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Deciphering the combined effect of bone morphogenetic protein 6 and calcium phosphate on bone formation capacity of periosteum derived cells-based tissue engineering constructs

Wei Ji^{a,b,1}, Greet Kerckhofs^{a,b,c,1}, Carla Geeroms^{a,b}, Marina Marechal^{a,b}, Liesbet Geris^{a,d,2}, Frank P. Luyten^{a,b,2,*}^a Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium^b Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium^c Biomechanics Lab, Institute of Mechanics, Materials, and Civil Engineering, UCLouvain, Belgium^d Biomechanics Research Unit, GIGA In silico Medicine, University of Liege, Liege, Belgium

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ABSTRACT

Cell based combination products with growth factors on optimal carriers represent a promising tissue engineering strategy to treat large bone defects. In this concept, bone morphogenetic protein (BMP) and calcium phosphate (CaP)-based scaffolds can act as potent components of the constructs to steer stem cell specification, differentiation and initiate subsequent *in vivo* bone formation. However, limited insight into BMP dosage and the cross-talk between BMP and CaP materials, hampers the optimization of *in vivo* bone formation and subsequent clinical translation. Herein, we combined human periosteum derived progenitor cells with different doses of BMP6 and with three types of clinical grade CaP-scaffolds (ChronOs[®], ReproBone[™], & CopiOs[®]). Comprehensive cellular and molecular analysis was performed based on *in vitro* cell metabolic activity and signaling pathway activation, as well as *in vivo* ectopic bone forming capacity after 2 weeks and 5 weeks in nude mice. Our data showed that cells seeded on CaP scaffolds with an intermediate Ca²⁺ release rate combined with low or medium dosage of BMP6 demonstrated a robust new bone formation after 5 weeks, which was contributed by both donor and host cells. This phenomenon might be due to the delicate balance between Ca²⁺ and BMP pathways, allowing an appropriate activation of the canonical BMP signaling pathway that is required for *in vivo* bone formation. For high BMP6 dosage, we found that the BMP6 dosage overrides the effect of the Ca²⁺ release rate and this appeared to be a dominant factor for ectopic bone formation. Taken together, this study illustrates the importance of matching BMP dosage and CaP properties to allow an appropriate activation of canonical BMP signaling that is crucial for *in vivo* bone formation. It also provides insightful knowledge with regard to clinical translation of cell-based constructs for bone regeneration.

Statement of Significance

The combination of bone morphogenetic proteins (BMP) and calcium phosphate (CaP)-based biomaterials with mesenchymal stromal cells represents a promising therapeutic strategy to treat large bone defects, an unmet medical need. However, there is limited insight into the optimization of these combination products, which hampers subsequent successful clinical translation. Our data reveal a delicate balance between Ca²⁺ and BMP pathways, allowing an appropriate activation of canonical BMP signaling required for *in vivo* bone formation. Our findings illustrate the importance of matching BMP dosage and CaP properties in the development of cell-based constructs for bone regeneration.

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1. Introduction

Long-bone fractures are common and are one of the most frequent non-fatal injuries sustained following trauma worldwide

* Corresponding author at: Herestraat 49, box 7003, 3000 Leuven, Belgium.

E-mail address: frank.luyten@uzleuven.be (F.P. Luyten).¹ These authors contributed equally to this work.² These authors share senior authorship.

[1–3]. In the United States, almost 8 million fractures are reported each year, and 5–10% of fractures fail to heal properly, resulting in delayed or non-unions [2,4]. As populations age, the incidence of non-unions also increase, owing to comorbidities such as diabetes [5]. The current state of the art to treat non-union fractures involves rigid fixation of the fractures and transplantation of autologous or allogenic bone grafts to stimulate the bone growth [6]. However, the inherent drawbacks of this approach, including insufficient autologous resources, pain and donor site morbidity, strongly urge clinicians and researchers to explore alternative therapeutic strategies.

With the advances of cell biology and material sciences, several alternative strategies are emerging to treat non-union fractures. One of these promising strategies is to develop advanced therapeutic medicinal product (ATMP) with stem cells laden in optimal carrier materials [3,7,8]. By using stem cells to initiate fracture healing and carrier materials (i.e., scaffolds) to guide and stimulate bone tissue formation [3,9], such cell-based ATMPs are envisioned to overcome the limited endogenous regenerative capacity of the patients [3,8].

Human periosteum derived cells (hPDCs) are of interest for bone regeneration owing to their key roles in fracture healing [10–13]. Moreover, recent studies demonstrate that postnatal hPDCs exhibit greater clonogenicity, differentiation capacity, and bone regenerative potential after transplantation compared to bone marrow stromal cells, although these two types of cells are derived from a common embryonic mesenchymal lineage [14]. On the other hand, calcium phosphate (CaP) materials have been widely used as synthetic bone substitutes in view of their excellent osteoconductivity as well as biodegradability [8,15,16]. Currently, the most frequently used clinically available CaP materials are hydroxyapatite (HA), β -tri-calcium phosphate (β -TCP) and combinations of HA and β -TCP, which display distinctive Ca^{2+} release rates due to their different Ca/P atomic ratio [17]. Our previous data has shown that the sufficient release of Ca^{2+} was an essential determinant for inducing osteogenic differentiation of hPDCs [18–20]. Furthermore, the chemical and structural properties of CaP also played an important role in determining the *in vivo* bone formation capacity of hPDCs/CaP constructs [21]. It is also important to note that bone formation in the aforementioned cell-based CaP constructs is a slow process (8 weeks) and until today, only limited amounts of bone and marrow formation have been obtained. Hence, the addition of osteoinductive factors such as bone morphogenetic proteins (BMPs) appears to be a straightforward approach to improve the bone forming capacity of hPDC/CaP constructs.

BMP2 (Infuse® or InductOs™) and BMP7 (Osigraft®) have been widely introduced to the clinic to treat non-healing bone defects or complicated bone fractures [22,23]. However, both BMP2 and BMP7 have been confronted with major issues such as early osteolysis, significant inflammation, heterotopic ossification, leading to less robust outcomes in clinics and limited use in the post-marketing period [24]. Recently, BMP6 has emerged as a unique and important BMP family member during skeletal development. Previous studies showed that BMP6 was more efficient in driving osteoblast differentiation of mesenchymal stem cells than BMP2 and BMP7 [25]. Vukicevic et al [24] reported that at 3 months of age, BMP6–/– mice showed 32% decrease of trabecular bone volume, and such effects could not be overcome by increased expression of other BMPs. Our previous results have also shown that exogenous BMP6 coated calcium phosphate scaffolds induced subcutaneous bone formation after 5 weeks, and there was a dominant role of BMP6 over the material in such ectopic bone formation process [26]. Although numerous studies have been performed to investigate biological functions of BMP6, the selection of BMP6 dosage is mainly experienced-based. Furthermore, the combined

effects of BMP6 (at different dosages) and CaP properties (at different Ca^{2+} release rates) on cell behavior, as well as on *in vivo* ectopic bone forming capacity are still largely unknown.

This study is aimed to decipher the importance and influence of BMP6 dosage and CaP properties, as well as their combination, on the biological performance of cell-based tissue engineering (TE) constructs. For this purpose, hPDCs were seeded into three different clinical-grade CaP-based scaffolds (with distinctly different Ca^{2+} release rates) combined with three doses of BMP6. The obtained constructs were evaluated for *in vitro* cell metabolic activity and signal activation after 16 h prior to implantation, as well as *in vivo* ectopic bone forming capacity after 2 weeks and 5 weeks after implantation. Finally, a multivariate statistical analysis was used to deduce key factors in the empirical data set. Our hypothesis was that the interactions between Ca^{2+} release rate and BMP6 dosage affect the *in vitro* cell behavior, hence influencing the *in vivo* skeletal tissue formation of hPDCs seeded TE constructs.

2. Materials and methods

2.1. Scaffolds preparation

Three different commercially available and clinically approved CaP-based materials were used, namely ReproBone™ (Ceramysis, Sheffield, England), ChronOS® (Depuy Synthes, Oberdorf, Switzerland), and CopiOs® (Zimmer Inc, Warsaw Indiana, USA). The characteristics and the composition of these materials, as provided by the supplier, can be found in Table 1. For each type of material, scaffolds (3 mm × 3 mm × 3 mm) were cut using a scalpel and a ruler. All preparations and handling of the scaffolds were accomplished in a sterile environment.

2.2. Expansion of human periosteum-derived cells (hPDCs)

Isolation and *in vitro* expansion of hPDCs were carried out as previously described [12,26]. The ethical committee for human medical research (KU Leuven) approved all procedures, and the patient informed consents were obtained. In brief, expanded cells from 4 different donors (29 ± 12 years old, detailed information in Supplementary Table S1) were pooled as a representative sample and expanded *in vitro* in culture media [DMEM-c; consisted of Dulbecco's Modified Eagle Medium (DMEM GlutaMax™, Invitrogen, Merelbeke, BE) supplemented with 10% Fetal Bovine Serum (Gibco, Merelbeke, BE), 1% antibiotics/antimycotics, and 1% sodium pyruvate (Invitrogen)].

2.3. Cell seeding and BMP coating of scaffolds

Recombinant human BMP6 was a generous gift provided by Prof. Slobodan Vukicevic (Centre for Translational and Clinical Research, University of Zagreb, Croatia). Upon confluence, hPDCs (Passage 7) were trypsinized, counted and re-suspended in phosphate buffered saline (PBS, pH 7.4, Gibco). Afterwards, 20 μL of cell suspension containing different dosage of BMP6 (ranging from 0 ng/mm³ to 210 ng/mm³) were drop seeded onto the aforementioned scaffolds that had been pre-wet with 1 mL of PBS. The loading dosage of cells and BMP6 were normalized to the open volume and the total volume space of the scaffold types, respectively, which were determined from the microCT-based image analysis in our previous work [21]. Table 2 listed the precise information of cell seeding density and BMP6 dosage for all the conditions. For samples loaded only with BMP6, 20 μL of PBS containing the equivalent amount of BMP6 was drop seeded into the scaffolds. After 1 h of incubation at 37 °C, 5% CO₂ and 95% relative humidity, 4.5 mL of DMEM-c was added. The seeded scaffolds were

Table 1

Composition and characteristics of different materials as provided by suppliers and our previous scanning [21].

Scaffold	Supplier	CaP chemistry	Matrix	Total volume (mm ³)	Open volume (mm ³)
ChronOs [®]	Depuy Synthes	100% β -Ca ₃ (PO ₄) ₂	N.A	27	17.55
ReproBone [™]	Ceramysis	Ca ₅ (PO ₄) ₃ (OH)/ β -Ca ₃ (PO ₄) ₂ (60%/40%)	N.A	27	20.08
CopiOs [®]	Zimmer	CaHPO ₄	Lyophilized bovine collagen I	27	21.78

Table 2

Cell seeding density and BMP6 loading amount for different scaffolds.

		ChronOs [®]	ReproBone [™]	CopiOs [®]
Cell seeding density (normalized to the open volume per scaffold)		50,000/mm ³	50,000/mm ³	50,000/mm ³
Theoretical cell amount per scaffold		0.9×10^6	1.0×10^6	1.1×10^6
BMP6 loading amount (normalized to the total volume per scaffold)	None	0 ng/mm ³	0 ng/mm ³	0 ng/mm ³
	Low	30 ng/mm ³	30 ng/mm ³	30 ng/mm ³
	Medium	120 ng/mm ³	120 ng/mm ³	120 ng/mm ³
	High	210 ng/mm ³	210 ng/mm ³	210 ng/mm ³
	None	0 ng	0 ng	0 ng
Theoretical BMP6 loading amount per scaffold	Low	810 ng	810 ng	810 ng
	Medium	3240 ng	3240 ng	3240 ng
	High	5670 ng	5670 ng	5670 ng

incubated overnight (approximal 16 h) to allow cell attachment before further *in vitro* and *in vivo* evaluation. The detailed information for all the experimental groups and sample preparation are listed in Table 3.

2.4. Lactate and glucose measurements

Part of the media from the cell-laden samples was collected to assess the lactate and glucose concentrations using two clinical diagnosis test strips; lactate Pro[™] test strip (ARKRAY, Europe B. V, NL) and Contour[®] blood glucose test strips (Bayer Consumer Care AG, CH), respectively, as described in our previous study [26]. Each sample was measured twice to minimize technical error. Blank medium was measured as control to calculate lactate production and glucose consumption. Thereafter, the ratio of lactate production to glucose consumption per cell was calculated to compare the metabolic activity of the cells upon seeding in different conditions.

2.5. Western blotting

Western blotting of the cell-laden samples was performed to assess the downstream pathway activation similar as our previous report [26]. Protein was extracted from cell-seeded scaffolds using Cell Extraction Buffer (Thermo Scientific) containing 0.3 M Phenylmethanesulphonyl fluoride and Protease Inhibition Cocktail (Sigma-Aldrich, Bornem, BE). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's instructions. 15 μ g of cell protein was loaded into and separated using a NuPAGE 4–12% Bis-Tris gel (Invitrogen). These proteins were subsequently electrotransferred to a Polyvinylidene fluoride membrane by semi-dry transfer (Invitrogen). The membranes were blocked with 5% BSA solution for 30 min followed by an overnight incubation with primary antibodies at 4 °C. The following primary antibodies were used: rabbit monoclonal phospho-SMAD1/5 (1:1000, Cell Signaling #9516), rabbit monoclonal SMAD1 (1:1000, Cell Signaling #6944), rabbit monoclonal phospho-Erk1/2 (1:1000, Cell Signaling #4377), rabbit monoclonal Erk1/2 (1:1000, Cell Signaling #4695) rabbit monoclonal phospho-p38 MAPK (1:1000, Cell Signaling #4511), rabbit monoclonal p38 MAPK (1:1000, Cell Signaling #8690), mouse monoclonal to GAPDH (1:1000, Cell Signaling #97166). The corresponding HRP-conjugated secondary antibodies were used at a

dilution of 1:2000 (Jackson, Pennsylvania, US) and images were developed by a LAS3000 Imaging System (FUJI) following the application of SuperSignal[®] West Femto reagent (Thermo Scientific, Illinois, US). Densitometry analysis, standardized to GAPDH as a control for protein loading, was carried out with ImageJ software (National Institutes of Health, USA). Triplicate samples were assessed for each condition (n = 3).

2.6. Subcutaneous implantation

In total, 24 NMRI-nu/nu mice (female, 8 weeks old) were used in the current study, and all procedures were performed according to the approved guidelines of the local ethical committee for Animal Research (KU Leuven). The obtained scaffolds as described in Section 2.3. were randomly implanted subcutaneously in the shoulder and the back area at the cervical region of animals. The remaining medium of the implanted scaffolds was collected and centrifuged. The pellet was then resuspended in RLT buffer (Qiagen, Venlo, The Netherlands) and stored at –80 °C for DNA quantification (Qubit system, Invitrogen, Belgium) to determine the cell seeding efficiency. After 2 weeks and 5 weeks of implantation, the mice were sacrificed and the implants were collected. Each explant was then fixed in 4% paraformaldehyde for 2 h and stored in PBS at 4 °C until further analyses.

2.7. Microfocus computed tomography and 3D image analysis

After 5 weeks of implantation, all explants were scanned using microCT. We applied a Phoenix NanoTom S (GE Measurement and Control Solutions, Germany) with a 180 kV/15 W high-performance microfocus X-ray tube. An operation voltage of 60 kV was used, along with a current of 162 μ A and a 0.3 mm filter of aluminum. We used an exposure time of 500 ms with a frame averaging of 1 and an image skip of 0 (i.e. fast scanning mode), and we acquired 2400 radiographs per sample, resulting in a scanning time of 20 min. The applied isotropic voxel size was 3 μ m.

As described in detail in previous work [21], prior to the 3D morphometric analysis, the explant was outlined automatically by a region of interest (ROI) using an in-house developed Matlab[™] tool (MorphingCity – ULg, Liege, Belgium). A three-level Otsu segmentation algorithm was used for segmenting bone from the CaP scaffold material (three classes of voxels i.e. background, bone and CaP). The binarized images were then denoised (closing

Table 3

Experimental design and group conditions.

	Group ^b	Evaluation	Investigation period	Analysis methods
Cell-laden samples ^a	ChronOs_c_none ChronOs_c_low ChronOs_c_medium ChronOs_c_high ReproBone_c_none ReproBone_c_low ReproBone_c_medium ReproBone_c_high CopiOs_c_none CopiOs_c_low CopiOs_c_medium CopiOs_c_high	<i>In vitro</i> & <i>in vivo</i>	<i>In vitro</i> : 16 h (n = 3) <i>In vivo</i> : 2 weeks (n = 3) & 5 weeks (n = 3)	<i>In vitro</i> : • lactate and glucose measurements • western blotting <i>In vivo</i> : • μ CT • (immuno)histology
Cell-free samples	ChronOs_low ChronOs_medium ChronOs_high ReproBone_low ReproBone_medium ReproBone_high CopiOs_low CopiOs_medium CopiOs_high	<i>In vivo</i>	<i>In vivo</i> : 2 weeks (n = 3) & 5 weeks (n = 3)	<i>In vivo</i> : • μ CT • (immuno)histology

^a For cell-laden samples, cell seeding density is 50,000/mm³, normalized to the open volume per scaffold as shown in Table 2.

^b The BMP6 loading amount ranging from none to high is normalized to the total volume per scaffold as listed in Table 2. The bare scaffolds without cell seeding or BMP6 loading, without ectopic bone formation capacity as shown from our previous studies [21,26], were not included in this study.

operation of 1, followed by despeckling of white and black speckles smaller than 200 voxels in 3D) and 3D morphometric analysis was done within the ROI using CTAn software (Bruker Micro-CT, Kontich, Belgium). We calculated the volume of bone in the total explant space as defined by the ROI as well as inside the scaffold only (defined by the borders of the scaffold material).

2.8. General histology & immunohistochemical staining

All explants were decalcified in an ethylene diamine tetra-acetic acid (EDTA)/PBS solution (pH 7.5) for 14 days and then embedded in paraffin. Sections (5 μ m thickness) were prepared using a microtome (Leica RM 2165, Leica Microsystems, Nussloch, Germany). The sections were stained with haematoxylin-eosin (HE) to assess the general histology. In addition, immunohistochemistry of human specific osteocalcin (hOCN) was performed to determine contribution of human donor cells to the new bone formation, as previously described [26]. In brief, the deparaffinized sections were quenched in 3% H₂O₂ followed by antigen retrieval in sodium citrate, 10 mM, pH 6, at 98 °C. Subsequently, sections were incubated with a primary anti-OCN guinea pig antibody at (1:1000, a generous gift from Dr. E. Van Herck, Legendo, KU Leuven) over night at 4 °C. A HRP-conjugated anti guinea pig secondary antibody was used at a dilution of 1:500 (Jackson) and 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) were used as a chromogenic substrate. Sections were photographed with a digital camera equipped microscope (IX83-P22F, Olympus).

2.9. Statistical analysis

Data are expressed as individual data points with bars representing the average value. Statistical analysis was performed using Graphpad Prism v.6 software. Analysis of variance (ANOVA) with a Tukey post hoc test was used for all multiple group experiments. Statistical significance is indicated on all graphs as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 and n = 3 if nothing else is indicated. Hierarchical clustering using Ward's method and principle component analysis (PCA) were performed in JMP[®] 14.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Cell metabolic activity

Glucose metabolism was investigated by measuring the ratio of lactate production to glucose consumption in conditioned media after 16 h *in vitro* culture (Fig. 1). Regardless of BMP6 dosage, the conversion rate of glucose to lactate almost reached two when cells were seeded in ChronOs[®], indicating the occurrence of anaerobic glycolysis. In contrast, the conversion of glucose to lactate significantly decreased when cells were seeded in ReproBone[™] and CopiOs[®]. Particularly, the conversion ratio of glucose to lactate dropped to one when cells were seeded in CopiOs[®], suggesting the occurrence of aerobic glycolysis (Fig. 1). The absolute concentrations of lactate production and glucose consumption per cell after 16 h *in vitro* culture are shown in Fig. S1.

3.2. Signal activation by western blotting

Next, protein lysates were prepared from cell-laden samples 16 h post seeding. Western blotting was performed to investigate canonical BMP-signaling (p-SMAD1/5) and non-canonical BMP-signaling (p-Erk & p-p38) downstream pathways (Fig. 2). Phosphorylation of SMAD1/5 was observed from three types of scaffolds laden with BMP6, however, cells seeded in scaffolds with medium or slow Ca²⁺ release rate (ChronOs[®] and ReproBone[™], respectively) showed higher phosphorylation of SMAD1/5 than the one with fast Ca²⁺ release rate (CopiOs[®]) when loaded with the same dosage of BMP6 (Fig. 2). Cells seeded in BMP6 loaded ChronOs[®] and ReproBone[™] tend to reached their peak of phosphorylation of p-SMAD1/5 (approx. two-fold) when loaded with a medium BMP6 dosage (Fig. 2). In comparison, cells seeded in BMP6 loaded CopiOs[®] showed a significant increase of p-SMAD only when BMP6 dosage exceeded medium dosage, and the phosphorylation of SMAD1/5 exhibited a linear tendency with the increase of BMP6 loading dosage (Fig. 2).

All the conditions showed an evident phosphorylation of Erk1/2 MAPK (Fig. 2), and cells seeded in CopiOs[®], regardless of BMP6 loading dosage, showed a significant higher phosphorylation of Erk1/2 than the ones seeded in ChronOs[®] and ReproBone[™].

Fig. 1. Lactate/glucose ratio from cell-laden samples after 16 h *in vitro* culture.

(Fig. 2). On the other hand, cells seeded in CopiOs® showed a significantly higher phosphorylation of p38 MAPK compared to the ones seeded on ChronOs® and ReproBone™. It is also noted that the phosphorylation of p38 tends to decrease when a high dosage of BMP6 was applied in the three types of scaffolds (Fig. 2).

3.3. Cell seeding efficiency for *in vivo* implants

Cell-laden constructs loaded with different doses of BMP6 were subcutaneously implanted to evaluate their ectopic bone formation capacity. The average cell seeding efficiency for ChronOs® and CopiOs® was $75.5 \pm 10\%$ and $82.1 \pm 4.95\%$, respectively, regardless of BMP6 loading dosage. (Fig. S2). It was noted that the average cell seeding efficiency for ReproBone™ decreased to $64.17 \pm 13.09\%$,

although there was no significant statistical difference compared to the other two types of scaffolds (Fig. S2).

3.4. Quantification of *in vivo* bone formation by microCT analysis

After 5 weeks subcutaneous implantation, hPDCs seeded on scaffolds without loading BMP6 hardly induced bone formation (Fig. 3). Instead, newly formed bone both inside and outside the scaffolds was only visible when BMP6 was applied (Fig. 3). With a low dosage of BMP6, evident bone formation was observed with hPDCs seeded in ChronOs® and ReproBone™ (Fig. 3). Yet, in CopiOs® only one of the triplicates showed a small amount of newly formed bone (Fig. 3). There was a clear trend in all three types of scaffolds that both the total volume of new bone and bone volume outside the scaffolds increased along with the increasing BMP6 dosage (Fig. 3). When a high dosage of BMP6 was applied, there was no statistical difference between scaffolds in terms of total volume of new bone formation (Fig. 3). Furthermore, the new bone formed inside the scaffolds was independent of BMP6 dosage but strongly influenced by the different types of scaffolds. Cells seeded in ChronOs® showed a relatively constant amount of newly formed bone inside the scaffolds ($\sim 1.5 \text{ mm}^3$), as compared to the other two conditions (Fig. 3). Cells seeded in ReproBone™ showed abundant new bone inside the scaffolds ($2.14 \pm 0.58 \text{ mm}^3$) when medium dosage of BMP6 was applied, while the low and high dosage of BMP6 led to a marginal amount ($\sim 0.68 \text{ mm}^3$) of new bone formed inside the scaffolds (Fig. 3). Particularly, for ChronOs® and ReproBone™ coated with low or medium dosage of BMP6, the bone formed inside the scaffolds was the major contributor to the total bone formation (Fig. 3). In

Fig. 2. Signal activation from cell-laden samples after 16 h *in vitro* culture. (a) Western blotting analysis and (b–d) densitometry of phosphorylation of SMAD, phosphorylation of Erk MAPK and phosphorylation of p38 MAPK. Asterisks above the bars indicate the comparisons between different BMP6 dosage and none BMP6 for each type of scaffold; Asterisks above the connected lines indicate the comparisons between different types of scaffolds with the same BMP6 dosage; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Fig. 3. MicroCT analysis of cell-laden explants after 5 weeks of subcutaneous implantation. (a) Representative cross-sectional microCT images through the middle of the explant. Bone formed outside and inside the scaffolds are labeled with green and red, respectively. Scale bars = 1 mm. (b) Total volume of new bone formation after 5 weeks; (c and d) Volume of newly formed bone located outside (c) and inside (d) the scaffolds. Asterisks above the bars indicate the comparisons between different BMP6 dosage and none BMP6 for each type of scaffold; Asterisks above the connected lines indicate the comparisons between different types of scaffolds with the same BMP6 dosage; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

contrast, in the case of CopiOs[®], the majority of new bone was formed outside the scaffold at both medium or high dosage of BMP6 (Fig. 3).

3.5. General histology

General histology of 5-week explants was assessed by HE staining. As shown in Fig. 4, hPDCs seeded in bare scaffolds without the addition of BMP6 only showed fibrous tissue infiltration within the explants, although osteoblast-like cells were observed in bare ChronOs[®] and ReproBone[™]. Bone formation was observed when hPDCs seeded in both ChronOs[®] and ReproBone[™] were combined with BMP6, which confirmed the microCT analysis results. Particularly, the newly formed bone osteoid (indicated as B) was in close

contact with the porous surface of scaffolds (indicated as S), which was consistent with those slightly basophilic stained osteoblast-like cells with spherical and large nucleus observed on ChronOs[®] and ReproBone[™] surfaces at 2 weeks (Fig. S3). Furthermore, when a high dosage of BMP6 was applied, blood vessel formation after 2 weeks (Fig. S3) and an abundant bone marrow formation after 5 weeks (Fig. 4) were observed in the three types of scaffolds seeded with hPDCs.

3.6. Immunohistochemical staining human specific osteocalcin

The contribution of the implanted cells to the newly formed bone was assessed by immunohistochemical staining of human specific osteocalcin (OCN) both after 2 weeks and 5 weeks of

Fig. 4. Representative images of HE staining from cell-laden constructs after 5 weeks subcutaneous implantation. S = scaffolds; B = newly formed bone. Scale bar = 50 μ m.

implantation. As shown in Fig. 5, positive human OCN staining, depicted by the brown stain in cell nuclei and newly formed bone matrix was mainly observed within the scaffolds, particularly on the surface of ChronOs[®] and ReproBone[™] combined with BMP6. The intensity of human OCN stain and staining area increased from 2 weeks to 5 weeks, suggesting the maturation of newly formed bone ossicles. Notably, hPDCs seeded in CopiOs[®] only showed a very faint positive staining of human OCN after 5 weeks within the scaffolds when high dosage of BMP6 was applied.

3.7. Multivariate statistical analysis of the empirical data

In order to decipher the combined effect of Ca^{2+} release rate and BMP6 dosage, we performed hierarchical clustering of the *in vitro* cellular response, Ca^{2+} release rate, and *in vivo* bone formation. This analysis displayed clustering of phosphorylation of SMAD with the bone volume inside the scaffolds (Fig. 6). Moreover, BMP6 dosage was closely clustered with total bone volume and bone volume outside the scaffolds (Fig. 6). Conversely, phosphorylation of p38 and ERK were closely clustered with Ca^{2+} release rate, which is directly related to different CaP properties (Fig. 6). Cluster analysis of different combinations of BMP6 dosage and scaffolds properties displayed clustering of cells seeding on ChronOs[®] and ReproBone[™]. A similar combined effect of BMP and Ca^{2+} was observed for these two types of cell-seeded scaffolds loaded with the different BMP6 dosages (Fig. 6). In contrast, the hPDCs seeding on CopiOs[®] with different BMP6 dosage were grouped together, and clearly separated from the other conditions (Fig. 6).

Principle component analysis (PCA) was performed to assess individual and combined effects of the different experimental conditions, as well as to retrieve a maximum amount of information from the entire dataset. The first principal component, which accounts for as much variation in the dataset as possible, is the linear combination of variables that has maximum variance among all linear combinations. The second principal component is the linear combination of variables that accounts for as much of the remaining variation as possible, with the constraint that the

correlation between the first and second component is 0. Upon analysis, different conditions and various biological readouts were separated by factors related to different BMP6 dosage and Ca^{2+} release rate. Particularly, Ca^{2+} release was strongly related with p38&Erk phosphorylation, whereas SMAD phosphorylation was highly correlated with bone volume inside the scaffolds (Fig. 6). Furthermore, Ca^{2+} release rate and SMAD phosphorylation were observed to have an inverse projection in component 1, indicating their intrinsic competing effect. On the contrary, in component 2, BMP6 dosage overrides the effect of Ca^{2+} release rate and appeared to be a dominant factor for total bone volume (Fig. 6).

4. Discussion

The aim of the current study was to comprehensively investigate the combined effect of BMP6 dosage and various types of clinical-grade calcium phosphate scaffolds (with different Ca^{2+} release rates) on the *in vivo* ectopic bone forming capacity of hPDC-seeded tissue engineered constructs. We hypothesized that the interaction between BMP6 and physicochemical properties of CaP scaffolds would affect *in vitro* cell behavior in terms of metabolic activity and signal pathway activation, hence influencing the *in vivo* skeletal tissue formation. For this purpose, cell-based constructs were prepared by seeding hPDCs into three different clinical-grades CaP-based scaffolds coated with three dosages of BMP6. The obtained constructs were analyzed for cell metabolic activity and signal activation assays, and then subsequently subcutaneously implanted in immune-deficient mice for 2 weeks and 5 weeks. Our main finding is that the interactions between CaP properties and BMP6 affect *in vitro* cell behavior and influencing the *in vivo* biological performance.

In this study, hPDCs showed different glycolysis pathways after 16hrs of seeding in the three types of clinical-grade CaP scaffolds, which have a clear difference in morphological structure as well and Ca^{2+} release rate. Specifically, ChronOs[®] and ReproBone[™] have a medium and slow Ca^{2+} release rate, and CopiOs[®] has a fast Ca^{2+} release rate, as both measured *in vitro* [27] and *in vivo* [21]. Cells

Fig. 5. Representative images of immunohistochemical staining of human specific osteocalcin (hOC) from cell-laden constructs after 2 & 5 weeks subcutaneous implantation. Scale bar = 50 μ m.

seeded in ChronOs[®] displayed an anaerobic glycolysis, where one glucose molecule generated almost two lactate molecules. In contrast, cells seeded in the other two types of scaffolds with higher porosity showed less lactate generation compared to the ones in ChronOs[®], indicating that an aerobic glycolysis (Warburg Effect) was predominant [28]. Anaerobic glycolysis is preferred by the differentiated cells when oxygen is limited, whereas the proliferating cells shift their metabolic machinery toward to aerobic glycolysis (Warburg Effect) with high levels of glucose uptake and lactate production [29]. In our previous study, ChronOs[®] was shown to have the lowest porosity (about 60%) among the three types of scaffolds [21]. The low porosity along with its medium Ca^{2+} release rate might result in a limited oxygen access within the ChronOs[®] constructs. In contrast, the high porosity (about 90%) along with the presence of Type I collagen matrix in CopiOs[®] might be favoring cell proliferation [30].

It is known that hPDCs express BMP Type I receptors (ALK3 & ALK6) and Type II receptors (BMPR2) [26], which are binding the BMP6 ligand [31]. Upon seeding into CaP scaffolds, hPDCs showed

an evident phosphorylation of p38 and ERK from all conditions regardless of BMP6 dosage and scaffold type. ERK and p38-activated MAPK pathways can be initiated by both non-canonical BMP pathways and Ca^{2+} signals [32,33]. Yet, our data showed that the phosphorylation of p38 and ERK was independent of BMP6 dosage, suggesting that both p38 and ERK-MAPK pathways were dominated by Ca^{2+} signals. Moreover, the chemical composition of CaP strongly affected the signal amplitude of p38 and ERK-MAPK. Cells seeded in CopiOs[®] (diphasic calcium phosphate) showed the highest phosphorylation of p38 and ERK above ChronOs[®] (β -TCP) and ReproBone[™] (mixture of β -TCP and hydroxyapatite). Diphasic calcium phosphate was shown to have a significantly faster Ca^{2+} release than β -TCP and hydroxyapatite [21], and we assume the faster Ca^{2+} release might result in a higher cumulative Ca^{2+} signaling to the hPDCs seeded in CopiOs[®], hence leading to a significantly greater activation of downstream p38 and ERK-MAPK pathways.

When BMP6 was present, hPDCs seeded in CaP scaffolds showed an evident phosphorylation of SMAD1/5/8, indicating the

Fig. 6. Multivariate statistical analysis of the empirical data. (a) Clustering of *in vitro* measurement from cell-laden conditions to *in vivo* bone formation. (b) PCA analysis display 2 components separating factors related to *in vivo* bone formation/BMP6 SMAD phosphorylation (component 1) and Ca^{2+} release/p38&Erk phosphorylation (Component 2).

canonical SMAD-dependent pathway activation [34]. Also, the SMAD1/5/8 signal amplitude gradually increased with the increments of BMP6 doses, which correlates with earlier experiments and modeling analyses of dynamics of TGF- β signaling [35]. When the same dosage of BMP6 was applied, the SMAD1/5/8 signal amplitude showed a tendency to decrease along with the increase of Ca^{2+} driven p38 and ERK phosphorylation, suggesting a cross-talk between canonical BMP pathway and Ca^{2+} signaling. BMP signaling pathways are strictly regulated by cross-talk and feedback-loops, leading to a controlled down-stream osteogenic process upon accurate pathway activation [36]. In consequence, the inappropriate Ca^{2+} signal activation may inhibit the activated canonical BMP signaling, leading to the decreased sensitivity to the low dosage of BMP6.

In this study, bone formation after 5 weeks of subcutaneous implantation was indeed observed with hPDCs seeded on BMP6 coated CaP scaffolds. Remarkably, in all three types of scaffolds, the total volume of newly formed bone increased along with the increasing BMP6 dosage. Such phenomenon suggests that BMP6 amplifies the stimulatory effect of CaP-scaffolds and acts as an important factor in driving bone formation, which corroborates with our previous study [26]. Furthermore, the increased BMP6 dosage resulted in an amplified volume of new bone outside the scaffolds, which is mainly formed by host cells. This might be caused by BMP6 diffusion from the scaffolds, which attracts host cells to proliferate and ossify at the peripheral site. In contrast, new bone formed inside the scaffold, which is primarily contributed by implanted cells evidenced by positive staining of human specific osteocalcin, is mainly dependent on material properties instead of BMP6 dosage. This suggests that the combined effect of BMP6 and CaP-stimulation of hPDCs resulted in new bone formation inside the scaffold following the intramembranous

pathway [37]. The bone formation within the scaffolds at early time points is important for clinical translation as such interconnected spongy bone structure is crucial to provides a stable structure for the fracture during the soft callus stage [38].

Considering the large and complex set of our empirical data, we applied multivariate statistical methods in order to gain further insight into the combined effects of multiple factors in the experimental design. Possibly, the BMP6-CaP scaffold interaction and the subsequent varied *in vivo* tissue formation could be caused by different downstream signaling activation. Interestingly, BMP6 dosage was clustered to total bone volume and bone volume outside scaffolds, while bone volume inside scaffolds and the elevated phosphorylation of SMAD1/5/8 were grouped together. This suggests the importance of appropriate activation of canonical BMP signaling pathway in hPDCs/BMP6/CaP constructs as the crucial driving force for cell differentiation and bone formation [39].

Due to their distinctively different chemical composition, the three types of CaP scaffolds in the current study were shown to have different Ca^{2+} release rate, namely CopiOs[®] > ChronOs[®] > ReproBone[™] [21]. As aforementioned, the cross-talk between canonical BMP pathway and Ca^{2+} signaling potentially influences the *in vivo* bone formation [18,26]. Therefore, the cluster analysis also allows us to understand the weight value of the CaP scaffold characteristics with regard to the *in vitro* cellular behavior and the *in vivo* outcome when combined with different dosages of BMP6. We show herein that when low or medium dosage of BMP6 was applied, cell-laden CopiOs[®] constructs were clustered to the cell-free CopiOs[®] scaffolds, distinctively separated from the ChronOs[®] and Reprobone[™]. This proposes that the fast Ca^{2+} release to be dominant over the low or medium dose of BMP6 stimulation on hPDCs, which is further supported by the PCA analysis. Concomitantly, cells seeding on these three types of scaffolds

combined with high dosage of BMP6 were clustered together, indicating the saturation of BMP6 is overriding the CaP scaffold material properties.

To conclude, in this study, we have developed hPDCs-based bone tissue engineering constructs by combining the stimulatory effects of BMP6 and CaP scaffolds. We have been able to decipher the individual and combined effect of BMP6 dosage and Ca²⁺ release rate on the *in vitro* and *in vivo* behavior of hPDCs/BMP6/CaP combination constructs. Cells seeded on CaP scaffolds with intermediate Ca²⁺ release rate combined with low or medium dosage of BMP6 demonstrated a robust new ectopic bone formation after 5 weeks contributed by both donor and host cells. This phenomenon might be due to the delicate balance between Ca²⁺ and BMP pathways, allowing an appropriate activation of the canonical BMP signaling pathway that is required for *in vivo* bone formation. Our findings illustrate the importance of matching BMP6 dosage and CaP properties in development of hPDCs/BMP6/CaP combination constructs. It also provides insights with regards to clinical translation of such cell-based constructs for bone regeneration.

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Conflict of interests

The authors declare that there is no conflict of interests.

Author contributions

W. Ji designed & planned the experiment, performed data collection & analysis, as well as manuscript preparation and editing. G. Kerckhofs designed & planned the experiment, performed data collection & analysis, as well as manuscript editing. C. Geeroms performed data analysis and manuscript editing. M. Marechal planned the experiment and performed manuscript editing. F.P. Luyten and L. Geris supervised the experiments, and assisted with the data interpretation and manuscript editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.actbio.2018.09.046>.

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