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Phosphatidylethanolamine biomimetic coating increases mesenchymal stem cell osteoblastogenesis

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Abstract

Previous observations (*e.g.*, decreased bacterial adhesion) have shed the light on the auspicious possibility to use phosphatidylethanolamine as biomimetic coating for metal implants. Additionally, it was experimentally shown that phosphatidylethanolamine induces bone formation, however, up to now no study was performed to understand this observation or to find an explanation. In an attempt to unveil how and why phosphatidylethanolamine can improve cell metabolism and osteogenic differentiation, primary cells (human umbilical cord perivascular cells) were cultured on native or phosphatidylethanolamine coated surfaces. Several parameters were followed on gene (real time polymerase chain reaction) and protein (*e.g.*, dot-blot and ELISA tests) levels. It was determined that phosphatidylethanolamine potentiates cell metabolism, osteogenic differentiation, and mineralisation early processes. By preventing biofilm formation while promoting new bone formation, phosphatidylethanolamine could be easily implemented as implant bio-mimicking coating. (132words)

Keywords: Biomimetic coating; Osteoblasts; Matrix mineralisation; Phosphatidylethanolamine; Stem Cells.

Introduction

Phospholipid (PL) coating is one of the biomimetic approaches to strengthen the intimate interface between metallic implant surface and bone tissue. Even if eukaryotic membranes exhibit variable compositions, approximately 40% of their dry weight is composed of lipids. Phospholipids are the most abundant among these lipids, and are also asymmetrically distributed in plasma membranes. The major phospholipids responsible for this dissymmetry are phosphatidylserine (PS) and phosphatidylethanolamine (PE), mainly located in the cytoplasmic leaflet, and phosphatidylcholine (PC), sphingomyelin (SM), and glycosphingolipids (mostly found in the exoplasmic leaflet) [1]. Lipids are not only responsible for the cell compartmentalisation (barrier function) and the adequate curvature required for (*e.g.*) mitosis but are also closely involved in signalling cascades [2]. Lipids have also a great importance in adequate embedded proteins function, leading to the lipid RAFT theory [3]. Lipids rafts are transient microenvironment enriched in saturated phospholipids, sphingolipids, cholesterol, and specific proteins within cell membrane in order to facilitate (*e.g.*) signal transduction. In bone and cartilage tissues, PL account for 20 to 30% of total lipids [4]. Therefore PL are excellent candidates for biomimetic coatings. PS, due to its property to bind calcium phosphate and its indirect role in bacteria adhesion inhibition [5-8] and PC, able to reduce platelet adhesion [9, 10], are the main studied PL. However, seldom studies are available on PE coating biological application. Incidence of PL coatings (POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine]), and POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1-glycerol))) on bacterial adhesion were tested on mirror polished metal (titanium-based) discs [11]. One Gram negative (*Escherichia coli*) and two Gram positive (*Staphylococcus carnosus* and *Bacillus subtilis*) strains were chosen. It was measured that PL coatings can reduce bacteria number by 80.6%, 73.7%, and 50.9% for POPC, POPG, and POPE, respectively. Additionally, the impact of the POPE-, POPC-, POPS-, and POPG-biomimetic coating on inflammatory reactions was investigated *in vitro* [11, 12] but also *in vivo* [13]. In the *in vitro* studies, RAW 264.7 (a murine leukaemic monocyte macrophage cell line) / phospholipids interactions exhibit only minor reactions in comparison to the primary positive control lipopolysaccharides (LPS). POPE was the only PL which did not induce any inflammatory reaction in terms of nitric oxide production [12]. Additionally, POPE induced tumour necrosis factor alpha (TNFA) cell release was even lower than the native metal surface one [11]. However TNFA release was the highest for POPS and POPG. These results are rather logical as PS is an apoptotic signal and PG a non-eukaryotic PL. Based on these results titanium plates coated with POPE were implanted in rats. It was concluded that POPE does not have a negative influence on the short- and long-term (study up to 56 days) inflammatory reactions in the peri-implant tissue [13]. Furthermore, it has

also been shown that POPE decreases MG-63 (a line derived from a human osteosarcoma) cell adhesion on smooth metal surfaces while increasing significantly cell metabolism (measured by MTT (3-(4,5-Dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide) [11]). Similar decreased adhesion was observed for human articular chondrocytes (HAC) [12]. Further studies were performed on the effect of POPE on 3-weeks induced chondrogenic and osteogenic differentiations. These experiments revealed that cartilage matrix production of HAC was significantly increased by about 30% on POPE-covered surfaces [12]. Similarly, improved osteopontin and collagen I gene expressions and bone nodules formation (osteocalcin (OC) immunocytochemistry) were observable for human mesenchymal stem cells only on POPE-coated surfaces [12]. Physical characterisation (*e.g.*, structure and stability) of the POPE coating was also studied [14, 15].

In an attempt to unveil how and why POPE can improve cell metabolism and osteogenic differentiation while reducing cell adhesion, primary cells (human umbilical cord perivascular cells or HUCPV) were culture on native or POPE coated surfaces and driven toward an osteoblastogenesis pathway for up to 5 weeks. Each week, several parameters, all targeting different aspects of osteogenic differentiation, were followed on gene level or/and protein level. The chosen cellular mechanisms can mainly be categorised in differentiation and mineralisation. Additionally, possible explanations are presented in the present study.

Materials and Methods

POPE coating

POPE (Avanti Polar Lipids, Inc.; Alabaster, Alabama) was dissolved in chloroform:methanol (80:20 v/v; Sigma-Aldrich Chemie GmbH, Munich, Germany) at a concentration of 1 mmol/L. This solution was directly dropped onto sterile borosilicate glass coverslips (500 µL solution per coverslips; 30 mm diameter, thickness 0.13 mm, and hydrolytic class 1; VWR International, Wien, Austria). Solvents were let evaporate for about 45 min and carriers were subsequently immerged in cell culture media for 2 h to allow phospholipids organisation. Afterwards, carriers were directly used for cell culture. It has to be mentioned here that mainly due to the deposition method (solvent used) glass coverslips, known to be suitable tissue culture dishes, were employed.

Cell culture

All biological tests were performed with primary cells, HUCPV, derived from Wharton's jelly of umbilical cords. HUCPV isolations were approved by the local ethical committee and performed as previously described [16]. For the experiments cells of the third to fifth passage were used. All biological evaluations, were performed on POPE-coated (+POPE) or non-coated (control, -POPE) coverslips. After POPE coating procedure, all coverslips (coated & control) were placed on pre-agarose coated 6-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). 80,000 cells in 50 µL medium were seeded on the coverslips and left adhered for

30 min. Thereafter the samples were incubated in 3 mL medium supplemented with 1% penicillin and 100 mg/mL streptomycin (Life Sciences, Karlsruhe, Germany). Cells were further cultured for 3 days before beginning of osteogenic differentiation. Culture medium was then supplemented with factors promoting osteogenic differentiation: 10^{-8} M dexamethasone, 5 mg/mL L-ascorbic acid 2-Phosphat and 10^{-8} M 1α,25-Dihydroxyvitamin D3 ($1,25(OH)_2D_3$; Sigma-Aldrich Chemie GmbH, Munich, Germany). After another week, 5 mM β-glycerolphosphate (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added. The cultures were then maintained for 2, 3, or 4 weeks and media was refreshed every 3 to 4 days. From 2 weeks on, supernatant aliquots were removed every week for 4 weeks and stored at -80°C until use. Time points were therefore named 2W, 3W, 4W, and 5W. Similarly, RNA, DNA, and protein extractions were performed as well as Alizarin red stainings (ARS) and quantification.

DNA extraction

Total DNA content (three independent experiments) was assessed at all-time points to normalise results of further biological tests in order to counteract variation of cell adhesion on covered and non-covered coverslips. All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany. Here cells were first digested over night at 60°C in a digestion solution containing 500 µL papain buffer solution (0.1 M NaH_2PO_4 and 5 mM EDTA, pH 6 in double distilled water (ddH_2O)), 5 µL β-mercaptoethanol, and 2.5 µL of papain solution (10 µg/mL papain in ddH_2O). On the next day samples were diluted 1:5 in DNA dilution buffer (2.5 M NaCl in 19 mM sodium citrate pH 7). Out of the latter solution 100 µL of diluted samples were pipetted in triplicate in 96-well plate, and 50 µL of DNA working buffer (2 M NaCl in 15 mM sodium citrate pH 7) as well as 50 µL of bisbenzimide solution (2 µg/mL bisbenzimide in DNA Working Buffer) were incubated 15 min in the dark. The reactions were fluorometrically measured (excitation: 355 nm, emission: 460 nm) with a VICTOR3 multilabel plate reader (Perkin Elmer, Massachusetts, USA). Unknown DNA concentrations of samples were obtained by plotting measured fluorescent emission to a DNA standard curve.

Ribonucleic acids (RNA) extraction – Reverse transcription real-time polymerase chain reaction (RT-qPCR)

RNA extractions (from three independent experiments) were performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The procedures were followed as outlined in the manufacturer's protocol. RNA concentrations (optical density – OD – at 260 nm) and purity (OD260/280) were measured by a NanoDrop 2000c (Thermo Scientific, Bonn, Germany).Total RNA of each sample was mixed with gDNA Wipeout buffer and RNase-free water and incubated at 42°C for 2 min. Subsequently, complementary deoxyribonucleic acid (cDNA) was synthesised cDNA were stored at -20°C until use. Primers and amplicon designs were carefully designed to ensure specific and efficient amplification (using Primer 3 version 4.0.0) or found in RTPrimerDB

database [17] and purchased from Eurofins MWG Operon (Ebersberg, Germany). Details on primers employed and amplicons can be found in supplementary material. qPCR was also assessed and validated (*e.g.*, optimal primer annealing temperature, reaction efficiency (cDNA tenfold dilution series over eight points), and specificity (PCR product checked on 1% agarose gel)). qPCR were performed with SsoFast EvaGreen supermix (Bio-Rad, Munich, Germany) and under the same conditions as described in[16]. PCR and amplification monitoring were run in triplicate for each sample using a CFX96 Touch real-time PCR detection system with CFX Manager software (Bio-Rad, Munich, Germany; version 3.0). A melting curve step was inserted to each run to confirm melting temperature (Tm) of PCR products, *i.e.*, qPCR specificity. Normalised gene expression ($\Delta\Delta C(t)$) [18] method was chosen. Reference genes (beta-2-microglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and polymerase (RNA) II (DNA directed) polypeptide A (*POLR2A*)) were carefully selected based on the geNorm algorithm method [19] automatically calculated with the CFX Manager software. Due to inherent biological variability between samples three biological and three technical replicates were performed. Differential expression between the two conditions (- or + POPE) was then statistically analysed with the *t*-test. Regulation threshold (expression fold change) and *p*-values (measure of the evidence against the null hypothesis in a statistical test) were set to 1.5 and 0.05, respectively.

Protein extraction

Total proteins (from three independent experiments) were extracted using NP-40/Igepal cell lysis buffer (150 mM NaCl, 1% NP-40/Igepal [v/v], in 50 mM Tris pH 8.0) freshly supplemented with 1x protease cocktail inhibitor (chemicals purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany). After centrifugation, the total protein concentration was determined in the supernatant with BCA Protein Assay Reagent (Thermo Scientific Pierce, Bonn, Germany), according to the manufacturer's protocol. Absorbances were acquired using an enzyme-linked immunosorbent assay (ELISA) reader (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany) at 562 nm. Unknown protein concentrations were determined and reported with reference to standards of bovine serum albumin (BSA).

Inorganic phosphate assay

Inorganic phosphate (Pi) is a central component involved in mineralisation process. Therefore Pi concentration was followed over 2 to 5 weeks of osteoblastic differentiation to check if POPE had an influence on mineralisation process. Pi content was measured with the PiPer Phosphate Assay Kit from Molecular Probes (Life Sciences, Karlsruhe, Germany; three independent experiments, each with n=5), according to the manufacturer's protocol. Total protein extracts were used to measure Pi content. Pi measurements were then normalised to DNA content, to avoid any inter-experiment variation.

Alizarin red S (ARS) staining and quantification

ARS can bind calcium-rich deposits and is therefore used to detect mineralisation. Chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany. ARS staining and quantification were performed with a method adapted from Gregory *et al*. [20]. After fixation (10% (v/v) formaldehyde) and staining (40 mM ARS; pH4.1), photographs were taken and samples were stored at -20°C prior to dye extraction. Incorporated dye was extracted with 10% (v/v) acetic acid. After centrifugation and neutralisation (10% (v/v) ammonium hydroxide) steps, extracted dye was measured (in quintuplicate) with a Tecan Sunrise at 532 nm and value for the samples were determined by comparison to a standard curve and normalised to DNA content.

Alkaline phosphatase (ALP)

ALP activity was assessed in the cell culture supernatants (QuantiChromeTM Alkaline Phosphatase Assay Kit; BioAssay Systems, Hayward, CA) (three independent experiments, each with n=5) after 2, 3, 4, and 5 weeks of induced differentiation), according to the manufacturer's protocol. This assay is based on the inherent catalytic and kinetic activity of ALP, *i.e*., removal of the phosphate group from ρ-nitrophenyl phosphate, resulting in the formation of a coloured product (ρ-nitrophenol; yellow), which is easily measured by a spectrophotometer (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany). The kinetic activity of the ALP enzymes contained in each material cell culture supernatant was normalised to a reference (tartrazine) as well as to sample total DNA content.

Dot Blot

Concentration of Phosphatase, orphan 1 (PHOSPHO1) and tubulin alpha (TUBA) proteins were estimated semi-quantitatively by using Dot-Blot in extracted proteins. 2 µL of samples were carefully spotted and let dried onto a nitrocellulose membrane (GE Healthcare Life science, Freiburg, Germany). After blocking, first antibodies anti-PHOSPHO1 (mouse monoclonal; Abnova GmbH, Heidelberg, Germany) and anti-tubulin alpha (rabbit monoclonal; clone EP1332Y; Merck KGaA, Darmstadt, Germany) were applied to the membrane for 30 min at RT. After 3 washing steps, membranes were incubated with the secondary antibodies (30 min at RT; horse radish peroxidase (HRP; goat anti-rabbit) or ALP (goat anti-mouse) conjugated; 1:10 000; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). After several washing steps, chromogenic substrates (3,3'-Diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) & X-phosphate disodium salt (BCIP), for HRP and AP, respectively; Sigma-Aldrich Chemie GmbH, Munich, Germany) were added to the corresponding blots. Blots were then documented using a ChemiDoc MP System (Bio-Rad, Munich, Germany). Quantification was performed using Image Lab 4.1 software (Bio-Rad, Munich, Germany). Pixel intensities of each spot were measured and signal intensity of PHOSPHO1 spots were normalised to TUBA corresponding one.

ELISA tests

OC and procollagen I N-terminal propeptide (PINP) releases (three independent experiments, each with n=5) in the cell culture media were measured by ELISA assay kits. The Human Osteocalcin Instant ELISA and the Enzyme-linked Immunosorbent Assay Kit for Procollagen I N-Terminal Propeptide were purchased from eBioscience, Frankfurt, Germany and Uscn Life Science Inc., Wuhan, China, respectively. The procedures were followed as outlined in the manufacturers' protocols. Absorbance of the formed coloured products was measured at 450 nm (620 nm reference wave length; with a Tecan Sunrise).

Statistical analysis

Statistics were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany; version 11.0). Standard analysis comparing two treatments was done by *t*-test. Statistical significance was accepted if the significance level was $p<0.05$. For RT-PCR, to detect differential expression, *t*-test method ($p<0.05$) was employed and directly calculated by CFX Manager Software (Bio Rad, Munich, Germany; version 3.0)

Results

RT-qPCR

To simplify data analyses, statistically significant differential gene expressions between the two different conditions (native/POPE surfaces) are presented in the Table 1. However, the complete gene expression patterns over time can be found in supplementary material.

Genes can be classified in the following categories (all denominated differences were statistically significant):

(I) *Apoptosis/survival.* Expression of B-cell CLL/lymphoma 2 (*BCL2*) and BCL2-associated X protein (*BAX*) were selected. *BAX* expression is upregulated on 2W and 4W while downregulated on 5W. *BCL2* remains stable except on 3W where its expression is downregulated.

(II) *Growth regulatory factors.* Targeted genes for this category were transforming growth factor, beta 1 (*TGFB1*) and bone morphogenetic protein 1, 2, 4, and 6 (*BMP1*, *2*, *4*, and *6*, respectively). On 2W *BMP2* and *TGFB1* are upregulated. On 3W, *BMP1* and *BMP6* are in turn upregulated. On 4W and 5W, no difference is observable.

(III) *Transcription factors and mitogen activated protein kinase 3 (MAPK3/ERK1).* Gene expression of activating transcription factor 4 (tax-responsive enhancer element B67) (*ATF4*), distal-less homeobox 5 (*DLX5*), FOS-like antigen 1 (*FOSL1*), runt-related transcription factor 2 (*RUNX2*), and Sp7 transcription factor (*SP7*) were studied over 5 weeks. Most of upregulations can be observed during the second week of differentiation. Therefore, an increase of *FOSL1* and *RUNX* can be observed on 2W. An increased expression of *SP7* is also

observable on 3W. On 4W and 5W, no differential expression can be observed. *MAPK3* expression is only upregulated after 2W culture.

(IV) *Adhesion and extracellular matrix (ECM) components.* To study the cell adhesion, cluster of differentiation 44 molecule (Indian blood group; *CD44*), intercellular adhesion molecule 1 (*ICAM1*), cadherin 11, type 2 (osteoblast –cadherin, *CDH11*), integrin alpha 1 (*ITGA1*), integrin alpha 2 (*ITGA2*), and integrin alpha 5 (*ITGAV*) were selected. In this group significant differences (for adhesion molecules) are again observable during 2W and 3W. On 2W, *CD44* and *ITGA2* are upregulated while *ICAM1* is downregulated on POPE substrate cultured cells.On 3W, *CD44* and *CDH11* are upregulated while *ICAM1* remains downregulated. Collagen 1A1 (*COL1A1*), *OC*, osteopontin (*OPN*), vitronectin (*VTN*) Fibrillin 1 (*FBN1*), fibronectin 1 (*FN1*), secreted protein, acidic, cysteine-rich (osteonectin; *SPARC*) and vinculin (*VCL*) were selected as substrate adhesion molecules (*i.e.*, ECM components). *OC* is upregulated on 2 and 3W. *OPN* is similarly upregulated but its expression decreases on 4W. *VTN* is strongly downregulated on 2Wand *VCL* is upregulated on 3W. *SPARC* is upregulated on 4W whereas its expression is downregulated on 3W and 5W.

(V) *Tumour necrosis factor (TNF) superfamily.* Osteoprotegerin (*OPG*), tumour necrosis factor receptor superfamily, member 11a, *NFKB* activator (*TNFRSF11A*, *RANK*), tumour necrosis factor (ligand) superfamily, member 11 (*TNFSF11*, *RANKL*), tumour necrosis factor receptor superfamily, member 1A (*TNFRSF1A*) and *TNFA* were selected as member of this category. The only differential expression could be observed at 2W, where TNFRSF1A is upregulated and at 3W, where *RANK* and *RANKL* are downregulated.

(VI) *Mineralisation.* In this category alkaline phosphatase, liver/bone/kidney (*ALPL*), ankylosis, progressive homolog (*ANKH*), ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*), phosphatase orphan 1 (*PHOSPHO1*), and solute carrier family 20 (phosphate transporter) member 1 (*PIT1*) were selected as target genes. Main gene regulations can be observed during 2W and 3W. At 2W, *ALPL*, *ENPP1*, *PHOSPHO1*, and *PIT1* are upregulated. However, on 3W and 4W, only *PIT1* and PHOSPHO1 are upregulated, respectively.

Inorganic phosphate assay

Phosphate, in the form of Pi, is a key component of bone mineral or hydroxyapatite (HA). In order to understand the effect of POPE coating on osteoblast (OB) differentiation, Pi content was followed in cell culture supernatant over the 5 weeks differentiation process. Pi content was normalised to total DNA content to account for possible variation of cell content. As presented in Fig.1 a significant increase of the Pi content was observable after 2 and 3 weeks induced osteoblastic differentiation with POPE coating.

Alizarin red S (ARS) staining and quantification

As calcium is the second key component of hydroxyapatite ARS assays were performed after 2, 3, 4, and 5 weeks induced differentiation to follow osteogenesis and mineralisation. Stainings were first documented and quantification was subsequently performed. Pictures and graph are presented in Fig.2. ARS release was normalised to DNA content to account for possible variation of cell content. After 2 and 3 weeks, calcium deposits are already visible; however, only on POPE coated surfaces cell condensations are observable. For ARS release, maximum staining was obtained on week 3. The decrease on week 4 and especially on week 5 is artefactual, as ARS was probably more tightly bound to calcium deposit and not completely released in the supernatant. The red colour was still observable in the cell pellet (data not shown). On POPE coated surfaces, significantly more ARS was found on week 3 and 4.

ALP

ALP hydrolyses organic phosphates at a high pH and is a validated biochemical marker of bone formation. Therefore, ALP activity was measured in culture supernatants during induced differentiation to see if POPE coating has an effect on mineralisation. The measured activities are presented in Fig.3. Here again, increased ALP activity were observed for cells cultures on POPE surfaces, especially on the second and third week.

Dot Blot

In order to explore if PHOSPHO1 has a role on the effect of POPE coating on mineralisation, its protein expression was measured over the weeks. As it can be observed in Fig.4 except on week 4, PHOSPHO1 relative quantities are higher in HUCPV cultured on POPE coated.

ELISA tests

To further investigate the effect of POPE on differentiation, OC and PINP protein expressions were quantified *via* ELISA tests Fig.5). OC (Fig.5A) is an established marker of bone formation. While OC levels are low at 2W, an increase of OC protein expression is visible at 3W especially for +POPE (+1359% and +2145%, for –POPE and +POPE, respectively). On the following weeks, the levels remain comparable to the 3W ones although +POPE levels are more in the range of –POPE ones. Statistically significant difference OC protein expression levels are measured between –POPE and +POPE on 3W and 4W. PINP (Fig.5B), propeptide of type I procollagen (N terminal PINP) is also a well-known marker of osteogenesis. Here levels of PINP are similar on 2W and 4W. On 3W, PINP is significantly higher for +POPE than for –POPE, however, the reverse effect is observed on 4W.

Discussion

Already in 1995, Hall and Miyake proposed that osteogenesis can be divided in subsequent sequence of events [21]. Shortly, mesenchymal stem cells commit to preosteoblasts. Then after condensation (which increases the

number of committed cells), preosteoblasts start to transform in osteoblasts. Afterwards, the ECM deposition begins followed by terminal differentiation and mineralisation. Therefore, in an attempt to partially reveal how POPE coating is influencing HUCPV differentiation several cellular mechanisms were followed either on gene and/or on protein levels while HUCPV were driven toward an osteoblastogenesis pathway.

BMP signalling pathway is one of the key signal is osteogenesis [22] and expression of several members have been observed to be up-regulated in this study. BMP2 and 4 induce new bone formation *in vitro* by promoting the differentiation of uncommitted mesenchymal stem cells [23] but also have a positive effect on committed osteoblasts, by increasing ALP activity, collagen 1A1 (*COL1A1*) synthesis, and *OC* expression [24]. BMP6 is also known to induce osteoblastic differentiation [25]. BMP1, is involved in fibril formation by cleaving propeptides of procollagen [26] and therefore involved in ECM organisation, BMP1 can be seen as a marker of a rather advanced OB differentiation. TGFB1 promotes proliferation and recruitment of new OB [27], and furthermore it is inhibiting osteoclast formation *in vitro* [28]. Analysing the growth regulatory factors expression, it seems that differentiation is increased on 2W and 3W for cells cultured on POPE substrate. Furthermore, BMP2 induces expression of transcription factors involved in OB differentiation/maturation such as RUNX2 [29], SP7 [30], and DLX5 [31]. It has also been reported than BMPs can activate MAPK3 [27]. MAPK3 has a pivotal role in the MAPK/ERK cascade. This cascade regulates numerous and various cellular processes such as differentiation. Moreover, MAPK3 can activates RUNX2 [32] and the expression of several bone-related proteins such as procollagen, OPN, and OC [27].

Several transcription factors involved in osteoblastogenesis were studied here. It is generally established that RUNX2, SP7, and ATF4 are key transcription factors involved in different steps of osteoblastic differentiation: commitment, proliferation, and matrix maturation, respectively [33, 34]. FOSL1 is generally involved in cell proliferation, differentiation, and transformation. It enhances osteoblast differentiation (regulate bone matrix formation) and activity [35]. RUNX2 is a multifunctional transcription factor. Its expression is upregulated in immature OB (and required for OB lineage determination) and it also upregulates several gene expressions (*e.g.*, bone matrix genes, *OC*, *COL1A1* [36]). RUNX2 may also be sufficient to induce OB differentiation [37]. SP7 (further directs the fate of OB cells (*e.g.*, by blocking their differentiation into chondrocytes [36]). DLX5 and ATF4 are rather late transcription factors [33]. DLX5 is generally coupling osteoblast/osteoclast activities [38].

Another category of studied genes referred to the adhesion molecules which mediate cell–matrix and cell–cell interactions. Three main families of adhesion molecules can be mentioned: (I) immunoglobulin (Ig), (II) cadherin, and (III) integrin families. From the Ig family, CD44 and ICAM1 were studied. CD44 is a

hyaluronan (one of the main component of ECM) and an OPN receptor [39]. Additionally, cells from the osteoblast lineage express CD44 in accordance to their morphological changes from osteoblast to osteocyte [39]. These data suggest that ECM is produced and that cells cultured on POPE are rather far in their differentiation processes. ICAM1 has a role in OB/osteoclast interaction [40], increasing the recruitment of osteoclasts [41]. The decrease of *ICAM1* may be seen as an inhibitor of osteoclast recruitment as the OB differentiation is rather fast. For cadherin, *CDH11* or OB cadherin [42] having a role in osteogenesis and in mineralisation [43], demonstrating again that POPE coating seems to accelerate differentiation compared to non-coated surface. Finally, several integrins were studied: ITGA1, ITGA2, and ITGAV. ITGA2 has a role in cell/matrix adhesion (*via – e.g.* – collagen), differentiation, and lead to the expression of osteoblastic differentiated genes such as *OC*, *ALPL* [44].

FN1 and *VTN* are genes coding for glycoproteins which can bind integrins. Additionally the glycoprotein SPARC has a role in collagen binding and in cell-matrix interaction. As SPARC and FN1 have a role in OB-matrix interaction and OB proliferation [45, 46], these genes downregulations may explain why generally less cells are detected on POPE coating. However, these results are somewhat contradictory to the adhesion molecules observations described above and should be elucidated in more detail.

During osteogenesis OB synthesise first the organic component of the matrix (mainly composed of COL1A1 *i.e.*, 90% [47]) and then other proteins *e.g.*, OPN and OC which will colonise the collagenous structure [48]. *COL1A1* is upregulated on POPE surfaces (2 and 3W) and PINP concentration increased on 3W +POPE. OC represents 20% of the non-collagenous protein. Its role remains unclear but as OC is generally closely associated with calcified area it seems to have a role in mineralisation. OC is a rather late (and classical) marker of osteoblast maturation [49]. The positive effect of POPE observed on *OC* expression was further proven on protein level as OC ELISA (Fig.5A) revealed an increased OC content on cells grown on POPE coated surfaces (especially on 3 and 4W).

RANKL is an osteoclast recruitment signal and is necessary for osteoclast differentiation while OPG (decoy RANKL/RANKL receptor) inhibits osteoclastogenesis. The *RANKL:OPG* ratio is a useful marker of bone turnover and mineralisation [50]. When there is bone resorption the ratio is superior to 1 and inferior to 1 when there is bone formation. After 2W induced differentiation, the ratios are low (0.51 and 0.82 for –POPE and +POPE, respectively). On 3W, -POPE ratio is high (1.70) while +POPE remains low (0.58). On 3 and 4W however they are always superior to 1.

Analysis of the expression of genes and proteins involved ECM components show clearly a positive effect of POPE on OB differentiation. A beginning of an explanation may be found in Bazzi *et al.* [51]. It was

demonstrated that PE enhance considerably membrane-protein associations by reducing calcium requirement for bounding. They explained therefore the primary location of PE on interior membranes. One of the employed proteins was protein kinase C (PKC) but the authors suggested that this mechanism could be generalised to other cytoplasmic proteins. PKC is a family of protein kinase enzymes which control various proteins by phosphorylating them. PKC regulates RUNX2 [52] and OC [53] and is involved in OB proliferation [54]. Furthermore, the use of $1,25(OH)_2D_3$ to differentiate HUCPV involved PKC and MAPK [55].

Additionally, the effect of POPE coating on bone matrix mineralisation was studied. As observed by ARS staining and quantification (Fig.2), a positive effect of POPE on mineralisation was measured. Matrix vesicles (MV) are essential for mineralisation initiation. MV are extracellular membrane vesicles secreted by osteoblasts. When ideal intra-vesicle concentrations of calcium (Ca) and inorganic phosphate (Pi) are reached, the first crystals of calcium hydroxyapatite mineral are generated [56]. In a subsequent step, crystals will pierce the MV membrane, will be release in the extracellular fluid, and will associate with collagen fibrils. Here, they will serve as template for the formation of new crystals, collagen being a scaffold for HA formation. Increase of intra-MV Ca is possible due to Ca-binding phospholipids, protein enrichment and action of Ca channels (*e.g.*, annexin V). Additionally, PE induces calcium uptake in bone *in vitro* [57]. For Pi, the mechanism is more complex. Three phosphatases ALP, ENPP1 and PHOSPHO1 have a fundamental role in controlling inorganic pyrophosphate (PPi):Pi ratio. PPi can adsorb on HA surface and inhibits crystal propagation while Pi induces mineralisation. ENPP1 ectoplasmically generates PPi by catabolising adenosine triphosphate (ATP) to PPi and AMP. Intracellular PPi is extracellularly exported by ANKH. In the extracellular space, ALP can degrade ATP and PPi to form Pi (and therefore regulates PPi:Pi ratio). Extracellular Pi is then transported into MV by PIT1. The key phosphatase involved in early mineralisation is PHOSPHO1 as it is responsible for initiating HA crystal formation inside MV. The striking characteristic of PHOSPHO1 is that PE is its principal substrate to produce Pi [58]. ALP can also use PE as template [59]. On gene level, *ALPL*, *ENPP1*, *PHOSPHO1*, and *PIT1* are upregulated on 2W +POPE (*ANKH* remains stable). The positive effect of POPE on mineralisation was also proved on protein level as ALP activity and Pi and PHOSPHO1 contents are higher on POPE coated surface

In conclusion, all together these results imply that (I) POPE potentiates HUCPV differentiation possibly *via* enhancing the osteogenic phenotype induced by $1,25(OH)_2D_3$ and PKC (II) POPE accelerates HUCPV mineralisation rate probably by its Ca affinity and its role as PHOSPHO1 substrate. These positive effects are observed early in the *in vitro* differentiation process as after 4 to 5 weeks no significant differences between cells grown on –POPE or +POPE can be measured. However, POPE coating concedes an advantage to new bone

formation being osteoinductive and may be really beneficial *in vivo* by enhancing the close contact between bone and implant and improving osseointegration at early implantation times, while decreasing the risk of infection.

Disclosure

The authors state that they have no conflicts of interest.

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Figure 1 Pi content normalised by DNA content in cell culture supernatants. Pi contents were measured up to 5W after induced osteoblastic differentiation of HUCPV cultured on native substrate (-POPE; black) and on POPE coated surface (+POPE; grey). Bars represent mean ±standard deviation (SD) of three independent experiments, each with n=5. Stars indicate statistically significant difference between two groups (*t*-test; *** $p \leq 0.001$).

Figure 2 ARS staining and quantification. (a) Stainings performed after 2 and 3 weeks; scale bar 200 µm. (b) Quantification, data are expressed in absolute amounts according to a standard curve and further normalised to respective DNA content. Black and grey represent native substrate (-POPE) and POPE coated surface (+POPE), respectively. Bars represent mean ±SD of three independent experiments, each with n=5. Significant difference (*t*-test) between two groups is indicated by an asterisk (** $p \leq 0.01$ and *** $p \leq 0.001$).

Figure 3 ALP activity measurements. ALP activity was assessed in cell culture supernatants (-POPE, black - +POPE; grey). ALP activities were further normalised by corresponding sample DNA content to prevent inter-sample variation. Bars represent mean ±SD of three independent experiments, each with n=5. Stars indicate statistically significant difference between two groups (*t*-test; *** $p \leq 0.001$).

Figure 4 PHOSPHO1 relative expression level. (a) Antibodies specificity to target protein (left panel) and dot blots of PHOSPHO1 and TUBA (right panel). Intensities of PHOSPHO1 were then normalised to their TUBA relative one and the relative expressions presented as column chart (b). Bars represent mean ±SD. Stars indicate statistically significant difference between two groups (*t*-test; * $p \leq 0.05$ and *** $p \leq 0.001$).

Figure 5 ELISA assays. OC (a) and PINP (b) protein expressions measured in supernatants of cell cultured with (grey) or without (black) POPE from 2 to 5W and further normalised by corresponding sample. Bars represent mean ±SD of three independent experiments, each with n=5. Stars indicate statistically significant difference between two groups (*t*-test; * $p \leq 0.05$ and *** $p \leq 0.001$).