ARTICLE
Journal of Cellular Biochemistry 109:347–355 (2010)

Journal of Cellular Biochemistry

Non-Resorbing Osteoclasts Induce Migration and Osteogenic Differentiation of Mesenchymal Stem Cells

L. Kreja,[1*] R.E. Brenner,[2] A. Tautzenberger,[1] A. Liedert,[1] B. Friemert,[3] C. Ehrnthaller,[4] M. Huber-Lang,[4] and A. Ignatius[1]

[1] *Institute of Orthopaedic Research and Biomechanics, Center of Musculoskeletal Research Ulm, University of Ulm, Ulm, Germany*

[2] *Department of Orthopaedics, Division of Biochemistry of Joint and Connective Tissuue Diseases, Center of Musculoskeletal Research Ulm, University of Ulm, Ulm, Germany*

[3] *Department of Surgery, Military Hospital, Ulm, Germany*

[4] *Department of Traumatology, Hand-, Plastic-, and Reconstructive Surgery, Center of Surgery, Center of Musculoskeletal Research Ulm, University of Ulm, Ulm, Germany*

ABSTRACT

Osteoclast activity has traditionally been regarded as restricted to bone resorption but there is some evidence that also non-resorbing osteoclasts might influence osteoblast activity. The aim of the present study was to further investigate the hypothesis of an anabolic function of non-resorbing osteoclasts by investigating their capability to recruit mesenchymal stem cells (MSC) and to provoke their differentiation toward the osteogenic lineage. Bone-marrow-derived human MSC were exposed to conditioned media (CM) derived from non-resorbing osteoclast cultures, which were generated from human peripheral blood monocytes. Osteogenic marker genes (transcription factor Runx2, bone sialoprotein, alkaline phosphatase (AP), and osteopontin) were significantly increased. Osteogenic differentiation (OD) was also proved by von Kossa and AP staining occurred in the same range as in MSC cultures stimulated with osteogenic supplements. Chemotactic responses of MSC were measured with a modified Boyden chamber assay. CM from osteoclast cultures induced a strong migratory response in MSC, which was greatly reduced in the presence of an anti-human platelet-derived growth factor (PDGF) receptor β antibody. Correspondingly, significantly increased PDGF-BB concentrations were measured in the CM using a PDGF-BB immunoassay. CM derived from mononuclear cell cultures did not provoke MSC differentiation and had a significantly lower migratory effect on MSC suggesting that the effects were specifically mediated by osteoclasts. In conclusion, it can be suggested that human non-resorbing osteoclasts induce migration and OD of MSC. While effects on MSC migration might be mainly due to PDGF-BB, the factors inducing OD remain to be elucidated. J. Cell. Biochem. 109: 347–355, 2010.

KEY WORDS: BONE REMODELING; OSTEOCLAST; MESENCHYMAL STEM CELL; CHEMOTAXIS; OSTEOGENIC DIFFERENTIATION; PDGF-BB; BMP-2

Skeletal mass homeostasis is maintained by the strictly coupled activities of bone-forming osteoblasts and bone-resorbing osteoclasts resulting in bone remodeling. The mechanisms by which bone formation might be coupled to bone resorption in the remodeling process is the object of intensive investigation but the precise molecular mechanisms that control bone remodeling remain insufficiently understood [Martin and Sims, 2005; Matsuo and Irie, 2008; Andersen et al., 2009]. The initiation phase of the bone remodeling cycle includes recruitment of osteoclast precursors, differentiation, and finally activation of osteoclasts before formation of new bone succeeds bone resorption.

Osteoclasts originate from hematopoietic cells of the monocyte/macrophage lineage. The key factors modulating osteoclastogenesis, receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and macrophage-colony stimulating factor (M-CSF), are expressed by osteoblasts. The RANK/RANKL/OPG and M-CSF/c-fms receptor regulatory axes couple osteoblast and osteoclast activity controlling the balance between bone formation and

L. Kreja and R.E. Brenner contributed equally to this work.

Grant sponsor: German Research Council (Deutsche Forschungsgemeinschaft); Grant number: KFO-200.

*Correspondence to: L. Kreja, Institute of Orthopaedic Research and Biomechanics, Center of Musculoskeletal Research Ulm, University of Ulm, Helmholtzstrasse 14, D-89081 Ulm, Germany. E-mail: ludwika.kreja@uni-ulm.de

Received 27 July 2009; Accepted 7 October 2009 • DOI 10.1002/jcb.22406 • © 2009 Wiley-Liss, Inc.
Published online 30 November 2009 in Wiley InterScience (www.interscience.wiley.com).

resorption [Boyle et al., 2003; Asagiri and Takayanagi, 2007; Boyce and Xing, 2008].

Osteoblasts derive from mesenchymal stem cells (MSC). Their differentiation and function is regulated by numerous factors like systemic hormones, nerve signals, and vascular agents. Moreover, paracrine cytokines and growth factors, among them transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and inflammatory cytokines such as interleukin-1β (Il-1β), Il-6, and tumor necrosis factor (TNF-α) are involved in osteoblast regulation [Katagiri and Takahashi, 2002; Huges et al., 2006; Matsuo and Irie, 2008]. The transition from bone resorption to formation during the remodeling process is mediated by osteoclast-derived "coupling factors" including factors directly secreted by osteoclasts, membrane bound factors like ephrinB2 ligand, and factors embedded in bone matrix and liberated by osteoclasts during the resorption process [Zhao et al., 2006; Matsuo and Irie, 2008]. All these factors might direct proliferation, differentiation, and activation of osteoblasts leading to formation of new bone in resorbed bone lacunae [Martin and Sims, 2005; Matsuo and Irie, 2008].

Osteoclast activity has traditionally been regarded as restricted to bone resorption [Teitelbaum, 2000; Bar-Shavit, 2007]. On the other hand, evidence suggests that also non-resorbing osteoclasts might influence osteoblast activity. This assumption is based on observations in patients with osteopetrosis, especially with autosomal dominant osteopetrosis Type II (ADOII) and also on studies with osteopetrotic animal models. Patients with ADOII have, despite reduced bone resorption, normal or even increased bone formation. Studies using osteopetrotic animal models indicate that bone formation could be independent of the resorptive capacity of osteoclasts [Karsdal et al., 2007; Segovia-Silvestre et al., 2009]. Recently, it was demonstrated by an in vitro study that conditioned media (CM), which were collected from non-resorbing osteoclasts, induced formation of bone-like nodules in a murine preosteoblastic cell line. The authors suggested, therefore, that osteoclasts secrete an anabolic signal, which is not derived from their resorptive activity [Karsdal et al., 2008].

The aim of the present study was to further investigate the hypothesis of the anabolic function of non-resorbing osteoclasts by investigating their capability to recruit MSC and to provoke their differentiation toward the osteogenic lineage. CM collected from non-resorbing human osteoclasts were proved for inducing the expression of a spectrum of osteogenic marker genes and the chemotactic activity of human-bone-marrow-derived MSC. Furthermore, the functional involvement of PDGF as a signaling factor was investigated.

MATERIALS AND METHODS

CULTURE OF OSTEOCLASTS

After approval by the ethics committee at Ulm University, the Red Cross Blood Bank Baden-Württemberg (Ulm, Germany) provided a buffy coat obtained from the blood of healthy anonymous donors. Peripheral blood mononuclear cells (PBMNC) were isolated using Histopaque®-1077 (Sigma–Aldrich, Germany; centrifugation 30 min at 400*g*) and 5×10^5 cells/cm^2 were seeded in tissue culture plastic multi-well plates (Nunc, Germany) in α-Medium (cat. No. F0925) supplemented with 1% L-Glutamine, 10% selected fetal calf serum (all from Biochrom, Germany), 20 ng/ml recombinant human (rh) RANKL and 10 ng/ml rh M-CSF (both from Chemicon International, USA), or 50 ng/ml RANKL and 25 ng/ml M-CSF. Osteoclast cultures were performed in 96-well plates with 100 µl medium/well or in 48-well plates with 250 µl medium/well. Cells were incubated at 37°C, saturation humidity, and 8.5% CO_2 resulting in medium pH 7.15. The medium was changed twice a week. After 21 days, cells were fixed with 4% buffered formaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using the Acid Phosphatase Leukocyte Kit (Sigma–Aldrich). Osteoclasts were identified as TRAP-positive multinucleated cells containing three or more nuclei [Buckley et al., 2005]. In absence of RANKL and M-CSF osteoclast formation did not occur.

COLLECTION OF SUPERNATANTS OF OSTEOCLAST CULTURES

Supernatants of osteoclast cultures, below referred to as CM, were obtained from 13 independent experiments with cryopreserved cells from two PBMNC donors. Each CM was prepared from separate osteoclast cultures performed with cryopreserved cells. CM were collected during each medium change, pooled, and stored at −70°C until they were added to MSC cultures in a concentration of 50% in MSC-expansion medium (see below) to investigate their capability to induce osteogenic differentiation (OD). As a negative control, MSC-expansion medium containing 50% of the above-mentioned osteoclast medium, however, not conditioned by osteoclast cultures (non-CM), was used.

CULTURE OF HUMAN MESENCHYMAL STEM CELLS

MSC were obtained from bone-marrow aspirates taken from surgical procedures of one male and four female healthy donors, aged 15–42 years during anterior cruciate ligament replacement or pelvic osteotomies in accordance with the terms of the ethics committee of Ulm University, Germany. MSC were isolated by density gradient centrifugation (Histopaque®-1077; Sigma–Aldrich) and adhesion to tissue culture plastic material (Nunc). Cells were expanded in MSC-expansion medium consisting of Dulbecco's Modified Eagle Medium (DMEM; F0415, Biochrom) supplemented with 10% fetal calf serum (FCS, Cambrex, Belgium), 1% L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Biochrom). The cultures were incubated at 37°C, saturation humidity, and 8.5% CO_2. Medium was changed twice a week. At subconfluence, cells were detached by 0.05% trypsin/0.02% EDTA (Biochrom) treatment and cryopreserved. The potential of isolated MSC to differentiate into osteoblasts, chondrocytes, or adipocytes was tested according to previously established protocols [Pittenger et al., 2001]. The cells expressed typical surface antigens CD9, CD54, CD90, CD166, and STRO-1 and were negative for CD34 and CD45 [Fickert et al., 2004]. The MSC were used in independent experiments in passage 1 or 2.

For testing the effect of the collected CM, 1×10^4 MSC/cm^2 (male donor) were seeded onto tissue culture plates (Nunc) in MSC-expansion medium. After 24 h medium was replaced by medium-containing 50% CM or 50% non-CM (negative control), respectively. To compare both treatments with successfully differentiated cells

(positive control), parallel cultures were treated with OD medium consisting of MSC-expansion medium with 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 0.2 mM ascorbate-2-phospate (all from Sigma–Aldrich).

ANALYSIS OF OSTEOGENIC DIFFERENTIATION

After exposition of MSC with the CM, non-CM (negative control), or medium with differentiation supplements (positive control) for 22 days mRNA expression of osteogenic marker genes was analyzed by quantitative real-time RT-PCR as previously described [Liedert et al., 2006]. Briefly, cells from triplicate cultures in 24-well plates were washed once with phosphate buffered saline (PBS; Biochrom), lysed, and total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was prepared from 1 μg total RNA and PCR amplification was performed. Specific primer pairs for bone sialoprotein (BSP), alkaline phosphatase (AP), osteopontin (OP), and transcription factor Runx2 (Table I) were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) and were synthesized by Thermo Electron (Ulm, Germany). Amplification products were cloned and used as standards for real-time RT-PCR in the iCycler system (Bio-Rad, Germany). Expression of each mRNA was normalized to the house-keeping gene GAPDH. Normalized values of gene expression of MSC treated with CM were related to that of MCS exposed to non-CM (negative control).

After fixing the cells in cold absolute methanol, calcium deposition was examined by von Kossa staining and AP was detected with the AP-staining kit (Sigma–Aldrich, 86R-1KT) following the manufacturer's recommendations.

CHEMOTAXIS ASSAY

To investigate if osteoclast CM induce migration activity in MSC (four female donors) a chemotaxis assay was performed. Chemotactic responses of MSC were measured with a modified Boyden chamber assay using a 48-well microchemotaxis chamber (Neuro-Probe, Inc., USA) and polycarbonate filters with 8 μm pores (Whatman Biometra, Germany) as described previously [Fiedler et al., 2006]. MSC were trypsinized and counted prior to the chemotaxis assay. The lower wells of the multiwell chemotaxis chamber were filled with MSC-expansion medium containing 50% CM or non-CM, respectively, and covered by the migration filter. Both CM and non-CM were ultrafiltrated prior to the chemotaxis assay using a molecular weight exclusion limit of 300 kDa (300k OMEGA Nanosep, Pall Corporation, USA) in order to concentrate on proteins of low molecular size and to eliminate large extracellular proteins with chemoattractive activity like fibronectin [Tribault et al., 2007]. 1×10^4 cells in 50 μl DMEM each were added to the upper wells. After incubation for 4 h, the filter was removed carefully. Non-migrated cells on the upper side were eliminated by rinsing the filter with cold PBS and scraping it over a rubber wiper. The remaining migrated cells on the lower side of the filter were fixed with 4% formaldehyde and counted after Giemsa staining was performed. All CM were tested in quadruplicate. The number of migrated cells in control wells (50% non-CM in MSC-expansion medium) and stimulated wells (50% CM in MSC-expansion medium) was counted in 12 random fields/well at 100-fold magnification. Results were expressed as total number of cells counted and the chemotactic index (CI) was determined as the average number of migrated cells in stimulated wells divided by the average number of migrated cells in control wells.

In addition, the effects of 10, 50, 100, and 1,000 pg/ml rh PDGF-BB (DPC Biermann, Germany) in filtrated non-CM and 50 pg/ml rh PDGF-BB in filtrated CM from mononuclear cell cultures without osteoclast formation were studied.

For analysis of an involvement of the PDGF-receptor β, MSC were preincubated for 30 min with 1 μg/ml goat anti-human PDGF-receptor β antibody (R&D, Germany) in DMEM as described previously [Fiedler et al., 2004]. Afterward, the cells were washed in DMEM, diluted in filtrated 50% CM derived from different cultures (CM1, CM2, CM8, CM10, CM12, Table II) in MSC medium and used for the chemotaxis assay in comparison to cells preincubated without PDGF-receptor β antibody. The specificity of the PDGF-receptor antibody was confirmed by testing basal migration (DMEM) and the effect of PDGF-BB (100 ng/ml in DMEM) without and after preincubation with the receptor antibody (0.01, 0.1, 1 μg/ml) as well as unspecific goat IgG (Sigma, St. Louis, 1 μg/ml).

PDGF-BB AND BMP-2 IMMUNOASSAY

BMP-2 and PDGF-BB concentrations in the CM were assessed in duplicate using the Quantikine BMP-2 and PDGF-BB immunoassay (R&D, Inc., Minneapolis) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

mRNA expression was investigated in MSC treated with CM derived from seven osteoclast cultures. A non-parametric Wilcoxon signed rank test was performed to evaluate differences between cells treated with conditioned and non-CM (negative control). Statistical significance was assumed if the *P*-value was 0.05 or lower.

The results of the chemotaxis assay for each tested CM are expressed as mean of quadruplicate determination ± standard deviation (SD). Statistical significance was determined by Student's *t*-test. Statistical differences of $P < 0.05$ were considered significantly different.

TABLE I. Human Primer Sequences Used for Real-Time RT-PCR

mRNA	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product size (bp)
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC	224
Runx2	GCAGTTCCC AGCATTTCAT	CACTCTGGCTTTGGGAAGAG	182
BSP	CGAGGGGGAGTACGAATACA	AGGTTCCCCGTTCTCACTTT	79
AP	CCTCGGAAGACACTCTGACC	CCACCAAATGTGAAGACGTG	61
OP	CTCAGGCCAGTTGCAGCC	GCCACAGCATCTGGGTATTT	177

TABLE II. Osteogenic Differentiation and Chemotactic Index (CI) of MSC After Exposition to Conditioned Media and PDGF-BB Concentrations Measured

	mRNA expression related to control (non-CM)				Staining			
MSC stimulation	Runx2	BSP	AP	OP	von Kossa	AP	CI	PDGF-BB (pg/ml)
Non-CM	1	1	1	1	Negative	Negative	1	0
OD medium	2.4 ± 1.7	7.4 ± 4.3	25.5 ± 16.2	2.3 ± 0.9	Positive	Positive	nt	nt
CM 1	5.9	10.1	3.4	0.6	Positive	Positive	5.5	nt
CM 2	1.6	3.2	3.3	1.7	Positive	Positive	6.9	16
CM 3	1.6	1.9	2.9	1.7	nt	nt	3.9	22
CM 4	4.0	15.1	1.6	0.6	nt	nt	3.0	75
CM 5	3.3	8.1	5.1	3.6	Positive	Positive	4.3	38
CM 6	4.0	2.6	3.5	2.1	Positive	Positive	nt	nt
CM 7	2.0	1.8	1.4	2.0	nt	nt	4.4	22
CM 8	nt	nt	nt	nt	Positive	Positive	6.8	49
CM 9	nt	nt	nt	nt	Positive	Positive	4.3	101
Mean ± SD	3.2 ± 1.6[a]	6.1 ± 5.1[a]	3.0 ± 1.3[a,b]	1.8 ± 1.0			4.9 ± 1.4[c]	46 ± 32[d]
CM 10	nt	nt	nt	nt	Negative	Negative	2.1	2
CM 11	0.9	0.7	0.9	1.3	Negative	Negative	2.1	6
CM 12	nt	nt	nt	nt	Negative	Negative	1.8	5
CM 13	nt	nt	nt	nt	Negative	Negative	2.2	1
Mean ± SD							2.1 ± 0.2	4 ± 2

nt, not tested.
mRNA expression of the osteogenic marker genes Runx2, BSP, AP, and OP in MSC exposed for 22 days with MSC-expansion medium containing 50% conditioned media derived from osteoclast cultures (CM1–CM9) or mononuclear cell cultures (CM10–CM13). Expression of each mRNA was normalized to the house-keeping gene GAPDH. Normalized values of gene expression of MSC treated with conditioned media were related to that of MCS exposed to non-conditioned medium (non-CM; negative control). As a positive control MSC were stimulated with medium containing osteogenic differentiation supplements (OD medium; n = 2 experiments in triplicates).
[a]Significant difference compared to cells treated with non-CM; $P < 0.05$.
[b]Significant difference compared to cells treated with OD medium; $P < 0.01$.
[c]Significant difference compared to cells treated with CM10–CM13; $P < 0.01$.
[d]$P < 0.05$.

RESULTS

CM were collected from cultures performed with PBMNC from two donors in 13 experiments (CM1–CM13, Table II). With PBMNC from one donor, formation of TRAP-positive multinuclear cells (osteoclasts) was successful. The treatment with RANKL and M-CSF resulted in characteristic inhomogeneous cultures consisting of a lot of osteoclastic cells (TRAP-positive multinucleated cells) and also remaining mononuclear cells (Fig. 1A). In the second donor, the generation of osteoclasts was not successful. The cultures obtained from this donor consisted only of mononuclear cells without any osteoclast formation (Fig. 1B). Individual variations in generation of osteoclasts from different donors are well known [Susa et al., 2004; Kreja et al., 2007]. For our experiments, we used the CM of cell cultures from both donors to distinguish between effects induced by cultures with successful osteoclast formation, which contained also mononuclear cells due to their typical inhomogeneity (CM1–CM9), or by mononuclear cells (CM10–CM13).

EFFECT OF CONDITIONED MEDIA ON OSTEOGENIC DIFFERENTIATION

CM of cultures with osteoclast formation (CM1–CM7) significantly induced OD of MSC (Table II). We found a significant up-regulation of the osteogenic marker genes Runx2, BSP, and AP after stimulation of MSC with CM1–CM7. mRNA expression of OP was also increased by trend (1.8-fold) compared to the control cultures, which were treated with non-conditioned osteoclast medium. The up-regulation of the osteogenic markers was in the same range as in MSC stimulated with the differentiation medium containing osteogenic supplements (positive control), except for AP, which was expressed on a higher level in the positive controls.

OD in cultures treated with CM from cultures with osteoclast formation was confirmed by cytochemical detection of calcified matrix by von Kossa staining as well as by AP staining (Table II, Fig. 2). MSC, which were exposed to CM derived from cell cultures without osteoclast formation (CM10–CM13) as well as MSC cultured in the presence of non-CM (negative control) did not show any positive AP and von Kossa staining. Expression of osteogenic marker genes was also not up-regulated (CM11, Table II). Positive von Kossa and AP staining was confirmed with CM of five additional donors with osteoclast formation demonstrating that the strong induction of OD was valid and not restricted to one individual.

EFFECT OF CONDITIONED MEDIA ON MSC MIGRATION

The results of the chemotaxis study are summarized in Table II. The CM, which stimulated MSC differentiation, induced increased migration activity as well. Mean CI obtained with CM1–CM9 (n = 8) was significantly higher compared to mean CI measured in cultures performed with CM10–CM13. Under basal conditions in the presence of non-CM (50% in MSC-expansion medium), migration of 65 ± 7 MSC (mean ± SD; n = 4 wells) was observed (Fig. 3). In comparison to these basal values, 8 CM from osteoclast cultures (CM1–CM9) induced increased migratory cell response in the range from 194 ± 20 migrated cells up to 446 ± 41 migrated cells, resulting in mean CI of 4.9 ± 1.4 (mean ± SD; n = 8 CM). In contrast, CM10–CM13 derived from mononuclear cell cultures without osteoclasts induced weak stimulation of MSC (range 117 ± 15 and 160 ± 22 migrated cells; mean ± SD; n = 4), resulting in mean CI of 2.1 ± 0.2.

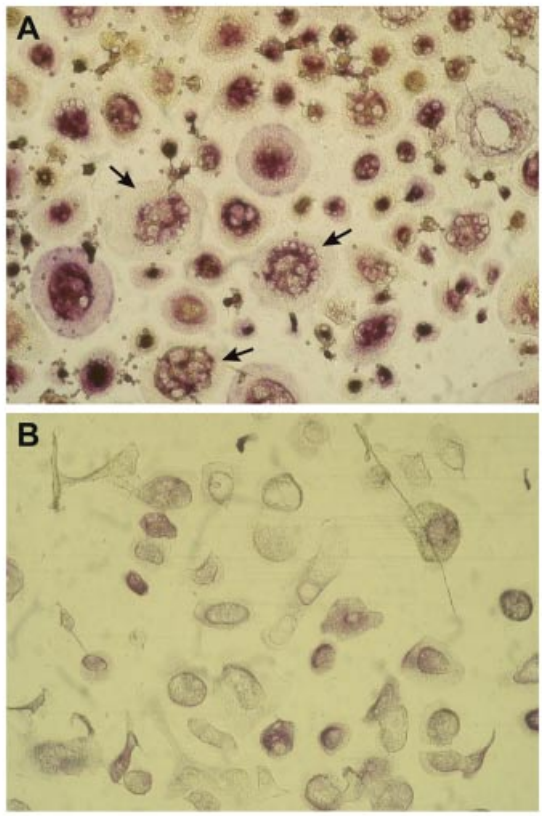

Fig. 1. Photomicrographs of TRAP-stained cells generated after 21 days culture of peripheral blood derived mononuclear cells in presence of 20 ng/ml RANKL and 10 ng/ml M-CSF. **A:** Multinucleated osteoclasts with more than three nuclei (indicated with arrows). **B:** Mononuclear cell cultures without osteoclast formation (magnification 200×).

FUNCTIONAL INVOLVEMENT OF PDGF

In order to prove the functional involvement of PDGF isoforms, a preincubation of MSC with an inactivating antibody against the PDGF-receptor β was performed and a significant decrease of the migratory cell response to CM secreted from osteoclast-forming cells was measured (Fig. 4). For three CM from osteoclast-forming cultures (CM1, CM2, CM8) the mean number of migrated MSC was 378 ± 62 without and 137 ± 15 after preincubation with the PDGF-receptor β antibody. Interestingly, with two CM from cultures without osteoclast formation (CM10, CM12) no major influence on cell migration was detected, indicating that most of the additional chemotactic activity secreted by osteoclast-forming cells was due to PDGF isoforms. In control experiments it could be shown that preincubation with unspecific goat IgG had no effect on basal migration (negative control) or stimulation of MSC migration with 100 ng/ml PDGF-BB (CI = 13.1 without and CI = 14.0 after preincubation with 1 μg/ml unspecific goat IgG). The effect of 100 ng/ml PDGF-BB was inhibited in a concentration-dependent manner by preincubation with the PDGF-receptor β antibody (CI values: 13.1 without, 8.9 with 0.01 μg/ml, 6.0 with 0.1 μg/ml, and 2.9 with 1 μg/ml PDGF-BB-receptor antibody) while basal migration activity was not affected.

The concentration of PDGF-BB in CM was determined with an immunoassay. In MSC-expansion medium with 50% CM from osteoclast cultures (CM2–CM9) the PDGF-BB concentrations ranged between 16 and 101 pg/ml. In media derived from cultures, which did not form osteoclasts (CM10–CM13), the concentration of PDGF-BB was significantly lower (Table II).

Effects of defined PDGF-BB concentrations in the range found in CM of osteoclast cultures on MSC migration were studied in further experiments. In filtrated non-CM we found an increase of migrated cells from a basal value of 38 ± 5 to 115 ± 13, 226 ± 21, 314 ± 33, and 474 ± 44 in presence of 10, 50, 100, and 1,000 pg/ml PDGF-BB, respectively. This was tested in quadruplicate with cells from one donor. MSC migration was also analyzed in response to CM of cultures without osteoclast formation (CM10–CM12) without and with addition of 50 pg/ml PDGF-BB. This concentration of PDGF-BB increased the number of migrated MSC from 118 ± 7 to 219 ± 7 ($n = 3$).

BMP-2, another possible candidate responsible for the observed effects, could not be detected in measurable amounts by ELISA technique. According to the minimal detectable dose given by the manufacturer, the BMP-2 concentration was below 11 pg/ml for all samples.

DISCUSSION

Osteoclast activity has been regarded as restricted to bone resorption; however, recent studies suggested that osteoclasts might release anabolic signals, which are not linked to their resorption activity [Karsdal et al., 2008]. The present study confirmed this hypothesis and demonstrated that supernatants collected from non-resorbing osteoclast cultures can induce OD of human MSC. Furthermore, it was shown for the first time that non-resorbing osteoclasts could recruit MSC and that PDGF-BB might be one functional candidate involved in this effect.

Karsdal et al. [2008] reported a concentration-dependent effect of CM from non-resorbing osteoclasts on the formation of bone-like nodules in a murine preosteoblastic cell line and suggested, therefore, that non-resorbing osteoclasts might secrete anabolic factors. Strengthening these findings, our results revealed that CM derived from non-resorbing osteoclasts induced a considerable up-regulation of osteogenic marker genes and mineralization in human MSC. Thereby, CM of osteoclast-forming cultures were as effective as the treatment with dexamethasone, β-glycerophosphate, and ascorbic acid, which are usually added in MSC cultures to induce OD [Pittenger et al., 1999]. Supernatants of mononuclear cell cultures with failing osteoclast formation, however, did not induce OD suggesting that osteoclasts and not the mononuclear cells remaining in the cultures released the factors affecting MSC differentiation. Our results also suggested that non-resorbing osteoclasts might recruit mesenchymal progenitor cells by secreting factors, which are highly chemoattractive. CM of osteoclast cultures induced a considerable migration activity, whereas supernatants of mononuclear

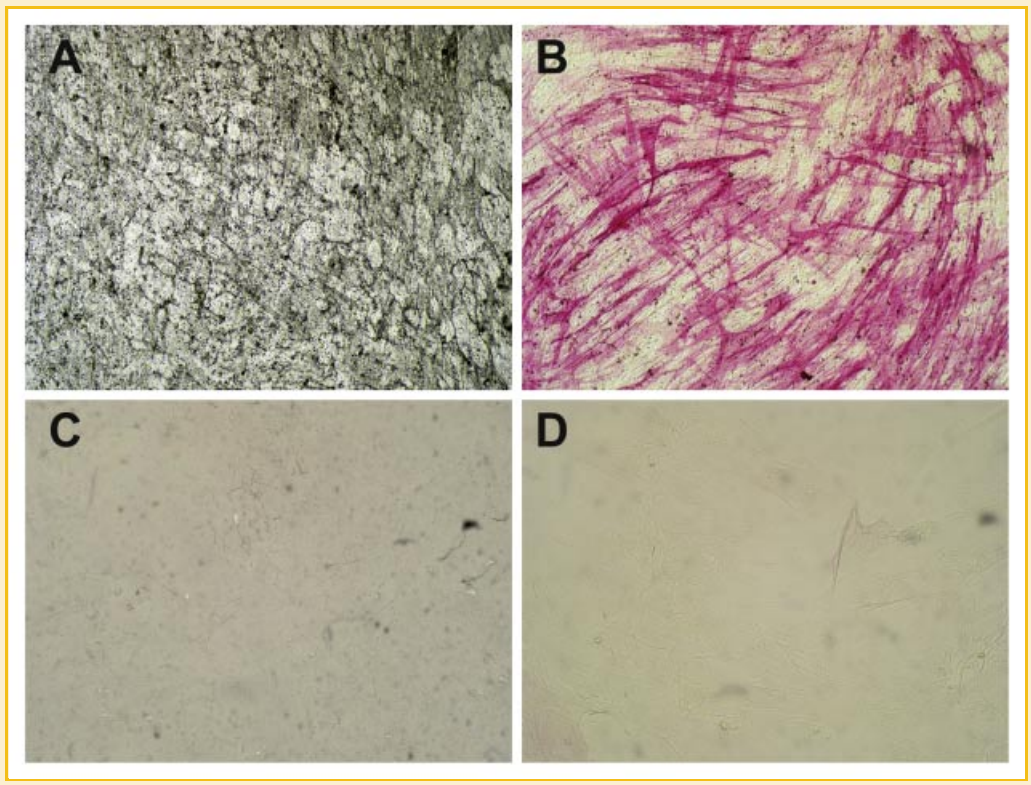

Fig. 2. Microscopic view of MSC cultures presented in Table II. **A:** Calcium deposition stained with von Kossa technique. **B:** AP expression in MSC after 22 days of culture in the presence of 50% osteoclast conditioned medium (CM2). Control culture in the presence of non-CM after **(C)** von Kossa staining and **(D)** AP staining (magnification 100×).

cells were significantly less attractive for MSC, indicating that the effect was as well induced by osteoclasts.

We focused our study on the question which molecular factors in the supernatants derived from non-resorbing osteoclasts could be responsible for the induction of MSC migration and differentiation. Series of cytokines, growth factors, and hormones are known to regulate OD, among them, e.g., BMPs, TGF-β, IGFs, bFGF, PDGF, and inflammatory cytokines such as Il-1β, Il-6, and TNF-α [Heng et al., 2004; Molchanova et al., 2008]. PDGF, BMP-2, bFGF, vascular endothelial growth factor (VEGF-A), IGF-I, IGF-II, placental growth factor-1 (PlGF-1), stromal-derived factor-1 (SDF-1), IL-8, Wnt3a, cystein-rich protein 61 (Cyr61), and Wnt I-inducible signaling pathway protein 3 (WISP3) are known to be chemoattractive for human MSC [Fiedler et al., 2002, 2005, 2006; Neth et al., 2006; Schmidt et al., 2006; Son et al., 2006; Ringe et al., 2007]. Epidermal growth factor (EGF), TGF-α, IGF-I, or hepatocyte growth factor (HGF) increased chemotactic effects of PDGF-BB [Ozaki et al., 2007].

Some of these mediators are known to be directly secreted by resorbing osteoclasts (e.g., PDGF-BB and HGF) or liberated from bone matrix during the resorption process (e.g., TGF-β, BMP, IGF-II) and acting on osteoblasts as ''coupling factors'' [Matsuo and Irie, 2008]. However, few is known about the activity of non-resorbing osteoclasts. Karsdal et al. [2008] hypothesized that mature osteoclasts secrete non-bone-derived signals independent of release of bone matrix signals but did not investigate the mechanisms further. It can be presumed that the CM of osteoclastic cell cultures contained several osteoinductive and chemotactic mediators.

In this study, we investigated the involvement of two possible candidates, BMP-2 as a potent inducer of MSC migration and OD, and PDGF-BB, which represents the most potent chemoattractive factor for human MSC known so far [Fiedler et al., 2004]. BMP-2 could not be detected in the CM, indicating that this important osteogenic growth factor was not responsible for the observed stimulatory effects on MSC migration and differentiation. However, PDGF-BB was found in osteoclast-derived supernatants in significantly higher concentrations compared to supernatants of mononuclear cells and therefore it was further investigated with respect to its functional role in the stimulation of MSC migration. Our results revealed that the chemotactic activity of osteoclast CM on MSC could be significantly diminished after inhibition of PDGF-receptor β. Therefore, it can be suggested that human osteoclasts in fact secrete PDGF-BB or even more PDGF isoforms, which are recognized by the PDGF-receptor β on the surface of MSC. Our results confirm a recent study, which reported a chemotaxis response of mouse preosteoblastic MC3T3-E1 cells to factors secreted by mice-derived osteoclasts. Using an siRNA-based strategy, PDGF-BB could be identified as a chemotactic factor [Sanches-Fernandez et al., 2008].

In the present study, most of the migratory response to CM from differentiated osteoclasts could be abolished after inhibition of PDGF-receptor β on MSC, while the basic effect of low molecular

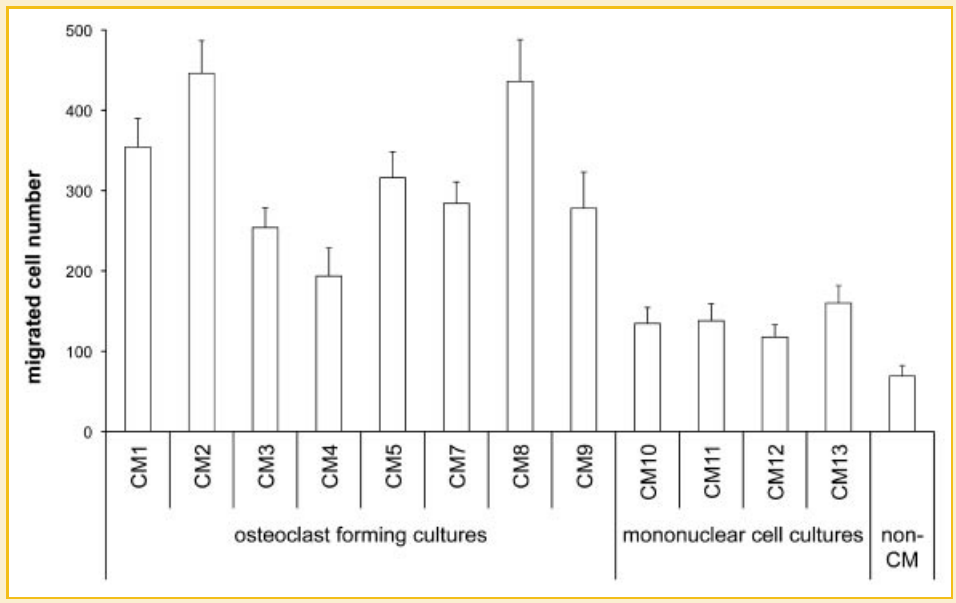

Fig. 3. Migratory response of MSC to MSC-expansion medium containing 50% conditioned media derived from osteoclast cultures (CM1–CM9), conditioned media derived from mononuclear cell cultures (CM10–CM13), or not conditioned osteoclast medium (non-CM) as a control. All samples were ultrafiltrated prior to the migration assay to concentrate on the molecular weight fraction below 300 kDa. The bars represent the number of migrated cells (quadruplicate determination, mean ± SD).

weight chemoattractive factors secreted in mononuclear cell cultures without osteoclast formation was not relevantly impaired, indicating that this effect might be mediated by other factors. Correspondingly, very low concentrations of PDGF-BB were measured in the media derived from mononuclear cells whereas the PDGF-BB concentration was significantly increased in osteoclast-forming cultures. This suggested that during osteoclast formation the synthesis of PDGF isoforms is up-regulated.

The importance of PDGF signaling in MSC migration [Fiedler et al., 2002, 2004; Ozaki et al., 2007] and proliferation [Ng et al., 2008] has already been reported. In the present study, the measured PDGF-BB concentrations in the CM were below 100 pg/ml raising

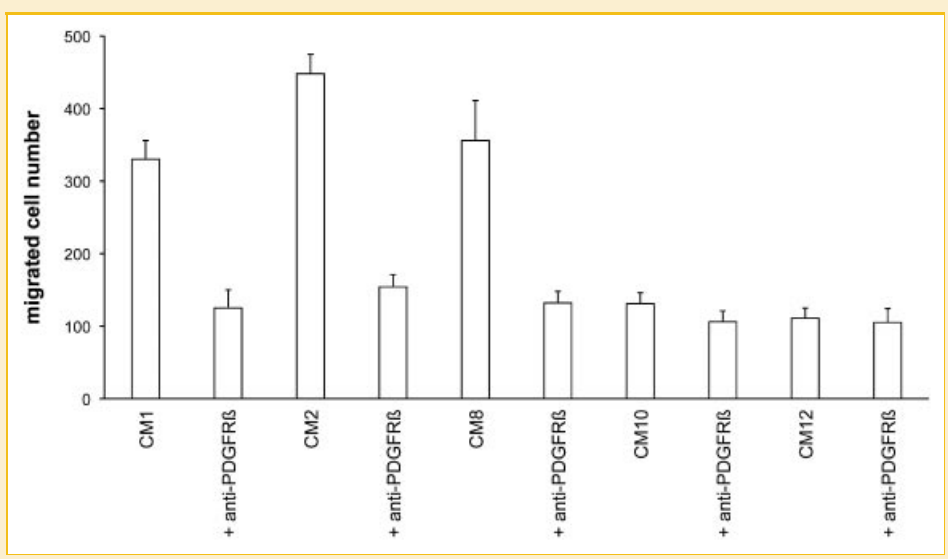

Fig. 4. Migratory response of MSC to MSC-expansion medium containing 50% conditioned media derived from osteoclast cultures (CM1, CM2, and CM8) and conditioned media derived from mononuclear cell cultures (CM10 and CM12) in the absence and presence (+anti-PDGFRβ) of an antibody blocking the PDGF-receptor β. All samples were ultrafiltrated prior to the migration assay to concentrate on the molecular weight fraction below 300 kDa. The bars represent the number of migrated cells (quadruplicate determination, mean ± SD).

the question if such low concentrations could in fact induce a migratory response in human MSC. Our results clearly show that this is the case even in the presence of other low molecular weight products secreted by mononuclear cell cultures without osteoclast formation.

The literature regarding the osteogenic effects of PDGF is controversial. PDGF-BB can promote bone healing in vivo probably due to its proliferative effect on precursor cells [Nash et al., 1994; Vikjaer et al., 1997; Lee et al., 2000]. Gruber et al. [2004] reported that PDGF could increase migration and proliferation, but decreased OD of mesenchymal progenitor cells under in vitro conditions. These data were supported by other authors [Kratchmarova et al., 2005]. Tamama et al. [2006] found positive but weak effects of PDGF on OD of bone-marrow-derived MSC. Recently, Goff et al. [2008] demonstrated that the PDGF pathway is regulated during osteogenesis in MSC and might modulate the expression of differentiation specific microRNAs. If PDGF-BB is also responsible for the strong induction of OD observed in this study seems to be questionable, as most of the literature suggested predominantly proliferative or chemoattractive effects on MSC. It has to be further investigated, which osteogenic mediators are secreted by non-resorbing osteoclasts. Possibly, PDGF isoforms act in concert with other factors.

In conclusion, the results of the present study revealed that human non-resorbing osteoclasts secrete mediator(s) inducing migration and OD of human MSC and suggested therefore an anabolic activity of non-resorbing osteoclasts on bone formation. While effects on MSC migration might be mainly due to PDGF-BB, the factor or the combination of factors inducing OD remains to be elucidated.

ACKNOWLEDGMENTS

This study was funded by the German Research Council (Deutsche Forschungsgemeinschaft; KFO-200). The excellent technical assistance of Mrs. S. Miller and Mr. G. Ravalli is gratefully acknowledged.

REFERENCES

Andersen TL, Sondergaard TE, Skorzynska KE, Dagnaes-Hansen F, Plesner TL, Hauge EM, Plesner T, Delaisse J-M. 2009. A physical mechanism for coupling bone resorption and formation in adult human bone. Am J Pathol 174:239–247.

Asagiri M, Takayanagi H. 2007. The molecular understanding of osteoclast differentiation. Bone 40:251–264.

Bar-Shavit Z. 2007. The osteoclast: A multinucleated, hemopoietic-origin, bone-resorbing osteoimmune cell. J Cell Biochem 102:1130–1139.

Boyce BF, Xing L. 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Biochem Biophys Res Commun 473:139–146.

Boyle WJ, Simonet WS, Lacey DL. 2003. Osteoclast differentiation and activation. Nature 423:337–342.

Buckley KA, Chan BYY, Fraser WD, Gallagher JA. 2005. Human osteoclast culture from peripheral blood monocytes: Phenotypic characterization and quantitation of resorption. Methods Mol Med 107:55–68.

Fickert S, Fiedler J, Brenner RE. 2004. Identification of subpopulations with characteristics of mesenchymal progenitor cells from human osteoarthritic cartilage using triple staining for cell surface markers. Arthritis Res Ther 6:R422–R432.

Fiedler J, Roderer G, Gunther KP, Brenner RE. 2002. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. J Cell Biochem 87:305–313.

Fiedler J, Etzel N, Brenner RE. 2004. To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF. J Cell Biochem 93:990–998.

Fiedler J, Leucht F, Waltenberger J, Dehio C, Brenner RE. 2005. VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. Biochem Biophys Res Commun 334:561–568.

Fiedler J, Brill C, Blum W, Brenner RE. 2006. IGF-I and IGF-II stimulate cell migration of bone-marrow-derived human mesenchymal progenitor cells. Biochem Biophys Res Commun 345:1177–1183.

Goff L, Boucher S, Fenstermacher S, Swerdel M, Chase LG, Adams CC, Chesnut J, Lakshmipathy U, Hart R. 2008. Differentiating human multipotent mesenchymal stromal cells regulate microRNAs: Prediction of microRNA regulation by PDGF during osteogenesis. Exp Hematol 36:1354–1369.

Gruber R, Karreth F, Kandler B. 2004. Platelet-released supernatants increase migration and proliferation and decreased osteogenic differentiation of bone marrow-derived mesenchymal progenitor cells under in vitro conditions. Platelets 15:29–35.

Heng BC, Cao T, Stanton LW, Robson P, Olsen B. 2004. Strategies for differentiation of stem cells into the osteogenic lineage in vitro. J Bone Miner Res 19:1379–1394.

Huges FJ, Turner W, Belibasakis G, Martuscelli G. 2006. Effects of growth factors and cytokines on osteoblast differentiation. Periodontology 2000 (41): 48–72.

Karsdal MA, Martin TJ, Bollerslev J, Christiansen C, Henriksen K. 2007. Are nonresorbing osteoclasts sources of bone anabolic activity? J Bone Miner Res 22:487–494.

Karsdal MA, Neutzsky-Wulff AV, Dziegiel MH, Christiansen C, Henriksen K. 2008. Osteoclasts secrete non-bone derived signals that induce bone formation. Biochem Biophys Res Commun 366:483–488.

Katagiri T, Takahashi N. 2002. Regulatory mechanisms of osteoblast and osteoclast differentiation. Oral Dis 8:147–159.

Kratchmarova I, Blogoev B, Sorensen MG, Kassem M, Mann M. 2005. Mechanism of divergent growth factors in mesenchymal stem cell differentiation. Science 308:1472–1477.

Kreja L, Liedert A, Schmidt C, Claes L, Ignatius A. 2007. Influence of receptor activator of nuclear factor (NF)-κB ligand (RANKL), macrophage-colony stimulating factor (M-CSF) and fetal calf serum on human osteoclast formation and activity. J Mol Histol 38:341–345.

Lee YM, Park YJ, Lee SJ. 2000. The bone regenerative effect of platelet-derived growth factor-BB delivered with a chitosan/tricalcium phosphate sponge carrier. J Periodontol 71:418–424.

Liedert A, Kaspar D, Claes L, Ignatius A. 2006. Signal transduction pathways involved in mechanical regulation of HB-GAM expression in osteoblastic cells. Biochem Biophys Res Commun 342:1070–1076.

Martin TJ, Sims NA. 2005. Osteoblast-derived activity in the coupling of bone formation to resorption. Trends Mol Med 11:75–80.

Matsuo K, Irie N. 2008. Osteoclast–osteoblast communication. Arch Biochem Biophys 473:201–209.

Molchanova EA, Payushina OV, Starostin VI. 2008. Effects of growth factors on multipotent bone marrow mesenchymal stromal cells. Biol Bull 6:555–570.

Nash TJ, Howlett CR, Martin C, Steele J, Johnson KA, Hicklin DJ. 1994. Effect of platelet-derived growth factor on tibial osteotomies in rabbits. Bone 15:203–208.

Neth P, Ciccarella M, Egea V, Hoelters J, Jochum M, Ries C. 2006. Wnt signaling regulates the invasion capacity of human mesenchymal stem cells. Stem Cells 24:1892–1903.

Ng F, Boucher S, Koh S, Sastry KSR, Chase L, Lakshmipathy U, Choong C, Yang Z, Vemuri MC, Rao MS, Tanavde V. 2008. PDGF, TGF-β and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): Transcriptional profiling can identify markers and signaling pathways important in differentiation of MSC into adipogenic, chondrogenic, and osteogenic lineages. Blood 15:295–306.

Ozaki Y, Nishimura M, Sekiya K, Suehiro F, Kanawa M, Nikawa H, Hamada T, Kato Y. 2007. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. Stem Cells Dev 16:119–129.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Pittenger MF, Mbalaviele G, Black M, Mosca JD, Marshak DR. 2001. Mesenchymal stem cells. In: Koller MR, Palsson BO, Masters JRW, editors. Human cell culture. Dordrecht: Kluwer Academic Publisher. pp 189–207.

Ringe J, Strassburg S, Neumann K. 2007. Towards in situ tissue repair: Human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2, and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. J Cell Biochem 101:135–146.

Sanches-Fernandez MA, Gallois A, Riedl T, Jurdic P, Hoflack B. 2008. Osteoclasts control osteoblast chemotaxis via PDGF-BB/PDGF receptor beta signaling. PLoS ONE 3:e3537.

Schmidt A, Ladage D, Schinkothe T. 2006. Basic fibroblast growth factor controls migration in human mesenchymal stem cells. Stem Cells 24:1750–1758.

Segovia-Silvestre T, Neutzsky-Wulff AV, Sorensen MG, Christiansen C, Bollerslev J, Karsdai MA, Henriksen K. 2009. Advances in osteoclast biology resulting from the study of osteopetrotic mutations. Hum Genet 124:561–567.

Son BR, Marquez-Curtis LA, Kucia M. 2006. Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells 24:1254–1264.

Susa M, Luong-Nguyen N-H, Cappellen D, Zamurovic N, Gamse R. 2004. Human primary osteoclasts: In vitro generation and applications as pharmacological and clinical assay. J Transl Med 2:6–17.

Tamama K, Fan VH, Griffith LG, Blair HC, Wells A. 2006. Epidermal growth factor as a candidate for ex vivo expansion of bone marrow-derived mesenchymal stem cells. Stem Cells 24:686–695.

Teitelbaum SL. 2000. Bone resorption by osteoclasts. Science 289:1504–1508.

Tribault MM, Hoemann CD, Buschmann MD. 2007. Fibronectin, vitronectin, and collagen I induce chemotaxis and haptotaxis of human and rabbit mesenchymal stem cells in a standardized transmembrane assay. Stem Cells Dev 16:489–502.

Vikjaer D, Blom S, Hjorting-Hansen E. 1997. Effects of platelet-derived growth factor-BB on bone formation in calvaria defects: An experimental study in rabbits. Eur J Oral Sci 105:59–66.

Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, Suda T, Matsuo K. 2006. Bidirectional ephrinVB2-EphB4 signaling controls bone homeostasis. Cell Metab 4:111–121.