Bone-site-specific responses to bisphosphonates

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Chapter 6.
Migration, fusion, and CXCL12-CXCR4-mediated chemoattraction of long bone and jaw osteoclast precursors

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Abstract

Bone is important to give the body support, but it also contains marrow that harbors mesenchymal stem cells and hematopoietic progenitors. An important chemoattractant for hematopoietic cell homing towards the bone marrow, CXCL12, was shown to inhibit expression of apoptosis related genes. Previously, it was shown that jaw osteoclast precursors express more anti-apoptotic genes than long bone osteoclast precursors. Also, jaw and long-bone marrow cells have a different osteoclastogenic potential. Here, we used time-lapse microscopy to follow long bone and jaw osteoclast precursors during three important steps of osteoclastogenesis, i.e. proliferation, migration, and fusion. Gene expression analyses were performed on markers of the different steps of osteoclastogenesis, and the directional migration of jaw and long-bone osteoclast precursors towards CXCL12 was studied. Long bone and jaw osteoclast precursors had similar rates of proliferation, random and directional migration, and fusion. Jaw osteoclast precursors expressed more CXCL12 and its receptors CXCR4 and CXCR7 than long bone osteoclast precursors. Therefore, we provide more evidence that osteoclast precursors are bone-site specific. Higher CXCL12 expression may explain the higher anti-apoptotic gene expression that was previously found in jaw cells than in long bone cells.

Introduction

Bone is continuously remodeled throughout life to keep it as strong as possible. Bone formation is carried out by osteoblasts derived from mesenchymal cells, whereas osteoclasts from hematopoietic origin are the bone-resorbing cells. When bone resorption is needed, osteoclast precursors are attracted from the blood or from the bone marrow and they migrate towards the sites where resorption has to take place. Upon contact with osteoblasts they fuse to become multinucleated osteoclasts due to stimulation by the cytokines macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) [1].

Several specialized cytokines called chemoattractants have been shown to be involved in the attraction of osteoclast precursors [2,3]. Also, the chemokine stromal cell-derived factor-1α (SDF-1α or CXCL12) and its receptor CXCR4 were shown to be involved in osteoclast precursor recruitment [4,5]. The expression of CXCL12 by stromal cells and the subsequent attraction of hematopoietic cells towards the bone marrow [6] can be counteracted by sphingosine-1-phosphate (S1P), a lipid that attracts osteoclast precursors, found at high concentrations in peripheral blood [7,8]. Moreover, CXCL12 has been associated with cell proliferation, cell survival, and tumor metastasis [4,9,10].

CXCL12 may play a prominent role in diseases such as bone cancer and osteoporosis, where excess of osteoclast activity is observed, leading to loss of bone mass and fragile bones. Bisphosphonates (BPs) such as pamidronate can be used to inhibit excessive bone resorption by causing osteoclast apoptosis and inhibiting their resorbing activity [11]. A specific type of BPs containing nitrogen (N-BPs) also inhibits the prenylation of small GTPases [12,13]. As these proteins are important for the cytoskeletal rearrangements necessary during adhesion and migration, it is likely that osteoclast precursor migration is affected by N-BPs [14,15].

Previously, we have shown that osteoclasts and their bone marrow precursors are different in long bones and jaws [16,17]. Long bone and jaw osteoclast morphology were shown to differ *in vitro* [18] and long bone osteoclasts differentiated faster than jaw osteoclasts, which was likely related to the cellular composition of the bone marrows [17,19]. Also, bone marrow cells from the jaw take up more BPs than those from long bone. Interestingly, this did not differently affect the number of osteoclasts formed from both cell types, indicating that the jaw osteoclasts or precursors may be less sensitive to BPs [20]. Here, we investigated whether osteoclast precursors from the jaw can compensate for cytotoxicity by forming more osteoclasts. Therefore, we studied the proliferation, migration and fusion of osteoclast precursors from jaw and long bones by time-lapse microscopy. Also, we compared the expression of CXCR4, CXCR7, and CXCL12, and studied the directional migration of long bone and jaw bone marrow cells towards CXCL12. With this study, we aim

to get more insight into the differences between long bone and jaw osteoclasts and their precursors regarding (i) proliferation, (ii) migration, and (iii) fusion, and the genes regulating these processes.

Materials and methods

Bone marrow cell isolation and cell culture

Animal experiments were approved by the Animal Welfare Committee of the VU University (Amsterdam, The Netherlands). Bone marrow cells were isolated from the mandibles and long bones (tibia and femur) as described previously [20]. Freshly isolated bone marrow cells were seeded and incubated with 150 µl α-MEM (GIBCO, Paisley, UK) complete culture medium with 5% fetal calf serum (FCS; Hyclone, Logan, UT, USA) and 1% antibiotic antimycotic solution containing 30 ng/mL recombinant mouse macrophage-colony stimulating factor (rmM-CSF; R&D Systems, Minneapolis, MN, USA) and 20 ng/mL recombinant mouse receptor activator of nuclear factor κB ligand (rmRANKL; R&D Systems) to stimulate osteoclastogenesis. For gene expression analyses, long bone and jaw bone marrow cells were seeded in 96 well plates (10^5 cells per well) and lysed after 1 or 3 days of culture. Freshly isolated bone marrow cells were also lysed for gene expression analyses and cell lysates were stored at -80°C until RNA isolation.

Time-lapse microscopy and image analyses

For time-lapse microscopy, cells ($8x10^5$ per well) were seeded in 2-well glass chamber slides (Nunc, Roskilde, Denmark) that were coated with carbon as described previously [21]. Cells were cultured for 3 days in the presence of 1 mL complete culture medium. Before live imaging started, cells were rinsed twice with PBS, the medium was replaced with fresh medium containing 10 µM pamidronate (PAM; Sigma-Aldrich, St. Louis, MO, USA) or control medium with vehicle, and this was covered with mineral oil (Sigma-Aldrich).

Cells were imaged using a Leica IR-BE (Leica Microsystems GmbH, Germany) inverted wide field microscope at 37°C in an atmosphere containing 5% CO_2. Phase contrast images, (3-5 locations per chamber) were acquired at 5, 6, or 10 minute time intervals using a x40 or x10 objective. Images were processed and analyzed using custom-made software and Image Pro Plus (Media Cybernetics, Carlsbad, CA, USA). For migration data, cell tracks were followed for 18 h after the start of imaging. The velocity was calculated by the total distance traveled divided by the time imaged, with a maximum of 18 h. For the analyses of cell division and cell fusion, cells were followed for 80 h in 3 independent experiments. Dividing and fusing cells were expressed as percentage of the total number of cells analyzed (4-23 per movie).

RNA isolation and real time quantitative PCR

RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and cDNA was synthesized using the MBI Fermentas cDNA

synthesis kit (Fermentas, Vilnius, Lithuania). Real time qPCR was performed on an ABI Prism 7000 using SYBR Green mastermix (Applied Biosystems, Foster City, CA, USA) as described previously [17], and primers are mentioned in Table 1. Gene expression was normalized for the housekeeping gene porphobilinogen deaminase (PBGD).

Table 1. Primer sequences used for real time quantitative PCR.

Gene	Primer sequence 5'→3'
PBGD	FW: AgTgATgAAAgATgggCAACt RV: TCTggACCATCTTCTTgCTgA
Ki67	FW: CAAAAGGCGAAgTggAgCTT RV: TgTTTCgCAACTTTCgTTTgTg
DC-STAMP	FW: TgTATCggCTCATCTCCTCCAT RV: gACTCCTTgggTTCCTTgCTT
TRACP	FW: gACAAgAggTTCCAggAgACC RV: gggCTggggAAgTTCCAg
NFATc1	FW: CATgCgAgCCATCATCgA RV: TgggATgTgAACTCggAAgAC
c-Fos	FW: TCACCCTgCCCCTTCTCA RV: CTgATgCTCTTgACTggCTCC
MafB	FW: AACgCgTCCAgCAgAAACAT RV: CTCAggAgAggAggggCTgT
F4/80	FW: gCACCAATgTACCAggCTCCTA RV: gATCCTTTTgCAgTTgAAgTTTCC
Runx2	FW: TgCCCAggCgTATTTCAg RV: TgCCTggCTCTTCTTACTgAg
CXCR4	FW: TggAACCgATCAgTgTgAgT RV: gggCAggAAgATCCTATTgA
CXCL12	FW: TgTgCATTgACCCgAAATTA RV: TCTCACATCTTgAgCCTCTTgT
CXCR7	FW: CTCACCgTCAggAAggCAAA RV: AggCTCTgCATAgTCAAACAAgTg

Transwell Migration Assay

The bottom chambers (24-well plate) were filled with 600 µL complete culture medium. Freshly isolated bone marrow cells were seeded into the upper chamber of cell culture inserts with 3 µm pore size (ThinCert; Greiner Bio-One, Monroe, NC, USA). Cells were incubated overnight to allow attachment to the ThinCerts. CXCL12 (R&D systems) was dissolved in 0.1% bovine serum albumin (BSA; Sigma) in PBS (0.1% PBS/BSA), filter sterilized, aliquoted, and stored at -20°C. Fresh medium was added in the bottom wells of new 24-well plates and contained the indicated concentration of CXCL12 or vehicle (PBS/BSA) as a control. ThinCerts were transferred to the wells containing fresh medium and incubated for 4 h at 37°C. The cells remaining in the upper chamber were swopped off the filter, were washed with PBS, and the bottom was fixed with 4% phosphate-buffered formaldehyde. Filters were stained with DAPI, cut from the ThinCerts, and covered with Vectashield on a microscope slide. Micrographs of 5 standardized areas were taken and analyzed using Image Pro-Plus Software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analyses

Migration was analyzed using a Kruskal-Wallis test followed by Dunn's Multiple Comparison tests. A Spearman correlation was used for the correlation between the number of cells and the velocity. Differences in cell division, fusion between long bone and jaw cells were analyzed with a Mann-Whitney U test. Gene expression was analyzed with a paired t-test. All data are expressed as means and standard deviations and differences were considered significant when $p<0.05$.

Results

Cell proliferation

An explanation why jaw osteoclasts or precursors may be less sensitive to BPs than those from long bone [20], may be that jaw osteoclast precursors exhibit an increased or prolonged precursor proliferation. We compared proliferation of long bone and jaw bone marrow cells using time-lapse microscopy and expressed the number of cells going through mitosis as a percentage of the total number of cells. Figure 1 shows the cumulative percentages at each time point, starting from day 3 of culture with M-CSF and RANKL. The majority of the dividing cells, divided within the first 18 hours of the experiment (Figure 1A). There was no difference between cell division in long bone and jaw bone marrow cells (Figure 1A). Gene expression of the proliferation marker Ki67 was also similar in long bone and jaw cells (Figure 1B). These data indicate that long bone and jaw osteoclast cultures have similar proliferation rates.

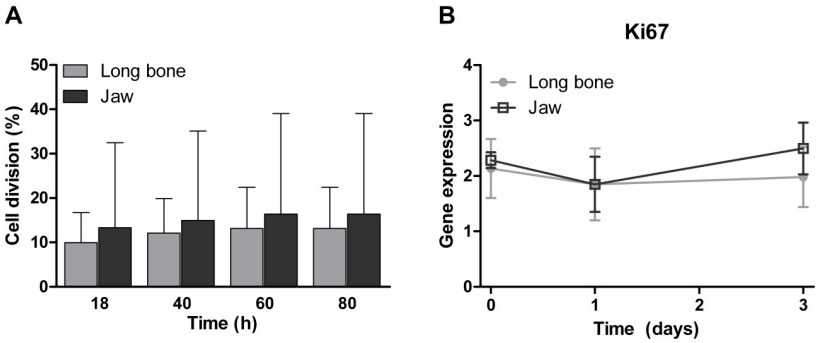

Figure 1. Long bone and jaw cell proliferation rates were similar. (A) The cumulative number of cells that had fused at the indicated time point after the start of imaging at day 3 of culture with M-CSF and RANKL, expressed as a percentage of the total number of cells in the field. A similar number of cell divisions was seen in long bone and jaw osteoclast precursors. Three to five movies (4-23 cells per movie) were analyzed in n=3 independent experiments. (B) mRNA expression of the proliferation marker Ki67 was similar in long bone and jaw bone marrow directly after isolation and after 3 days of culture with M-CSF and RANKL (n=3 for t0, n=8 for t1 and t3).

Long bone and jaw osteoclast precursors migrate at the same velocity

Using time-lapse microscopy, we investigated the random migration of long bone and jaw bone marrow cells that had been primed towards the osteoclast lineage with M-CSF and RANKL for 3 days. Cells were followed for up to 18 hours. There was no significant difference between the migration velocity of long bone and jaw osteoclast precursors (Figure 2A). The bisphosphonate PAM (10 μM) did not affect the migration velocity of both cell types (Figure 2A).

Dynamics of fusing cells

In Figure 2A, the effect of PAM on the migration velocity of all visible cells is depicted. When we distinguished between mononuclear and multinucleated cells in the control condition, multinucleated cells moved slightly slower than mononuclear cells, both in long bone and in jaw cultures (not significant, Figure 2B). Also, fusing cells, i.e. the mononuclear and multinucleated cells that fused within the time span of the experiment, moved slightly slower than the cells that did not fuse, both in long bone and in jaw cultures (Figure 2C). Furthermore, there was a negative correlation (Spearman's r_s= -0.47 and p=0.05 for long bone, Spearman's r_s= -0.45 and p=0.09 for jaw) between the number of cells per field and the velocity (Figure 2D), suggesting that in the presence of more potential fusion partners, cells may move slower. Altogether, our results suggest that cells migrate faster when they do not fuse or when fewer cells are in their vicinity. We also observed that not all cells adjacent to each other fused, whereas these cells were able to fuse with other cells, further away (Figure 3A-C), indicating that they did not reach a fusion competent status yet or that they were not fusion compatible. However, after fusion with other cells, they were in close contact with each other without fusing again (Figure 3D). These data indicate that fusion is a selective process and that active migration is necessary to come into contact with fusion competent and compatible cells.

Cell fusion

The fusion capacity of long bone and jaw osteoclast precursors was assessed in time-lapse movies from day 3 of culture in the presence of M-CSF and RANKL, until 80 hours after the start of imaging. The cumulative percentage of cells that had fused at that time point is depicted in Figure 4a. There was no difference between the fusion rate in long bone and jaw bone marrow cells (Figure 4A). The number of multinucleated cells present at the start of imaging was similar in long bone and jaw cultures (Figure 4B).

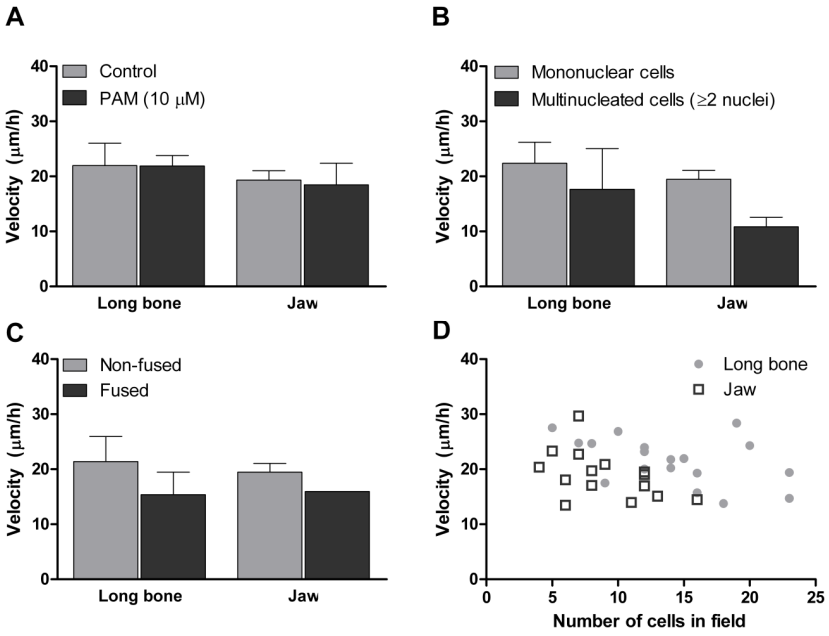

Figure 2. Long bone and jaw osteoclasts precursors migrate at a similar velocity. Migration velocity was expressed as μm/h and measured for a maximum of 18 h after the start of imaging at day 3 of culture. (A) The velocity of long bone and jaw osteoclasts and precursors was similar and unaffected by 10 μM pamidronate (PAM), as measured in 3 or 5 movies (4-23 cells per movie), performed in n=4 (control) or n=3 (PAM) independent experiments. (B) Multinucleated cells moved 1.3 times (long bone) and 1.8 times (jaw) slower than mononuclear cells (not significant, n=2-4). (C) Fusing cells (n=1 for jaw) moved 1.4 times (long bone) and 1.2 times (jaw) slower that non-fusing cells (n=3-4). (D) Negative correlation (Spearman) between the velocity and the number of cells in each field, r_s=-0.47 and p=0.05 for long bone, and r_s=-0.45 and p=0.09 for jaw.

We also measured the gene expression levels of the osteoclast marker tartrate-resistant acid phosphatase (TRACP), and dendritic cell-specific transmembrane protein (DC-STAMP), an essential fusion protein [22]. At day 3, when fusion measurements were started, long bone cells expressed significantly more TRACP and DC-STAMP than jaw cells (Figure 5A,B). This may indicate that they were further in differentiation and had a higher fusion capacity. Also, expression of the transcription factor NFATc1, was higher in the long bone cells at this time point (Figure 5C). c-Fos expression was similar in the long bone and in the jaw cells at day 3, whereas it was higher in the jaw cells at day 1 of culture (Figure 5D). Altogether, in line with previously reported data [17], these data indicate that

differentiation of long bone osteoclasts happens faster than jaw osteoclast differentiation. Yet, this was not represented by a difference in fusion rate between long bone and jaw osteoclast precursors (Figure 4A).

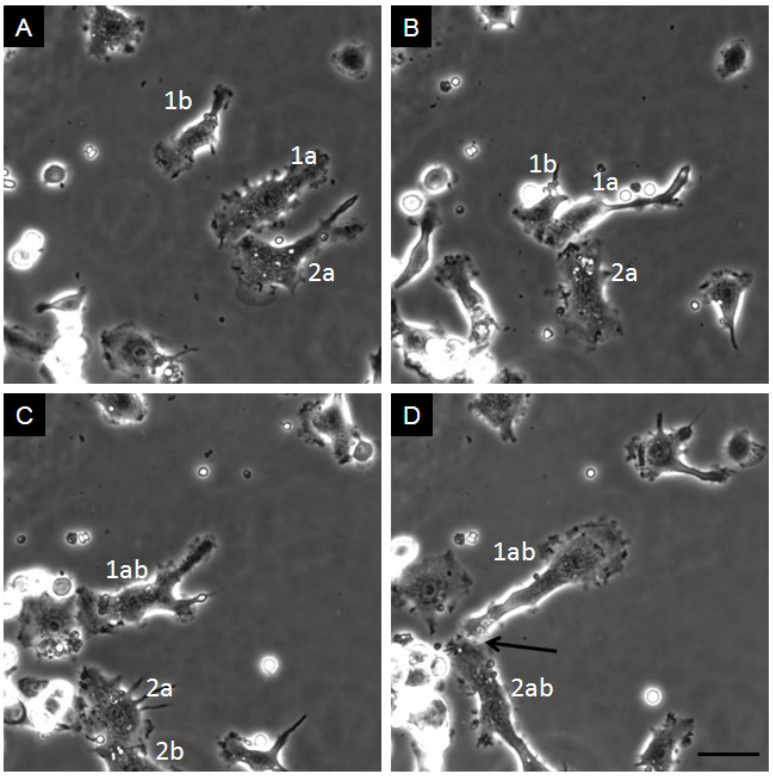

Figure 3. Selective fusion of long bone osteoclast precursors. Cells 1a and 2a are in contact (A), but do not fuse. Yet, they fuse at a later time-point where they fuse with cells 1b (B) and 2b (C), respectively. Later on, the fused cells 1ab and 2ab are in contact again without fusing (D). Bar: 20 µm.

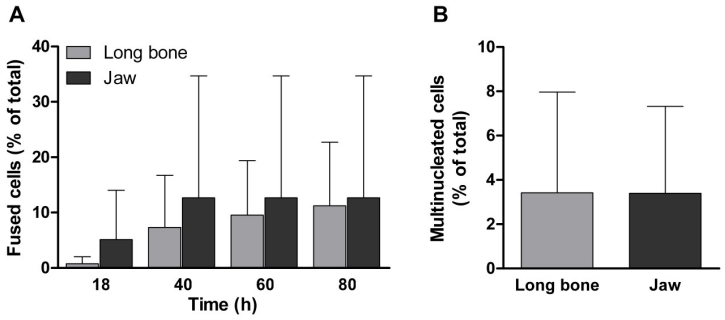

Figure 4. Long bone and jaw cell fusion rates were similar. (A) The number of cells that fused as a percentage of the total number of cells in the field at the indicated time points. (B) The percentage of multinucleated cells at the start of imaging was similar for long bone and jaw cells. 3 or 5 movies were analyzed in 3 independent experiments.

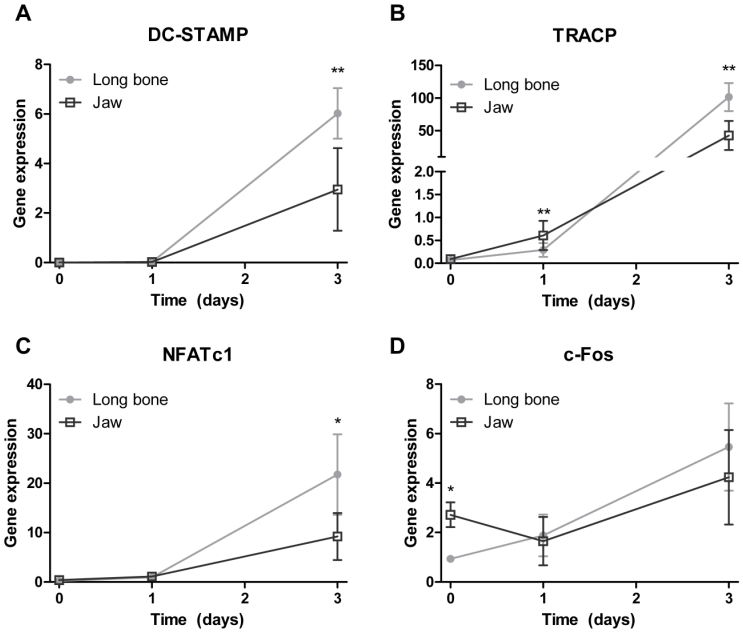

Figure 5. Gene expression of osteoclast markers. (A) DC-STAMP was absent directly after isolation and higher in long bone osteoclast precursors than in jaw osteoclast precursors after 3 days of culture with M-CSF and RANKL. At this time point, (B) TRACP and (C) NFATc1 expression were also higher in the long bones. (D) c-Fos expression was higher in the jaw cells directly after isolation, however this difference disappeared after culture with M-CSF and RANKL. *p<0.05, **p<0.01, using a paired t-test, n=3 for t0 and n=8 for t1 and t3.

Non-osteoclast differentiation

To further investigate why fusion rates in long bone and jaw cultures did not correlate with markers for osteoclastogenesis, we measured gene expression of differentiation markers of other cell types present in the bone marrow, i.e. macrophages and osteoblast precursors. Expression of the macrophage markers MafB and F4/80 was significantly higher in jaw than in long-bone marrow cells cultured with M-CSF and RANKL (Figure 6A,B). Expression of the early osteoblast marker Runx2, was slightly higher in jaw cultures than in long bone cultures, however, this was only significant at the start of culture, i.e. before M-CSF and RANKL were added (Figure 6C). These results indicate that the presence of other cell types such as macrophages, may, at least in part, explain the relatively low expression of osteoclastogenesis markers in jaw bone marrow cultures compared to long bone cultures.

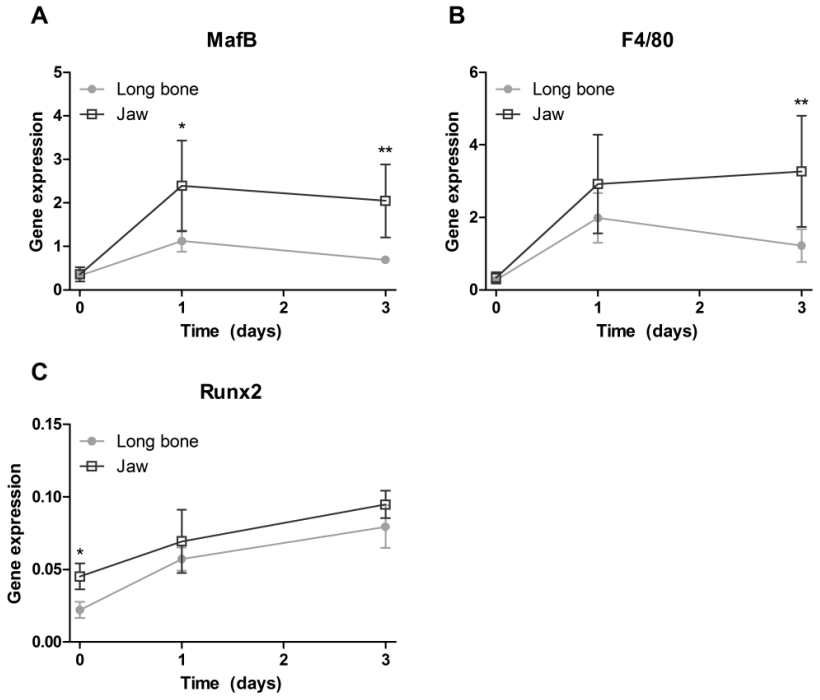

Figure 6. Gene expression of macrophage and osteoblast markers. (A) MafB and (B) F4/80 expression were higher in jaw than in long bone osteoclast cultures with M-CSF and RANKL. (C) Jaw bone marrow cells expressed more Runx2 than long-bone marrow cells directly after isolation. *p<0.05, **p<0.01 using a paired t-test, n=3 for t0 and n=8 for t1 and t3.

Directional migration

There was no difference between the migration velocity of long bone and jaw osteoclast precursors (Figure 2A). However, attraction of osteoclast precursors towards the resorption site is also an important step during osteoclastogenesis. Therefore, we analyzed gene expression of CXCL12 and CXCR4, an important chemoattractant-receptor pair that may be involved in osteoclasts precursor attraction [4,5]. Jaw cells expressed more CXCR4 (Figure 7A) and CXCL12 (Figure 7B) than long bone cells. This was significant for CXCL12 after 1 day of culture and for both genes after 3 days of culture with M-CSF and RANKL.

To assess whether higher CXCR4 expression resulted in an increased attraction of jaw cells towards CXCL12 we performed a Transwell Migration Assay. Both long bone and jaw bone marrow cells that were pre-cultured for 1 day with M-CSF and RANKL migrated towards CXCL12 in a dose dependent manner (Figure 7C). However, migration towards CXCL12 did not differ between long bone and jaw bone marrow cells, indicating that those cells similarly responded to CXCL12. Possibly, this could be attributed to higher expression of the decoy receptor for CXCL12, CXCR7, that accompanied higher CXCR4 expression in jaw cells (Figure 7D).

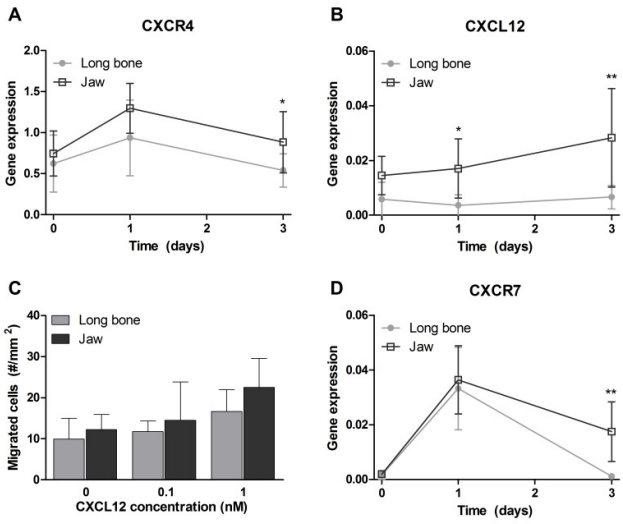

Figure 7. Jaw osteoclast precursors express more CXCR4, CXCR7, and CXCL12 than those from long bone. (A) Gene expression of the chemoattractant-receptor pair CXCR4 and (B) CXCL12 was higher in the jaw than in long-bone marrow cells cultured with M-CSF and RANKL. (C) Long bone and jaw bone marrow cells migrated towards CXCL12 in a similar, dose dependent manner. (D) Jaw bone marrow cells expressed more CXCR7 than long-bone marrow cells after culture with M-CSF and RANKL. *p<0.05, **p<0.01 using a paired t-test, n=5-7 for t0 and n=8 for t1 and t3.

Discussion

Previously, we have shown that despite an enhanced BP uptake in jaw osteoclast precursors, similar numbers of osteoclasts differentiate from jaw and long bone osteoclast precursors after exposure to BPs [20]. This could be attributed to a higher capacity of jaw osteoclast precursors to prevent apoptosis. Another explanation could be that jaw osteoclasts compensate for cell death by increasing formation *in vitro* by an altered (i) proliferation, (ii) migration, and/or (iii) fusion. Here, we showed that jaw and long bone cells did not have a different capacity in either of those parameters. Yet, we found that long bone and jaw bone marrow cells express different levels of CXCL12, a regulator of osteoclast precursor attraction and an inhibitor of pro-apoptotic protein expression [4].

By undergoing fission, we have already shown that osteoclast formation is a very flexible process [23]. We showed here that osteoclast fusion is also a selective process, and that osteoclast precursor migration is an important step during osteoclastogenesis *in vitro*. Both long bone and jaw cells were flexible, and able to adapt their velocity to the situation, i.e. they migrated faster when they were mononuclear, when they did not fuse, and when fewer potential fusing partners were present. Together with previous studies which showed that human osteoclast precursors migrate further in an early stage of differentiation than at a later stage [24], our data indicate that both long bone and jaw osteoclast precursors are actively migrating, probably in search for appropriate fusion partners that are fusion competent and compatible.

As the small GTPases that are affected by N-BPs are important for the cytoskeletal rearrangements necessary during adhesion and migration, it is likely that osteoclast precursor migration is affected by N-BPs [14,15]. Surprisingly, pamidronate (10 μM) did not affect the migration velocity of the cells. Perhaps, the concentration used in these experiments was too low to cause an effect on small GTPase function. Yet, a higher concentration of 50 μM caused cell death within 6 hours after addition (not shown). These data may indicate that PAM does not cause an effect on migration of these osteoclast precursors without being toxic to those cells.

Previous data have shown that long bone osteoclasts differentiate faster than jaw osteoclasts *in vitro*, possibly due to higher numbers of committed osteoclast precursors, i.e. myeloid blasts, in long bone marrow [17]. In the present study, we confirmed that osteoclast marker expression was higher in the long bones than in the jaw during early differentiation. Surprisingly, this was not represented by a higher fusion rate in the long bone marrow cells. Perhaps, lower osteoclast marker expression was the result of the abundance of macrophages in jaw cultures, as shown by high expression of the macrophage markers MafB and F4/80. This would lead to a relatively lower expression of osteoclast markers in the jaw cultures, not due to the presence of fewer osteoclasts, but due to the

presence of other cell types in the culture. Another explanation for the discrepancy between osteoclast marker expression and fusion rates could be attributed to the different culture substrates. Gene expression data are based on cells growing on plastic whereas fusion was studied on carbon-coated glass chamber slides.

Interestingly, jaw bone marrow cells expressed more CXCR4 than long bone cells after 3 days of culturing with M-CSF and RANKL. Since both long bone and jaw bone marrow cells migrated towards CXCL12, CXCR4 was likely present on those cells as well. Yet, there was no difference in the migration towards CXCL12 between long bone and jaw bone marrow cells after 1 day of culture with M-CSF and RANKL. Likely, this was the result of the higher expression of the decoy receptor for CXCL12, CXCR7, leading to an opposite, chemorepulsive effect [25,26].

Next to CXCR4, jaw bone marrow cells also expressed more CXCL12 than long bone marrow cells. This might indicate that jaw bone marrow may have a stronger chemoattracting potential than long bone marrow cells. Therefore, it would be of interest to investigate whether jaw bone marrow has a stronger chemoattracting potential towards other bone marrow cells or hematopoietic precursors from the blood. Also, this may indicate that the egress of hematopoietic precursors from the bone marrow to the blood [7] may occur preferably from the long bones, and further research is required to investigate whether the jaw and long bone marrows play a different role in terms of the hematopoietic stem and progenitor cell pool.

Wright et al. (2005) showed that CXCL12 can, besides attracting osteoclast precursors, inhibit the pro-apoptotic protein Bim, and increase the anti-apoptotic/pro-apoptotic mRNA ratio, thereby stimulating osteoclast survival [4]. The high CXCL12 expression in the jaw cells that we found in this study, may therefore explain the higher expression of the anti-apoptotic genes Bcl-2 and Bcl-xL that we found in jaw than in long-bone marrow cells in the previous study [20]. Currently, it is unknown through which receptor the anti-apoptotic effect of CXCL12 may be mediated. Altogether, these results may indicate that jaw osteoclast precursors express a higher level of survival factors than long bone osteoclast precursors, and therefore, they may be less sensitive to BP-induced apoptosis.

In conclusion, we showed that long bone and jaw bone marrow osteoclast precursors do not differ in proliferation, migration, and fusion rates. However, jaw bone marrow cells express more CXCL12 than long-bone marrow cells, possibly explaining why jaw bone marrow cells had a higher anti-apoptotic gene expression than long-bone marrow cells. Our data provide additional evidence that the osteoclast precursors from long bone and jaw are different.

Acknowledgments

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