contrast with chemotaxis when the signal is a soluble factor [8,23]. However, the ECM peculiarities of these eroded surfaces, defined as reversal surfaces [4,24], are poorly investigated. The same holds true for their interaction with the cells colonizing them, defined as reversal cells [4,24]. Still, close interactions of reversal cells with the resorption zone beneath the OCs have been reported [19,25,26]. Analyses at high magnification especially draw the attention on interactions between reversal cells and demineralized collagen fibers [19], suggesting that these fibers might be one of the signals attracting reversal cells. Accordingly, insoluble collagen was reported to induce haptotaxis of human and rabbit MSCs [8].

How insoluble collagen is recognized by osteoprogenitors, and how this recognition may induce cell movement, has not been investigated. In this respect, it is of interest that OB lineage cells were reported to express the collagen receptor uPARAP/Endo180, and that inactivation of this receptor leads to impairment of bone formation and skeletal deformities [27,28]. This receptor is able to internalize collagen which is delivered to lysosomes via endosomal trafficking [29], whereas uPARAP/Endo180 is recycled to the plasma membrane [30]. Importantly, endocytic recycling pathways are suspected to be involved in cell migration [31], and accordingly, the absence of uPARAP/Endo180 resulted in impaired migration of fibroblasts concomitantly with deficient collagen internalization [32,33]. It is also of interest that uPARAP/Endo180-mediated collagen uptake is associated with collagenolysis, not only at the lysosomal level, but also at the extracellular level [34,35], and may be part of the previously reported cleaning activity of reversal cells [19].

These data prompted us to investigate the haptotactic response of human OB lineage cells to insoluble collagen, compared with other ECM molecules. We used therefore a standardized transmembrane migration assay and both human bone-derived cells (HBDCs) obtained from bone outgrowths and adipocyte derived stem cells differentiated into OBs (ADSC-OBs). We also addressed the mechanism of collagen-induced haptotaxis by investigating the involvement of the endocytic collagen receptor, uPARAP/Endo180 in this process.

Materials and methods

Cells for haptotaxis and collagen internalization assays

Human OB-like cells of two distinct origins were used to mimic reversal cells in vitro: 1. Primary adipocyte-derived stem cells (ADSCs) (kit obtained from: Invitrogen, Franklin Lakes, NY, USA) were seeded at a density of 1700 cells per bovine cortical bone slice (96-well) (IDS Nordic, Herlev, Denmark and Boneslices.com, Jelling, Denmark) using culture conditions and medium as described by the cell-supplier. Culture media were refreshed after 4 days of culture. Starting from the seventh day and for an additional 14 days ADSCs were differentiated by supplying the culture medium with 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone. The media were refreshed twice a week. Differentiation into an early osteoblastic phenotype was verified by Q-RT-PCR where collagen type I and osteopontin expressions were increased about 2-fold and RANKL by 5-fold, furthermore bALP activity was increased by 5-fold. In addition, expression of MMP13 was increased by 17-fold. This profile matches well the characteristics of a reversal cell model as an early osteoblastic phenotype with collagenolytic potential. ADSCs differentiated to early OBs (ADSC-OBs) either were used for collagen internalization assay, or were fluorescently labeled with 10 μM green Vybrant for 15 min (V12883, Molecular Probes, Eugene, OR, USA). Labeled ADSC-OBs were detached by using 0.1% (w/v) collagenase (*Clostridium histolyticum*, Wako, Tokyo, Japan) for 30 min followed by 0.05% (v/v) trypsin–EDTA (Gibco, Invitrogen, Franklin Lakes, NY, USA) for 10 min, and finally by mechanical detachment. Detached ADSC-OBs were washed with excess serum, resuspended in serum free medium, and used for haptotaxis assays. 2. The second source of OB-lineage cells was obtained from outgrowths from explants taken from the proximal femur of osteoarthritis patients undergoing total hip replacement (Danish Ethical Committee approval, project-ID: S-20110114). The explants were cultured as previously described [36]. The outgrowing "human bone-derived cells" (HBDCs) were cultured until confluence (after 4–5 weeks), then fluorescently labeled with 10 μM green Vybrant for 15 min as explained for the ADSCs, and finally detached using Accutase (PAA Laboratories, GE Healthcare, Piscataway Township, NJ, USA) for 10 min. Detached HBDCs were resuspended in serum free medium and used for haptotaxis assays.

Materials for haptotaxis and collagen internalization assay

The materials for haptotaxis and collagen internalization assays were cell matrix type I-A (porcine collagen type I) (Nitta Gelatin, Osaka, Japan); human vitronectin (BD Biosciences, San Jose, California, USA); bovine fibronectin (Wako, Tokyo, Japan); bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA); calf osteopontin (generous gift from Dr. W.T. Butler, University of Texas, Houston, USA); gelatin prepared by heating collagen type I at a concentration of 1.5 mg/mL serum free medium for 30 min at 60 °C; and cysteine-proteinase inhibitor E64d dissolved in ethanol (Calbiochem, San Diego, CA, USA). The monoclonal mouse uPARAP/Endo180 antibody 5f4 used for the haptotaxis and internalization assays, and its isotype-matched control anti-trinitrophenyl (TNP) antibody, and also the other monoclonal mouse uPARAP/Endo180 antibody 2h9F12 used for immunohistochemistry were generated by the Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark [37].

Haptotaxis assay

The haptotaxis assays were performed in a multiwell 24 well insert system (BD Falcon FluoroBlok, BD Biosciences, San Jose, California, USA) with an 8 μm pore size membrane impervious to light. The experimental procedure used was essentially as previously described [8]. The lower side of the membrane was coated with 28 μL matrix protein dissolved in serum free medium containing the same number of molecules of collagen type I, gelatin, vitronectin, osteopontin and fibronectin (42 μg collagen type I, gelatin, or BSA, 10.5 μg vitronectin, 8.34 μg osteopontin, or 28 μg fibronectin). They were placed in the incubator for 30 min at 37 °C in order for the matrix protein coating to adhere to the transwell membrane. A 250 μL cell suspension containing 75,000 fluorescently labeled ADSC-OBs or HBDCs in serum free medium was added to the insert (upper chamber), and 750 μL of medium was added to the lower chamber. After an overnight incubation at 37 °C and 5% CO_2 in the incubator, the migrated ADSC-OB or HBDC on the lower surface of the transwell membrane was washed in PBS, fixed with 3.7% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, and washed. The membranes were carefully cut out, placed on glass slides with their lower face up, mounted in ProLong Gold with DAPI (Molecular Probes, Eugene, OR, USA) and stored in the dark at 4 °C until analysis. The number of fluorescently labeled cells and nuclei on the lower face of the membranes was counted over the whole surface using an Axio Imager Z1 microscope (Carl Zeiss, Jena,

Germany). The results were recorded as the total number of migrated cells per transwell.

Collagen internalization assay

Collagen internalization in ADSC-OBs was performed essentially as previously described [38]. In brief, bone slices with ADSC-OBs were incubated with 20 μM E64d (Calbiochem, San Diego, CA) to prevent degradation of internalized collagen and either 10 μg/mL uPARAP/Endo180 blocking antibody (5f4) or no antibody at 37 °C for 4 h. 20 μg/mL of labeled collagen (Oregon green-labeled gelatin, Invitrogen, Franklin Lakes, NY, USA) was added and cells were then incubated overnight at 37 °C to allow the cells to internalize collagen. Excess and cell surface bound collagen ligand was removed by trypsinization for 3 min and replaced with fresh medium. Bone slices were subsequently transferred to a 14 mm glass dish (P35G-0-14-C MatTek, Ashland, MA, USA) and imaged immediately with a Zeiss LSM 510 confocal microscope. Cell outlines were visualized using DIC (differential interference contrast) microscopy. Alternatively, following the overnight incubation with the collagen ligand, ADSC-OBs were detached from the bone slices as explained previously. Serum free medium was supplemented with E64d 10 μM during all detachment steps. ADSC-OBs were then washed, and resuspended in PBS containing 5 μg/mL WGA–Alexa 594 (wheat germ agglutinin, Invitrogen, Franklin Lakes, NY, USA) to stain the cell surface. Thereafter they were washed, resuspended in PBS, centrifuged onto a coverslip using a Shandon chamber cytospin system (Thermo Scientific, Langenselbold, Germany), fixed for 10 min in 4% paraformaldehyde, mounted onto an objective glass in ProLong Gold with DAPI (Molecular Probes, Eugene, OR, USA) and imaged using a Zeiss LSM 780 confocal microscope.

Histological assessment of adult human cancellous bone

Paraffin-embedded iliac crest biopsies from 9 patients (Danish Ethical Committee approval, project-ID: 20010082) with no skeletal diseases were used for immunohistochemistry. The immunohistochemical staining of uPARAP/Endo180 was performed by using a monoclonal mouse IgG 2h9F12, which was detected with horseradish peroxidase (HPR)-conjugated goat anti-mouse IgG1 subtype-specific secondary antibody (Jackson Immunovision, Baltimore Pike, West Grove, PA, USA). This immunostaining was conducted as a double immunostaining, so as to also localize the OC marker Tartrate Resistant Phosphatase (TRAcP). This procedure was previously described [10]. Reversal cells and bone lining cells (BLCs) were recognized by their position on respectively broken or unbroken lamellae, which were visualized in polarized light. All the first and second uPARAP/Endo180-positive reversal cells or BLCs neighboring to an OC (see Fig. 3A) were quantified. The results were expressed as the percentage of reversal cells or BLCs which were stained positive for uPARAP/Endo180.

Transmission electron microscopy (EM) on bone biopsies from primary hyperparathyroidism (PHPT) patients (Danish Ethical Committee approval, project-ID: S-20070121) was performed as previously described [5]. PHPT biopsies were chosen due to their increased activation frequency of bone remodeling which makes the detection of bone remodeling sites at the EM level easier.

Cytochemistry of osteoclasts cultured on bone slices

OCs were generated from buffy coats donated by healthy volunteers (Danish Ethical Committee approval, project-ID: 2007-0019) and cultured on bovine bone slices (IDS Nordic, Herlev, Denmark) for 72 h as previously described [39] They were processed for staining of collagen and actin as reported [40]. Collagen was stained with a rabbit antibody directed against collagen type 1 (ab34710 Abcam, Cambridge, UK), followed by an incubation with an anti-rabbit antibody (Alexa Flour 568, Invitrogen, Franklin Lakes, NY, USA). F-actin was stained with phalloidin (Alexa Flour 488, Invitrogen). Confocal pictures were taken using an Olympus FluoView FV10i (Olympus Corporation, Shinjuku, Tokyo, Japan).

Statistical analyses of the data

For in vitro haptotaxis experiments, all experiments were performed at least twice. The parameters measured in the different conditions were compared using unpaired Student's t-test when the data were Gaussian distributed; otherwise Mann–Whitney Rank sum test was used. While in the case of in vivo quantification of uPARAP/Endo180, paired Student's t-test was used when comparing Gaussian distributed differences between two parameters within the same patient. Results are represented as mean ± standard deviation (SD). All graphs and statistics were done using GraphPad Prism version 5 (GraphPad Software, San Diego, California, USA). $P < 0.05$ was considered statistically significant. The final figures were assembled using the CorelDraw package (x4) (Corel Corporation, Ottawa, Ontario, Canada).

Results

Non-degraded demineralized collagen I is present on eroded bone surfaces

The analysis of eroded surfaces at high resolution shows OCs and mononucleated cells at their immediate vicinity, as reported by others [25]. Recent studies showed that these cells belong to the OB lineage [4], and were called reversal cells [4,24]. The alterations made by the OC on the bone surface may represent signals attracting these cells after the departure of the OC. Figs. 1A–D show that non-digested demineralized collagen represents one of these alterations, since it is present beneath both the OC and the reversal cells. The likelihood that denuded collagen is left behind the OC when it moves away from the resorption site is also supported by the presence of demineralized collagen at the back-side of the resorption trenches made by OCs cultured on bone slices (Figs. 1F, G). Furthermore, we found evidence of enwrapment of these collagen fibers by the plasma membrane of these reversal cells, and the plasma membrane was more electron-dense at these enwrapment sites (Fig. 1E). This indicates active interactions between reversal cells and collagen. Together, this suggests that the collagen demineralized by the OC might serve as a signal attracting reversal cells.

Collagen type I exerts a high haptotactic effect on osteoblast-lineage cells compared with other ECM molecules

In order to test the aforementioned hypothesis, we developed an in vitro model where ADSC-OBs and HBDCs were seeded on the upper-surface of a porous membrane whose lower surface was coated with native collagen, or with osteopontin, vitronectin, or fibronectin, which are ECM molecules that might be relevant to eroded surfaces generated in vivo. As control molecules, we also included BSA and gelatin representing denatured collagen. Fig. 2 shows that during an overnight culture, cells migrated to the lower surface of the membrane, and that migration levels were significantly higher in response to native collagen and fibronectin, compared to all other proteins, including gelatin. Fibronectin was actually as efficient as collagen. Interestingly, the migration patterns of ADSCs-OBs and HBDCs were similar.

The endocytic collagen receptor uPARAP/Endo180 is frequently present in early reversal cells, and is involved in haptotaxis of osteoblast lineage cells to collagen

The haptotactic response to collagen shows that these cells recognize collagen and suggests the involvement of a collagen receptor. As explained in the Introduction, uPARAP/Endo180 is a collagen receptor involved in cell migration, associated with clathrin-coated pits, is

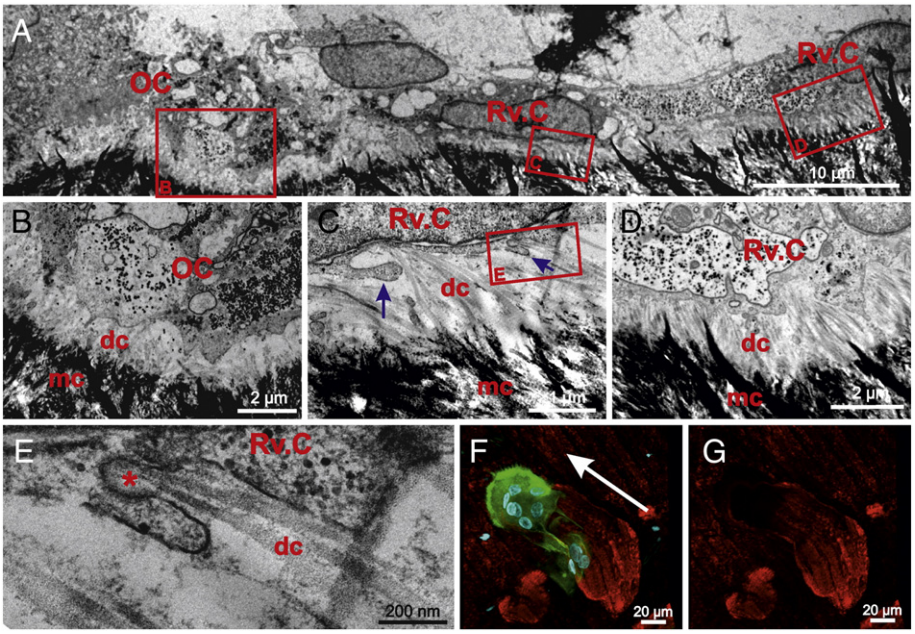

Fig. 1. Non-degraded demineralized collagen I is present on eroded bone surfaces. (A) Electron micrograph showing an eroded surface with an osteoclast (OC) and two reversal cells (Rv.C). (B, C, D) Higher magnification of the boxed areas shown in (A). The interface between the bone-facing surface of the OC or reversal cells and the eroded surface shows demineralized collagen fibers (dc) on top of black mineralized fibers (mc). Gradual decrease in density of mineral indicates the presence of OC activity [19]. Note the presence of cellular extensions of reversal cells in (C) (blue arrows). (E) Higher magnification of the boxed area in (C). Note the enwrapment (asterisk) of demineralized collagen fibers (dc) as well as the electron dense plasma membrane at the enwrapment site. (F) Confocal image of an OC making a resorption trench on a bone slice, where F-actin is stained in green, non-degraded demineralized collagen in red and nuclei in blue with DAPI. The white arrow indicates the direction of movement of the OC. (G) The same view as in (F), showing only red non-degraded demineralized collagen left by the OC at the backside of the resorption trench.

present in OB-lineage cells and participates in bone formation through an unknown mechanism. Fig. 3A shows immunoreactivity for uPARAP/Endo180 in OC-surrounding cells on the bone surface. These cells can be categorized into BLCs on quiescent surfaces, and early reversal cells which have colonized eroded surfaces. We quantified the proportion of uPARAP/Endo180-positive cells in each of these categories, limiting our analysis to the two surface cells at each side of the OCs. This quantification revealed an average of 75% of uPARAP/Endo180-positive early reversal cells, and an average of 50% of uPARAP/Endo180-positive BLCs proximal to OCs (Fig. 3B). These measurements show a significant increase in uPARAP/Endo180 levels in early reversal cells compared to BLCs, and are compatible with a role of uPARAP/Endo180 in early reversal cells.

In order to investigate whether the presence of uPARAP/Endo180 in these reversal cells might reflect a role in haptotaxis to collagen, we performed the haptotaxis assay in the presence and absence of an antibody, 5f4, which has been shown to decrease uPARAP/Endo180 availability [38]. Fig. 4 shows that compared to a control isotype antibody, 5f4 reduced the migration of both ADSC-OBs and HBDCs to the collagen- and gelatin-coated surfaces, but the effect was statistically most significant for collagen. The level of reduction was about 30% in the case of collagen, and a dose response curve showed that the present concentration of 5f4 corresponds to a maximum inhibition (not shown). These observations suggest that uPARAP/Endo180 contributes to some degree to migration towards native collagen.

uPARAP/Endo180 is involved in collagen internalization

Next we investigated the mechanism responsible for the promigratory role of uPARAP/Endo180. One of the possible mechanisms relates to its

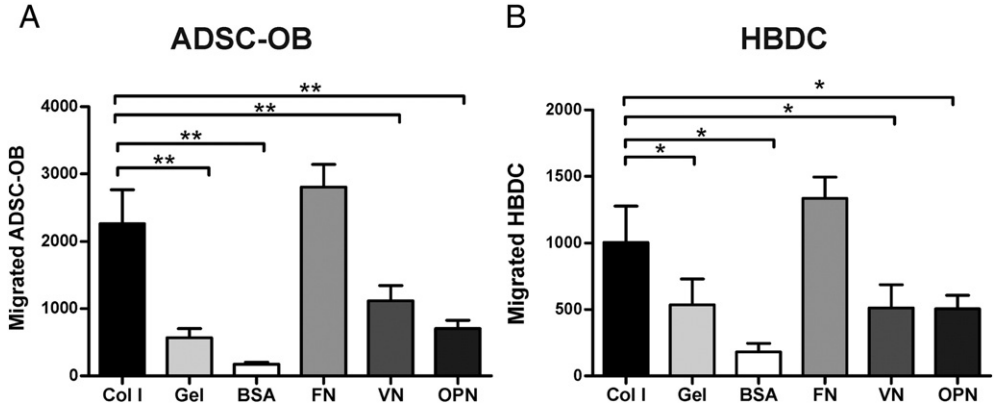

Fig. 2. Higher haptotactic migration of ADSCs-OBs (A) and HBDCs (B) towards collagen type I and fibronectin, compared with other extracellular matrix proteins. The migration towards collagen type 1 (Col1), gelatin (Gel), BSA, fibronectin (FN), vitronectin (VN), and osteopontin (OPN) was compared using the haptotaxis assay. Each bar represents the mean ± SD of the number of migrated cells per well, counted in 6 (A) or 4 (B) wells. The haptotactic responses to collagen and the other matrix molecules were compared by using the Mann–Whitney Rank sum test. ns: not significant, *$P < 0.05$, **$P < 0.01$.

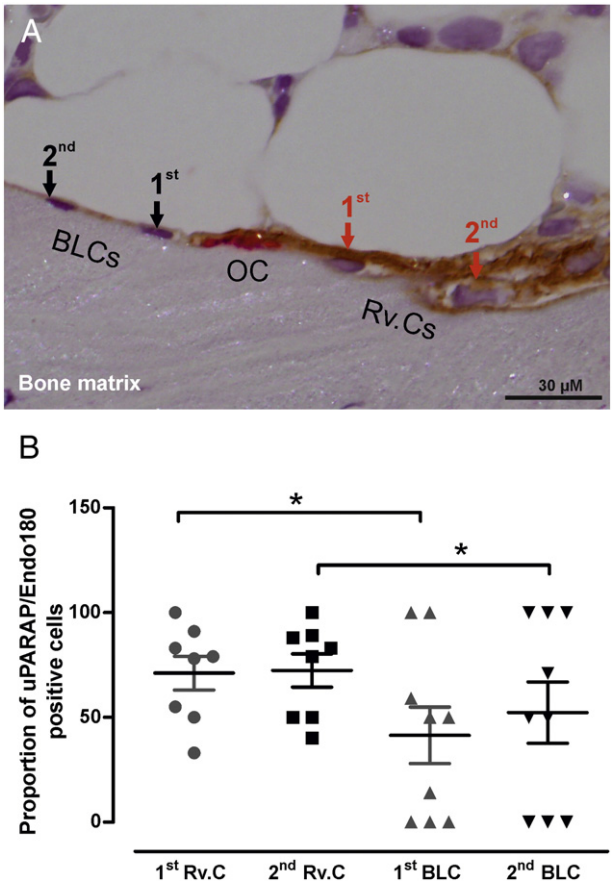

Fig. 3. The endocytic collagen receptor uPARAP/Endo180 is expressed in reversal cells and BLCs in vivo. (A) Histological appearance of double immunostained sections with OC marker TRAcP (red) and collagen receptor uPARAP/Endo180 (brown). uPARAP/Endo180-positive BLCs (black arrows) on a quiescent surface, and uPARAP/Endo180-positive reversal cells (red arrows) are shown on an eroded surface at both sides of a TRAcP-positive OC (asterisk). (B) The two bone surface cells at each of the sides of an OC (see A) were sorted in BLCs and reversal cells, depending on whether they were on unbroken or broken lamellae, respectively (see Materials and methods). The percentage of those expressing uPARAP/Endo180 was calculated for each biopsy. Each of these percentages is shown by a dot. The horizontal bars are the means ± SD of the 9 determinations corresponding to each biopsy. The percentages of uPARAP/Endo180-positive BLCs and Rv.Cs were compared by using the paired t-test. *$P < 0.05$.

ability to internalize collagen [31–33]. Accordingly, the 5f4 antibody which reduces uPARAP/Endo180 bioavailability has been shown to prevent collagen uptake in a number of cell types [38]. Here we show that ADSCs-OBs incubated with collagen readily internalized collagen, but not if the incubation was performed in the presence of 5f4 (Fig. 5). Thus, 5f4 affects both collagen internalization and haptotaxis to collagen.

Discussion

OB recruitment is mandatory for coupling bone resorption to formation during bone remodeling. The first manifestation of this recruitment is the colonization of eroded surfaces by reversal cells, which are actual progenitors of bone forming OBs [4,5]. Cell recruitment is determined by a number of environmental-specific activities, all strictly coordinated in time and space. Most of these activities are poorly known in the case of the OB recruitment occurring during bone remodeling. Here we focus on insoluble factors exposed by the OC on eroded bone surfaces, and propose that demineralized collagen is one of the agents attracting reversal cells from sites contiguous to the resorbed bone surface, as shown in the model of Fig. 6. In this way, BLCs may recolonize the resorbed bone surface, like in a wound-repair process [20,22]. Our proposal is based on complementary observations: presence of collagen at freshly eroded surfaces where reversal cells have to be recruited; prevalence of the collagen receptor uPARAP/Endo180 on these reversal cells; actual ability of collagen to induce recruitment of OB lineage cells; and involvement of uPARAP/Endo180 in this process.

Demineralized collagen proves to be present in the resorption zone of resorbing OCs, and to be associated with reversal cells. These features were previously already reported in adult rabbit cancellous bone and in mouse calvaria [19,26]. They are now also shown in adult human cancellous bone. Of note, the presence of non-degraded demineralized collagen in resorption lacunae is a logical consequence of the mechanism of OC bone resorption [41]. This mechanism consists in solubilization of mineral and degradation of collagen only once collagen is uncovered by demineralization. Importantly, both mineral solubilization and cathepsin K-mediated collagen degradation require acidification, which means that once the OC stops acidification, the local raise in pH renders the acid-requiring cathepsin K ineffective for degrading the freshly demineralized collagen. Accordingly, pit assays show that the rate of degradation of collagen by isolated OCs proves to be slower than the rate of solubilization of mineral [42]. The reason why collagen left-over is often overlooked in vivo is that the surrounding cells are in close contact with the resorption area [19,25] and contribute to quickly removing this collagen left-over through a mechanism involving MMPs, which do not require acidic pH [19,41,43]. As explained in the Introduction, many of these so-called reversal cells are likely to originate from BLCs [13–16] and progressively mature in bone forming OBs [4].

The present transmembrane assays indicate that matrix-bound collagen is able to induce recruitment of OB-lineage cells. Compared with other ECM molecules, including osteopontin which is typical of reversal surfaces [44], collagen's potency for haptotaxis was superior and equaled only by fibronectin. The same migration pattern was obtained with the two OB cell types used in our assays, one of which consists of outgrowths from bone surfaces of human biopsies which are rich in BLCs, i.e. one of the cell types suspected to be recruited on eroded surfaces (see Introduction and Fig. 6). Our data are also in line with those of Thibault et al. [8], who showed similar relative haptotactic efficiency of bound collagen, fibronectin, and vitronectin towards human MSCs. Thus, matrix-bound collagen proves to be an effective haptoattractant, irrespectively of the type of OB lineage cell and of the laboratory where the experiments were performed.

Of note, the relevance of this in vitro observation is supported by the close physical proximity between the OC resorption zone and the surrounding cells as shown in Fig. 1 and in other reports [19,25]. However, this relevance is stressed even more by in vivo studies where excessive amounts of demineralized collagen were allowed to accumulate in the OC resorption zone. Treatment of rabbits with the cathepsin K inhibitor odanacatib resulted in impaired collagen degradation and increased reversal cell recruitment, as based on measurements of collagen accumulation in the resorption zone and quantifications of densities of reversal cells on bone surfaces of the vertebrae [26]. Previously, it was stressed that inhibition of cathepsin K in mouse bone explants resulted in impaired OC collagen degradation and active recruitment of reversal cells closely interacting with this collagen [19]. This collagen-induced reversal cell recruitment may be one of the explanations why bone formation is favored in situations where cathepsin K activity is decreased, either by odanacatib [45], or by increasing the pH of the OC–bone interface [46].

An important issue is the mechanism whereby collagen might induce recruitment of reversal cells. Collagen is not only a structural component of the matrix, but also a well-known signaling molecule able to define cell behavior through specific collagen receptors [47]. In the present study, we made several observations fitting the hypothesis that the endocytic collagen receptor uPARAP/Endo180 contributes to the mechanism inducing recruitment of reversal cells. We showed that uPARAP/Endo180 is expressed in the majority of the reversal cells next to OCs and to a lesser extent in BLCs. uPARAP/Endo180 was also recently reported in the canopy cells covering the OCs and believed to be a

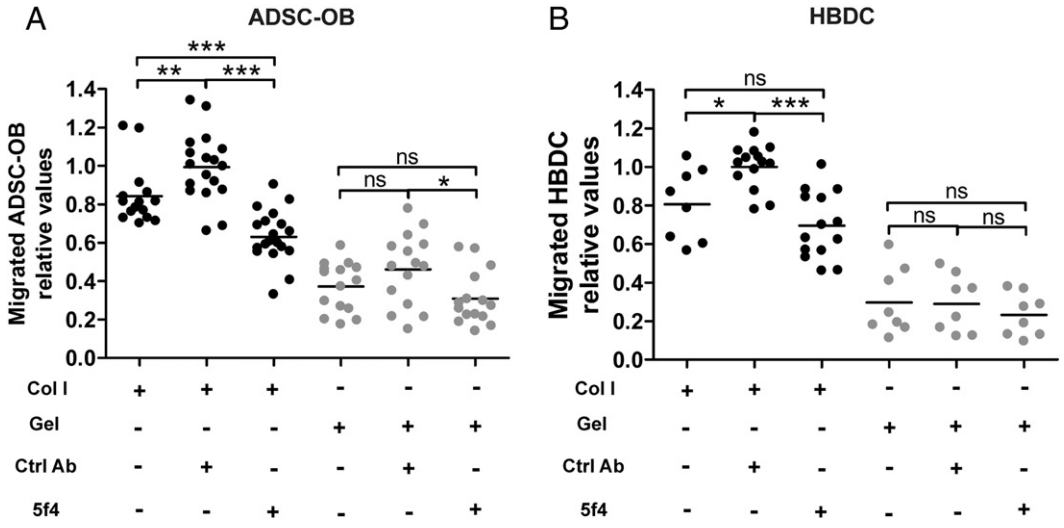

Fig. 4. Anti-uPARAP/Endo180 mAb 5f4 inhibits the haptotactic migration of ADSCs-OBs (A) or HBDCs (B) towards collagen type I. The haptotactic migration towards collagen type I and gelatin was measured as explained in the legend of Fig. 2, in the presence or absence of anti-uPARAP/Endo180 5f4 (20 μg/mL) or of its isotype matched control antibody anti-TNP 20 μg/mL. Prior to the assay, the cells were already preincubated with the antibodies for 30 min at 37 °C. Between two and four experiments were performed, each with at least four cultures per condition. The graphs show the pooled data of all experiments, as number of migrations normalized to the number of migrations obtained in the presence of the control antibody. Migration numbers did not cluster depending on the experiments. Each dot represents the number of migrated cells in one culture-well. The horizontal bars represent the mean ± SD of the number of migrated cells in the respective experimental conditions. The haptotactic responses in these different conditions were compared by using the Mann–Whitney Rank sum test, *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant.

reservoir of osteoprogenitors [48]. Thus uPARAP/Endo180 expression coincides with the cell population involved in colonization of eroded surfaces and collagen cleaning [19,41,43]. Furthermore, we observed clathrin-coated pits in the plasma membrane of reversal cells exactly at the sites of collagen enwrapment, and uPARAP/Endo180 is known to be associated with clathrin-coated pits in the plasma membrane [49]. Furthermore, we show that an antibody preventing recycling of uPARAP/Endo180 to the plasma membrane reduces the migration of OB-lineage cells to immobilized collagen in the transmembrane assay. This holds true for the two OB cell types we tested. The combination of these in vivo and in vitro observations makes us conclude that uPARAP/Endo180 contributes to reversal cell recruitment. Future research should address whether this role contributes to impaired bone formation and skeletal deformities induced by the absence of uPARAP/Endo180 [27,28].

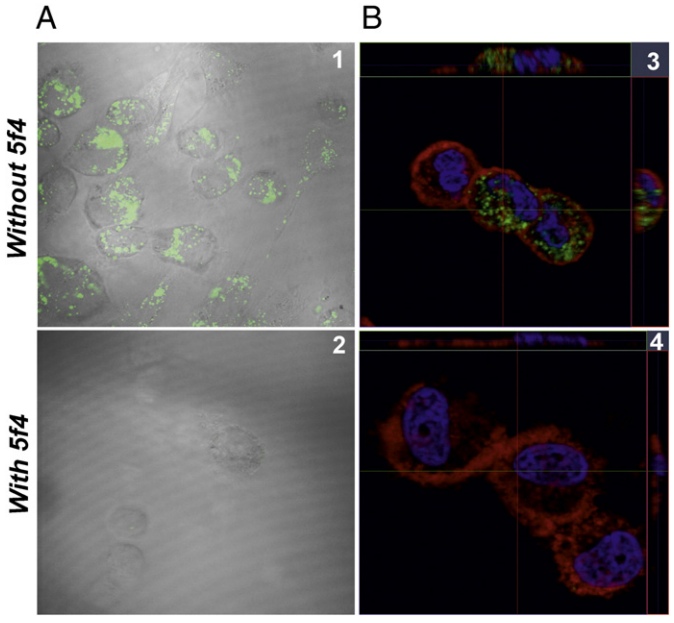

Fig. 5. Anti-uPARAP/Endo180 mAb 5f4 inhibits internalization of fluorescently-labeled collagen by ADSCs-OBs. ADSC-OBs on bone slices were incubated with green-labeled collagen in the absence (1, 3) and presence (2, 4) of uPARAP/Endo180 blocking antibody 5f4 10 μg/mL. (A) Confocal images of the cells on the bone slices were taken immediately after incubation. The cell outlines were visualized with DIC. (B) Confocal images of the cells after detachment and visualization of nuclei with DAPI (blue) and the cell outlines with WGA–Alexa 594 (red). In (A) and (B) collagen internalization (green) is seen in the absence of anti-uPARAP/Endo180 (1, 3), but not in its presence (2, 4). In (B), cross-sections at the level of the indicated axes were reconstructed from the stack of horizontal sections. Note the green signal appears intracellular whatever the direction of the section.

How uPARAP/Endo180 is involved in migration is far from clear [29,30,32,50,51]. It can be speculated that this involvement relates to cell adherence, clustering of critical plasma membrane factors to the points where the cell contacts collagen, or activation of an endocytic recycling pathway, since endocytic recycling is suspected to be involved in cell migration [31]. As explained in the Introduction, the latter possibility was particularly tempting to consider because early reversal cells colonizing resorption lacunae also clear eroded surfaces of collagen remnants [19]. Thus, we had reasoned that uPARAP/Endo180 could perhaps support at the same time two functions required at this step of the remodeling cycle: OB recruitment and removal of collagen remnants. However, even if we could show that OB lineage cells internalize collagen through uPARAP/Endo180, other observations indicate the absence of a strict link between collagen internalization and migration. Importantly in this respect, denatured collagen (i.e. gelatin) is much less efficient than native collagen in attracting OBs (Fig. 2), although gelatin is more efficiently internalized by uPARAP/Endo180 compared to native collagen [34]. The importance of the native characteristics of collagen might rather indicate a link between migration and cell adherence, since uPARAP/Endo180-mediated cell adherence was reported to require a triple helical conformation of collagen [52].

Another important conclusion of this study is that additional collagen receptors appear to be involved in the colonization of the resorption site by reversal cells, since anti-uPARAP/Endo180 was far from providing a full inhibition of migration. β1 integrins and the discoidin domain receptors (DDR) are examples of other collagen receptors present in OB lineage cells [53,54]. A cooperation between β1 integrins and uPARAP/Endo180 has been reported both for collagen uptake [35] and for cell adherence where uPARAP/Endo180 was only involved as an early modulator [32]. β1 integrins and DDRs have been implicated in cell spreading, migration and adhesion, all aspects that might relate to haptotaxis to demineralized collagen [55]. Interestingly, these collagen receptors

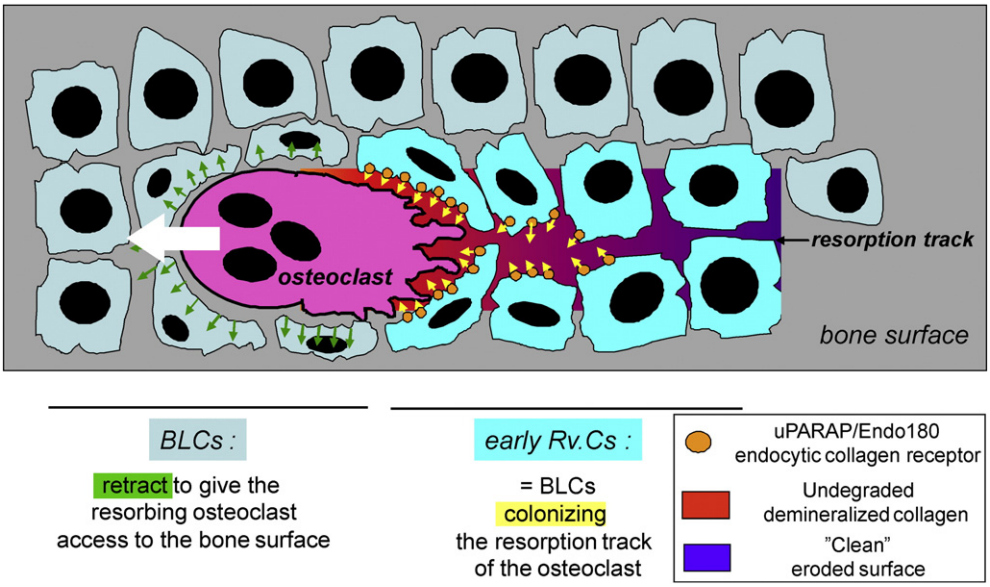

Fig. 6. Model for recruitment of Rv.Cs into vacated resorption lacunae. The scheme shows a top view of a bone surface (gray). The access of the OC (pink) to the bone matrix requires retraction of the BLCs (pale blue). Once the OC has resorbed and moves away, the new epitopes exposed on the eroded surface (red) contribute to initiate a series of steps leading to bone formation. The first step is cell recruitment. The cells colonizing the resorbed surface are called reversal cells (bright blue), and include BLCs and osteoprogenitors from other sources (not shown in the figure) [5]. We show here that demineralized collagen (red) left behind by the OC contributes in driving this recruitment. Furthermore, this collagen-driven process appears to involve the endocytic collagen receptor uPARAP/Endo180 (orange). After this recruitment step, local signals will induce specific activities making progressively the surface of the bone matrix propitious to bone formation (purple), and allowing maturation of the reversal cells into mature bone forming OBs [4].

require native collagen characteristics [55], and this is in line with the strong haptotaxis to collagen compared with gelatin shown in the present study. Of note, the absence of DDRs also results in skeletal abnormalities [55]. These data indicate the need of investigating to what extent other collagen receptors are involved in the colonization of resorption sites by reversal cells.

In summary, it is often proposed that chemoattractants diffusing into the bone marrow recruit osteoprogenitors to the bone surface [2,3,7,8]. Here we show that demineralized collagen generated by OCs represents a likely signal attracting osteoprogenitors positioned contiguously to bone remodeling sites [5,9–16]. We also suggest that uPARAP/Endo180 is one of the receptors involved in this process (Fig. 6). The present study should open the way to investigate the possible involvement of other receptors and matrix-bound molecules triggering this recruitment.

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References

[1] Parfitt AM. Bone-forming cells in clinical conditions. In: Hall BK, editor. Bone, The osteoblast and osteocyte. Caldwell, NJ: The Telford Press; 1990. p. 351–429.
[2] Dirckx N, Van Hul M, Maes C. Osteoblast recruitment to sites of bone formation in skeletal development, homeostasis, and regeneration. Birth Defects Res Part C 2013;99:170–91.
[3] Teti A. Mechanism of osteoclast-dependent bone formation. BoneKey Reports, 2; 2013. p. 449. http://dx.doi.org/10.1038/bonekey.2013.183 Article number.
[4] Andersen TL, Abdelgawad ME, Kristensen HB, Hauge EM, Rolighed L, Bollerslev J, et al. Understanding coupling between bone resorption and formation: are reversal cells the missing link? Am J Pathol 2013;183:235–46.
[5] Kristensen HB, Andersen TL, Marcussen N, Rolighed L, Delaisse J-M. Osteoblast recruitment routes in human cancellous bone remodeling. Am J Pathol 2014;184:778–89.
[6] Bianco P, Sacchetti B, Riminucci M. Stem cells in skeletal physiology and endocrine diseases of bone. Endocr Dev 2011;21:91–101.
[7] Mundy GR, Rodan SB, Majeska RJ, DeMartino S, Trimmier C, Martin TJ, et al. Unidirectional migration of osteosarcoma cells with osteoblast characteristics in response to products of bone resorption. Calcif Tissue Int 1982;34(6):542–6.
[8] Thibault MM, Hoemann CD, Buschmann MD. Fibronectin, vitronectin, and collagen I induce chemotaxis and haptotaxis of human and rabbit mesenchymal stem cells in a standardized transmembrane assay. Stem Cells Dev 2007;16:489–502.
[9] Bi LX, Mainous EG, Yngve DA, Buford WL. Cellular isolation, culture and characterization of the marrow sac cells in human tubular bone. J Musculoskelet Neuronal Interact 2008;8:43–9.
[10] Andersen TL, Sondergaard TE, Skorzynska KE, Dagnaes-Hansen F, Plesner TL, Hauge EM, et al. A physical mechanism for coupling bone resorption and formation in adult human bone. Am J Pathol 2009;174:239–47.
[11] Jensen PR, Andersen TL, Soe K, Hauge EM, Bollerslev J, Amling M, et al. Premature loss of bone remodeling compartment canopies is associated with deficient bone formation: a study of healthy individuals and patients with Cushing's syndrome. J Bone Miner Res 2012;27:770–80.
[12] Andersen TL, Hauge ElM, Rolighed L, Bollerslev J, Kjærsgaard-Andersen P, Delaisse J-M. Correlation between absence of bone remodeling compartment canopies, reversal phase arrest, and deficient bone formation in post-menopausal osteoporosis. Am J Pathol 2014;184:1142–51.
[13] Kim SW, Pajevic PD, Selig M, Barry KJ, Yang J-Y, Shin CS, et al. Intermittent parathyroid hormone administration converts quiescent lining cells to active osteoblasts. J Bone Miner Res 2012;27:2075–84.
[14] Chow JWM, Wilson AJ, Chambers TJ, Fox SW. Mechanical loading stimulates bone formation by reactivation of bone lining cells in 13-week-old rats. J Bone Miner Res 1998;13:1760–7.
[15] Dobnig H, Turner RT. Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. Endocrinology 1995;136:3632–8.
[16] Nöth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS. Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. J Orthop Res 2002;20:1060–9.
[17] Ferrier J, Xia S-L, Lagan E, Aubin JE, Heersche JNM. Displacement and translocation of osteoblast-like cells by osteoclasts. J Bone Miner Res 1994;9:1397–405.
[18] Karsdal MA, Fjording MS, Foged NT, Delaisse J-M, Lochter A. Transforming growth factor-β-induced osteoblast elongation regulates osteoclastic bone resorption through a p38 mitogen-activated protein kinase- and matrix metalloproteinase dependent pathway. J Biol Chem 2001;276:39350–8.
[19] Everts V, Delaissé J-M, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P, et al. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res 2002;17:77–90.
[20] Jones SJ, Gray C, Boyde A. Simulation of bone resorption–repair coupling in vitro. Anat Embryol 1994;190:339–49.

[21] Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science 1997;277:225–7.
[22] Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. J Cell Biol 1997;137:1445–57.
[23] Eccles SA, Box C, Court W. Cell migration/invasion assays and their application in cancer drug discovery. Biotechnol Annu Rev 2005;11:391–421.
[24] Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, et al. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR histomorphometry nomenclature committee. J Bone Miner Res 2013;28:2–17.
[25] Domon T, Suzuki R, Takata K, Yamazaki Y, Takahashi S, Yamamoto T, et al. The nature and function of mononuclear cells on the resorbed surfaces of bone in the reversal phase during remodeling. Ann Anat 2001;183:103–10.
[26] Jensen PR, Andersen TL, Pennypacker BL, Duong LT, Delaissé J-M. The bone resorption inhibitors odanacatib and alendronate affect post-osteoclastic events differently in ovariectomized rabbits. Calcif Tissue Int 2014;94:212–22.
[27] Wagenaar-Miller RA, Engelholm LH, Gavard J, Yamada SS, Gutkind JS, Behrendt N, et al. Complementary roles of intracellular and pericellular collagen degradation pathways in vivo. Mol Cell Biol 2007;27:6309–22.
[28] Fasquelle C, Sartelet A, Li W, Dive M, Tamma N, Michaux C, et al. Balancing selection of a frame-shift mutation in the MRC2 gene accounts for the outbreak of the Crooked Tail Syndrome in Belgian Blue Cattle. PLoS Genet 2009;5:e1000666.
[29] Wienke D, MacFadyen JR, Isacke CM. Identification and characterization of the endocytic transmembrane glycoprotein Endo180 as a novel collagen receptor. Mol Biol Cell 2003;14:3592–604.
[30] Howard MJ, Isacke CM. The C-type lectin receptor Endo180 displays internalization and recycling properties distinct from other members of the mannose receptor family. J Biol Chem 2002;277:32320–31.
[31] Jones M, Caswell P, Norman J. Endocytic recycling pathways: emerging regulators of cell migration. Curr Opin Cell Biol 2006;18:549–57.
[32] Engelholm LH, List K, Netzel-Arnett S, Cukierman E, Mitola DJ, Aaronson H, et al. uPARAP/Endo180 is essential for cellular uptake of collagen and promotes fibroblast collagen adhesion. J Cell Biol 2003;160:1009–15.
[33] East L, McCarthy A, Wienke D, Sturge J, Ashworth A, Isacke CM. A targeted deletion in the endocytic receptor gene Endo180 results in a defect in collagen uptake. EMBO Rep 2003;4:710–6.
[34] Madsen DH, Engelholm LH, Ingvarsen S, Hillig T, Wagenaar-Miller RA, Kjøller L, et al. Extracellular collagenases and the endocytic receptor, urokinase plasminogen activator receptor-associated protein/endo180, cooperate in fibroblast-mediated collagen degradation. J Biol Chem 2007;282:27037–45.
[35] Shi F, Harman J, Fujiwara K, Sottile J. Collagen I matrix turnover is regulated by fibronectin polymerization. Am J Physiol Cell Physiol 2010;298:C1265–75.
[36] Gallagher JA. Human osteoblast culture. In: Helfrich MH, Ralston SH, editors. Bone research protocols, methods in molecular biology. New Jersey: Humana Press; 2003. p. 3–18.
[37] Sulek J, Wagenaar-Miller RA, Shireman J, Molinolo A, Madsen DH, Engelholm LH, et al. Increased expression of the collagen internalization receptor uPARAP/Endo180 in the stroma of head and neck cancer. J Histochem Cytochem 2007;55:347–53.
[38] Madsen DH, Ingvarsen S, Jürgensen HJ, Melander MC, Kjøller L, Moyer A, et al. The non-phagocytic route of collagen uptake a distinct degradation pathway. J Biol Chem 2011;286:26996–7010.
[39] Soe K, Delaissé J-M. Glucocorticoids maintain human osteoclasts in the active mode of their resorption cycle. J Bone Miner Res 2010;25:2184–92.
[40] Soe K, Andersen TL, Hobolt-Pedersen AS, Bjerregaard B, Larsson LI, Delaissé J-M. Involvement of human endogenous retroviral syncytin-1 in human osteoclast fusion. Bone 2011;48:837–46.
[41] Delaisse J-M, Andersen TL, Engsig MT, Henriksen K, Troen T, Blavier L. Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. Microsc Res Tech 2003;61:504–13.
[42] Soe K, Merrild DMH, Delaissé J-M. Steering the osteoclast through the demineralization–collagenolysis balance. Bone 2013;56:191–8.
[43] Andersen TL, MdC Ovejero, Kirkegaard T, Lenhard T, Foged NT, Delaissé J-M. A scrutiny of matrix metalloproteinases in osteoclasts: evidence for heterogeneity and for the presence of MMPs synthesized by other cells. Bone 2004;35:1107–19.
[44] Dodds RA, Connor JR, James IE, Rykaczewski EL, Appelbaum E, Dul E, et al. Human osteoclasts, not osteoblasts, deposit osteopontin onto resorption surfaces: an in vitro and ex vivo study of remodeling bone. J Bone Miner Res 1995;10:1666–80.
[45] Duong LT. Therapeutic inhibition of cathepsin K-reducing bone resorption while maintaining bone formation. Bonekey Rep 2012;67:1–8.
[46] Schaller S, Henriksen K, Sveigaard C, Heegaard AM, Hélix N, Stahlhut M, et al. The chloride channel inhibitor NS3736 prevents bone resorption in ovariectomized rats without changing bone formation. J Bone Miner Res 2004;19:1144–53.
[47] Vogel WF. Collagen-receptor signaling in health and disease. Eur J Dermatol 2001;11:506–14.
[48] Jensen PR, Andersen TL, Pennypacker BL, Duong le T, Engelholm LH, Delaissé JM. A supra-cellular model for coupling of bone resorption to formation during remodeling: lessons from two bone resorption inhibitors affecting bone formation differently. Biochem Biophys Res Commun 2014;443:694–9.
[49] Isacke CM, van der Geer P, Hunter T, Trowbridge IS. p180, a novel recycling transmembrane glycoprotein with restricted cell type expression. Mol Cell Biol 1990;10:2606–18.
[50] Hillig T, Engelholm LH, Behrendt N. The endocytic collagen receptor, uPARAP/Endo180, in cancer invasion and tissue remodeling. In: Edwards D, Hoyer-Hansen G, Blasi F, Sloane BF, editors. The cancer degradome, proteases and cancer biology. New York: Springer; 2008. p. 245–58.
[51] Sturge J, Wienke D, Isacke CM. Endosomes generate localized Rho-ROCK-MLC2-based contractile signals via Endo180 to promote adhesion disassembly. J Cell Biol 2006;175:337–47.
[52] Thomas EK, Nakamura M, Wienke D, Isacke CM, Pozzi A, Liang P. Endo180 binds to the C-terminal region of type I collagen. J Biol Chem 2005;280:22596–605.
[53] Gronthos S, Simmons PJ, Graves SE, Robey PG. Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 2001;28:174–81.
[54] Zhang Y, Su J, Yu J, Bu X, Ren T, Liu X, et al. An essential role of discoidin domain receptor 2 (DDR2) in osteoblast differentiation and chondrocyte maturation via modulation of Runx2 activation. J Bone Miner Res 2011;26:604–17.
[55] Leitinger B. Transmembrane collagen receptors. Annu Rev Cell Dev Biol 2011;27:265–90.