DEVELOPMENT OF AN OSTEOBLAST/OSTEOCLAST CO-CULTURE DERIVED BY HUMAN BONE MARROW STROMAL CELLS AND HUMAN MONOCYTES FOR BIOMATERIALS TESTING

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Abstract

The communication of bone-forming osteoblasts and bone-resorbing osteoclasts is a fundamental requirement for balanced bone remodelling. For biomaterial research, development of *in vitro* models is necessary to investigate this communication. In the present study human bone marrow stromal cells and human monocytes were cultivated in order to differentiate into osteoblasts and osteoclasts, respectively. Finally, a cultivation regime was identified which firstly induces the differentiation of the human bone marrow stromal cells followed by the induction of osteoclastogenesis through the osteoblasts formed – without the external addition of the factors RANKL and M-CSF. As a feedback on osteoblasts enhanced gene expression of BSP II was detected for modifications which facilitated the formation of large multinuclear osteoclasts. Phenotype characterization was performed by biochemical methods (DNA, LDH, ALP, TRAP 5b), gene expression analysis (ALP, BSP II, RANKL, IL-6, VTNR, CTSK, TRAP, OSCAR, CALCR) as well as light microscopy, confocal laser scanning microscopy, and scanning electron microscopy. After establishing this model on polystyrene, similar positive results were obtained for cultivation on a relevant bone substitution material – a composite xerogel of silica, collagen, and calcium phosphate.

Keywords: human bone marrow stromal cells, osteoblasts, human monocytes, osteoclasts, osteoclastogenesis, co-cultivation, biocompatibility, bone remodelling, cross talk, biomaterial, xerogel

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Introduction

Owing to the lack of autologous bone, the development of innovative artificial bone substitution materials is of high importance for surgery and orthopaedics (Gelinsky and Heinemann, 2010). In addition to certain mechanical properties, these materials have to exhibit several biocompatibility features. In order to finally achieve replacement of the substitution material by healthy bone, interaction with the cell types concerned must be considered. The corresponding bone remodelling process – including the cross-talk or coupling as its crucial part – involves bone-building cells (osteoblasts) and bone-resorbing cells (osteoclasts) at a balanced activity level (Matsuo and Irie, 2008).

Osteoblasts (Ob) derive from bone marrow stromal cells (BMSC) which are also known as mesenchymal stem cells (MSC). BMSC are stimulated *in vitro* by ascorbic acid 2-phosphate, β-glycerophosphate, dexamethasone, and as appropriate, 1,25-dihydroxyvitamin D3 to develop the osteoblastic phenotype (Jaiswal *et al*., 1997). Osteoblasts synthesise cytokines and growth factors that regulate formation, activity, and survival of osteoclasts. These multinuclear cells are developed by fusion of monocytes that originate from haematopoietic stem cells. Osteoclasts (Oc) are regulated by various cytokines and hormones such as interleukin IL-1, IL-6, IL-11, LIF, and parathyroid hormone (Suda *et al*., 1992). Two osteoclastogenesis activators play a key role in osteoclast differentiation: macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). M-CSF is important for osteoclast precursor cell survival, migration, and cytoskeletal reorganisation (Yoshida *et a*l., 1990; Lagasse and Weissman, 1997). RANKL binds to its receptor RANK on the surface of osteoclast precursor cells leading to the activation of several signalling pathways which are crucial for the development of mature osteoclasts (Lacey *et al.*, 1998). The effects of RANKL are inhibited by osteoprotegerin (OPG) which acts as a decoy receptor (Hadjidakis and Androulakis, 2006). Both M-CSF and RANKL are required to induce expression of genes that typify the osteoclast lineage, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK), osteoclast-associated receptor (OSCAR), and calcitonin receptor (CALCR) (Boyle *et al*., 2003). *In vivo* and *in vitro* M-CSF and RANKL are expressed by cells of the osteoblastic lineage as well as stromal cells and finally induce osteoclastogenesis dominantly by cell-cell-contact (Mundy *et al*., 1995; Haynesworth *et al*., 1996; Matsuo and Irie, 2008). Furthermore, Majumdar *et al*. showed

that human bone marrow stromal cells (hBMSC) differentiating along the osteogenic linage express cytokine genes and support haematopoiesis similar to that observed for primary osteoblasts (Majumdar *et al*., 2000).

Up to now, *in vitro* cell culture experiments have been performed predominantly using osteoblasts of murine or human origin. Furthermore, corresponding precursor cells have been used in order to study the differentiation behaviour on particular substrates. In this context, the differentiation of hBMSC into osteoblasts is state of the art. Several studies have been published concerning the formation and cultivation of osteoclasts on biomaterials *in vitro*. Success was achieved for the use of monocyte-derived $CD14^+$ precursors (Botelho *et al*., 2006; Spence *et al*., 2009) as well as murine (Narducci and Nicolin, 2009) and human monocytes (Detsch *et al*., 2008a; Heinemann *et al*., 2010) cultivated on artificial biomaterials. Perrotti *et al*. facilitated the differentiation of human peripheral blood mononuclear cells (hPBMC) into osteoclasts on a xenogenous bone mineral (Perrotti *et al*., 2009). The differentiation of preosteoclasts from minipigs in mineralized porous polymer foams was microscopically proven by Nakagawa *et al*. (2004). Owing to the mono-cultivation of the cells, RANKL and M-CSF were added in these cases.

Co-cultures are attractive for *in vitro* biomaterial testing because they are one step closer to natural conditions and allow elucidation of some aspects of the complex interactions between bone-building and bone-resorbing cells. Concerning the evaluation of the potential application of biomaterials for bone tissue engineering, on the one hand optimal conditions for the proliferation and differentiation of osteoblasts must be maintained, and on the other hand it is necessary to ensure that osteoclasts are able to develop and keep their typical functions. Recent studies reporting on co-cultures of osteoblasts and osteoclast used different cell combinations. Some authors report on successful cultures of osteoblast cell lines or primary osteoblasts in combination with PBMC or isolated monocytes. The cells were of murine or human origin and were cultivated with or without the addition of M-CSF and RANKL (Bloemen *et al*., 2009; Greiner *et al*., 2009; Jones *et al*., 2009; Tortelli *et al*., 2009; Bernhardt *et al*., 2010). Only few publications dealing with co-cultures based on BMSC are known. Nakagawa *et al*. performed experiments using porcine BMSC and haematopoetic cells from bone marrow (Nakagawa *et al*., 2004), whereas Mbalaviele *et al*. used hBMSC and $CD34^+$ bone marrow haematopoietic progenitors on polystyrene (Mbalaviele *et al*., 1999).

To the best of our knowledge, studies on human co-cultures of osteoblasts/osteoclasts derived by human bone marrow stromal cells and human monocytes for biomaterial testing have not been published until now. In the present article we describe the development of such a novel co-culture system. Therefore, three modifications of cell culture media regimes were tested on tissue culture polystyrene and two modifications of hBMSC/hOb mono-cultures were used as references. Finally, the modification identified as optimal was applied for testing of a relevant bone substitution biomaterial and first results were reported. Previous studies on the composite xerogels used for that purpose, demonstrated osteogenic differentiation of hBMSC as well as osteoclast formation when mono-cultures were seeded directly on the material (Heinemann *et al*., 2007; Heinemann *et al*., 2009).

Materials and Methods

Isolation of human monocytes

HMc were isolated from buffy coats (purchased from the German Red Cross blood donation service) obtained from the blood of healthy anonymous donors. The isolation is based on the OptiPrep™ density-gradient media technique with some modifications. OptiPrep™ (ProGen Biotechnik, Heidelberg, Germany) was mixed with alpha-MEM (Biochrom, Berlin, Germany) to obtain an OptiPrep™ working solution (WS), a 1.078 g/ml and 1.068 g/ml gradient solutions. The target density was calculated and checked using a densimeter (Mettler Toledo Densito 30PX; Mettler Toledo, Greifensee, Switzerland). The buffy coats were centrifuged at 450 g for 20 min without brake and the leukocyte-rich fraction (LRF) at the interface was collected. OptiPrep™ WS was mixed with the leukocyte-rich fraction to obtain a density of 1.1 g/ml. In a 50 ml Falcon tube, the OptiPrep™ WS/LRF mixture was placed under a layer of 1.078 g/ml lymphocyte-specific gradient solution. A layer of HEPES buffered saline (HBS) was placed on top and centrifuged at 700 g for 20 min without brake. The PBMC fraction was collected, washed with phosphate-buffered saline (PBS) containing 2 mM EDTA and 0.5% BSA, and centrifuged at 400 g for 10 min. OptiPrep™ WS was mixed with the PBMC fraction to obtain a density of 1.1 g/ml and was covered by layers of 1.078 g/ml gradient solution, 1.068 g/ml gradient solution and HBS in that order. The solution was centrifuged at 600 g for 25 min without brake. The monocyte-enriched PBMC fraction floating in the 1.068 g/ml layer was collected and washed with PBS/EDTA/BSA. Finally, monocytes were purified via magnetic activated cell sorting by negative selection using Monocyte Isolation Kit II (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions and counted.

Co-culture on Polystyrol

HBMSCs, isolated from bone marrow aspirates, were kindly provided by Prof. Bornhäuser and co-workers, Medical Clinic I, Dresden University Hospital (Oswald *et al*., 2004). The cells were expanded in Dulbecco's modified Eagle's medium (DMEM), low glucose, supplemented with 10% foetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin in a humidified atmosphere at 37°C and 7% CO_2. Medium and all supplements were obtained from Biochrom. HBMCs in passage 5 were seeded at a density of $8x10^3$ per 48-well and cultured in cell culture medium containing 50 µM ascorbic acid 2-phosphate (Sigma, Taufkirchen, Germany) referred to as (-)-medium. On day 3, one fraction was osteogenically induced by the addition of 10 nM dexamethasone (Sigma),

Table 1. Schedule of the cultivation modifications differing in media composition: (-) without osteogenic supplements is equal to DMEM with 50 µM ascorbate; (+) 10 nM dexamethasone, 50 µM ascorbate, 10 mM β-glycerophosphate, 10 nM vitamin D3; (++) 10 nM dexamethasone, 50 µM ascorbate, 10 mM β-glycerophosphate.

Modification	d0	d1 → d2	d3 → d12	d13/d0	d14/d1 → d21/d7 → d28/d14
I	seeding of hBMSC	(-)	(+)	(++)	(++)
II		(-)	(+)	seeding of hMc	(++)
III		(-)	(+)		(-)
IV		(-)	(-)		(-)
V		(-)	(-)	(-)	(-)

Table 2. Primer sequences and corresponding annealing temperatures applied for RT-PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	T_A [°C]
GAPDH	GGTGAAGGTCGGAGTCAACGG	GGTCATGAGTCCTTCCACGAT	55
ALP	CACGGGCACCATGAAGGAAAA	ATTCTCTCGTTCACCGCCCAC	55
BSP II	AATGAAAACGAAGAAAGCGAAG	ATCATAGCCATCGTAGCCTTGT	55
RANKL	CCAAGATCTCCAACATGACT	TACACCATTAGTTGAAGATACT	50
IL-6	CTGCTCCTGGTGTTGCCT	CCTGCAGCCACTGGTTCT	57
VTNR(α_v)	AAGTTGGGAGATTAGACAGAGG	CTTTCTTGTTCTTGAGGTGG	57
VTNR(β_3)	TGCCTCAACAATGAGGTCATCCCT	AGACACATTGACCACAGAGGCACT	59
CTSK	GATACTGGACACCCACTGGGA	CATTCTCAGACACACAATCCAC	57
TRAP	TTCTACCGCCTGCACTCCAA	AGCTGATCTCCACATAGGCA	57
OSCAR	GAGTAGCTGAAAGGAAGACGCGAT	CAGAGCGCTGATTGGTCCATCTTA	59
CALCR	GCAATGCTTTCACTCCTGAGAAAC	CAGTAAACACAGCCACGACAATGAG	57

10 mM β-glycerophosphate (Sigma), and 10 nM 1,25-dihydroxy vitamin D3 (Calbiochem/Merck, Darmstadt, Germany) to the medium (+). Co-culture was initiated on day 13 and medium was changed to DMEM supplemented with 7.5% foetal calf serum (FCS), 7.5% human serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM ascorbic acid 2-phosphate without (-) or with (++) the addition of 10 nM dexamethasone and 10 mM β-glycerophosphate according experimental parameters presented in Table 1. Subsequently, hMc isolated as described were plated at a density of $3x10^5$ per well on the formed hBMSC cell layer.

Preparation of the xerogels

Sol-gel composite xerogels were prepared without any thermal treatment as described previously (Heinemann *et al.*, 2009). In the present study a three component modification consisting of 50% silica, 30% fibrillar bovine collagen, and 20% hydroxyapatite was used. The composite hydrogels had an initial volume of 800 µl, and shrunk during drying to disc-shaped xerogels with diameters of about 5 mm and a height of about 3 mm.

Co-culture on biomaterial

Composite xerogel samples were γ-sterilized (25 kGy) and soaked in cell culture medium (DMEM supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin and 50µM ascorbic acid 2-phosphate) for 24 h before cell seeding. After removing the medium, 250 µL of hBMSC cell suspension (5000 cells per µL) were seeded per well. Osteogenic differentiation was started at day 3 by addition of 10 nM dexamethasone, 10 mM β-glycerophosphate and 10 nM Vitamin D3 to the medium. On day 13, a monocyte cell suspension with $4x10^5$ cells per well was seeded on the top of the sample in the 96-well-plates and co-culture cultivated for up to 4 weeks in (-)-medium (Medium composition corresponded to modification III of the experiments on polystyrene).

Colorimetric measurements

All measurements were performed with cell lysates obtained after 1, 14, 21, and 28 days of cultivation. Cell lysis was achieved with 1% Triton X-100 (Sigma) in PBS. For all colorimetric measurements, a SpectraFluor Plus microplate reader (Tecan, Crailsheim, Germany) was used.

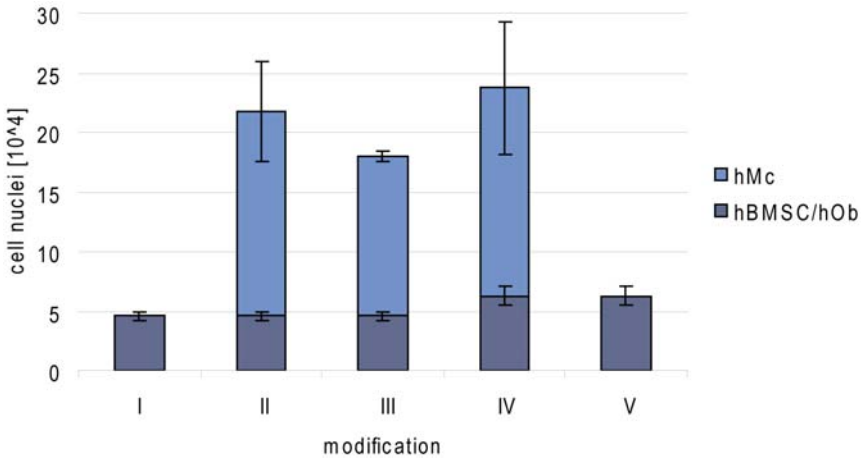


Fig. 1. Number of cell nuclei at d14/d1 calculated from DNA measurements.

DNA assay

Examination of DNA amount was carried out using Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen, Darmstadt, Germany). The DNA amount was correlated with the number of cell nuclei using calibration lines of cell lysates of pure hBMSCs and pure monocytes with defined cell numbers.

LDH activity assay

Cell proliferation was determined through the total activity of LDH in the cell lysates using an LDH Cytotoxicity Detection Kit (Takara, Saint-Germain-en-Laye, France). An aliquot of cell lysate was mixed with LDH substrate buffer, and the enzymatic reaction was stopped after 30 min with 0.5 M HCl. The absorbance was read at 492 nm. The LDH activity was correlated with the cell number using a calibration line of cell lysates with defined cell number.

ALP activity assay

Osteoblast differentiation was evaluated by the measurement of ALP activity. An aliquot of cell lysate was added to ALP substrate buffer, containing 2 mg/mL p-nitrophenyl phosphate (Sigma), 0.1 M diethanolamine, 1 mM $MgCl_2$, 0.1% Triton X-100 (pH 9.8), and the mixture was incubated at 37 °C for 30 min. The enzymatic reaction was stopped by the addition of 0.5 M NaOH, and the absorbance was read at 405 nm. A calibration line was constructed from different concentrations of p-nitrophenol.

TRAP 5b activity assay

Osteoclast differentiation was evaluated by the measurement of TRAP5b activity using naphthol-ASBI phosphate (N-ASBI-P, Sigma) as a substrate according to a slightly modified protocol of Janckila *et al.* (Janckila *et al.*, 2001). Cell lysates were added to the TRAP 5b reaction buffer consisting of 2.5 mM N-ASBI-P in 100 mM Na-acetate (Sigma) buffer containing 50 mM Na-tartrate (Sigma), 2% NP-40 (Sigma) and 1% ethylene glycol monomethyl ether (EGME, Sigma) adjusted to pH 6.1 and the mixtures were incubated at 37 °C for 1 h. The enzymatic reaction was stopped by adding 0.1 M NaOH and fluorescence was measured at an excitation wavelength of 405 nm and emission wavelength of 535 nm. The relative fluorescence units were correlated to a TRAP 5b standard.

Statistics

All measurements were collected in triplicate and expressed as mean ± standard deviations. Analysis of variance (ANOVA) was employed to assess the statistical significance of results. *P* values less than 0.01 (**) were considered very significant for all analyses.

Microscopy

Light microscopy of cell-seeded PS was performed using a Zeiss Axiovert 25 (Zeiss, Jena, Germany) equipped with a digital camera (Canon, Tokyo, Japan). Confocal laser scanning microscopy (cLSM) was applied to evaluate adhesion, differentiation, and multinuclearity of the cells. After washing with PBS and fixing with 3.7% formaldehyde the cells were permeabilized with 0.2% Triton-X-100 in PBS and blocked with 1% bovine serum albumin (BSA, Sigma) for 30 min. Cytoskeletal actin was stained with AlexaFluor 488®-Phalloidin (Invitrogen, Carlsbad, CA, USA), cell nuclei with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Microscopy was carried out on an upright Axioscop 2 FS mot equipped with a LSM 510 META module (Zeiss) controlling an argon-ion (Ar^+) laser and NIR-femtosecond titanium-sapphire laser for 2-photon excitation (Coherent (Santa Clara, CA, USA) Mira 900F). Excitation of AlexaFluor 488® was carried out at 488 nm (Ar^+ laser). The NIR-fs-laser was used for excitation of DAPI at 750 nm (2 photon excitation) and fluorescence was recorded at 461 nm. After finishing the cLSM experiments, cell-seeded silica-collagen xerogels were dehydrated, critical-point dried, mounted on stubs, and sputtered with gold. Scanning electron microscopy (SEM) was carried out using a Philips (Eindhoven, The Netherlands) environmental scanning electron microscope (ESEM) XL 30 in Hi-Vac mode by applying an acceleration voltage of 3 kV and detecting secondary electrons for imaging.

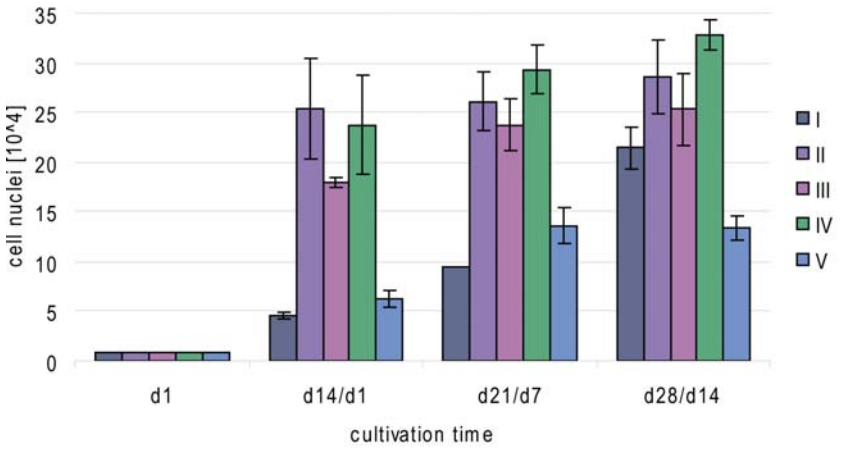


Fig. 2. Number of cell nuclei during 28 days of cultivation calculated from DNA measurements.

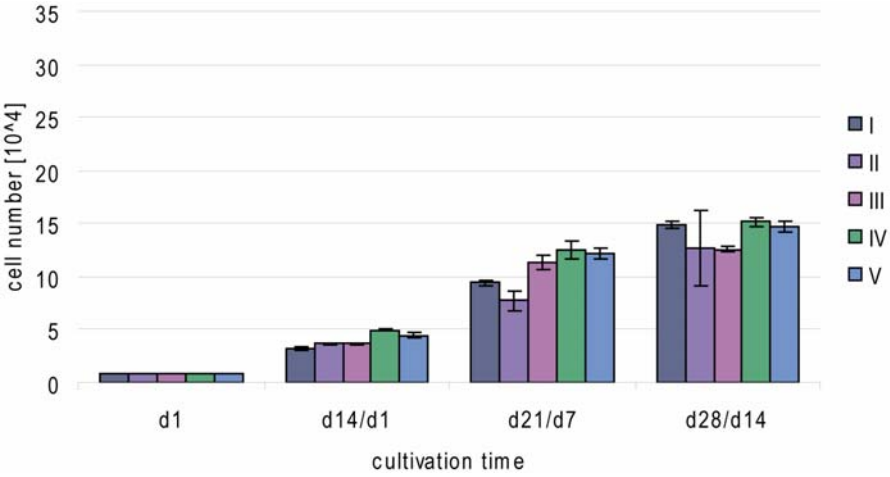


Fig. 3. Cell numbers calculated from LDH activity. Taking into account the negligible LDH activity of non-proliferating hMc/hOc, the results predominantly represent the proliferation of hBMSC/hOb in mono-culture as well as in co-culture.

RT- PCR

For analysis of gene expression by reverse transcription-polymerase chain reaction (RT-PCR), cells on culture dishes as well as on xerogels were washed twice with PBS and immediately used for RNA preparation. Total RNA isolation was performed using the peqGOLD MicroSpin Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA concentration was measured using a Nanodrop ND 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was transcribed from 250 ng of total RNA in a 20 µL reaction mixture containing 200 U of Superscript II Reverse Transcriptase (Invitrogen), 0.5 mM dNTP (Invitrogen), 12.5 ng/µL random hexamers (MWG Biotech, Ebersberg, Germany), and 40 U of RNase inhibitor RNase OUT (Invitrogen). For cDNA synthesis, the reaction mixtures were incubated for 50 min at 42°C followed by 15 min at 70°C in a Thermocycler (Peqlab). For PCR experiments, 1 µL of cDNA was used as a template in a 20 µL reaction mixture containing specific primer pairs to detect transcripts of alkaline phosphatase (ALP), bone sialoprotein II (BSP II), receptor activator of nuclear factor κB ligand (RANKL), interleukin-6 (IL-6), α_V- and β_3-subunits of vitronectin receptor (VTNR), cathepsin K (CTSK), tartrate resistant acid phosphatase (TRAP), osteoclast-associated receptor (OSCAR), calcitonin receptor (CALCR), and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively, for each sample. The primer sequences (MWG Biotech) and annealing temperatures are summarized in Table 2. After the initial activation step at 95°C for 4 min, 25-40 PCR cycles were run with each a denaturation step at 95°C for 45 s, an annealing step at 50-59°C for 45 s, and a synthesis step at 72°C for 1 min followed by a final synthesis step at 72°C for 10 min in a Primus 25 Advanced Thermocycler (Peqlab). The same single stranded cDNA was used to investigate the expression of all genes described. The resulting PCR-products were analyzed using the FlashGel™ Dock and documentation system (Cambrex Bio Science, East Rutherford, NJ, USA). Expression of the markers was normalised to the expression of the housekeeping gene by image analysis using the BioImaging System Gene Genius with the acquisition software GeneSnap and the GeneTools software (SynGene, Cambridge, UK).

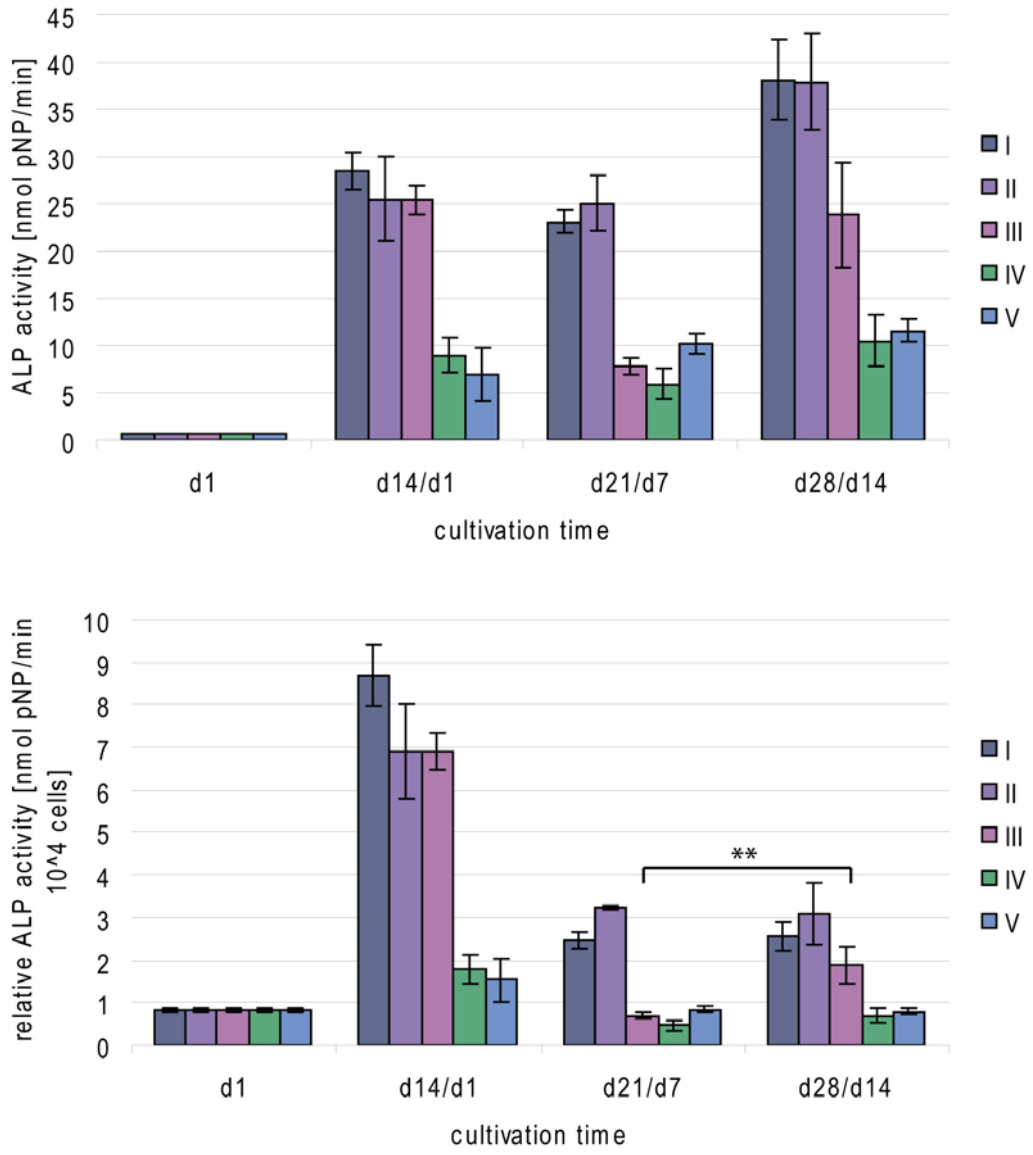


Fig. 4. Absolute ALP activity and relative ALP activity based on LDH activity measurements of the hBMSC/hOb measured for mono-cultures and co-cultures.

Results

Co-culture testing on polystyrene

Cultivation of the co-culture was performed using three media modifications and two hBMSC fractions in mono-culture as a reference. The schedule of the cultivation modifications and corresponding media is presented in Table 1.

During the first 13 days, hBMSC cultured in (+)-medium (modification I) proliferated from $0.8x10^4$ to about $5x10^4$ per well, whereas for hBMSC cultivated in (-)-medium (modification V) proliferation was slightly higher (Fig. 1). In co-cultures (modifications II-IV) DNA analysis measures the total of hBMSC/hOb and hMc. Therefore an analytical distinction is not possible and the results are considered as the number of cell nuclei instead of cell numbers. Furthermore, that denotation regards fusion of several hMc to form multinuclear osteoclasts. On the supposition that modifications I-III and IV-V are identical up to d13, using the hBMSC mono-cultures as references, the total can be arithmetically divided into the two cell types. Consequently, after one day in culture and after removal of non-adherent cells, about $13\text{-}18x10^4$ hMc are adherent next to the hBMC/hOb on the co-culture modifications. That amount corresponds to the half of the applied cell numbers.

During further cultivation the increasing DNA amounts on d21/d7 and d28/d14 (Fig. 2) are ascribed to the proliferation of hBMSC/hOb, because hMc/hOc belong to postmitotic cell types (Nijweide *et al.*, 1986). LDH activity measurements were performed in order to prove DNA results. Comparison of separate calibration lines revealed the LDH-activity of hMc to be about 1/60 of that of hBMSC (data not shown). Taking that into account, LDH activity of hMc/hOc is negligible in co-culture with hBMSC/hOb although the applied cell number of hMc is higher by a factor of about 3.5-5 compared to hBMSC/hOb. Therefore, cell numbers calculated from LDH activity predominantly represent the hBMSC/hOb cell fraction also in co-culture (Fig. 3). The results of the mono-cultures

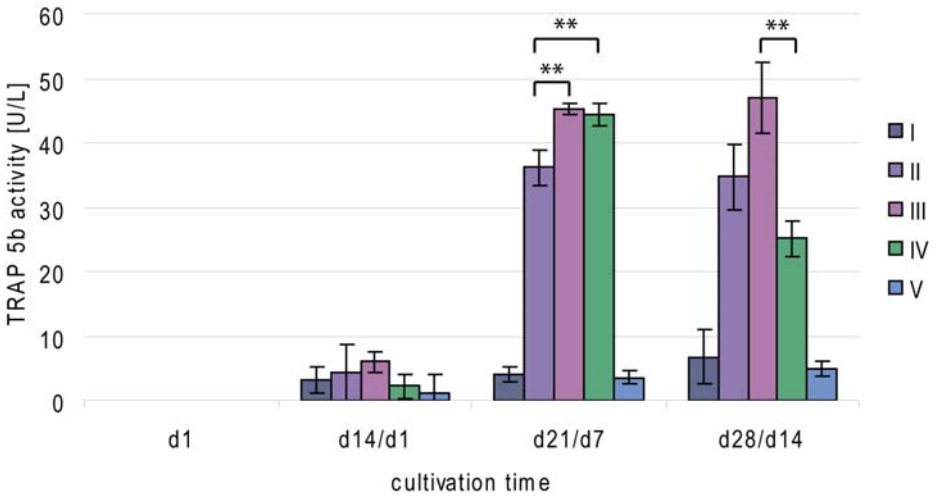


Fig. 5. TRAP 5b activity of the hMc/hOc measured during the cultivation time.

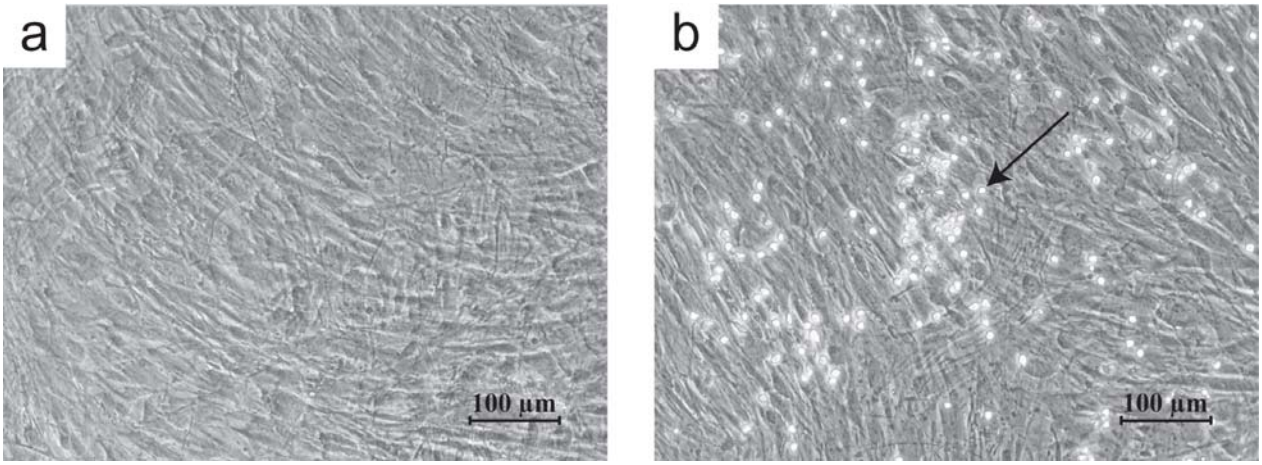


Fig. 6. Light microscopy images of the hBMSC/hOb mono-culture (**a**, modification I) and the co-cultivation with hMc/hOc (**b**, modification II) on d14/d1. The black arrow indicates a monocyte.

(modifications I and V) correspond well with the DNA analysis over the whole cultivation period. The combination of both methods to determine proliferation is suitable to obtain more information from the co-culture. The comparison of the LDH activity of the different modifications suggests that the proliferation behaviour of the hBMSC/hOb does not seem to be influenced by the presence of the hMc/hOc, independently of the media used.

Fig. 4 shows the ALP activity measured for all modifications. Since hMc/hOc have no ALP activity, the values are attributed to the hBMSC/hOb. HBMSC/hOB of modifications I-III (osteogenically induced) and IV-V (not induced) are identically cultured up to d13 which results in similar ALP activities also on d14/d1. Addition of osteogenic supplements to the media results in typically increasing ALP activity for modifications I-III. ALP activity of the HBMSC/hOb passes the typical maximum, which characterises cell maturation and beginning matrix mineralization. This is confirmed by relative ALP activities (ALP/LDH) calculated for modifications I and V. Assuming similar hOb cell numbers, this course is also suggested for the co-culture modifications II and III. For modifications IV-V the typical base level of ALP activity of the hBMSC is detected. Since the values are not relative to the initial cell number, increasing ALP activity is due to proliferation in these cases. Owing to the similar cell culture media regime, the ALP activities of modifications I and II as well as IV and V are similar. This confirms that differentiation of hBMSC/hOb is not affected by the hMc/hOc from d13. Since d13, hBMSC/hOb of modification III have been cultured without osteogenic supplements which results in decreased ALP activity, as measured on d21/d7. The level is similar to modifications IV and V which have been cultured without osteogenic supplements since the beginning. Surprisingly, the ALP level of modification III significantly increases again on d28/d14 which might be attributed to the interaction with hMc/hOc. The same effect can be speculated for modification IV without being significant.

TRAP 5b activity was measured to determine the osteoclast differentiation status of the hMc/hOc. The results are shown in Fig. 5. At d14/d1 hMc have undergone one day of cultivation in the co-culture and show low TRAP 5b activity. Modifications I and V reveal the base level determined according to the measurement method. During osteoclastogenesis in co-culture TRAP 5b activity clearly

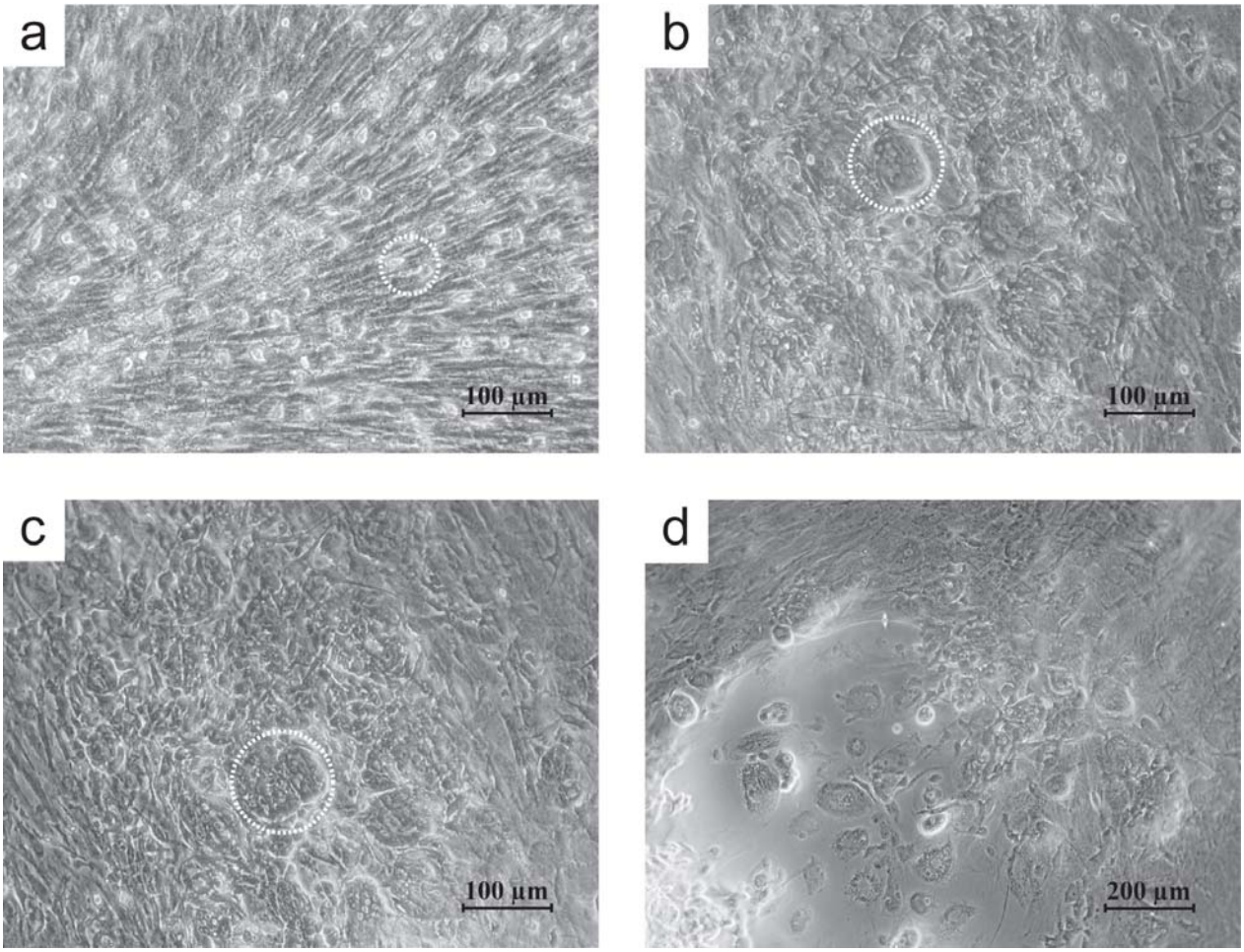


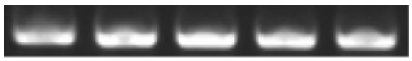
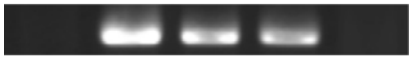
Fig. 7. Light microscopy images of modifications II (**a**), III (**b**), and IV (**c**, **d**) on d28/d14. Dotted circles indicate cells of the osteoclastic lineage embedded in the dense layer of spread hBMSC/hOb.

increases up to d21/d7 and maintains the corresponding levels. On d21/d7 the TRAP 5b activities of modifications III and V are significantly higher compared to modification II. For modification IV a slight decrease to the late time point is detected and TRAP 5b activity is significantly lower compared to modification III.

Light microscopy of the cells was performed to evaluate cell behaviour and formation of characteristic patterns, especially multinuclear osteoclasts in the co-culture. Image (a) in Fig. 6 confirms the dense cell layer formed during proliferation of the hBMSC/hOb during the first 14 days of mono-culture. In addition to that layer, round-shaped monocytes (an example of which is indicated by the black arrow) can be identified on d14/d1 in the co-culture (image b). Owing to direct physical contact the cell types should be able to establish typical cell-cell communication. The images of Fig. 7 show the results on d28/d14. The cell distribution of the hBMSC/hOb monoculture did not change compared to Fig. 6 during further cultivation and is not shown for that reason. The image of modification II reveals that hMc migrated to become evenly distributed over the surface. The cell shape changed from small spherical to larger adherent (Fig. 7a). The cell size distribution is considerably regular. No large multinuclear cells could be found. In contrast, large multinuclear osteoclasts with cell diameters of up to about 100 µm have been formed on modifications III and IV. These cells are closely embedded in the hBMSC/hOb cell layer. In addition to these large cells some smaller ones similar to that of modification II can be identified. Image d shows the edge of the hBMSC/hOb cell layer at lower magnification revealing increased number of large osteoclasts. It has to be highlighted that these cells formed without addition of RANKL and M-CSF.

To further characterize the phenotypes of differentiated hBMSC as well as hMc, gene expression was analyzed performing RT-PCR on d28/d14 (Table 3). Markers assigned to hBMSC/hOb are ALP, BSP II, RANKL, and IL-6. Markers assigned to hMc/hOc are TRAP, OSCAR, and CALCR. CTSK and VTNR subunits are also used to characterize osteoclasts but are also expressed by stem cells (Silva *et al*., 2003; Mandelin *et al*., 2006; Del Fattore *et al*., 2008). Expression of GAPDH was determined to verify the usage of similar amounts of RNA for RT-PCR. After 28 days of cultivation gene expression of ALP was detected for all modifications and was highest for modification II. BSP II was also detected for all modifications but was clearly higher for modifications III and IV. RANKL expression levels were similar for all samples. The expression levels of IL-6 and VTNR (β_3) were reduced

Table 3. Gene expression of ALP, BSP II, RANKL, IL-6, VTNR (α_v), VTNR (β_3), CTSK, TRAP, OSCAR, and CALCR for the five modifications on d28/d14. Expression of the markers was normalised to the expression of the housekeeping gene GAPDH. For comparison, highest values were set to 1.

gene	I	II	III	IV	V	I	II	III	IV	V
GAPDH										
ALP						0.8	1	0.4	0.2	0.2
BSP II						0.5	0.4	0.8	1	0.3
RANKL						1	0.8	0.9	0.7	0.8
IL-6						0.6	0.6	1	0.8	0.7
VTNR (α_v)						0.8	0.7	0.9	1	0.8
VTNR (β_3)						0.2	0.6	0.9	1	0.6
CTSK						0.5	0.9	1	0.9	0.4
TRAP						0	1	0.7	0.6	0
OSCAR						0	0.5	0.6	1	0.1
CALCR						0	0.2	1	0.6	0

for modifications I and II compared with modifications III-V. VTNR (α_v) gene expression was similar for all modifications. CTSK was detected with similarly elevated levels in the co-cultures, but also with much lower levels in the hBMSC/hOb mono-cultures of modification I and V. The osteoclastic markers TRAP, OSCAR, and CALCR were detected with varying rankings for all co-culture modifications.

Co-culture on biomaterial

The reported co-culture is designed to be a technique for the characterization of a biomaterial in terms of its ability to maintain or even influence the cross-talk between the cells involved in bone remodelling. Therefore, we tested the co-culture on a bone replacement material that we are currently developing. In this connection, we previously reported on biocompatibility experiments on silica-collagen based xerogels using hBMSC (Heinemann *et al*., 2007) and differentiation of hMc to osteoclasts directly on the surface of the xerogels (Heinemann *et al*., 2009). Now, the procedure of modification III was used for co-cultivation of hBMSC/hOb and hMc/hOc directly on this material. Fig. 8 shows corresponding cLSM and SEM images of the samples on d42/d28. During the cultivation time the hBMSC adhered, proliferated and formed dense cell layers of differentiated osteoblasts covering the whole sample. Multinuclear osteoclasts can be identified amongst the osteoblast cell layer by typical morphological features such as spherical morphology and typical actin ring. This seals the resorption zone in which acid and enzymes are secreted in order to resorb underlying matter (Detsch *et al*., 2008b). The osteoclast (b), depicted as an example, was formed by fusion of four single monocytes and has a diameter of about 50 µm. Podosomes as part of the adhesion apparatus are visible as dots in the inner of the cell. On the outer edge of the cell additionally thin filopodia are visible. SEM imaging revealed similar outer morphology of the osteoclasts (c). Cell nuclei and actin ring are not visible in that case; however filopodia and typical blebbing on the cell surface were recognized.

Discussion

The evaluation of new bone graft materials initially focuses on the response to osteoblasts and their precursors to the respective material, by analyzing biocompatibility, osteoconductivity or osteoinductivity. However, the success of an implant not only depends on the interaction with osteoblasts. Osteoblasts are responsible for the generation of bone extracellular matrix but also regulate the differentiation and activity of osteoclasts. The coordinated action of osteoblasts and osteoclasts is critical for bone remodelling. Therefore the investigation of osteoclast differentiation and function on a new biomaterial is of great interest. Osteoclastic resorption may affect bone formation and is a crucial step for the conversion of the bone graft material into native bone. A fast remodelling

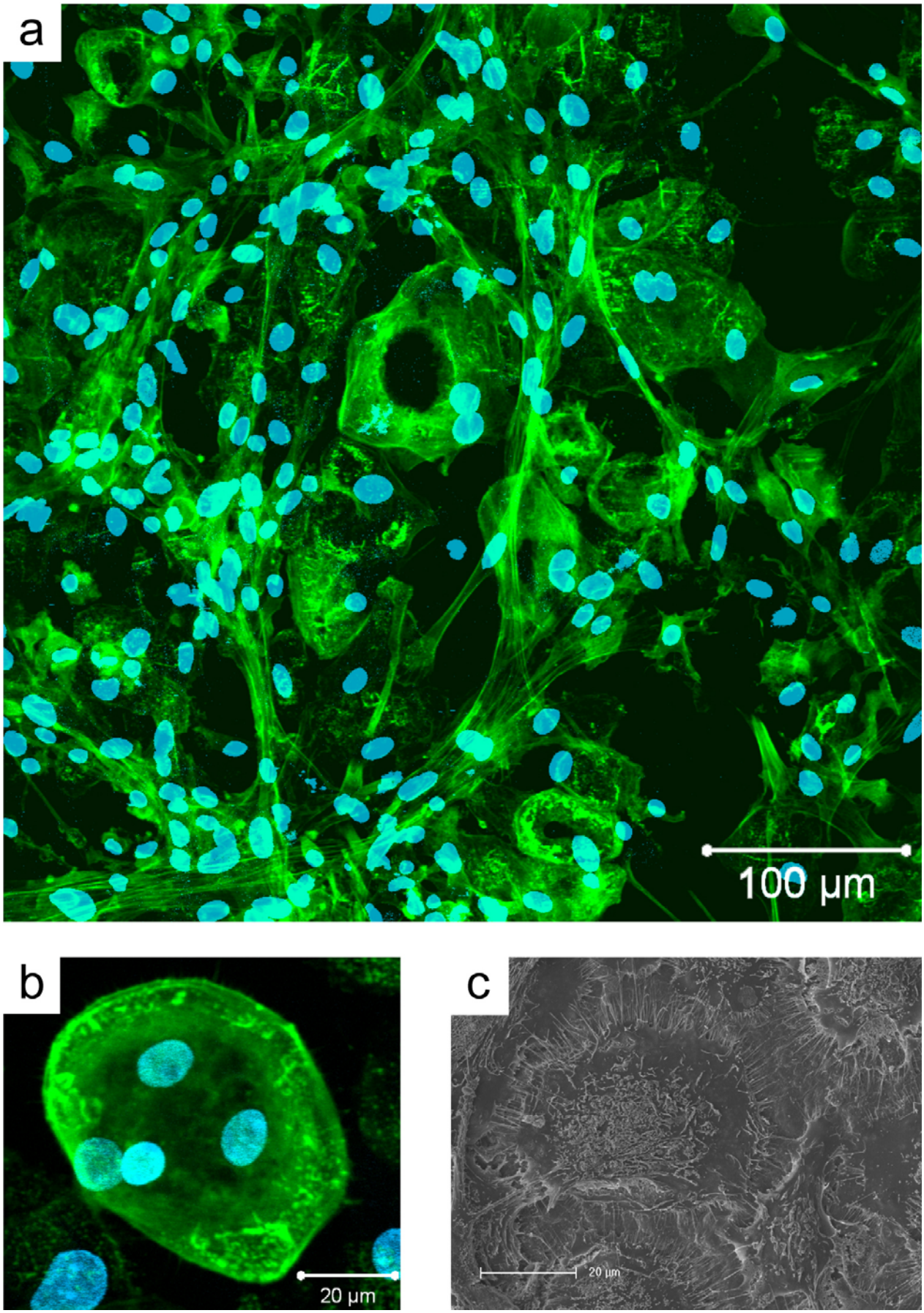


Fig. 8. 3D reconstructions from cLSM image stacks (**a**, **b**) and SEM image (**c**) after 28 days of co-cultivation on silica-collagen-calcium phosphate composite xerogels. The actin skeletons (green) and the nuclei (blue) are visible.

process of the bone graft is desirable and strongly depends on the conductivity of the material to both osteoblasts and osteoclasts. In the present study, a procedure for co-cultivation of osteoblasts/osteoclasts derived by hBMSC and hMc was successfully established. We found significant influence of medium composition on the proliferation and differentiation of hBMSC as well as hMc and now provide an optimal strategy for the present system.

During the first 13 days of hBMSC/hOb mono-cultivation the non-induced and osteogenically induced cell fractions showed similar cell proliferation. Owing to the fact that hMc/hOc are postmitotic cells, their contribution to proliferation values is constant over cultivation time (Nijweide *et al*., 1986). In co-culture DNA measurements are useful to detect the whole amount of nuclei of both cell types, whereas LDH activity measurements can be useful to focus on hBMSC/hOb proliferation due to the significantly lower LDH activity of hMc/hOc. Compared to the LDH activity measurements in mono-cultures, for all co-culture modifications the hMc/hOc behaviour seems not to influence hBMSC/hOb proliferation.

The differentiation of the hBMSC into osteoblast-like cells was confirmed by the increase of ALP activity which is an early marker relevant in matrix mineralization (Hughes and Aubin, 1997; Hessle *et al*., 2002). In the present study, an increase of ALP activity was observed for hBMSC due to modifications I-III during cultivation on PS in the presence of osteogenic supplements whereas ALP activity did not rise in the absence of osteogenic supplements (modifications IV and V). The activity reaches the typical maximum around day 14 followed by falling values representing osteocytic differentiation (Robins, 1997). Modifications I and II as well as IV and V show similar courses of absolute and relative ALP activity suggesting that hMc/hOc do not influence osteogenic differentiation. Interestingly, absolute and relative ALP activity of modification III significantly increases again from d21/d7 to d28/d14. This effect can be attributed to inductive activity of hMc/hOc which already has been reported by Fong *et al*. (Fong *et al*., 2008). For all five modifications the relative proportions on d28/d14 were supported by corresponding RT-PCR ALP expression levels.

Owing to the detection of RANKL gene expression for all modifications, induction of hMc to form osteoclasts should appear for modification II-IV (Hsu *et al*., 1999). The differentiation of the monocytes in co-culture was evaluated by their TRAP activity as well as multinuclearity and gene expression of osteoclastic markers. Two isoforms of TRAP circulate in human blood, known as TRAP 5a and TRAP 5b. Especially the TRAP 5b isoform is derived from osteoclasts (Halleen *et al*., 2000; Halleen *et al*., 2002; Yam and Janckila, 2003). Since both isoforms derive from post-translational processing of a common gene product, gene expression analyses are not sufficient to distinguish between them. Therefore we additionally performed a TRAP 5b activity assay, which clearly showed the presence of TRAP 5b in the co-cultures. RT-PCR analyses showed the highest expression level for TRAP (total of isoforms 5a and 5b) of modification II, whereas biochemical analysis revealed the highest level of isoform 5b for modification III. This finding can be explained by referring to the light microscopy images. For modification II from d14/d1 to d28/d14 a morphological change of the small spherical hMc to adherent round cells appears, however, large multinuclear osteoclasts were not detected. Possibly, the presence of osteogenic supplements causes changes in the expression profile of hBMSC/hOb cytokines which may result in inhibitory effects on migration and/or fusion of mononuclear monocytes. Haynesworth *et al*. found that osteogenic supplements cause decreasing the expression of IL-6 and IL-11, which are well-known regulators of osteoclastogenesis *in vitro* (Haynesworth *et al*., 1996). The decrease of IL-6 expression for modifications I and II compared to modifications III-V was also detected in the present study. Further effects were discussed by Takeyama *et al*. who reported on the inhibitory effect of extracellular phosphate on osteoclastogenesis while osteoclast cell numbers declined with increasing concentration of β-glycerophosphate in the culture medium (Takeyama *et al*., 2001). Furthermore, dexamethasone can influence the osteoclast formation from monocytes by several mechanisms. On the one hand dexamethasone is known to promote osteoclastogenesis by inhibition of OPG mRNA expression and stimulation of RANKL mRNA expression by osteoblasts in a dose-dependent manner (Sivagurunathan *et al*., 2005; Kondo *et al*., 2008). Hozumi *et al*. confirmed similar effects of dexamethasone on RANKL and OPG mRNA expression, however the size of single cells and the number of nuclei per cell were recognized to be smaller than for the control group (Hozumi *et al*., 2009). In contrast, Kim *et al*. reported that expression of RANKL and OPG was not affected by the presence of dexamethasone, but rather the formation of multinucleated osteoclasts was inhibited by down-regulation of vitronectin receptor (β_3 subunit) expression. Moreover, dexamethasone treatment influenced the fusion of mononuclear precursors, but not the formation of TRAP-positive prefusion osteoclasts (Kim *et al*., 2006). The observations of Kim *et al*. correspond exactly with our findings for co-culture modification II, where TRAP-positive mononuclear cells in combination with reduced β_3 integrin expression were observed.

In contrast to modification II, microscopy revealed fewer mononuclear cells, but more large multinuclear cells for modifications III and IV. These cells stand out as a silhouette from the cell layer of hBMSC/hOb which is most obvious on the edges of the cell layers where monocytes could migrate more freely. These modifications provide optimal conditions for the development of mature osteoclasts. All common osteoclastic markers were detected at an elevated expression level. Whereas CTSK and VTNR are also expressed by hMSC/osteoblasts (Silva *et al*., 2003; Mandelin *et al*., 2006), the genes for TRAP, CALCR, and OSCAR are highly specific markers. A distinct feedback of the osteoclasts on osteoblasts is shown for the gene expression of BSP II, which is clearly most intensive for modifications III and IV – the only modifications which facilitated the formation of large

multinuclear osteoclasts. Similar results have been recently published by Kreja *et al*. whereby BSP II mRNA expression was up-regulated after stimulation of hBMSC with conditioned media collected from human osteoclasts (Kreja *et al*., 2010).

Taking into account both the differentiation of hBMSC and hMc, modification III seems to be best-suited for co-cultivation of human osteoblasts and osteoclasts in the present case. Performing a cultivation strategy starting with osteogenic supplements in order to differentiate hBMSC followed by replacement of osteogenic supplements during osteoclastogenesis similar to our modification III, Mbalaviele *et al*. discovered negative effects on the number of osteoclast-like cells compared to co-culture without osteogenic supplements and explained that effect by decreased expression of IL-6 and IL-11, LIF (Mbalaviele *et al*., 1999). Although cultivation strategies were similar, opposing results might be ascribed to the different cell types used. Mbalaviele used $CD34^+$ bone marrow haematopoietic progenitors isolated by positive selection. In contrast we used monocytes isolated by negative selection, whereby non-monocytes are labelled using antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a. In our case $CD34^+$ cells cannot be excluded, however performing flow cytometry using an anti-CD14-FITC antibody, we detected the purity of negatively isolated monocytes to be about 90%.

Finally, modification III was chosen for first tests on a relevant bone substitution material. The results concerning cell proliferation and differentiation could be confirmed and were completed by cLSM and SEM which revealed the presence of osteoblasts and osteoclasts being adherent next to each other directly on the biomaterial's surface. The formation of an actin ring is the most significant characteristic to confirm the osteoclastic phenotype (Monchau *et al*., 2002). Furthermore cLSM visualized podosomes – structures by which osteoclasts adhere to the surface via an intramembranous integrin. These molecules act as an intermediate and the intracellular cytoskeletal actin microfilaments. These microfilaments bind to integrins through complex focal adhesion contacts containing several proteins in a ring shape at the periphery of the cell (Lakkakorpi *et al*., 1989). All these structural elements were confirmed for the modification III co-culture. The feature of the biomaterial to support the proliferation and differentiation of osteoblasts as well as the formation of multinuclear osteoclasts provides proper conditions for the material to be resorbed and/or degraded and finally be replaced by newly formed bone. Extensive experiments using modification III as a human osteoblast/osteoclast co-culture on various biomaterials will be a topic of future studies in our group.

Conclusion

The formation of a working co-culture of human osteoblasts and osteoclasts derived by hBMSC and hMc respectively was demonstrated in the study. The *in vitro* experiments demonstrated a significant influence of the medium composition and confirmed that osteogenically differentiated hBMSC were able to induce osteoclastogenesis of hMc without external addition of RANKL and M-CSF. *Vice versa* osteoclasts stimulated BSP II gene expression of osteoblasts. An optimal cultivation regime for co-cultivation of both cell types was identified by detection of several differentiation markers. The method was successfully adapted for preliminary testing of a biomaterial composed of silica, collagen, and calcium phosphate.

Acknowledgements

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Discussion with Reviewers

Reviewer I: The authors should explain why it is worth taking the pain of going through BMSC isolation and subsequent enrichment with monocytes and not using whole bone marrow which already comprises monocytes and has long been used as an osteoblast/osteoclast mixed model. What are the advantages?

Authors: One main requirement of the present study was the use of human precursor cells. The aim of the study was to establish a co-culture model with specified starting conditions. Therefore, isolation of the precursor cells (hBMSC and hMc) was carried out carefully. The study design was intended to exclude the most of uncontrollable influencing factors in order to enable us to make a clear conclusion.

Of course, bone marrow is closer to *in vivo* conditions. However, its donor-dependent quality, inherent mixture of cell types of varying differentiation levels, the mixture of factors and cytokines, was not considered to be suitable.

Reviewer I: The authors discuss several components of their medium and their effects on osteoclast formation but never mention and discuss Vitamin D, which they use and which was the first (or one of the first) osteoclast-inducing agents used. They might do this on purpose, but the net sensation is that of an awkward omission.

Authors: We are well aware that vitamin D3 plays a crucial role in osteoclastogenesis. A corresponding study on this topic was performed in our lab and is intended to be published in the near future. However, the effect of vitamin D3 on osteoclastogenesis is discussed controversially in the literature. In the present study vitamin D3 was only used for osteogenic induction (up to day 12) – it was NOT supplied in combination with monocytes or osteoclasts (see Table 1). Therefore, we feel that it is not necessary to stress this controversial topic at this point.

Reviewer I: The authors examine and extensive panel of genes, including all the main osteoclastic genes. They do not, however, consider osteocalcin, among the osteoblastic markers, although it is often considered one of the best markers of a mature phenotype. It would be interesting to examine osteocalcin expression in the culture conditions tested.

Authors: It is known that osteocalcin expression can be repressed due to dexamethasone (Jaiswal *et al*., 1997, text reference). Therefore, osteocalcin expression analysis could have been influenced by that in the present study. Nevertheless, we also observed osteocalcin expression for the osteogenically differentiated hBMSC. According to our experience, BSP II which was investigated in the present study is also a very accepted and reliable marker for mature osteoblasts – and seemed to be more appropriate in the present case.

Reviewer II: Was any remodelling of the scaffold material observed after co-culture?

Authors: We tried to visualize resorption appearance after removal of the cells by trypsinisation. However the inherent surface roughness of the xerogels hampered unambiguous identification of resorption lacunae. Further studies using polished surfaces of these xerogels are currently being initiated to go further into this question.