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Erstveröffentlichung in / First published in:

Biomaterials Advances. 2022. 134, Art. Nr. 112692. Elsevier. ISSN 2772-9508.

DOI: https://doi.org/10.1016/j.msec.2022.112692

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Impact of degradable magnesium implants on osteocytes in single and triple cultures

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Abstract

In vitro triple cultures of human primary osteoblasts, osteocytes and osteoclasts can potentially help to analyze the effect of drugs and degradation products of biomaterials as a model for native bone tissue. In the present study, degradation products of Magnesium (Mg), which has been successfully applied in the biomedical field, were studied with respect to their impact on bone cell morphology and differentiation both in osteocyte single cultures and in the triple culture model. Fluorescence microscopic and gene expression analysis, analysis of osteoclast- and osteoblast-specific enzyme activities as well as osteocalcin protein expression were performed separately for the three cell types after cultivation in triple culture in the presence of extracts, containing 5 and 10 mM Mg^{2+}. All three cell species were viable in the presence of the extracts and did not show morphological changes compared to the Mg-free control. Osteoblasts and osteoclasts did not show significant changes in gene expression of ALPL, BSPII, osteocalcin, TRAP, CTSK and CA2. Likewise on protein level, no significant changes in ALP-, TRAP-, CTSK- and CAII activities were detected. Osteocytes showed a significant downregulation of MEPE, which codes for a protein playing an important role in regulation of phosphate homeostasis by osteocytes. This study is the first to analyze the effects of Mg degradation products on primary osteocytes in vitro, both in single and triple culture. Even if promoting effects on the three examined bone cell species were not found in the applied triple culture setup, it was shown, that Mg degradation products do not interfere with the activity of osteoblasts, osteoclasts and osteocytes in vitro.

1. Introduction

Magnesium (Mg) has been successfully applied as orthopedic implant material for a long time due to its biocompatibility and biodegradability, providing the possibility to create temporary implants, which gradually degrade with emerging bone regeneration (1–3). Furthermore, the mechanical properties of Mg-based materials are very close to natural bone tissue, overruling that of other metallic implant materials like stainless steel and titanium alloys. The potential of Mg-based implants for fracture repair has been tested in different animal models and some clinical trials (4,5). Proteomics studies revealed, that Mg changed the expression of some osteogenesis-related proteins (6). In contrast to non-degradable materials, a possible interference of Mg degradation products with metabolical pathways has to be considered (7). High concentrations of Mg^{2+} have cytotoxic effects in vitro as demonstrated

by Wang and co-workers for four different cell lines (8). On the other hand, Mg^{2+} is essential for various metabolic processes in cells, as it acts as cofactor for more than 600 enzymes and plays a pivotal role in DNA repair and replication fidelity (9). Therefore, it was hypothesized, that degradation products of Mg implants are the reason for the osteoconductivity of these materials. Different in vitro studies analyzed the effect of Mg salts and Mg extracts on bone-derived cells and revealed partly contradictory results: An upregulation of osteoblast markers like ALPL, BSPII, and osteocalcin was detected in different osteoblast cell lines in the presence of Mg ions (10,11). On the other hand, numerous studies have demonstrated, that Mg^{2+} significantly suppresses the crystallization of hydroxyapatite, which leads to a decreased production of osteoblast mineralized extracellular matrix and, in some cases in a decreased ALP activity (12–16). Considering the effects of magnesium ions on osteoclasts, an increased resorption activity was found, when osteoclasts were differentiated in vitro from peripheral blood mononuclear cells (PBMC) in the presence of Mg ions and Mg extracts (17). Co-cultures of osteogenically differentiated MSC with osteoclasts revealed an increased expression of osteoclast marker genes and increased TRAP activity in the presence of Mg extracts (18). An increased expression of osteoclast marker genes as well as an increased osteoclast number was also detected in a study on osteoclasts which were derived from monocytic U937 cells in the presence of 1-10 mM Mg (15). Furthermore, recent studies showed immunomodulatory properties of both Mg^{2+} (19–21) and degradable Mg (22,23). To the best of our knowledge, no studies have been conducted on the influence of degradable Mg implants on osteocytes. However, to understand the impact of biomaterials, which are intended to be used as bone graft materials, not only the interaction with bone-forming osteoblasts and bone-resorbing osteoclasts is worth investigating, but also the influence on osteocytes, which orchestrate bone turnover by osteoblasts and osteoclasts via signaling pathways (24). Recently, we developed an in vitro bone model comprising all three bone-related cell types to analyze the impact of bioactive molecules and biomaterials extracts on the cross-talk between osteocytes, osteoblasts and osteoclasts (25). The aim of the present study was to analyze the influence of degradation products of Mg implants on those triple cultures. We furthermore analyzed the differentiation of osteocytes directly cultivated on the surface of degradable Mg.

2. Materials and Methods

2.1. Preparation of degradable Mg discs and generation of extracts

The Mg extract was prepared as described in (26). Magnesium specimens were cut from a cast ingot of pure Mg (99.95%; Helmholtz-Zentrum hereon, Geesthacht, Germany) in cuboid form (1 cm × 1 cm × 0.5 cm). The direct assay was performed with casted and extruded pure Mg (99.94 wt.% purity, Luxfer MEL Technologies, Magnesium Electron, Manchester, UK)in form of discs (1.4 cm height and 0.9 cm diameter). The pure Mg discs were wet-ground using the silicon carbide (2500 grit silicon carbide) abrasive paper at 50 rotations per minute with a twin-wheel grinder and polisher (Saphir 360, ATM

Qness GmbH, Mammelzen, Germany). All materials (cubes and discs) were cleaned ultrasonically for 20 min in n-hexane, 20 min in acetone, and 3 min in 100% ethanol (all chemicals, Merck KGaA, Darmstadt, Germany). The Mg discs were gamma sterilized and kept under vacuum until use.

The cubic samples were sterilized ultrasonically in 70% ethanol for 20 min, dried under sterile conditions and extracted (0.2 g/1 mL extraction medium) in Eagle's minimum essential medium, Alpha modification (α-MEM) (Life Technologies GmbH, Karlsruhe, Germany) without supplements. Extraction was performed for 72h under cell culture conditions (37°C, 5% CO_2 and humidified atmosphere). The extract containing degradation products were harvested under sterile conditions and stored at 4°C until further use. An aliquot was used for the determination of Mg content by atomic absorption spectrometry as described previously (27).

2.2. Cell culture

2.2.1. Osteoblasts and osteocytes

Human primary pre-osteoblasts were isolated from human femoral heads of osteoarthritic patients undergoing total hip replacement at the University Hospital *Carl Gustav Carus* Dresden (Germany) after informed consent (approval by the ethics commission of TU Dresden) as previously described (28). For differentiation into osteocytes, cells were cultivated for 7-10 days in osteogenic medium (α-MEM with glutamax containing 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin (PS) (Gibco), 10^{-7} M dexamethasone, 10 mM β-glycerophosphate and 12.5 µg/ml ascorbic acid-2-phosphate, all osteogenic supplements were from Sigma-Aldrich), harvested using Trypsin/EDTA (Gibco) and embedded in collagen gels as previously described (25). 8 parts of collagen solution (4 mg/ml rat tail collagen, Meidrix Biomedicals Esslingen, Germany), were mixed with one part of 10x HBSS, 10 mM β-glycerophosphate, 12.5 mM ascorbic acid-2-phosphate and neutralized with 1 N NaOH. Cells were resuspended in this mixture in a final concentration of $8*10^4$ cells/ml. Each 500 µl of this collagen/cell suspension was pipetted either to 12-well transwell inserts with 0.4 µm pore size (Sarstedt) (for triple culture experiments) or directly to Mg discs (d= 9 mm), which had been immersed into cell culture medium for 24 h before. Gelation of collagen was performed in the incubator for 30 min. After the collagen gels had formed, α-MEM with glutamax containing 2% FCS, PS, 10 mM β-glycerophosphate and 12.5 µg/ml ascorbic acid-2-phosphate was added (500 µl to the transwell insert and 2 ml to the well plate; 1 ml to the gels which were placed directly on the Mg discs). The constructs were incubated for 14 days for the differentiation of osteocytes from osteoblasts as described previously (29). Medium was changed after 7 days for the transwell inserts and every 3-4 days for the direct cultures.

2.2.2. Osteoclasts

Mature osteoclasts were differentiated and detached as previously described (30). In brief, peripheral blood mononuclear cells (PBMC) isolated from buffy coats by density gradient centrifugation were seeded to 25 cm^2 ultra-low attachment cell culture flasks (Corning) and differentiated in the presence

of 25 ng/mL MCSF and 50 ng/ml RANKL (both from Peprotech) for 5 days. Cells were detached using 2 mM EDTA in PBS containing 0.5 % BSA on a shaker. After centrifugation cells were resuspended in medium. An amount of cells corresponding to an initial monocyte number of $5*10^5$ was used to seed each sample of the triple culture. Silicon grids were used to separate osteoblasts from osteoclasts during cell adhesion (25).

2.2.3. Osteocyte culture in the presence of Mg - based materials

Mg discs were immersed in cell culture medium for 24 h and the medium was removed afterwards. Human primary osteoblasts, suspended in neutralized collagen solution (see 2.2.1., 500 µl suspension per sample) were directly applied to the discs or to 48-well tissue culture plates as control group and allowed to gel over 30 min in the incubator. After formation of the collagen gels, 1 ml α-MEM with glutamax containing 2% FCS, PS, 10 mM β-glycerophosphate and 12.5 µg/ml ascorbic acid-2-phosphate was added. Medium was changed after 1, 5, 8, 12, 15, 19 and 21 days of cultivation and the concentration of Mg was detected using ICP OES (Plasma Quant Elite, Analytik Jena).

2.2.4. Triple cultures in the presence of Mg extracts

Triple cultures of collagen gel-embedded osteocytes with osteoblasts and osteoclasts were performed in transwell inserts as previously described (25) (figure 1). In brief, osteocytes were differentiated from human osteoblasts (three donors, see table 1), embedded in collagen gels in transwell inserts (see 2.2.1.) ($8*10^4$ cell/ml). Differentiation medium consisted of α-MEM with glutamax containing 2% FCS, PS, 10 mM β-glycerophosphate and 12.5 µg/ml ascorbic acid-2-phosphate. After 14 days of osteocytic differentiation, osteoblasts ($5*10^4$, same donors as osteocytes) and osteoclasts (derived from $5*10^5$ monocytes, PBMC of four donors) were seeded onto the apical side of the transwell membrane in different compartments, separated by custom-made silicon grids. After initial attachment of osteoblasts and osteoclasts, specimens were cultured in the presence of Mg extracts (see 2.1.) with a Mg concentration adjusted to 5 and 10 mM as well as medium without extracts for 7 days without further medium change.

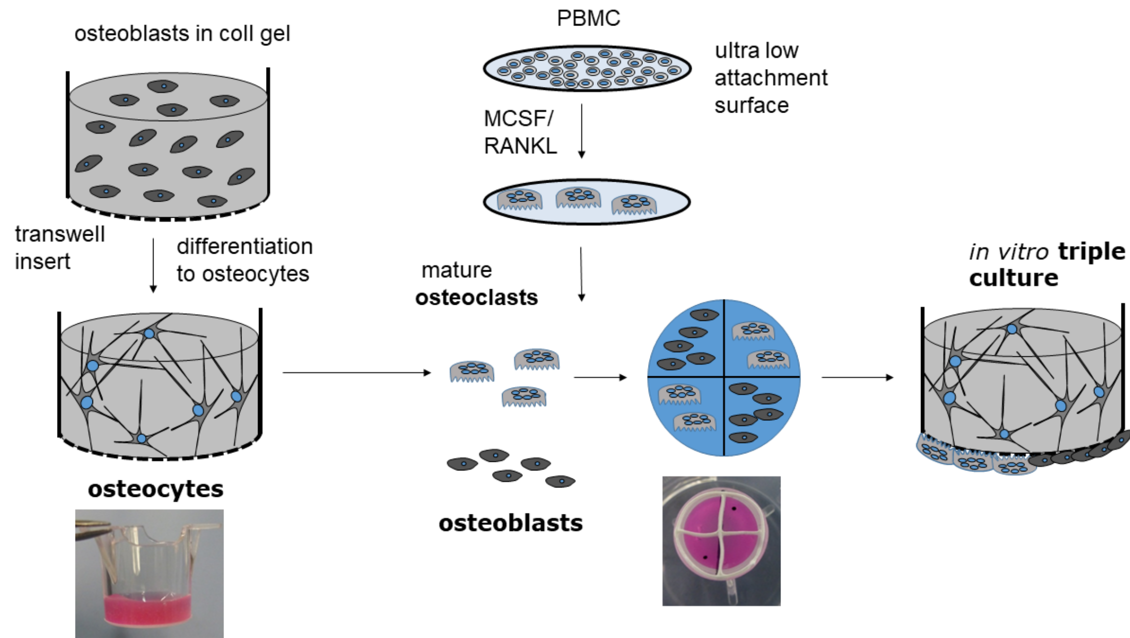

Figure 1: Workflow for the preparation of in vitro triple cultures of osteoblasts, osteocytes and osteoclasts

2.2.4. Sources of osteoblasts/osteocytes and osteoclasts for the different experiments.

In total, eight independent experiments were performed (five triple culture experiments and three osteocyte single culture experiments). In triple cultures, osteoblasts and osteocytes of the same donor were used for every single experiment. For osteocyte single cultures osteoblasts were differentiated into osteocytes. PBMC were obtained from blood donations, four different donors were used: donor A for triple cultures I and III, donor B for triple culture II, donor C for triple culture IV and donor D for triple culture V. Information on sex and age of the donors were not provided by the German Red Cross.

Donor information for human pre-osteoblasts, which were used for the differentiation of osteoblasts and osteocytes are provided in table 1.

Donor information	Experiments
female 75 years	osteocyte single culture III
female 56 years	osteocyte single culture I, triple culture III
female 67 years	triple cultures IV and V
male 65 years	osteocyte single culture II, triple cultures I and II

2.3. Fluorescence microscopy

Osteocyte-containing collagen gels (both from triple and single cultures) and membranes, seeded with osteoblasts and osteoclasts were fixed with phosphate buffered formaldehyde (4 %). After treatment with 0.1% Triton X-100 in PBS for 5 min the specimens were washed five times with PBS, followed by an incubation in 1 % BSA in PBS for 30 min (membranes) or 60 min (gels). Staining was performed with Phalloidin-iFluor 488 (Abcam) and DAPI with an incubation time of 30 min (membranes) and overnight (collagen gels), respectively. Specimens were again washed with PBS and imaged using a Keyence BZ-X810 fluorescence microscope.

2.4. RNA isolation, cDNA synthesis and PCR

For gene expression analysis, osteocyte-containing gels were removed from the transwell inserts and Mg discs, respectively and incubated with collagenase II solution (3 mg/mL collagenase II in α-MEM, 10% FCS, 2 mM L-glutamine, PS, 3 mM $CaCl_2$) for 1 h at 37 °C. The digests were transferred to 15 mL tubes, washed with PBS and centrifuged. The supernatant was discarded.

PET membranes from transwell inserts were cut into quarters according to the different regions, seeded with osteoblasts and osteoclasts (25).

RNA was isolated from pellets and membrane pieces using a commercially available kit (peqGOLD MicroSpin Total RNA Kit, Peqlab, Erlangen, Germany). cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions.

PCR reactions were set up using the TaqMan Fast Advanced Master Mix (Applied Biosciences) and TaqMan Gene Expression Assays for the following genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), bone gamma-carboxyglutamate protein (osteocalcin, BGLAP), podoplanin (E11/g38; PDPN), phosphate regulating endopeptidase homolog, X-linked (PHEX), matrix extracellular phosphoglycoprotein (OPF 45, MEPE), receptor activator of NF-κB Ligand (RANKL, TNFSF11), alkaline phosphatase (ALPL), bone sialoprotein II (BSP II), tartrate-resistant acid phosphatase (ACP5), cathepsin K (CTSK) and carbonic anhydrase II (CAII) (Applied Biosystems), according to manufacturer's instructions.

PCR was run with an Applied Biosystems® 7500 fast Real-Time PCR system. Relative gene expression (fold-change) was calculated by using the $2^{-\Delta\Delta Ct}$ method and normalized to the Mg free control.

2.5. Measurement of enzyme activities

For osteoclasts in triple cultures the activities of TRAP, cathepsin K and CA II, and for osteoblasts in triple cultures the activity of ALP were measured and normalized to the DNA content of the respective sample. Frozen membrane sections were thawed and treated with 1% Triton X-100 in PBS for 50 min on ice with a sonication step for 10 min in between. Activities of TRAP, CAII and CTSK were quantified as previously described [57]. Briefly, ALP and CAII activity were determined using the

cleavage of p-nitrophenyl phosphate to p-nitrophenol in different buffers and quantified by absorption measurement at 405 nm. For the analysis of TRAP activity the cleavage of Naphthol ASBI phosphate at acidic pH in the presence of tartrate was quantified by fluorescence measurements at an excitation and emission wavelength of 405/520 nm. For the analysis of CTSK activity the cleavage of Z-LR-AMC (Enzo Life sciences) was quantified by the fluorescence measurement at an excitation/emission wavelength of 365/440 nm. DNA concentration of the lysates was quantified using the Quantifluor One dsDNA kit (Promega) according to manufacturer´s instructions.

2.6. Osteocalcin ELISA

Osteocalcin protein production was assessed in supernatant samples of cell culture experiments. A solid phase sandwich ELISA kit (Human Osteocalcin DuoSet ELISA # DY1419-05; R&D Systems, USA) was used for analyzing osteocalcin content. After detection and development, absorption measurements were conducted at a wavelength of 450 nm with a wavelength correction at 540 nm using a spectrofluorometer infinite M200pro (Tecan Trading AG, Switzerland). Final concentrations were calculated by non-linear regression via a standard calibration curve in the range of 0.3-10 ng/ml.

2.7. Statistics

Five independent experiments of triple cultures were performed including cells of three osteoblast/osteocyte donors and four PBMC donors (Table 1). Within the single experiments, all samples were seeded in triplicates for each analysis. Kruskal-Wallis test, followed by Dunn's multiple comparisons test was performed in Graphpad Prism 8.0. For osteocyte single cultures in direct contact to the Mg discs, osteoblasts of three different donors were differentiated into osteocytes (Table 1). Samples were again seeded in triplicates for each analysis. One way ANOVA, followed by Tukeys multiple comparisons test was performed in Graphpad prism 8.0.

3. Results

3.1. Cultivation of gel-embedded osteocytes in the presence of Mg discs

To analyze the impact of Mg degradation products on osteocytes, we performed experiments with single cultures of osteocytes, which were differentiated from osteoblasts embedded in collagen gels. To this end, predifferentiated osteoblasts were embedded in collagen gels and placed on top of the degradable Mg implants. The first two weeks of cultivation were performed for the osteoblast to osteocyte differentiation (29). After this, osteocytes were cultivated for another 7 and 14 days to be comparable to the cultivation time of the triple cultures, which have a setup of 2 weeks osteocyte differentiation plus 1 week final cultivation.

The release of Mg to the cell culture medium over time remained quite stable around 5 mM (figure 2B). The pH of the cell culture medium increased to around 8 as indicated by the color of the phenolic red dye, which was included into the cell culture medium. Off-gel release studies in a different cell culture medium with serum supplementation have shown a burst release in the first 3 days of immersion with concentrations of 22 mM (± 6 mM; day 1) and 11 mM (± 2 mM; day 3) (data not published). After 28 days of cultivation, the Mg discs showed distinct signs of degradation (figure 2 A). Elemental mapping of the Mg surface showed, as expected, besides Mg the elements Ca and P, as well as traces of K and S, the latter probably originating from proteins, containing cystein (Figure 2 A). There were no differences between samples, which were in contact with collagen gels and cell-laden collagen gels. Cell-laden gels stuck the whole cultivation time on top of the metallic samples and did not start floating. After 21 days of cultivation, all gel-embedded cells showed osteocyte-like morphology and there were no differences between gels, which were cultivated separately, compared to gels in contact to the Mg samples (figure 3).

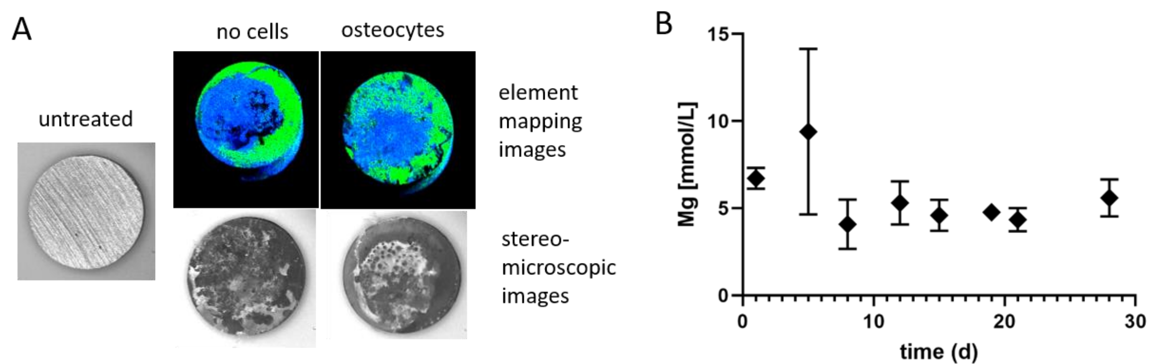

Figure 2: A) images of MG discs (9 mm diameter), untreated and after cultivation in the presence of empty collagen gels and osteoblast/osteocyte-laden collagen gels. Color code for element mapping images: Mg: green, P: dark blue, Ca: cyan, exemplary images out of n=3 for cell-free and n=9 for cell containing discs, B) Mg concentration in the supernatant of Mg discs, cultivated in the presence of osteoblast/osteocyte-laden collagen gels, quantified by ICP-OES (n=6).

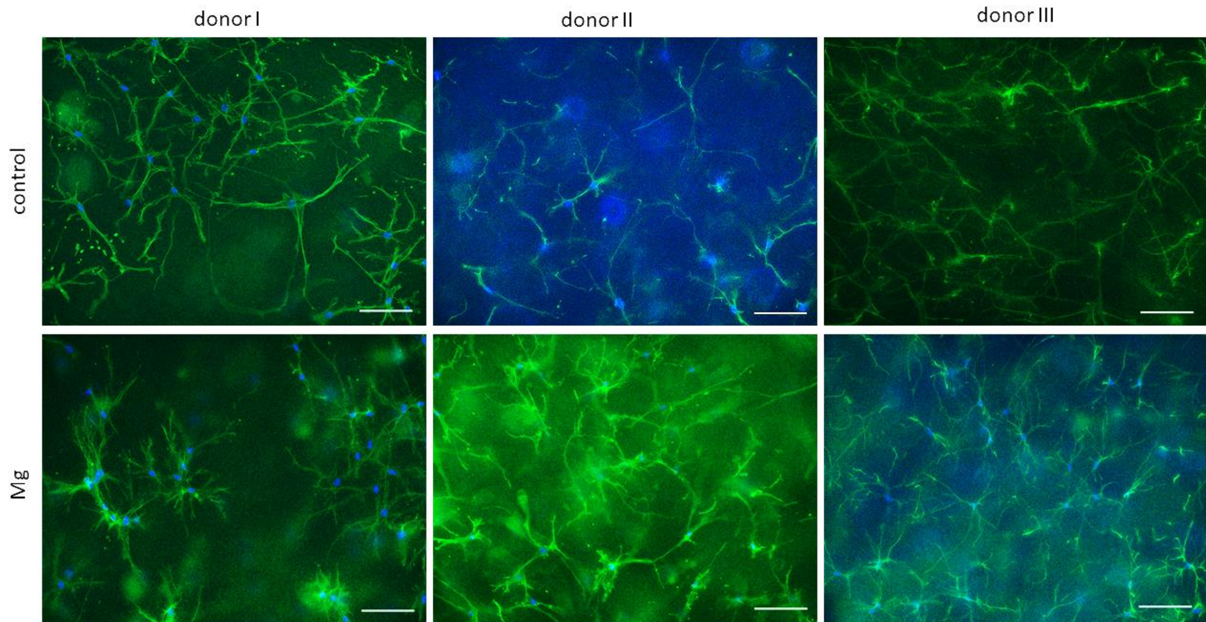

Figure 3: Fluorescence microscopic images of osteocytes, embedded in collagen gels, cultivated in contact to Mg discs or in tissue culture plates (control), cytoskeleton appears green (iFluor488 phalloidin staining) and nuclei appear blue (DAPI staining), scale bars represent 100 µm

Expression of osteocyte marker gene E11 did not change in the presence of the Mg degradation products. Likewise, the mRNA expression of osteocalcin (BGLAP) and RANKL, which are both markers of late osteoblasts and early osteocytes, did not change in the presence of the material. On protein level, however, the expression of osteocalcin was significantly downregulated in the presence of the Mg (figure 4F). Furthermore, we observed a significant downregulation of PHEX expression after 28 days cultivation in the presence of Mg. We did not observe expression of the osteocyte marker MEPE in these three experiments. Interestingly, the mRNA expression of ALP, which is an early osteoblast marker, significantly decreased in the presence of Mg, but only after four weeks of cultivation (Figure 4).

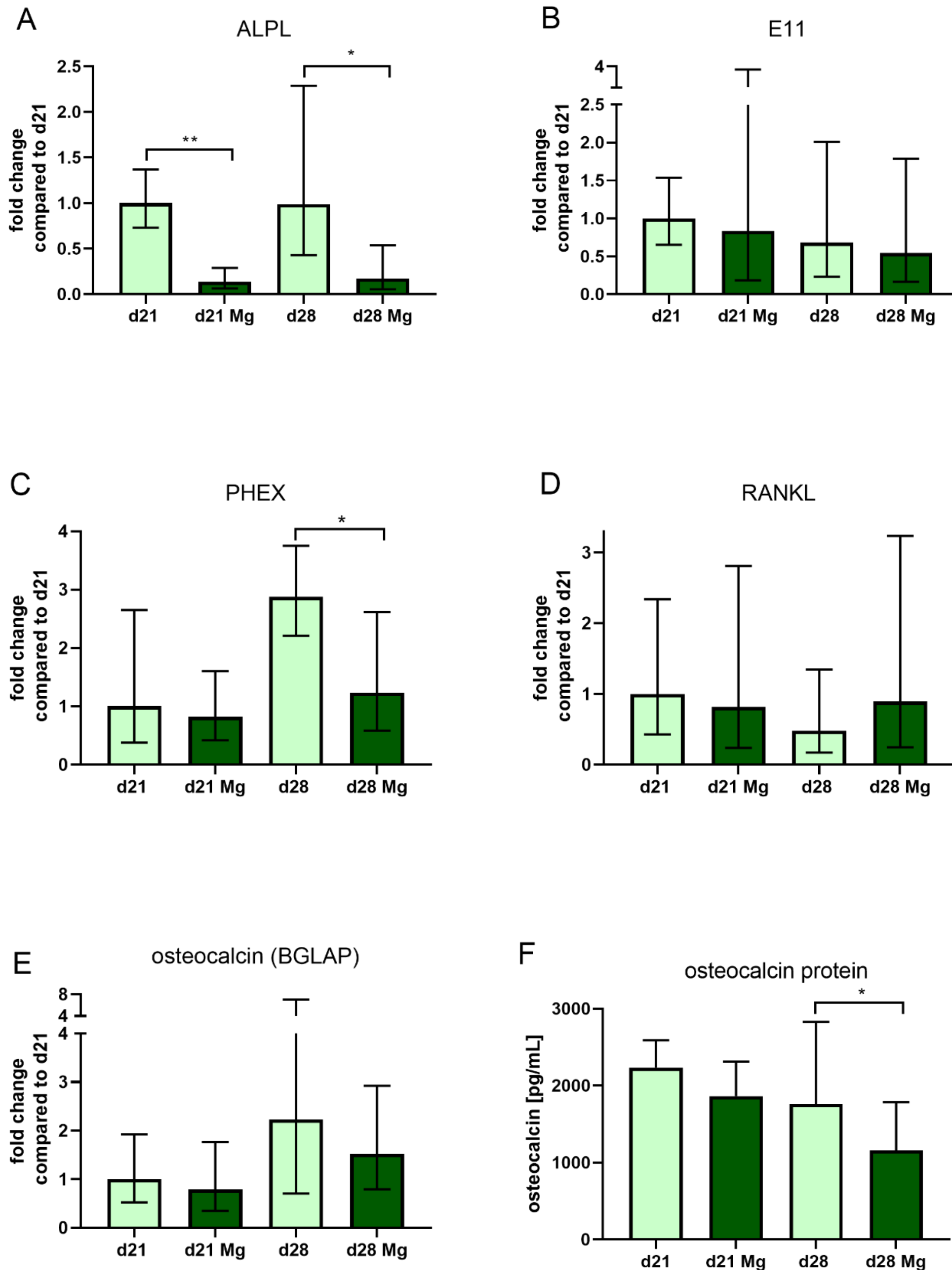

Figure 4: A-E Gene expression of osteocyte markers osteocalcin (BGLAP), E11, PHEX and RANKL, as well as expression of the osteoblast marker ALPL were analyzed for osteocytes, differentiated from osteoblasts of three donors (each n=3, n=9 in total, see table 1). Diagrams show fold changes compared to the control (without contact to Mg discs) +/- upper and lower limit, F: osteocalcin expression was quantified on protein level using ELISA. Diagram shows averages +/- standard deviation (n=9). One way ANOVA followed by Tukey post-hoc test, *p<0.05, ** p<0.01 compared to the control.

3.2. Impact of Mg extracts on triple cultures of osteocytes, osteoblasts and osteoclasts

To investigate gene expression and activity as well as morphology of osteocytes, osteoclasts and osteoblasts in triple culture in response to Mg degradation products the cultures were treated with Mg extracts containing 5 and 10 mM Mg^{2+} in comparison to a Mg free control for 7 days. Osteocytes had been differentiated for 14 days before starting the triple cultures, so the cultivation times between single and triple culture were chosen in a comparable time frame.

3.2.1. Cell morphology in triple cultures in response to Mg

All three bone cell species showed their typical morphology in triple culture and there were no changes in the presence of metal extracts containing 5 and 10 mM Mg (figure 5).

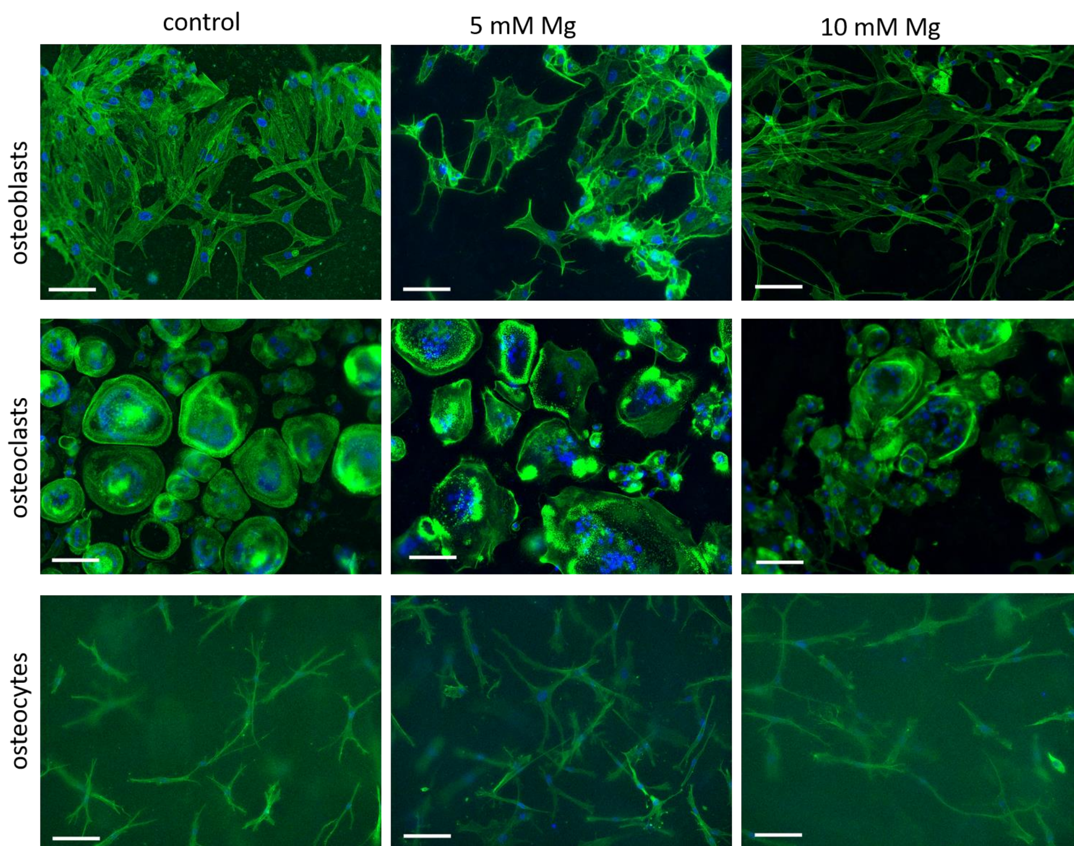

Figure 5: Fluorescence microscopic images of osteoblasts, osteoclasts and gel-embedded osteocytes in triple cultures in the presence and absence of magnesium extracts, cytoskeleton appears green (iFluor488 phalloidin staining) and nuclei appear blue (DAPI staining), scale bars represent 100 µm.

3.2.2. Gene expression and osteocalcin production of osteocytes in triple culture in response to Mg

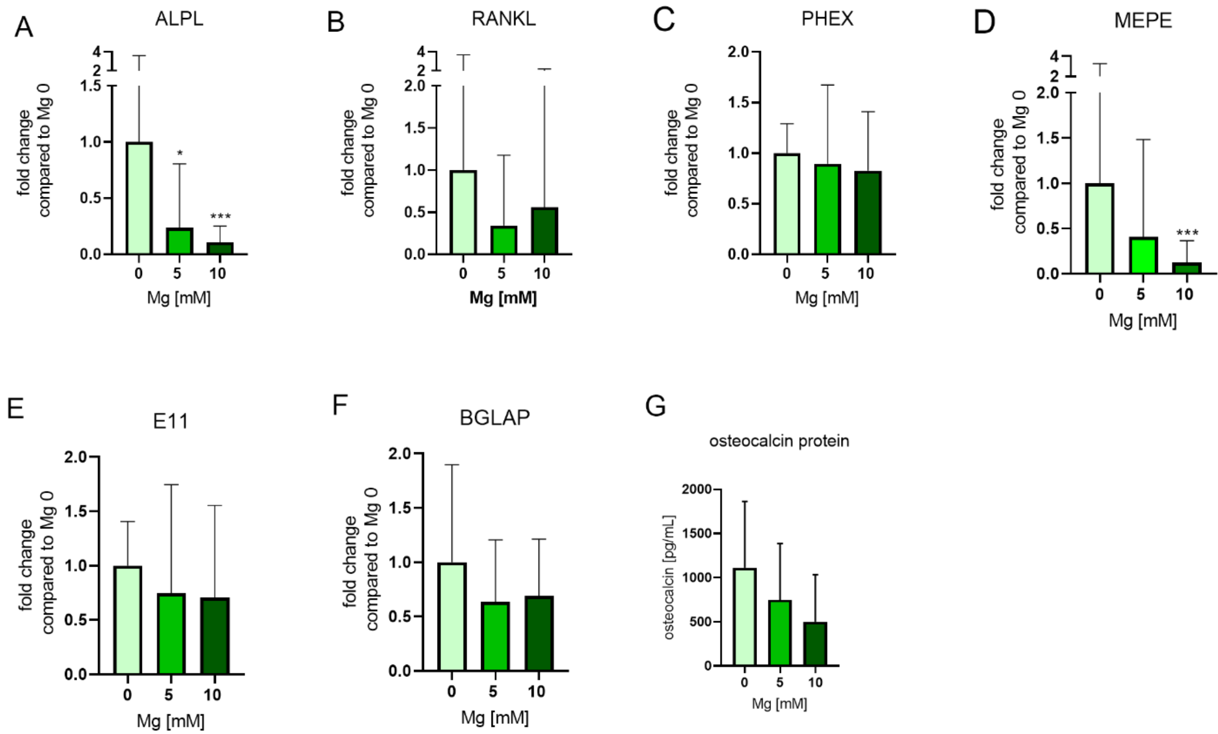

Figure 6: A-F Gene expression of osteocyte markers RANKL, PHEX, MEPE, E11 and BGLAP (osteocalcin), and of the osteoblast marker ALPL was analyzed in five independent experiments involving osteoblasts and osteocytes of three donors and osteoclasts of four donors, each n=3, n=15 in total (see table 1). . Diagrams show fold changes compared to the control (without Mg), +/- upper and lower limit, * p<0.05, ***p<0.001. High upper and lower limits occur due to the combination of the five different experiments due to variations between the different donors. Therefore, gene expression for the single experiments is shown in figure S1. F: Osteocalcin concentration in the supernatant of the cultures was analyzed by ELISA for two independent experiments. Data of the single experiments are shown in figure S1. Average +/- standard deviation (n=6).

Osteocyte marker genes BGLAP, E11, RANKL, PHEX and MEPE were analyzed in osteocytes in the triple cultures. While E11, RANKL, PHEX and BGLAP expression was not significantly changed in the presence of Mg extracts, gene expression of MEPE was significantly reduced in the presence of 10 mM Mg (figure 6). Gene expression of the osteoblast marker ALPL, was downregulated as well, as already observed in the single osteocyte cultures (see figure 4).

Furthermore, the protein expression of osteocalcin was analyzed. No significant changes were detected, when the cultures were treated with Mg extracts in comparison to the control (figure 6 G). It has to be taken into consideration, that osteocalcin concentration was quantified in the supernatants of the triple cultures, containing released osteocalcin of both osteoblasts and osteocytes.

3.2.3. Gene expression and activity of osteoclasts in triple culture in response to Mg

Osteoclasts in triple culture with osteocytes and osteoclasts expressed typical markers TRAP (ACP5), CTSK and CA2 both on gene and protein level. However, those expressions were not changed in the presence of Mg extracts of 5 mM and 10 mM Mg concentration (figure 3).

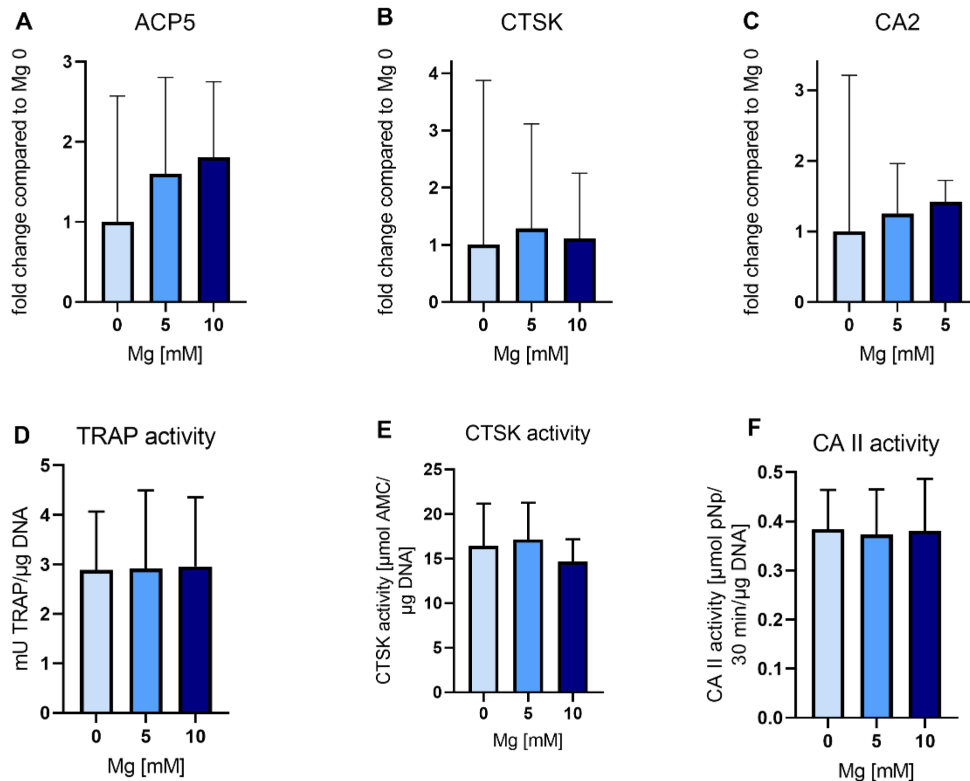

Figure 7: A-C Gene expression of osteoclast markers ACP5 (TRAP), CTSK and CA2 (A-C) and activities of the related osteoclast-specific enzymes TRAP, CTSK and CAII (D-F) were both analyzed in four independent experiments involving osteoblasts and osteocytes of three donors and osteoclasts of three donors, each n=3, n=12 in total (see table 1). A-C: Diagrams show fold changes compared to the control (without Mg), +/- upper and lower limit. High upper and lower limits occur while combining of the four different experiments due to variations between the different donors. Therefore, gene expression for the single experiments is shown in figure S2. D-F diagrams show mean values +/- standard deviations (n=12). One way ANOVA followed by Tukey post-hoc test did not reveal significant differences between the groups. .

3.2.4. Gene expression and activity of osteoblasts in triple culture in response to Mg

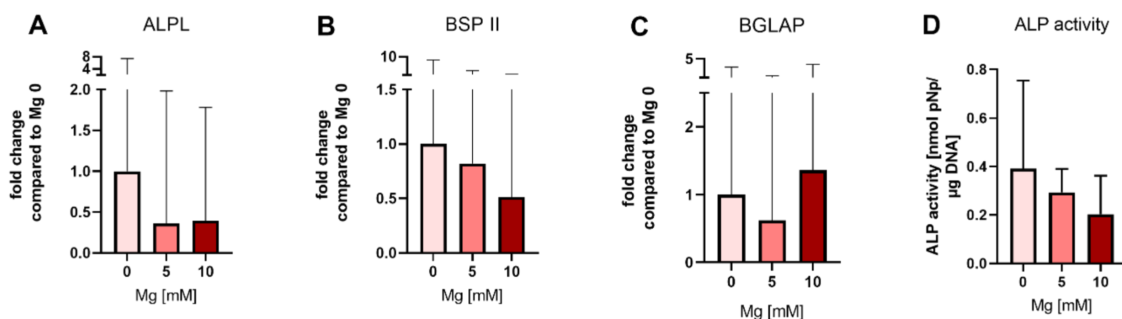

Figure 8: Gene expression of osteoblast markers ALPL, BSP II and osteocalcin (BGLAP) (A-C) and ALP activity (D) were analyzed in five independent experiments involving osteoblasts and osteocytes of three donors and osteoclasts of four donors, each n=3, n=15 in total (see table 1). A-C Diagrams show fold changes compared to the control (without Mg), +/- upper and lower limit. High upper and lower limits occur while combining of the five different experiments due to variations between the different donors. Therefore, gene expression for the single experiments is shown in figure S3. D: Diagram shows mean values +/- standard deviations (n=15). One way ANOVA followed by Tukey post-hoc test did not reveal significant differences between the groups.

Osteoblasts in triple culture with osteocytes and osteoclasts expressed typical marker ALP (both on gene and protein level) (figure 8 A,D), as well as markers BSP II and osteocalcin (BGLAP) on mRNA level (figure 8 B,C). There were no significant changes in the presence of Mg extracts of 5 mM and 10 mM Mg concentration.

Discussion

Osteocytes are the most abundant bone cell species and play a pivotal role in the regulation of bone turnover (31). Therefore, the interaction of these cells with bone graft materials needs to be considered in addition to cytocompatibility analyses with osteoblasts and their precursors. However, isolation of primary osteocytes is quite tedious, since the cells are surrounded by mineralized bone matrix. Alternatively, osteocytes can be differentiated by embedding osteoblasts in collagen gels and cultivating them under low serum conditions (32,33). Those in vitro differentiated osteocytes show osteocyte-like morphology and express early osteocyte markers like E11, PHEX, MEPE and osteocalcin (BGLAP). Nevertheless, as we have found in several experiments, the expression of late osteocyte markers sclerostin and DMP1 is not always detectable in osteocytes derived from osteoblasts in vitro (33) (plus unpublished data). Possibly the potential of human primary osteoblasts to further differentiate into osteocytes is donor dependent. In the present study, we were able to differentiate osteocytes from primary osteoblasts in collagen gels in the presence of degradable Mg-based materials. Due to the release of hydroxide (OH^-) during the degradation process, the medium condition changed to an alkaline environment (34). The elevated pH in the presence of the Mg discs, did not affect viability and differentiation of the cells in our study. It has been shown before, that Mg ions even have the potential to protect cells from alkaline stress (35).

Collagen gels, which were placed on top of the Mg discs, considerably slowed down the release of Mg ions from the discs. While materials without collagen gels showed a burst release followed by decreasing concentrations of released Mg, we detected a sustained Mg release in the presence of collagen gels in the range of 5 mM, which is far below the cytotoxic range (8) and has shown to support cell proliferation (26). The release of Mg from metal implants has been recently demonstrated to be decreased as well, when only cells are seeded on the samples (36), indicating, that both cell monolayers and collagen gels are able to form a barrier, protecting the material from fast degradation.

To the best of our knowledge, this study is the first to examine the influence of Mg degradation products on osteocytes. Osteocytes did not show morphological changes in the presence of Mg degradation products, and most of the examined osteocytic markers were not differentially expressed. However, there was a significant decrease of ALPL gene expression compared to osteocytes of the control. The transition of osteoblasts to osteocytes is characterized by down regulation of collagen I and ALP (37,38), indicating, that Mg might accelerate osteocytic transition. Contradictory, PHEX, an osteocyte marker gene was also significantly decreased after 28 days of cultivation. Also in triple cultures with osteoblasts and osteoclasts, ALPL expression of osteocytes was significantly down regulated, however osteocytic gene expression was likewise downregulated in the presence of Mg degradation products. Especially the gene expression of MEPE was significantly downregulated. MEPE belongs to the small integrin-binding ligand, N-linked glycoproteins (SIBLINGs) of the extracellular matrix and contains an acidic serine aspartate-rich motif (ASARM) which associates after its enzymatic release with hydroxyapatite crystals and therefore regulates mineralization (39). Thus, MEPE, along with other proteins of the SIBLING family, plays an important role in the regulation of phosphate homeostasis. During osteoblast-osteocytes transition MEPE is expressed later than E11 and osteocalcin (37). The present study shows no clear impact of Mg and its degradation products on osteocyte differentiation, since both osteoblast and some osteocyte marker genes are downregulated. Nevertheless, Mg did not inhibit the differentiation of osteocytes from primary osteoblasts, since both groups showed the same, typical osteocytic morphology.

Expression of osteoclast markers TRAP, CTSK and CA2, both on mRNA and protein level was not significantly changed in triple cultures with osteoblasts and osteocytes in the presence of Mg degradation products. This was a bit surprising, since earlier studies had demonstrated different effects of Mg on osteoclasts: TRAP activity was decreased in osteoclast single cultures in the presence of Mg extracts, but resorption was increased at the same time (17). In contrast, osteoclasts, which were cultivated in the presence of Mg salts, showed an upregulation of osteoclast-specific genes (15). When osteoclasts were co-cultivated with osteogenically differentiated MSC, TRAP activity as well as gene expression of osteoclast markers were increased in the presence of Mg extracts (18).

One explanation for this discrepancy could be the fact, that the present study applied mature osteoclasts, which were differentiated before adding both Mg extracts and the other two cell types. This experimental setup was used due to different cell culture media requirements for (1) differentiation of PBMC to osteoclasts (10 % serum) and (2) maintenance of osteocytic differentiation state (2 % serum). Therefore, development of an in vitro protocol, allowing the differentiation of osteoclasts under conditions, which do not lead to a re-differentiation of osteocytes to osteoblasts, would be highly beneficial to improve the triple culture setup and to analyze the impact of bioactive substances already during osteoclastogenesis in triple culture. Another hypothesis on the missing

effect of Mg extracts on the expression and activity of osteoclast markers in triple culture is that through the interaction with osteoblasts and/or osteocytes, osteoclasts behave differently compared to single cultures of osteoclasts. As already mentioned before, there were also huge differences in TRAP activity of osteoclast single cultures (17) and co-cultures with osteogenically differentiated MSC (18) in the presence of Mg extracts. However, these experiments were not performed within the same study and therefore also donor variation could be the reason for the different outcome of the two studies. In the present study, we did not perform investigations with osteoclasts in single culture, which would have been beyond the scope of the study.

Osteoblasts in triple cultures did not show significant changes in osteogenic ALP, osteocalcin and BSPII expression and no significant changes in ALP activity in the presence of Mg degradation products. Many other studies have demonstrated effects of Mg ions and Mg extracts on differentiation of osteoblasts and their precursors. An increased ALP activity was found for osteogenically differentiated MSC (14) as well as different osteoblast cell lines (11,40) in the presence of 3-10 mM Mg. Furthermore, an upregulation of osteogenic gene markers ALPL, osteocalcin and BSPII has been demonstrated for different osteoblast cell lines in the presence of Mg ions and Mg extracts (10,41). Zhang and co-workers (35) investigated the molecular regulation mechanisms of osteoblast differentiation induced by Mg ions and demonstrated an activation of the PI3K signaling pathway via TRPMK ion channel and protein kinase. A comprehensive study on the impact of Mg extracts on the osteoblastogenesis of mesenchymal stem cells revealed regulation of different genes, including TRPM7, ALPL, osteopontin and osteocalcin (26). Especially the latter study revealed a complex interaction of Mg with differentiating osteoblasts both positively and negatively influencing the respective pathways. Since the setup of in vitro triple cultures is unique, the results can barely be compared to other studies involving Mg ions or extracts and osteoblasts. However the triple culture was applied to reflect the in vivo situation and an interplay between all three cell types is a unique approach to study Mg-related effects. To make the system less complex, we performed an additional experiment to analyze the effect of Mg extracts on the osteogenic differentiation of human osteoblasts in single cultures (figure S 4). Also in this experiment, which used cells of the same donors and experimental setup (osteogenic pre-differentiation with dexamethasone, low-serum conditions for cultivation in the presence of Mg extracts) we did not see a significant change of ALP activity in the presence of Mg extracts. After 7 days of cultivation, the specific ALP activity was even decreased in the presence of Mg extracts. Finally, we can conclude, that the cultivation of osteoblasts in co-culture with osteocytes and osteoclasts did not change the ALP activity compared to single cultures in a similar experimental setup. An osteogenesis promoting effect of Mg extracts under this experimental conditions was not observed. This leads to the assumption, that the present in vitro model with respect to the selected cell species and cultivation conditions was not suitable to demonstrate an effect of Mg extracts on osteogenic transformation. In

this context, it has to be considered, that experimental conditions in vitro have a huge impact on the observed effects. The following examples of Mg effects on ALP activity and gene expression of ALP and osteocalcin demonstrate, how different experimental settings can determine the outcome of the respective experiments: Luthringer an co-workers cultivated human MSC in the presence of Mg extracts, and showed an upregulation of osteocalcin only in the first week of differentiation, while it was not upregulated after 2 and 3 weeks; a similar finding was reported for ALP expression, which was only upregulated after 1 and 3 weeks of differentiation, but not after two weeks (26). Another study applied Mg extracts to four different osteoblast lines, and after one week of differentiation, the ALP expression was not upregulated in three of them (41). Only human osteoblasts showed an upregulated ALP expression after one week and osteocalcin expression was upregulated only in the presence of dexamethasone (41). Although the effect of Mg ions on osteoblast differentiation has been elucidated and confirmed by many authors, it is not possible to see an enhanced osteogenic differentiation in every experimental setup, including the present study.

Nevertheless, the present study is, to the best of our knowledge, the first analyzing the effect of Mg degradation products on primary osteocytes. We were able to demonstrate, that transition of osteoblasts to osteocytes is not hampered by Mg degradation products and we hypothesize an accelerated transition, due to the significantly decreased ALPL expression in presence of the Mg degradation products. The decreased mRNA expression of the osteocyte markers PHEX and MEPE, in contrary, suggests a slower osteocytic differentiation in the presence of Mg extracts. Additional experiments, possibly involving also osteocytic cell lines, will be necessary to unravel potential effects of Mg degradation products on osteocyte differentiation and signaling.

Acknowledgements: The authors thank the German Research Foundation (DFG) for financial support (grant number BE 5139/3-2). Furthermore, we thank Max von Witzleben for generating custom-made silicon grids and Katharina Wirsig for the osteocalcin ELISA.

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