Catabolic activity of osteoblast lineage cells contributes to osteoclastic bone resorption in vitro

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ABSTRACT

Osteoblast lineage cells in human bone were recently shown to colonize eroded bone surfaces and to closely interact with osteoclasts. They proved to be identical to reversal cells and are believed to differentiate into bone-forming osteoblasts thereby coupling resorption and formation. However, they also exert catabolic activity that contributes to osteoclastic bone resorption, but this has not received much attention. Herein, we used co-cultures of primary human osteoblast lineage cells and human osteoclasts derived from peripheral blood monocytes to investigate whether a catabolic activity of osteoblast lineage cells could impact on osteoclastic bone resorption. Through a combination of immunofluorescence, in situ hybridization and time-lapse experiments, we show that MMP-13-expressing osteoblast lineage cells are attracted to and closely interact with bone-resorbing osteoclasts. This close interaction results in a strong and significant increase in the bone resorptive activity of osteoclasts – especially those making trenches. Importantly, we show that osteoclastic bone resorption becomes sensitive to inhibition of matrix metalloproteinases in the presence, but not in the absence, of osteoblast lineage cells. We propose that this may be due to the direct action of osteoblast-lineage-derived MMP-13 on bone resorption.

KEY WORDS: Bone resorption, MMP-13, Osteoclast, Reversal cell, Co-culture, MMP, Matrix metalloproteinase

INTRODUCTION

Existing bone is renewed through bone remodeling, which involves removal of old bone (bone resorption) by multinucleated osteoclasts, and the formation of new bone by osteoblasts (bone formation). Studies based on histomorphometry have shown that early osteoblast lineage cells are recruited to freshly eroded bone (Abdelgawad et al., 2016; Kristensen et al., 2014; Andersen et al., 2013; Delaisé, 2014; Everts et al., 2002; Lassen et al., 2017) and are intermixed with osteoclasts on eroded surfaces (Lassen et al., 2017). These cells are classically called ‘reversal cells’ (Dempster et al., 2013). They were recently shown to contribute to a ‘mixed reversal-resorption’ phase rather than to a pure reversal phase as it is usually presented (Dempster, 2017; Lassen et al., 2017). The identification of this mixed reversal-resorption phase has helped clarifying how the anabolic signals of the osteoclasts are transferred to the osteoblast lineage cells, thereby promoting their differentiation into bone-forming osteoblasts. In addition to the anabolic activity of the reversal cells, which involves secretion of regulating factors that prevent bone resorption but promote bone formation, it has been suggested that these cells also have catabolic activities during bone remodeling (Bord et al., 1996; Delaissé et al., 2003; Mulari et al., 2004; Domon et al., 2001; Abdelgawad et al., 2016; Andersen et al., 2013; Delaissé, 2014; Everts et al., 2002). Reversal cells on eroded bone surfaces next to osteoclasts have been shown to produce a collagenolytic matrix metalloproteinase (MMP) identified as MMP-13 (Andersen et al., 2004; Fuller and Chambers, 1995; Nakamura et al., 2004; Abdelgawad et al., 2016; Delaissé et al., 2003). MMP-13 can cleave the triple helix of type I collagen, and MMP activity was shown to be the collagenase that reversal cells use to remove collagen debris from eroded surfaces (Everts et al., 2002; Mulari et al., 2004), a process that is important to allow subsequent bone formation (Everts et al., 2002; Mulari et al., 2004). However, it is still unknown whether there is a direct involvement of reversal cells in osteoclastic bone resorption. In the case of murine cathepsin K-knockout models, it seems that cathepsin K deficiency induces the expression of proteinases such as MMP-13 in osteoblast lineage cells (Kiviranta et al., 2005). Under such circumstances, it might be that osteoblast lineage cells can contribute to osteoclastic bone resorption and this possibly explains why the bone phenotype of pycnodysostosis patients or cathepsin K-knockout mice is not more severe.

So far, various studies have investigated the involvement of reversal cells and MMP-13 in the ‘cleaning’ process following bone resorption (Abdelgawad et al., 2016; Domon et al., 2001; Everts et al., 2002). However, knockout experiments show that MMP-13 is rate-limiting for bone resorption itself (Inada et al., 2001), and the presence of MMP-13 in the sub-osteoclastic resorption zone (Andersen et al., 2004) strongly suggests that reversal cells/osteoblast lineage cells might directly contribute to collagen degradation during osteoclastic bone resorption itself. Our current study further investigates this hypothesis.

Towards this aim, we established co-cultures of osteoclasts and osteoblast lineage cells using primary cells from a human source in an attempt to mimic the human in vivo microenvironment. We used human osteoblast lineage cells isolated directly from human bone outgrowths as a model for reversal cells and co-cultured them with osteoclasts derived from human peripheral blood monocytes. We found that in the absence of serum and recombinant receptor activator of nuclear factor κB ligand (RANKL, also known as TNFSF11), osteoclastic bone resorption was stimulated by the presence of osteoblast lineage cells and especially favored osteoclasts making trenches. Furthermore, we found that a
significant fraction of this stimulation was mediated through MMP activity originating from the osteoblast lineage cells.

RESULTS

Osteoblast lineage cells expressing MMP-13 are closely associated with multinucleated bone-resorbing osteoclasts

Human osteoclasts derived from peripheral blood and osteoblast lineage cells derived from human trabecular bone outgrowths were seeded on cortical bovine bone slices and co-cultured for 72 h in the absence of serum and recombinant RANKL. Chromogenic in situ hybridization (CISH) was used to visualize the collagen type I mRNA-expressing osteoblast lineage cells (Fig. 1A), intermixed with pre-osteoclasts/osteoclasts devoid of such an expression (Fig. 1A, marked with blue and black arrows, respectively). In comparison to collagen type I expression, the expression of MMP-13 was only detected in a fraction of the mono-nucleated cells (Fig. 1B). At higher magnification, strong expression of MMP-13 was detected in mono-nucleated cells (Fig. 1C, dark blue arrows) closely associated to multinucleated osteoclasts (Fig. 1C, black arrow) but also within the resorption cavity. No background staining was detected in the negative control (Fig. 1D). Based on previous reports (Delaissé et al., 2002; Abdelgawad et al., 2016; Andersen et al., 2004; Everts et al., 2002), it is reasonable to conclude that the MMP-13-positive cells represent a fraction of the osteoblast lineage cells. This conclusion is consistent with real-time reverse transcription quantitative PCR (RT-qPCR) analyses showing that MMP-13 expression is not detected in osteoclast cultures, but is clearly present in the osteoblast lineage cells (data not shown). Thus, based on this knowledge and observations such as shown in Fig. 1C, we conclude that the osteoblast lineage cells express MMP-13 and are in close proximity to osteoclasts in the co-culture model as also observed in vivo (Abdelgawad et al., 2016; Andersen et al., 2004; Delaissé et al., 2003).

Osteoblast lineage cells are attracted to actively bone-resorbing osteoclasts resulting in close contact

In order to better visualize the close interaction between bone-resorbing osteoclasts and osteoblast lineage cells, we used multiplex fluorescence in situ hybridization (FISH) to label both MMP-13-expressing osteoblast lineage cells and TRAcP5 (also known as ACP5)-expressing osteoclasts and analyzed them by confocal microscopy. Fig. 2A clearly shows a MMP-13-expressing osteoblast lineage cell in close contact with a TRAcP5-positive bone-resorbing multinucleated osteoclast. In fact, the osteoblast lineage cell is within the resorption cavity and underneath the osteoclast (Fig. 2A). Time-lapse recordings were used to investigate whether osteoblast lineage cells end up in such close contacts with osteoclasts by chance or due to a specific attraction. Fig. 2B and Movie 1 shows an example of such time-lapse recording and highlights that osteoblast lineage cells are indeed attracted to actively resorbing osteoclasts. Note that the osteoblast lineage cells end up in close contact with the osteoclast through a clear directional movement (as shown in Fig. 2A). Movie 2 shows an enlargement of the recording, showing the labeled cells in order to better visualize the movements of the osteoblast lineage cells.

Osteoblast lineage cells promote osteoclastic bone resorption – especially those making trenches

In order to examine the effect of osteoblast lineage cells on bone resorption, osteoclasts from one donor and osteoblast lineage cells from another donor were cultured in the absence of RANKL and serum on cortical bone for 72 h. Fig. 3 shows a representative result of such an experiment where the eroded surface (excavations) relative to bone surface was quantified for four different endpoints. First, the total eroded surface, which showed a significant increase...
by 3.4-fold in co-cultures compared to osteoclast mono-cultures (Fig. 3A). The total eroded surface was subsequently split into eroded surfaces made by osteoclasts in pit- or trench-mode (Søe and Delaissé, 2010; Søe and Delaissé, 2017). Second, the pit-surface relative to bone surface, which showed a significant increase, but only by 1.4-fold in co-cultures compared to mono-cultures (Fig. 3B). Third, we assessed the trench surface relative to bone surface; this showed a strong significant increase by 9.1-fold (Fig. 3C). Finally, the trench surface relative to total eroded surface: as a consequence of the results shown in Fig. 3B,C the relative contribution of trenches to the total eroded surface increased significantly from 28% in mono-cultures to 70% in co-cultures (Fig. 3D). Fig. 3E shows representative images of resorption pits and trenches in mono- and co-cultures in the absence of serum and RANKL. While Fig. 3 only shows results from a single experiment, Fig. 4 shows that these results are clearly reproduced in 13 independent experiments. The total eroded surface is systematically increased in co-cultures (on average by 4.9-fold) (Fig. 4A), while osteoclast-mediated pit formation on average was stimulated by 4.3-fold (Fig. 4B), and trench formation increased by 8.8-fold (Fig. 4C) in the presence of osteoblast lineage cells. In 11 out of the 13 experiments, we found a selective stimulation of osteoclasts in trench mode, resulting in a significant average increase in trench surface relative to eroded surface of 2.4-fold (Fig. 4D). When comparing mono- and co-cultures in the presence of serum and recombinant RANKL, we did not observe any stimulation of osteoclastic bone resorption by the osteoblast lineage cells (data not shown).

Osteoblast lineage cells cause osteoclasts to make larger trenches and in general increase the number of resorption events

The increased bone resorption activity of osteoclasts in co-culture with osteoblast lineage cells shown in Figs 3 and 4 could be due to: (1) an increased number of resorption events, (2) an increased size, or (3) both. Therefore, a systematic evaluation of the number of resorption events appearing as pits and trenches was conducted. The total number of resorption events was found to be significantly increased in co-cultures compared to mono-cultures (Fig. 5A). The number of pits was only increased 1.5-fold (Fig. 5B) while the number of trenches was increased 3.5-fold (Fig. 5C). By measuring
the average length of trenches per bone slice, we found that the length was increased significantly in the co-culture, by 1.2-fold (Fig. 5D). Furthermore, we combined the information on the eroded bone surfaces covered by pits and trenches with the number of resorption events on the same bone slices. In this way, we estimated that the size of pits was not different in co-cultures to that in mono-cultures (Fig. 5E), but that there was clearly an increase in the case of trenches (Fig. 5F). Thus, we conclude that the presence of osteoblast lineage cells increases the number of pits but not their size, while it both increases the number of trenches as well as their length/size.

It has been shown in many studies that expression of RANKL by osteoblast lineage cells stimulates osteoclastic bone resorption (Burgess et al., 1999; Fuller et al., 1998; Lacey et al., 1998). Owing to the inhibitory effect of osteoprotegerin (OPG) on the resorptive activity of osteoclasts in our co-cultures we can confirm these findings (Fig. 6). Interestingly, inhibition of RANKL signaling by treatment with OPG only suppressed the elevated bone resorption by 80% [Fig. 6A,B, compare OC (osteoclast mono-culture) with OC+OB (osteoclast and osteoblast co-culture)+OPG]. This suggests that factors other than RANKL may contribute to the stimulation of bone resorption in presence of osteoblast lineage cells.

Osteoblast lineage cells stimulate osteoclastic bone resorption through action of MMPs

We found that osteoblast lineage cells in close contact with bone-resorbing osteoclasts express MMP-13 (Figs 1 and 2). Therefore, we compared the effect of GM6001 (a broad MMP inhibitor) on

![Fig. 3. Osteoblast lineage cells promote osteoclastic bone resorption in vitro in the absence of recombinant RANKL and serum.](image)

Human osteoclasts (OC) from one donor were cultured on bovine cortical bone slices (n=5) in the presence or absence of osteoblast lineage cells (OB) (each obtained from separate donors) for 72 h (in the absence of recombinant RANKL and serum). (A–D) Data showing different bone resorption endpoints. The percentage of (A) total eroded surface relative to bone surface (BS), (B) pit surface relative to BS, (C) trench surface relative to BS, and (D) trench surface relative to eroded surface (ES) is shown. (E) Representative images of resorption cavities in mono- and co-cultures (*, trenches; #, pits). Scale bars: 100 µm. **P=0.0079 (A); *P=0.0397 (B); **P=0.0079 (C); **P=0.0079 (D) (two-tailed Mann–Whitney test).

![Fig. 4. Stimulated bone resorption in the presence of osteoblastic lineage cells is a general property amongst the different donors/experiments.](image)

Human osteoclasts (OC) obtained from different blood donors (n=13) were cultured on bovine cortical bone in the absence and presence of osteoblastic lineage cells (OB) obtained from different bone donors (n=10). (A–D) Different resorption endpoints were analyzed. Each pair of dots on the graphs represent single experiments (n=13) and show the mean of the measurements obtained from four to five bone slices for each culture. Data obtained from the same experiment are connected with lines. The percentage of (A) total eroded surface relative to bone surface (BS), (B) pit surface relative to BS, (C) trench surface relative to BS, and (D) trench surface relative to eroded surface (ES). ***P=0.0002 (A); ***P=0.0007 (B); ***P=0.0002 (C); **P=0.0012 (D) (two-tailed Wilcoxon matched-pairs signed rank test).
osteoclastic bone resorption in mono- and co-cultures. Data from one experiment is presented in Fig. 7A–D, while representative images of bone resorption excavations in the presence or absence of GM6001 are shown in Fig. 7E. Bone resorption was not significantly affected by GM6001 in the osteoclast mono-culture (Fig. 7A–D, OC). Interestingly, however, under co-culture conditions, GM6001 significantly reduced the total eroded surface relative to bone surface by 85% (Fig. 7A), pit surface relative to bone surface by 75.8% (Fig. 7B) and trench surface relative to bone surface by 89% (Fig. 7C). No significant effect was observed with respect to trench surface relative to eroded surface (Fig. 7D). The inhibitory effect of GM6001 on bone resorption only in the presence of osteoblast lineage cells was confirmed in eight different experiments (Fig. 8A–C), while it was also confirmed that the trench surface relative to eroded bone surface was unaffected (Fig. 8D). Finally, the inhibitory effect of GM6001 on bone resorption in co-culture could only be observed on native bone, but no longer on inorganic bone (Fig. S1). All in all, these results suggest that MMPs (possibly MMP-13) delivered by osteoblast lineage cells directly contribute to collagenolysis during osteoclastic bone resorption.

**DISCUSSION**

Osteoclastic bone resorption is a complex process, and studies using bone explants have demonstrated that cysteine proteinases and MMPs are involved in osteoclastic bone resorption (Everts et al., 1992; Everts, 1998; Delaissé et al., 2003). However, in isolated osteoclast cultures, only inhibition of cysteine proteinases decreases bone resorption and not inhibition of MMPs (Delaissé et al., 1987). Furthermore, bone explants from MMP-13-knockout mice were found to have diminished bone resorption (Inada et al., 2001). Another study reported that the murine osteoclastic cell line MC3T3 renders bone resorption of rabbit osteoclasts sensitive to MMP inhibition (Delaissé et al., 2002). It has also been shown that osteoblast lineage cells juxtaposed to osteoclasts express MMP-13, and that MMP-13 protein is delivered directly to the resorptive site underneath the osteoclast (Andersen et al., 2004). Taken together, these data suggest that MMPs originating from osteoblast lineage cells directly contribute to osteoclastic bone resorption and that especially MMP-13 might be involved (Delaissé et al., 1993, 2003; Fuller and Chambers, 1995; Nakamura et al., 2004; Delaissé, 2014).

In vivo hybridization (ISH) and time-lapse recordings show that osteoblast lineage cells are selectively attracted to bone-resorbing osteoclasts and end up in direct contact. This close contact results in a strongly increased osteoclastic bone resorption. In addition, a detailed analysis of resorption excavations highlighted that this was caused by an increased size of trenches and a general increase in the number of resorption events. However, most importantly, we found that this stimulation of osteoclastic bone resorption by osteoblast lineage cells could be inhibited by an MMP inhibitor while not affecting osteoclast mono-cultures.

In vitro, osteoclasts are surrounded by osteoblast lineage cells, such as reversal cells, canopy cells and bone-lining cells. When an osteoclast has left behind an eroded surface, these cells colonize the eroded surface (Abdelgawad et al., 2016; Andersen et al., 2013; Delaissé, 2014; Everts et al., 2002; Kristensen et al., 2014). Some studies have suggested that these cells are phagocytic macrophage-like cells, owing to their ability to phagocyte collagen, or they were suggested to be mono-nucleated osteoclasts (Eriksen et al., 1984). Recent studies have, through immunohistochemical staining, identified these cells as osteoblast lineage cells (Abdelgawad et al., 2016; Andersen et al., 2013; Lassen et al., 2017).
1997; Abdelgawad et al., 2016; Everts et al., 2002) and they were suggested to be able to digest collagen. Although the possible contribution of osteoblast lineage cells in bone resorption has been addressed in several studies (Delaissé et al., 2003, 2002; Everts et al., 2002; Holliday et al., 1997), a functional study based on human cells was still lacking. We herein use human primary osteoclasts and osteoblast lineage cells, and find a strong significant impact of osteoblast lineage cells on osteoclastic bone resorption.

Since we do not add recombinant RANKL to our cultures, one could think that the stimulatory effect of bone resorption was caused solely by RANKL produced by the osteoblast lineage cells. The interaction of RANKL, produced by osteoblasts, and its receptor, RANK (also known as TNFRSF11A), is a strong inducer of osteoclastogenesis as well as bone resorption and osteoclastic migration (Henriksen et al., 2003; Wada et al., 2006; Burgess et al., 1999; Everts et al., 2002). We herein show that inhibition of RANKL by OPG significantly reduced the stimulatory effect of osteoclastic bone resorption. Nevertheless, the presence of RANKL inhibitor did not fully inhibit the elevated bone resorption in the presence of osteoblast lineage cells. Therefore, it would be expected that the addition of osteoblast lineage cells to osteoclast cultures would affect the resorptive activity in response to MMP inhibition. Taking all of these observations together, and the results from the present study, supports the hypothesis that the resorptive activity is sensitive to MMP inhibition only in the presence of osteoblast lineage cells. MMP-13 is able to cleave the native triple helix of collagen molecules at one site into a three-quarter and one-quarter length fragment (Billinghurst et al., 1997). However, other studies performed with cells directly isolated either from calvaria or long bones suggest that osteoclasts from calvarial bone depend on MMP activity for collagen degradation while the action of osteoclasts from long bones is independent of MMP activity (Everts et al., 1999). However, it was not addressed whether this result may have been influenced by the possible contribution of non-osteoclastic cells to osteoclastic resorption. Other studies suggest that MMP-13 could be...
involved in initiation of bone resorption (Holliday et al., 1997) whereas others suggest that MMP-13 could be involved both before and after bone resorption by osteoclasts (Everts et al., 2002). However, in the present study, we show that MMPs from osteoblast lineage cells (i.e. MMP-13) are also involved in collagenolysis during the bone resorption process itself.

An earlier study has stressed, in a systematic analysis of mice and rabbit bone, that reversal cells next to osteoclasts expressed MMP-
13 mRNA and that the MMP-13 protein diffused into the sub-
osteoclastic resorption zone (Andersen et al., 2004). Moreover, other studies using electron microscopic examination of resorbing osteoclasts showed reversal cells extending beneath osteoclasts and that are thus in direct contact in vivo (Abdelgawad et al., 2016). The present study was able to repeat these observations in vitro showing that MMP-13-positive osteoblast lineage cells are not only in close proximity to osteoclasts but are also located underneath them. This, together with previous reports (Abdelgawad et al., 2016; Everts et al., 2002), indicates that this close interaction between osteoblast lineage cells and osteoclasts is important for the proper activity of osteoblastic MMPs. Furthermore, time-lapse recording of a resorbing osteoclast revealed that osteoclast lineage cells sometimes actively follow the resorbing osteoclasts. It is of course interesting to investigate what may trigger the osteoblast lineage cells to follow the osteoclasts. Osteoclasts produce cathepsin K as TRACP that can affect neighboring cells like osteoblast lineage cells and may trigger them to follow the resorbing osteoclasts (Metz-Bruck et al., 2012; Sheu et al., 2003; Karsdal et al., 2007), but a direct haptotactic attraction to collagen type I is also a possibility (Abdelgawad et al., 2014). However, further investigations are needed to address this issue in more detail.

In our resorption assays, we observed two types of differently shaped resorption cavities, pits and trenches. The variation in shapes of excavations has been observed in several studies both in vitro and in vivo (Gentzsch et al., 2003; Rumpler et al., 2013; Mosékilde, 1990; Jones and Boyde, 1994). However, it was only in recent studies (Søe et al., 2013; Søe and Delaissé, 2017) that these were characterized and it was demonstrated that they reflect two different resorption modes of osteoclasts, intermittent in pit-mode and continuous in trench-mode. Herein, we show that the presence of osteoblast lineage cells mainly favors osteoclasts conducting continuous resorption appearing as trenches. The elongated trench excavations are characterized by the absence of collagen remnants, long duration of resorption and high erosion speed – all reflecting high levels of collagenolysis. In contrast, pits reflect an intermittent resorption with collagen remnants (Panwar et al., 2016; Søe et al., 2013; Søe and Delaissé, 2010, 2017). Finally, we show that osteoclastic bone resorption performed in co-culture with osteoblast lineage cells is only sensitive to GM6001 on native, but not on inorganic, bone. Taking all of these observations together, one possible explanation for this finding may be that the osteoblastic MMPs act to slightly increase the rate of collagenolysis and thereby balancing it with the rate of demineralization, since this is known to favor trench formation (Søe et al., 2013). Moreover, we also found that co-cultures resulted in longer trenches. Since trenches are characterized by high erosion speed (Søe and Delaissé, 2017), the extension of trenches herein may be due to an increased resorption speed or an earlier onset of trench formation in co-cultures compared to in-cultures.

In conclusion, we find that addition of osteoblast lineage cells to osteoclast cultures stimulates bone resorption by favoring the trench resorption mode. This stimulation was inhibited in the presence of MMP inhibitor, and thereby suggests that MMPs of osteoblast lineage cells contribute directly to osteoclastic bone resorption.

**MATERIALS AND METHODS**

**Generation of osteoclasts and osteoblast lineage cells**

**Generation of osteoclasts**

Human CD14<sup>+</sup> cells were isolated from blood donors as previously described (Møller et al., 2017) (approved by the local ethical committee, 2007-0019; written consent obtained from each donor) and seeded at a density of 5 × 10<sup>6</sup> cells/T75 culture flask (Greiner, InVitro, Fredensborg, Denmark) with α-MEM (Invitrogen, Taastrup, Denmark) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, F9665-500 ml), 25 µg/ml human macrophage-colony stimulating factor (M-CSF) (R&D Systems, Abingdon, UK). The cells were cultured for 2 days at 37°C and 5% CO<sub>2</sub> in a humidified incubator. Thereafter, they were differentiated into mature osteoclasts through addition of 25 ng/ml RANKL and 25 ng/ml M-CSF (R&D System, Minneapolis, MN) for 7 days and the medium was changed twice.

**Generation of osteoblast lineage cells**

Human osteoblast lineage cells were obtained from bone of osteoarthritis patients undergoing hip replacement surgery (approved by the local ethics committee, S-2011-0114) as previously described (Galagher, 2013). Informed consent was obtained for all tissue donors. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. In brief, bone was cut into smaller pieces and cleaned with PBS twice through vigorous shaking. Five to six bone slices were carefully placed in each well of 12-well plates in DMEM (Invitrogen) containing 10% FBS, 2 mM L-glutamine, 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 10<sup>−8</sup> M dexamethasone with a metal grid placed on top of the bone slices to prevent them from moving. The plates were placed in an incubator for 7 days at 37°C and 5% CO<sub>2</sub>. After 7 and 14 days, the medium was renewed and the outgrowth cells from the surface of the bone fragments started to migrate from the bone and onto the culture plate (after 7–10 days of culture). The metal grid and the bone pieces were removed after 14 days of culture whereas the medium was change twice a week. The outgrowths were cultured until they reached near confluency (a total of ~35 days).

**Bone resorption assay**

Matured osteoclasts (~50,000 cells/96-well) were seeded on 0.4-mm-thick bovine cortical bone slices (BoneSlices.com, Jelling, Denmark) with α-MEM containing 25 ng/ml M-CSF for 4 h. Subsequently, osteoblast lineage cells (~12,500 cells/96-well, for co-culture) or only medium (for mono-culture) were seeded on top of osteoclasts in the presence or absence of the MMP inhibitor GM6001 (6 µM) (Merck Life Science, Hellerup, Denmark). For each experiment, four or five bone slices were used, as indicated in the figure legends, and the resorption excavations were visualized by means of TRACP, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. For Fig. S1, eight bone slices were used per condition. NaOCl treatment of bone slices for Fig. S1 was performed as previously described (Søe et al., 2013). The total eroded surface, subdivided into pit and trench surfaces, was evaluated as previously reported (Søe and Delaissé, 2010). Pits were characterized as round excavations with well-defined edges where the ratio between length and width was less than two. Trenches were defined as elongated excavations with well-defined edges that were at least twice longer than wide and with clear signs of continued resorption. The prevalence of trenches was also calculated as percentage trench surface relative to total eroded surface. The total number of pits and trenches was determined as described previously through a random systematic count (Merrild et al., 2015). The average length of trenches was measured on 100 trenches per bone slice as previously described (Vanderoost et al., 2013) using CellensEntry 1.7 software (Olympus Corporation, Shinjuku, Tokyo, Japan). The calculated size of trenches and pits was calculated by relating the percentage of eroded bone surface to the number of resorption events.

**In situ hybridization**

**Singleplex chromogenic ISH**

Mature osteoclasts were cultured with osteoblast lineage cells on 0.2 mm bovine cortical bone slices for 12 h as explained in the section entitled ‘Bone resorption assay’. ISH analysis was performed using the BaseScope in situ hybridization protocol (product number: 323900, Advanced Cell Diagnostics, Hayward, CA), as previously described (Abdelgawad et al., 2016). Cells were fixed with 3% paraformaldehyde and 2% sucrose diluted in PBS for 30 min at room temperature and pretreated with a blocker of endogenous peroxidases (pretreatment I) and proteinase III (pretreatment III). The pretreated cells were hybridized overnight at 40°C with 20ZZ probes.
specifically targeting either human MMP-13 mRNA (7003731, Advanced Cell Diagnostics) or human collagen type I mRNA (705321, Advanced Cell Diagnostics) diluted 1:1 with probe diluent (300041, Advanced Cell Diagnostics), or with probe diluent alone (negative control). The amplification was conducted according to instructions by the supplier and probe signals were visualized using Liquid Permanent Red (K0640, DAKO, Glostrup, Denmark) and stained with hematoxylin to visualize the nuclei (Merck Millipore, Denmark). Finally, images were acquired using an Olympus BX-50 microscope with an UC30 Olympus camera.

Multiplex fluorescent ISH
As described in the section entitled ‘Bone resorption assay’, the mature osteoclasts were co-cultured with osteoblast lineage cells on 0.2 mm bovine cortical bone slices for 72 h. The multiplex fluorescent ISH was performed using a modified version of that described in the supplier’s user manual (RNAscope Multiplex Fluorescent v2 assay, Advanced Cell Diagnostics). Cells were fixed and pretreated as described for singleplex chromogenic ISH. The pretreated cells were hybridized overnight at 40°C with 20Z2Z probes specifically targeting both human MMP-13 (Channel 3 probe, 703731, Advanced Cell Diagnostics) and TRACP5 (Channel 1 probe, 427211, Advanced Cell Diagnostics) mRNA mixed 1:1. In accordance with the instructions provided by the supplier, both probes were amplified together in three steps followed by a sequential florescence labeling of each channel. Channel 1 was labeled with TSA Plus Fluorescein (NEL741E001KT, PerkinElmer, Skovlunde, Denmark) diluted 1:1500, and channel 3 was labeled with TSA Plus Cyanine 3 (NEL744E001KT, PerkinElmer) diluted 1:1500. Samples were mounted with ProLongGold containing DAPI (P36931, Molecular Probes) and covered by glass coverslips and sealed with white nail polish. The confocal images were obtained using an Olympus Fluoview FV10i1 microscope (Olympus Corporation) in z-stack mode. The confocal aperture was set at 1.0 (z-stack depth: 12.2 µm) using a 60× objective. Images were evaluated using Imaris version 7.6.5 (Bitplane AG, Zürich, Switzerland).

Time-lapse recording of cells in co-culture
Time-lapse recording was performed as previously described (Søe and Delaissé, 2017). Osteoblast lineage cells and osteoclasts were seeded on 0.4-mm-thick bone slices as mentioned in the section entitled ‘Bone resorption assay’ with αMEM containing 25 ng/ml M-CSF, 100 nM SiR-actin and 10 µM verapamil for 4 h. Subsequently, these bone slices were transferred to a chambered cover-glass (Nunc Lab-Tek II, ThermoFisher Scientific, Waltham, MA) and placed upside down as previously described and transferred to a chambered cover-glass (Nunc Lab-Tek II, ThermoFisher Scientific, Waltham, MA) and placed upside down as previously described (Søe and Delaissé, 2017) with αMEM containing M-CSF, SiR-actin and verapamil as aforementioned. Time-lapse images were taken with an Olympus Fluoview FV10i1 microscope (Olympus Corporation) as described previously (Søe and Delaissé, 2017).

Statistical analysis
All graphs and statistical analyses were performed using GraphPad Prism software version 6 (GraphPad Software, LA Jolla, CA). The level of significance was set for P<0.05. The normal distribution of datasets was tested through a D’Agostino and Pearson omnibus normality test. The following statistical tests were used: Mann–Whitney test (Figs 3, 5, 6 and 7), Wilcoxon matched-pairs signed rank test (Figs 4 and 8), and one-way ANOVA Sidak’s multiple comparisons test (Fig. S1). All P-values are based on two-tailed P-values.

Acknowledgements
We thank Kaja Søndergaard Laursen for excellent assistance with in situ hybridization and Jane Schwartz Leonardt for excellent collaboration on recruiting patients to donate bone used for generating primary osteoblast lineage cells.

Competing interests
The authors declare no competing or financial interests.

Author contributions

Funding
This work was supported by the Region of Southern Denmark (grant number 13/ 27663), Vejle Hospital/Lillebaelt Hospital and the University of Southern Denmark.

Supplementary information
Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.229351.supplemental

References


Fig. S1. MMPs from osteoblast-lineage cells only stimulate osteoclastic resorption on collagen-containing substrate. Data show a representative experiment of two, where the effect of 6 µM GM6001 (GM) on osteoclastic bone resorption ("OC" osteoclasts) was tested in the presence of osteoblast-lineage cells (OB) when seeded on bone or NaOCl-treated bone. The latter removes all organic components including collagen. Bone resorption was performed over 72h. ES/BS: eroded surface/bone surface. Bars show the mean ± SD, n=8 (except for "bone:OC+OB+BM" where n=7 because an outlier was removed based on Grubbs' test α=0.05). Statistics: one-way ANOVA and Sidak’s multiple comparisons test.
Movie 1

Movie 1 shows the time-lapse recording from which snapshots shown in Fig. 2B were taken. Time-lapse recording was made with using a 10x objective with a confocal aperture of 2.0 (z-plane depth of 21.2 µm). The total recording time was 70h showing and images were taken every 21 min. The movie shows a resorbing osteoclast making a trench that is successively caught up by at least two osteoblast-lineage cells. The trench-making osteoclast can be recognized by the crescent shaped actin ring at the leading edge and is marked with a white arrow. The osteoblast-lineage cells can be recognized by the presence of stress fibers and are marked by white and blue arrows. These arrows move just prior to the migration of osteoblast-lineage cells towards the osteoclast. The bone surface is labeled with rhodamine and cells are labeled with SiR actin (stains f-actin).
Movie 2

Movie 2 shows an enlargement of the video showing the SiR actin labeled cells from Movie 1 to better visualize the directed movement of osteoblast-lineage cells onto the bone resorbing osteoclast.