Peri-Prosthetic Tissue Cells Show Osteogenic Capacity to Differentiate Into the Osteoblastic Lineage

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ABSTRACT: During the process of aseptic loosening of prostheses, particulate wear debris induces a continuous inflammatory-like response resulting in the formation of a layer of fibrous peri-prosthetic tissue at the bone-prosthesis interface. The current treatment for loosening is revision surgery which is associated with a high-morbidity rate, especially in old patients. Therefore, less invasive alternatives are necessary. One approach could be to re-establish osseointegration of the prosthesis by inducing osteoblast differentiation in the peri-prosthetic tissue. Therefore, the aim of this study was to investigate the capacity of peri-prosthetic tissue cells to differentiate into the osteoblast lineage. Cells isolated from peri-prosthetic tissue samples ($n = 22$)—obtained during revision surgeries—were cultured under normal and several osteogenic culture conditions. Osteogenic differentiation was assessed by measurement of Alkaline Phosphatse (ALP), mineralization of the matrix and expression of several osteogenic genes. Cells cultured in osteogenic medium showed a significant increase in ALP staining ($p = 0.024$), mineralization of the matrix ($p < 0.001$) and ALP gene expression ($p = 0.014$) compared to normal culture medium. Addition of bone morphogenetic proteins (BMPs), a specific GSK3β inhibitor (GIN) or a combination of BMP and GIN to osteogenic medium could not increase ALP staining, mineralization, and ALP gene expression. In one donor, addition of GIN was required to induce mineralization of the matrix. Overall, we observed a high-inter-donor variability in response to osteogenic stimuli. In conclusion, peri-prosthetic tissue cells, cultured under osteogenic conditions, can produce alkaline phosphatase and mineralized matrix, and therefore show characteristics of differentiation into the osteoblastic lineage. © 2016 The Authors. *Journal of Orthopaedic Research* published by Wiley Periodicals, Inc. on behalf of Orthopaedic Research Society. J Orthop Res 35:1732–1742, 2017.

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Aseptic loosening is the most common long-term cause of failure in total joint arthroplasty.[1] The process of loosening is thought to be a complex interplay between mechanical as well as biological factors. Particulate wear debris, continuously generated by articulating motion at the bearing surfaces, has been implicated as one of the primary causes initiating peri-prosthetic bone loss.[2] Subsequently, a fibrous-like peri-prosthetic tissue layer with poor mechanical properties is formed, triggering bone resorption and prosthesis displacement.[3]

The current treatment for aseptically loosened prostheses is invasive revision surgery, which consists of removal of the old prosthesis together with the peri-prosthetic tissue, and insertion of a new prosthesis. Sometimes, in addition, bone augmentation has to be done with allograft bone, depending on the severity of bone loss. This procedure is highly demanding for the patient and is associated with blood loss, complications, and morbidity, especially in elderly patients with a poor general health condition.[4–6] Furthermore, revision prostheses display poorer clinical and functional performance than that of the primary prosthesis.[7–9] Therefore, less demanding therapies alternative to extensive revision surgery but with adequate functional performance are necessary. Currently, several minimally invasive techniques are being investigated to remove the peri-prosthetic tissue and stabilize the loosened prosthesis by subsequent bone cement injection.[10,11]

Another approach would be to promote bone formation in the peri-prosthetic tissue in order to compensate peri-prosthetic bone loss and subsequently stabilize the loosened prosthesis. This could be accomplished in a minimally invasive way by percutaneous, local introduction of osteogenic factors at the peri-prosthetic space, which will drive osteogenic differentiation of peri-prosthetic tissue cells.

However, little is known about the role of peri-prosthetic tissue cells in bone formation. Most effort so far has been concentrated on understanding the role of these cells in bone resorption around the implant. The few studies investigating their role in bone formation show that peri-prosthetic tissue cells produce factors that suppress osteoblast function and induce production of inflammatory cytokines.[12,13] Moreover, wear particles and metal ions can directly affect osteoblasts by reducing type one collagen production[14,15] and decreasing alkaline phosphatase activity as well as calcium deposition.[16] In addition, wear particles have been shown to decrease osteoblast proliferation,[15,17] change the phenotype of mature osteoblasts,[18] and stimulate osteoblasts to secrete inflammatory cytokines.[14–16,19] Remarkably, one study revealed that cells from the peri-prosthetic tissue produce several osteoblastic proteins themselves.[20] In agreement, histological assessment of peri-prosthetic tissue demonstrated that this tissue exhibits osteogenic characteristics as shown by the presence of intramembranous formation of osteoid,[21,22] an increased mineral

apposition rate and bone formation rate with active osteoblast lining, and production of immature bone matrices with poor bone quality.[22] Furthermore, an increased expression of several bone morphogenetic proteins (BMPs) in several cell types of the peri-prosthetic tissue was found.[23] As BMPs are regulators and potent inducers of osteoblast differentiation,[24] the local increase of BMP synthesis in peri-prosthetic tissue could be an attempt to regenerate or maintain implant fixation. However, to our knowledge, nothing is known about the capability of peri-prosthetic tissue cells to (re) generate bone themselves. Therefore, the aim of this study is to investigate whether cells within the peri-prosthetic tissue are able to differentiate into the osteoblastic lineage.

MATERIALS AND METHODS

Peri-Prosthetic Tissue Samples

Peri-prosthetic tissue samples harvested from aseptically loosened femoral stems or acetabular components of 22 patients were obtained during revision surgery of total hip replacements. The peri-prosthetic tissue was collected as "waste" material and as such should not be traceable to specific patients according to Dutch Medical Ethics laws and legislation. Therefore, only limited donor characteristics are available (see Table 1). Collected samples were kept in sterile NaCl 0.9% at 4°C, for a maximum of 24 h, until they were processed. This study was approved by the Medical Ethics Committee of Leiden University Medical Center (C12-107).

Cell Culture

Collected tissue samples were minced and incubated at 37°C for 2 h in α-MEM (Gibco, Carlsbad, CA) with collagenase I A (2 mg/ml; Sigma–Aldrich, St Louis, MO). The cell suspension was then centrifuged and washed twice in α-MEM supplemented with 10% Fetal Calf Serum (FCS; Greiner Bio One, Kremsmünster, Austria). Cells were cultured in petri-dishes in α-MEM supplemented with 10% FCS, 1% Glutamax (Gibco), 3% penicillin and streptomycin (Gibco), and 25 µg/ml Amphotericin B (Gibco) for 72 h. Thereafter, cells were cultured in the same medium but without Amphotericin B. When cultures reached 90% confluence, the cells were transferred to 75 cm² flasks. For the experiments cells from passage 1 or 2 were used.

The human osteosarcoma cell line SaOS-2 (ATCC, Manassas, VA) and the human dermal fibroblast cell line (HDF-a; ScienCell, Carlsbad, CA) were cultured in DMEM (Gibco) supplemented with 10% FCS and 1% penicillin and streptomycin (Gibco). These cell lines were used as positive and negative controls in the experiments, respectively.

Osteogenic Differentiation Experiments

Peri-prosthetic tissue cells were seeded at a density of 8,650 cells/cm². To induce osteoblast differentiation, normal culture medium (NM, as described above) was supplemented with freshly added ascorbic acid (50 µg/ml; Merck Inc., Kenilworth, NJ), β-glycerophosphate (5 mM; Sigma–Aldrich), and dexamethasone (0.1 µM; Sigma–Aldrich) with or without recombinant human BMP-2, BMP- 6 (50 ng/ml; R&D Systems, Minneapolis, MN) or a specific GSK3β inhibitor (GIN; 10nM; kindly provided by Dr. Rawadi, Prostrakan, France; Engler et al.[25]) Cell cultures were subjected randomly to either BMP-2 or BMP-6. The culture medium was replaced every 3 to 4 days.

Alkaline Phosphatase Activity

Alkaline Phosphatase (ALP) activity was assessed by both histochemical staining and a colorimetric assay. For ALP

Table 1. Demographic Data From Included Peri-Prosthetic Tissue Samples

Donor	Sex	Age	Fixation	Bearing	Cup/Stem	Years in Situ	Visible Presence of Wear Debris
1	F	81	Cemented	Metal/PE	Cup	>5	Yes
2	M	74	Cementless	Metal/PE	Cup	>5	No
3	F	85	Cemented	Metal/PE	Cup	>5	Yes
4	M	59	Cemented	Metal/PE	Stem	>5	Yes
5	M	81	Cemented	Metal/PE	Cup	>5	No
6	F	79	Cemented	Metal/PE	Stem	>5	Yes
7	M	55	Cementless	Metal/Metal	Cup	>5	Yes
8	F	80	Cemented	Metal/PE	Cup	>5	No
9	F	73	Cementless	N/A	N/A	>5	Yes
10	F	75	Cementless	Ceramic/PE	N/A	>5	No
11	F	N/A	Cemented	Metal/PE	Cup	>5	Yes
12	M	79	Cemented	Metal/PE	Cup + Stem	>5	No
13	F	80	Cementless	Metal/PE	Cup	>5	No
14	F	86	Cemented	Ceramic/PE	Cup	>5	Yes
15	M	32	Cemented	Metal/PE	Cup	2–5	Yes
16	F	76	Cementless	Ceramic/PE	Cup	>5	No
17	F	74	Cemented	Metal/PE	Cup + Stem	>5	No
18	M	69	Cemented	Metal/PE	N/A	>5	Yes
19	M	84	Cemented	Metal/PE	Stem	>5	Yes
20	F	71	Cementless	Metal/PE	N/A	>5	No
21	M	66	Cemented	Metal/PE	Cup	>5	Yes
22	F	80	Cemented	Metal/PE	Stem	2–5	Yes

F, Female; M, Male; PE, Polyethylene; N/A, Not available.

staining, cells were cultured for 18/21 days after which they were fixed in 3.7% buffered formaldehyde and stained with a solution containing 0.1 mg/ml naphthol ASMX phosphate (Sigma), 0.5% N, N-dimethylformamide, 2 mM $MgCl_2$, and 0.6 mg/ml of fast blue BB salt (Sigma) in 0.1 mM Tris–HCl (pH 8.5) for 5 min. Thereafter, ALP staining was completely washed out of the cell layer with a freshly prepared solution of 50 mM NaOH in EtOH. The absorbance was measured at 500 nM using a VERSAmax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). Enzymatic ALP activity was measured after 4, 6, 13, 18, 21, 25, 28, and 32 days of culture using p-nitrophenyl phosphate (pNPP), as described by van der Horst et al.[26] Briefly, the cells were lysed and ALP activity was measured kinetically using 6 mmol/L pNPP at 405 nm using a VERSAmax Tunable Microplate Reader. DNA concentration in the cell lysate was measured using the fluorescent dye bisBenzimide H 33,258 (Hoechst 33,258, Sigma) and was calibrated against a DNA standard (0.5–10 µg/ml herring sperm DNA). ALP activity was corrected for the amount of DNA in the culture.

Mineralization
Mineralization of the cultures was assessed using the fluorescent dye Bonetag (Li-COR Biosciences, Lincoln, NE), as described previously.[27] Briefly, 24 h before fixation with 3.7% buffered formaldehyde, 2 nM Bonetag 800 was added to the culture medium. The fixed cells were scanned with the Odyssey Infrared Imaging System (Li-COR) at a resolution of 42 µm, intensity 6.5 and medium quality. Integrated intensity (counts/mm^2) of each well was calculated by the Odyssey software.

Quantitative RT-PCR
Total RNA was isolated from cells using RNA-Bee (Tel-Test Inc., Friendswood, TX). cDNA was synthesized using M-MLV reverse transcriptase (Promega, Fitchburg WI) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the Quantitect SYBRgreen PCR kit (Qiagen, Venlo, The Netherlands) with an iQ5 PCR cycler (BioRad, Hercules, CA). For used primer sets, all spanning at least one intron, see Table 2. Data were normalized relative to GAPDH expression. Levels of gene expression in differentiation experiments were expressed as fold-change relative to expression in SaOS-2 and HDFA cells using the $2^{-\Delta\Delta Ct}$ method. Basal levels of gene expression at beginning of experiments were expressed as fold-change relative to expression in positive controls (e.g., SaOS-2 cells, HDFA cells, human endothelial cells, and human monocytes).

Statistical Analysis
Statistical analysis was performed using SPSS (IBM SPSS Statistics 23). To account for treatment clustering within donors during osteogenic differentiation, linear mixed-effects modelling was used to analyze the ALP activity staining, mineralization, and gene expression data, while two-way (mineralization) or three-way (ALP cell layer) ANOVA was used for the outcome measures in the time-dependent experiments.

Linear regression was performed for testing the relation between variability levels of ALP activity and/or mineralization and patient- and implant characteristics as well as between levels of mineralization and basal gene expression levels of "cell-specific" genes. For all tests, a p-value of <0.05 was regarded as statistically significant. Values represent mean ± SD, unless stated otherwise.

RESULTS
The effect of osteogenic stimuli on osteogenic differentiation of peri-prosthetic tissue cells was studied in 22 donors after 18–21 days of culture. When cultured in normal medium (NM), cells displayed ALP activity (Fig. 1A). Stimulation of cells with osteogenic medium (OM) significantly increased ALP activity ($p = 0.024$). OM supplemented with either BMP-2, GIN and BMP-2 + GIN or, alternatively, supplemented with BMP-6, and BMP-6 + GIN did not significantly increase ALP activity (Fig. 1B). In all culture conditions, variation in the level of ALP activity was observed between donors (Figs. 1A,B). These variations between donors makes it impossible to assess differences between BMP-2 and BMP-6. Mineralization of the matrix was studied using the fluorescent dye Bonetag. When cells were cultured in NM, little to no fluorescence was observed, whereas OM induced mineralization in 15 out of 22 donors, although to (very) different extents (Figs. 2A,B). In 5 out of 15 donors mineralization could be increased compared to OM when BMP, GIN or BMP + GIN was added (Supplementary Fig. S1). Interestingly, in donor 3, mineralization could not be induced by OM alone, but addition of GIN was needed to induce mineralization. Furthermore, in donor 19, mineralization could not be induced by OM alone nor OM with additives (Supplementary Fig. S1). Overall, addition of BMP, GIN or BMP + GIN could not significantly increase mineralization compared to OM

Table 2. Oligonucleotides Used in RT-PCR

| Gene	Forward	Reverse
GAPDH	5′-GACAGTCAGCCGCATCTTC-`3	5′-GCAACAATATCCACTTTACCAGAG-`3
ALP	5′-TAAAGCAGGTCTTGGGGTGC-`3	5′-GGGTCTTTCTCTTTCTCTGGCA-`3
Col1a1	5′-TTTGGATGGTGCCAAGGGAG-`3	5′-CACCATCATTTCCACGAGCA-`3
OCN	5′-CCCAGCGGTGCAGAGTC-`3	5′-TCAGCCAACTCGTCACAGTC-`3
S100A4	5′-TTGGTTTGGTGCTTCTGAGATGT-`3	5′-TCACCCTCTTTGCCCGAGTA-`3
Vimentin	5′-CCAAACTTTTCCTCCCTGAACC-`3	5′-CGTGATGCTGAGAAGTTTCGTTGA-`3
Endoglin	5′-TCACCACAGCGGAAAAAGGT-`3	5′-CAGGAACTCGGAGACGGATG-`3
Pecam	5′-AGACGTGCAGTACACGGAAG-`3	5′-CTTTCCACGGCATCAGGGA-`3
CD68	5′-AGGCTGGCTGTGCTTTTCTC-`3	5′-TCTCTGTAACCGTGGGTGTC-`3
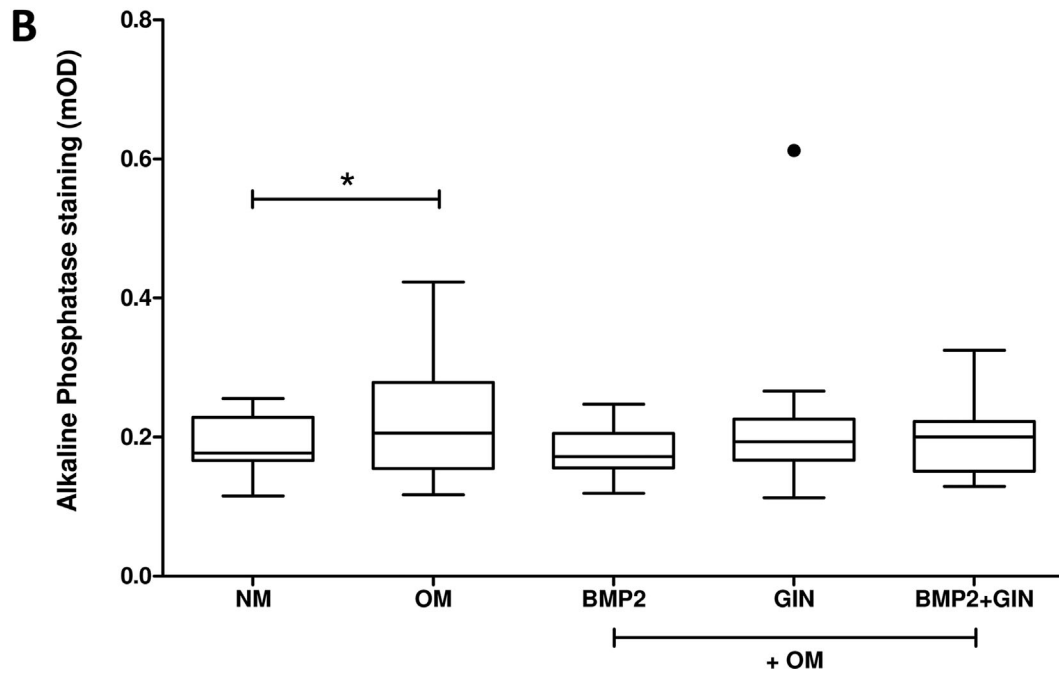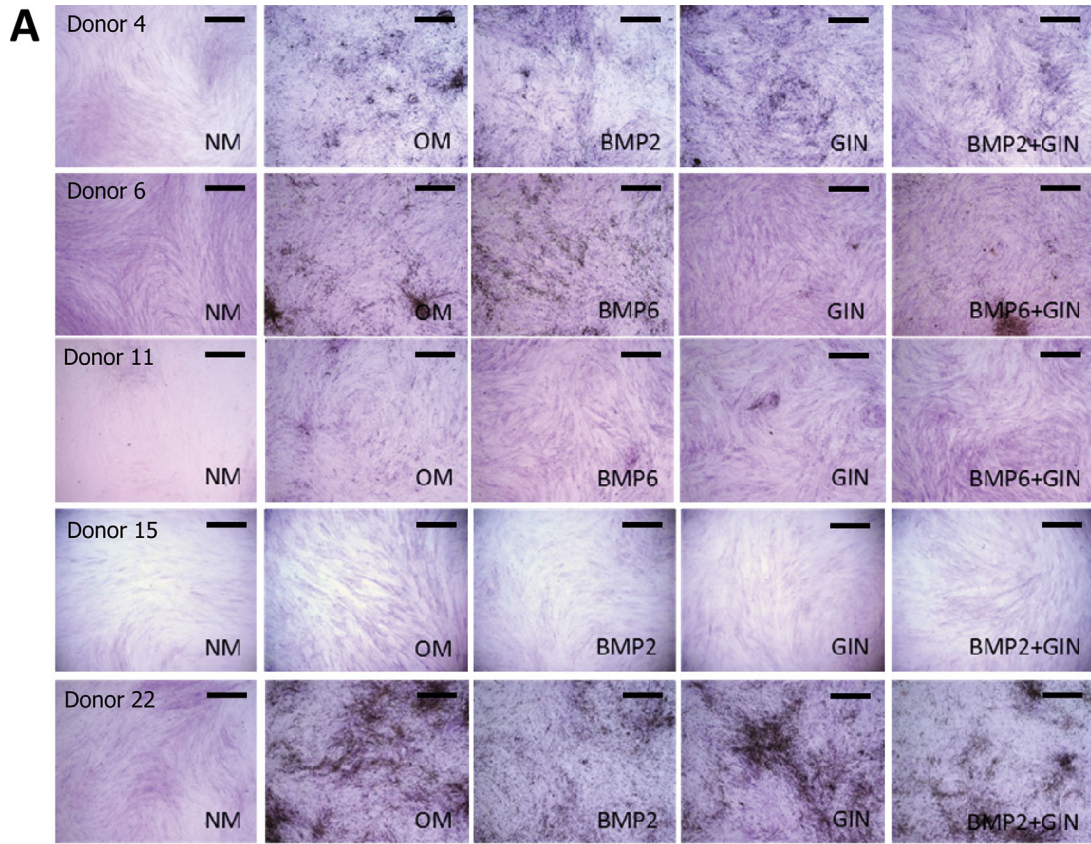
Figure 1. Alkaline Phosphatase (ALP) staining. (A) Pictures of ALP staining of cell cultures of five representative donors cultured in NM or stimulated with OM or OM supplemented with either BMP-2 or BMP-6, GIN and BMP-2 or BMP–6 + GIN. Bars represent 1 mm. (B) Boxplot showing the mOD after extraction of ALP staining from the cultures of twenty-two donors cultured in NM, OM or OM supplemented with either BMP-2, GIN or BMP–2 + GIN. Values represent mean ± SD, dots represents 1.5*IQR. NM: normal medium; OM: osteogenic medium; BMP: bone morphogenetic protein; GIN: GSK3β inhibitor; IQR: Inter Quartile Range. *$p < 0.05$ compared to NM.
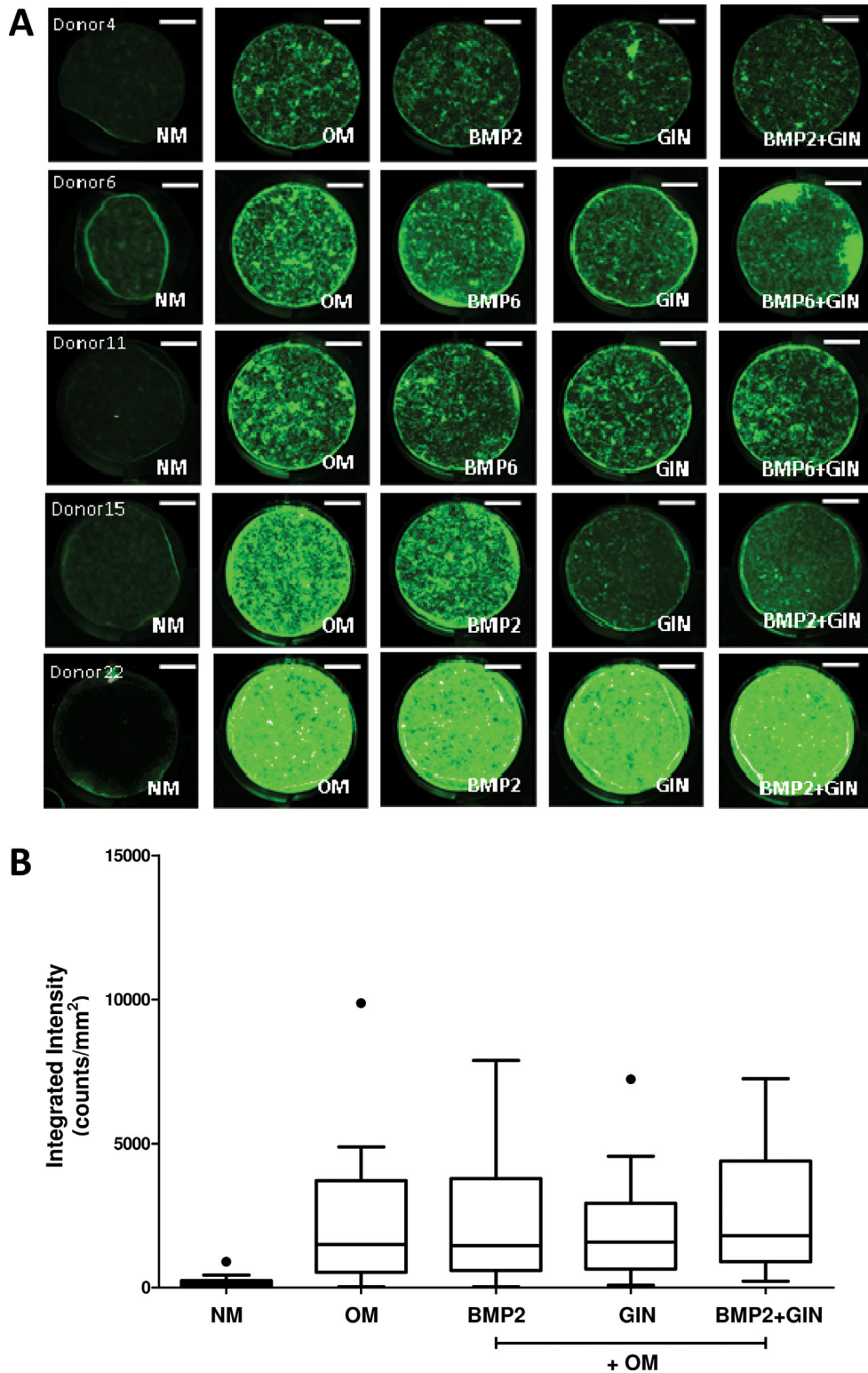
Figure 2. Mineralization of the matrix as measured by Bonetag (fluorescent dye). (A) Pictures of Bonetag fluorescent staining of cultures of five representative donors stimulated with OM or OM supplemented with BMP-2 or BMP-6, GIN and BMP-2 or BMP–6 + GIN. Normal culture medium served as a negative control. Bars represent 5 mm. (B) Boxplot showing the Integrated Intensity of the fluorescence of twenty-two donors stimulated with OM or OM supplemented with either BMP-2, GIN or BMP–2 + GIN. Values represent mean ± SD, dots represents 1.5*IQR. NM: normal medium; OM: osteogenic medium; BMP: bone morphogenetic protein; GIN: GSK3β inhibitor; IQR: Inter Quartile Range.

(Fig. 2B). Subsequently, we also investigated the response to continuous stimulation with BMP-6. However, there were no significant differences in ALP activity and mineralization compared to single stimulation at day 4 (data not shown).

The effect of osteogenic stimuli on ALP, Alpha-1 type I collagen (Col1a1) and osteocalcin (OCN) gene expression was investigated in 19 donors (Fig. 3). OM and OM supplemented with GIN or BMP-2 + GIN could significantly increase ALP gene expression (resp. $p = 0.014$, $p < 0.001$, $p = 0.023$) compared to NM. Col1a1 and OCN gene expression were not significantly different between culture conditions.

We investigated whether the observed variation in levels of ALP activity and mineralization between donors could be explained by patient- and implant characteristics. Type of fixation (ALP: $\beta = 0.026$, $p = 0.538$; Mineral: $\beta = -174.30$, $p = 0.875$), gender (ALP: $\beta = 0.06$, $p = 0.187$; Mineral: $\beta = 80.21$, $p = 0.947$) or age (ALP: $\beta = 0.001$, $p = 0.693$; Mineral: $\beta = -21.14$, $p = 0.661$) could not explain the found variations in ALP activity and mineralization.

Next, we tested whether variation observed in levels of ALP activity and mineralization between donors could be explained by degree of responsiveness to osteogenic stimuli. Since ALP activity and mineralization as described above were only measured at a single time-point (e.g., after 18–21 days of culture), variations observed in these markers for osteogenic differentiation could be explained by differences in response-rate/degree of responsiveness of the cells from different donors to osteogenic conditions. Therefore, in five donors, we investigated the response to osteogenic stimuli over time. Since there was almost no additional effect of BMPs and/or GIN compared to OM alone, we decided to investigate only the response to OM over time. In all donors, at each time-point, we observed a significant difference ($p < 0.001$) between culture conditions in ALP activity in the cell layer (Fig. 4A). For mineralization, all donors showed a significant different pattern in mineralization when cultured with OM, with mineralization observed from day 13, 21, or 28 onwards (Figs. 4B,C). In all cases, mineralization occurred directly after a decrease in ALP activity was seen (Fig. 4A).

Furthermore, we investigated whether the observed variation in osteogenic responsiveness could be explained by the heterogeneous composition of the cell population at the start of experiments. Therefore, we selected several "cell-specific" genes to compare cell populations between donors. All samples showed the presence of macrophage, fibroblast, osteoblast, and endothelial cells, however, a high-inter-donor variability in basal gene expression levels of peri-prosthetic tissue was observed (Fig. 5). No significant associations were found between levels of Integrated Intensity (mineralization at day 18–21) and gene expression levels of ALP ($\beta = -474.89$, $p = 0.91$), OCN ($\beta = -72.15$, $p = 0.115$), S100A4 ($\beta = -141.80$, $p = 0.49$), Vimentin
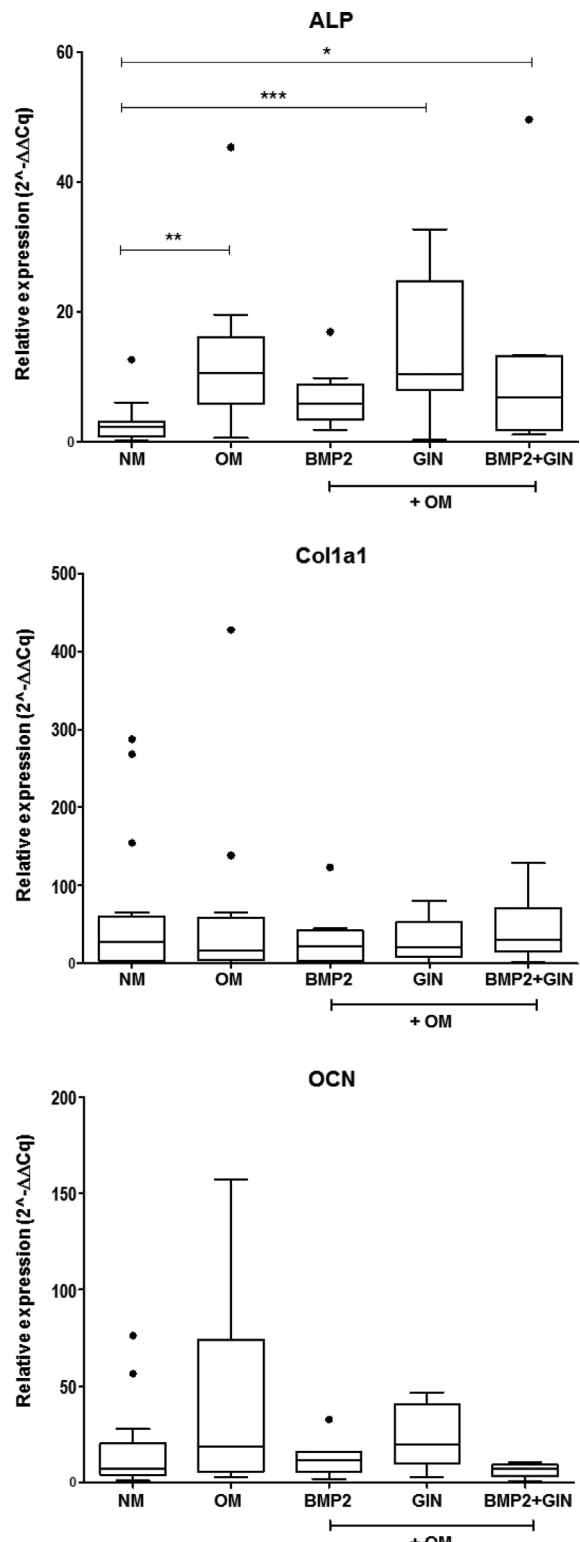

Figure 3. Relative gene expression ($2^{-\Delta\Delta Cq}$) of cells stimulated with osteogenic stimuli. Expression of alkaline phosphatase (ALP), alpha-1 type I collagen (Col1a1) and osteocalcin (OCN) was corrected for internal control GAPDH and the geometric mean of calibrators SaOS-2 and HDFA. Boxplot showing the mean ± SD, dots represent 1.5*IQR. NM: normal medium; OM: osteogenic medium; BMP: bone morphogenetic protein; GIN: GSK3β inhibitor; IQR: Inter Quartile Range. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
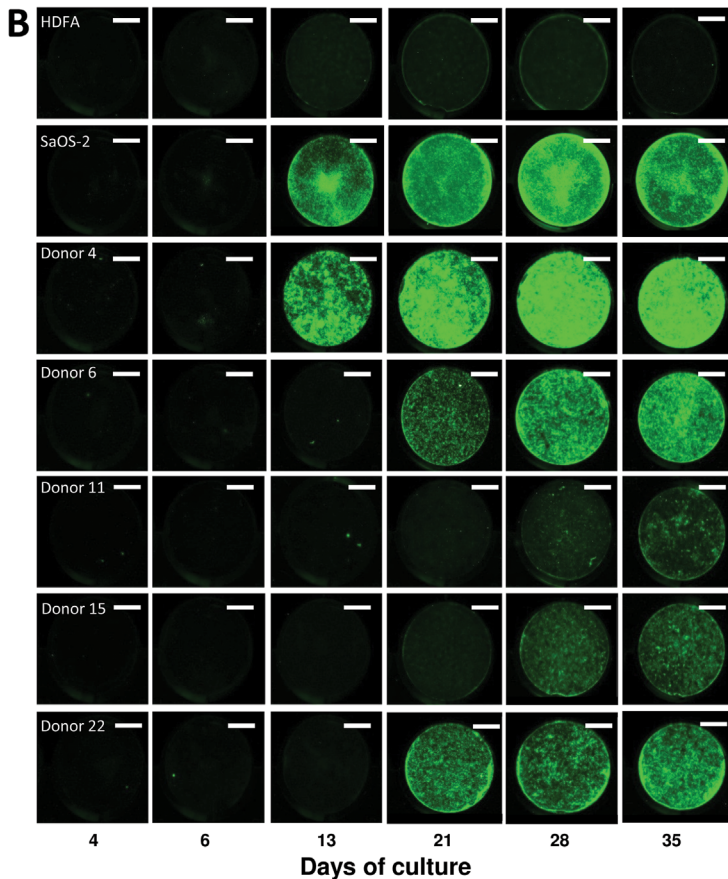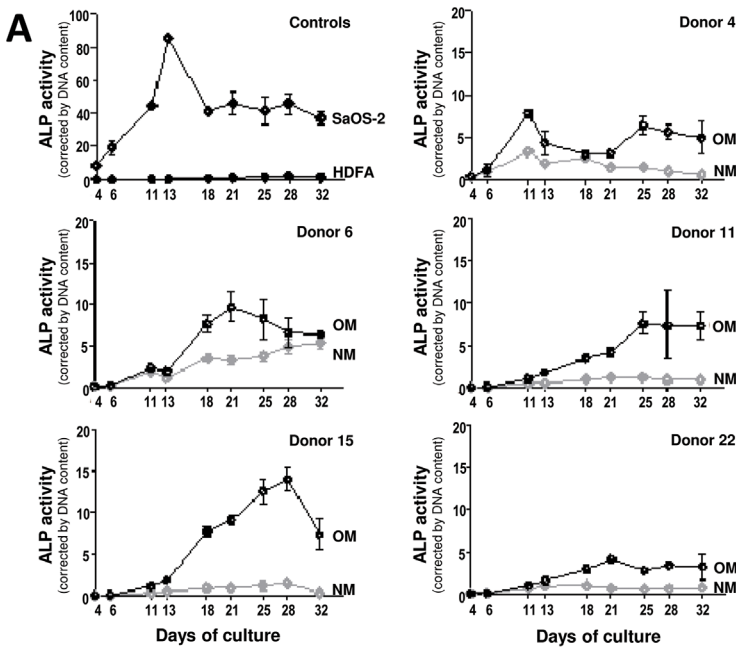
Figure 4. Analysis of differentiation of time course experiment. (A) ALP activity measured kinetically over time in cell cultures of five donors cultured in normal medium (NM) or stimulated with osteogenic medium (OM). Pictures (B) and Integrated Intensity (C) of mineralization of the matrix over time of these cultures measured using the fluorescent dye Bonetag. SaOS-2 cells and HDFA cells served as positive and negative control, respectively. Values represent mean ± SD. Bars represent 5 mm.

($\beta = -562.25$, $p = 0.72$), Endoglin ($\beta = -13287.76$, $p = 0.194$), Pecam-1 ($\beta = -251941.78$, $p = 0.067$) or CD68 ($\beta = -148.69$, $p = 0.59$).

DISCUSSION

In the current study, we subjected peri-prosthetic tissue cells to osteogenic stimuli to investigate whether these cells could differentiate into the osteoblastic lineage. We observed an increase in alkaline phosphatase (ALP) activity and a production of a mineralized matrix upon induction/stimulation with osteogenic stimuli. Increase of ALP activity in early stages of osteoblast differentiation is assumed to reflect the number of progenitor cells
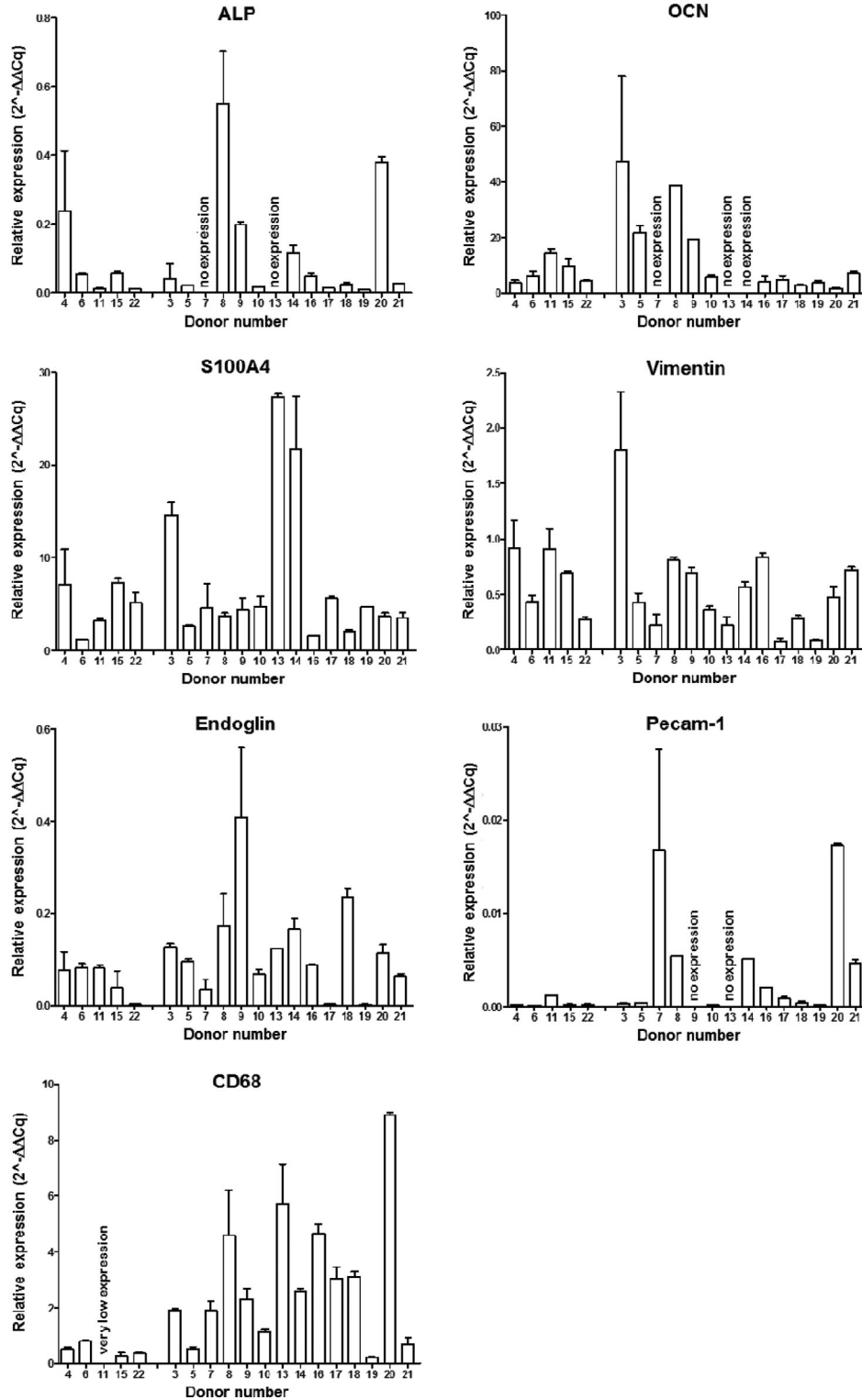
Figure 5. Relative gene expression $(2^{\wedge}-\Delta\Delta Cq)$ of peri-prosthetic tissue cells. Expression of alkaline phosphatase (ALP) and osteocalcin (OCN) was corrected for internal control GAPDH and relative gene expression in SaOS-2 cells. Expression of S100A4 and vimentin was corrected for internal control GAPDH and relative gene expression in HDFA cells. Expression of Endoglin and Pecam-1 was corrected for internal control GAPDH and relative gene expression in human endothelial cells. Expression of CD68 was corrected for internal control GAPDH and relative gene expression in human monocytes.

committed to osteogenic differentiation in a cell population.[28] In general during differentiation, ALP first increases and then decreases when mineralization is well progressed.[28] In agreement, in peri-prosthetic tissue cultures ALP activity reached a peak at the onset of mineralization. We also observed cells stained for ALP activity after 18–21 days of culture in NM. Furthermore, in one donor, we noticed an increase in enzymatic ALP activity over time when cells were cultured in NM. In a study by Heinemann et al.[29] it was found that cells obtained from granuloma explants from endoprosthetic revisions stained positive for ALP activity, indicating that in peri-prosthetic tissue a population of cells could be present that is already committed to the osteoblastic lineage.

Mineralization of the matrix describes the final stage of osteoblast/osteogenic differentiation. Instead of using conventional staining techniques like Alizarin Red S, we have chosen Bonetag to assess the level of mineralization in our cultures, since we previously showed Bonetag to be more sensitive to small changes in mineralization.[27] In the current study, we observed an increase in mineralization when cells were cultured with OM. Moreover, since BMPs are known inducers of osteogenic differentiation,[24] and a local increase of synthesis of several BMPs in the peri-prosthetic tissue has been reported,[23] we also stimulated cells with OM supplemented with either BMP-2 or BMP-6. Several BMPs, including BMP-2 and BMP-6, have shown positive effects on bone formation, fracture healing, and implant osseointegration in several in vitro and in vivo animal models.[30–33] BMP-2 is even used in clinical practice for accelerated healing of fractures and for spinal fusions.[30,34] In our recent study using the murine cell-line KS483, a combination of BMP-4 and GIN (a stimulator of Wnt-signalling) was found to enhance mineralization and decrease the expression of Sclerostin (an inhibitor of bone formation) compared to BMP-4 alone.[35] Therefore, we also stimulated cells with OM supplemented with either GIN or a combination of GIN and BMP-2 or BMP-6. Our results show large-inter-donor differences in response to these stimuli, ranging from no additional effect to necessity for mineralization to occur. When all data were put together no significant increase of mineralization using BMP, GIN or BMP + GIN was observed. Therefore, we were not able to ascertain a standard formula or one common pathway which guarantees osteogenic differentiation in peri-prosthetic tissue cells.

To confirm our hypothesis that there was an inter-donor degree of responsiveness to osteogenic stimuli, we performed a time-series experiment using five representative donors from the first set of experiments. Indeed, we observed an inter-donor degree of responsiveness as expressed by differences in peak-height of ALP activity and differences in time till onset of mineralization. Furthermore, we noticed an intra-donor difference in degree of mineralization at day 21

between both types of experiments. We speculate that this results from differences in cell densities or the fact that the cells were at different passages when cells were subjected to osteogenic stimuli, and therefore, responses were slower or faster.

The RT-PCR results obtained in this study should be interpreted with care, since we observed variations between osteogenic and non-osteogenic conditions regarding the expression of our reference gene (data not shown). The variation could not be explained by any type of technical error. Literature, presenting RT-PCR data of peri-prosthetic tissue cells, shows the use of several different reference genes like GAPDH, 18S, β-actin, PBGD, HPRT, and RPL32.[36–39] We tested all these genes, widely used as reference genes in peri-prosthetic tissue samples, but all showed differences in expression between osteogenic and non-osteogenic conditions in at least some donors. Variation in the gene expression levels of reference genes under different experimental conditions has already been found in other studies,[40,41] indicating that it is not surprising we were not able to find the optimal reference gene for these types of cells (under these types of experimental conditions). Because of the variation in the expression level of the reference genes, calculation of cellular proportions was not possible. Therefore, in future studies, for example FACS analysis could be used to obtain data on percentages of different cell types within each sample.

In this study, we used peri-prosthetic tissue cells from early passages, since at higher passages the risk of substantial in vitro growth selection exists.[42,43] To our opinion, a heterogeneous population of cells could reflect a more in vivo-like response to osteogenic stimuli. We tried to characterize the cell content of the tissue by investigating gene expression patterns of peri-prosthetic tissue cells at the beginning of the experiments (day 0). Since there is no suitable tissue to serve as a control for peri-prosthetic tissue, we used different control cell lines to determine the relative expression. A high-variation in the gene expression levels between donors was observed, indicating heterogeneous populations of cells, which is in line with several studies evaluating the cellular content of peri-prosthetic tissue.[43–45] Although we did not find an association between the responsiveness to osteogenic stimuli and the cell content, it is not excluded that the high-inter-donor variability in gene expression levels might account for the high-inter-donor variability in response to osteogenic stimuli.

Besides tissue characteristics (such as cell content of the tissue), implant and/or patients characteristics, might explain some of the observed variability in the osteogenic responsiveness between the peri-prosthetic tissue of the patients. In our study, prosthesis characteristics (i.e., type of fixation) could not explain the variability in response to osteogenic stimuli. However, other studies showed that surface characteristics of implants can influence the inflammatory response to

implant material and subsequently affect the expression of bone formation markers (e.g., BMP-2 and ALP).[46,47] Therefore, in future studies, the effect of different implant materials and surface characteristics on osteogenic differentiation of peri-prosthetic tissue cells should be investigated. In addition, potentially relevant patient- and tissue-related characteristics, such as type of bone loss (linear vs. erosive) and in situ location of the tissue should be taken in to account. Despite our relatively large number of samples for an in vitro study, the sample size was still too small to perform proper association studies between different clinical groups. Therefore, in future studies, larger sample sizes of peri-prosthetic tissue with more detailed patient-, implant- and tissue characteristics might be essential to explain the inter-donor variability in response to osteogenic stimuli. As the primary aim of this study was to determine whether or not peri-prosthetic cells are capable to differentiate into the osteoblastic lineage, the experimental set-up was relatively simple. Further study is needed to determine the influence of number, size and type of particles, as well as immune cells, on the osteogenic capacity of the cells.

To our knowledge, this is the first (in vitro) study investigating the response of peri-prosthetic tissue cells to osteogenic stimuli. Altogether, our results indicate that these cells, cultured under osteogenic conditions, show characteristics of differentiation into the osteoblastic lineage (i.e., over time increase of ALP activity and production of a mineralized matrix), although a standard formula inducing osteogenic differentiation was not found in this study.

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AUTHORS' CONTRIBUTIONS
All authors meet the criteria for authorship and all authors have read and approved the final submitted manuscript.

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SUPPORTING INFORMATION

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