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Xenohybrid Bone Graft Containing Intrinsically Disordered Proteins Shows Enhanced In Vitro Bone Formation

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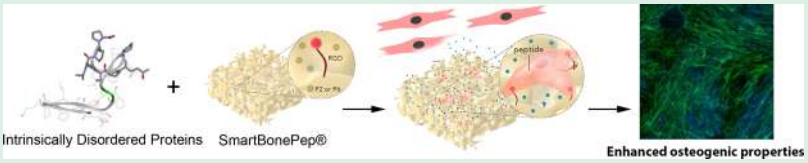

ABSTRACT: Bone defects are a significant health problem worldwide, as bone is the second-most transplanted tissue after blood. Although a myriad of bone grafts (BGs) have been used to treat bone repairs, none of them possesses all the desirable characteristics. An approach to improve BGs is to add bio-active components, however often difficult as BG production may disrupt the biological activities of such molecules. Here, we present a composite xenohybrid BG, SmartBonePep, with a type of biomolecule inspired by intrinsically disordered proteins (IDPs). These synthetic peptides (named P2 and P6) are physically entrapped into the polymer matrix of the composite BG. The effects of SmartBonePep on human osteoblasts were tested. Results showed that SmartBonePep enhanced proliferation and osteogenic effects. In order to verify the bioactivity of P2 and P6, these peptides were tested indirectly by being added to cell culture media too. Here, P2 or P6 exhibited promoting effects on osteogenic-related gene expressions. In this study, we showed highly effective osteoinductive synthetic peptides P2 or P6, which possess proline-rich and intrinsically disordered structural characters. This use of IDPs may provide promising bone enhancement biomolecules for clinical usage.

KEYWORDS: *bone graft, xenograft, intrinsically disordered, bone regeneration, osteogenesis, proline-rich*

1. INTRODUCTION

Challenging bone defect remains as a major health concern for public health.[1] Self-healing ability of the human body is limited in severe cases and biomaterials are thereafter introduced into this area.[2] Multiple categories of biomaterials were developed for bone regeneration in reconstructive surgeries such as oral, maxillofacial, craniofacial, orthopedics, and traumatology applications, where most of the biomaterials could be classified as degradable and nondegradable.[3] Although plenty non-degradable substitutes have shown great integration with the human body and proved as safe and effective implants, for example, titanium and titanium alloys, the demand for degradable biomaterials remains strong, as in some cases, the requirement of the surgery could not be fulfilled by these nondegradable bone substitutes.[4] The selection of implanted biomaterials is particularly relevant in bone defect of paediatrics because, for example, the cranial bone of young children still needs to undergo growth and development after surgical interference.[5] Synthetic materials are relatively safe implants from the perspective of immunogenicity, but most of them fail to mimic the real structure of human bone. Placement of bone graft (BG) is one of the better options for treatment of cranial bone defect during developmental stages.[6,7] Because autograft is quite limited, not to say almost impossible in pediatric cases, BG from other resources is useful for such clinical cases. It is a generally accepted paradigm that bone substitutes should resemble naturally occurring human cancellous bone as closely as possible, and a very commonly used source of bone matrixes is animal-derived bones, where bovine xenografts are mostly used in clinical practice, distantly followed by equine and porcine ones.[8−10] It is acknowledged that bovine-derived cancellous BGs are the closest xenograft to regenerate human bone and are safe products daily used in clinical practice, where bone regeneration is needed in reconstructive surgeries.[11,12] Although this type of BG provides proper mechanical support and a suitable platform for cell growth, and other factors are still needed for attachment and promoting the process,[2,13,14] some of which are met by xenohybrid composites.[15,16] Actually, bone healing is inherently linked to surface interactions and mineral deposition, and chemical reactions underlying development of self-replicating complex organic molecules could only develop at surfaces that templated and catalyzed topochemical reactions.[17]

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Table 1. Sequence and Characters of Selected NuPep

peptide	sequence (N terminus to C terminus)	polar amino acids	hydrophobic amino acids
P2	PLVPSQPLVPSQPLVPSQPQPPLPP	7 (S, Q)	18 (P, L, V)
P6	PHQPMQPQPPVHPMQPLPPQPPLPP	7 (H, Q)	18 (P, M, V, L)

Bio-active molecules have ascended as a promising alternative to assist in challenging bone regeneration. Growth factors and other biomolecules that regulate this complex physiology, for example, bone morphogenic proteins (BMPs), platelet-derived growth factor, and vascular endothelial growth factor, have been suggested to be used alone or in combination with a BG material for therapeutic use in bone regeneration.[18,19] Long-term effects of BMPs have been heavily debated in the current literature and thus not yet clearly identified; this also prevents BMPs from being FDA-approved for pediatric treatment.[20−22] This paves the road for use of smarter biomolecules.

This study uses a new and rapidly popular protein family called "intrinsically disordered proteins" (IDPs).[23] IDPs have been shown to be involved in biomineralization, particularly through signaling and regulation of the direction and extend of mineral crystal growth.[24] It possesses high-specificity/low-affinity interactions which play a crucial role and its unique structural feature enables the IDPs to participate in both one-to-many and many-to-one signaling.[23,25−27] A naturally occurring IDP that is currently used for regeneration of periodontal defects is amelogenin (AMEL).[28−30] AMEL is the main component in EMD/Emdogain: a large number of clinical studies endorse the clinical effect of Emdogain.[31−35] Inspired by the idea of IDPs, artificial peptides based on the common characteristics of several proline-rich "disorganized" regions present in hard tissue extracellular matrix proteins have been developed.[36,37] These peptides (also named P1−P6) have been shown to improve cell viability and growth, stimulate pre-osteoblast differentiation, induce osteogenic gene expression in progenitor cells, and were fully biocompatible even in very high concentrations,[36,38,39] suggesting potential applications in regenerative medicine for bone and dentine formation and biomineralization.

The purpose of the current study was to investigate the effect of short proline-rich IDPs (here, the most promisingly investigated are P2 and P6) on human osteoblasts and investigate its potential for use in a new composite bone substitute for the regeneration of bone defects in and losses in pediatric and oncological applications. Although the peptide sequence P6 used in this study is identical to part of the AMEL sequence, P2 is a consensus peptide based on the common characteristics of the proline-rich regions in hard tissue extracellular matrix proteins.[40−42] Two different in vitro cell-testing methodologies were introduced: (1) direct exposure of P2 or P6 on human osteoblasts and (2) physical entrapment of P2 or P6 inside SmartBone, a commercially available composite xenograft−synthetic bone substitute developed for bone regeneration in reconstructive surgeries.[15,16,43] Given intrinsic subject variability, three different donors of human osteoblasts were used and examined using various assays such as lactate dehydrogenase (LDH), real-time polymerase chain reaction (PCR), multiplex protein quantification, and confocal analysis.

2. MATERIALS AND METHODS

2.1. Direct Effect of Biomimetic Synthetic Peptides on Osteoblasts. The sequences of the biomimetic synthetic peptides P2 and P6 (designed by Corticalis AS, Oslo, Norway, and supplied by Pepmic Co., Ltd., Jiangsu, China) are available in Table 1, as previously described.[36] Both peptides were prepared as stock solutions at 4 mM in 0.1% acetic acid and stored at −20 °C. The commercially available enamel matrix derivative (Emdogain, EMD, Straumann AG, Basel, Switzerland) was used as the positive control for testing against the synthetic biomimetic peptides. EMD was prepared as a stock solution at 10 mg/mL in 0.1% acetic acid and stored at −20 °C. The concentration of 2.5 μM was used for both EMD and P2 and P6.

2.1.1. Cell Culturing, Expansion, and Seeding. Commercially available normal human osteoblasts (Lonza, Germany) were utilized for cell experiments. Cells from three different donors were tested. Detailed information is shown in Table 2. Cells were cultured in osteoblast growth medium (Promocell, Germany) in a standard cell-culturing environment as 37 °C humidified atmospheres with 5% CO_2. The medium was changed every 3 days, and cells between passage 5 and 7 were used for experiments. Bone cells were seeded in a 24-well plate with a density of 5000 cells/cm^2, followed by the addition of EMD, P2, and P6 (Figure 1). These peptides were added to the culture media during every media change (twice per week).

Table 2. Detailed Information of the Donors

donor number	gender	age	position
1	male	32	distal femur
2	male	13	femur bone end
3	female	20	ulna/radius

2.1.2. Cytotoxicity. LDH activity in the culture media was used to indicate the cytotoxicity. All the measurements were performed according to the manufacturer's kit instructions (Roche Diagnostics, Mannheim, Germany). LDH activity was determined spectrophotometrically after 30 min of incubation at 25 °C of 50 μL of the culture and 50 μL of the reaction mixture by measuring the oxidation of nicotinamide adenine dinucleotide at 490 nm in the presence of pyruvate. Results were presented normalized to the control. Cells cultured in growing media and treated with phosphate-buffered saline (PBS) were set as the negative control, while cells treated with 1% Triton X-100 were set as the positive control.

2.1.3. Determination of Alkaline Phosphatase Activity. Alkaline phosphatase (*ALP*) activity was determined from cells after 21 days of culturing. Briefly, on day 21, cells were washed twice with PBS and solubilized with 0.1% Triton X-100. Then, cell lysates were incubated with an assay mixture of *p*-nitrophenyl phosphate (pNPP). Cleavage of pNPP (Sigma, Saint Louis, Missouri, USA) in a soluble yellow end product which has absorbance at 405 nm was used to assess *ALP* activity. In parallel, a standard curve with calf intestinal alkaline phosphatase (CIAP) (Promega, Madison, USA) was constructed; 1 μL from the stock CIAP was mixed with 5 mL of ALP buffer (1:5000 dilution) and subsequently diluted 1:5.

2.1.4. Chamber Migration Assay. For the chamber migration assay, cells were seeded in dual-chamber culture inserts (ibidi, Munich, Germany) and allowed to grow for one day. Then, the inserts were removed, creating a wound, and the cells were stimulated with the different peptides. Pictures were taken after 1 and 24 h and analyzed with the manufacturer's software to calculate wound closure.

2.1.5. Mineralization. The mineralization effects of P2 and P6 was tested using OsteoImage Mineralization Assay (Lonza, Cologne,

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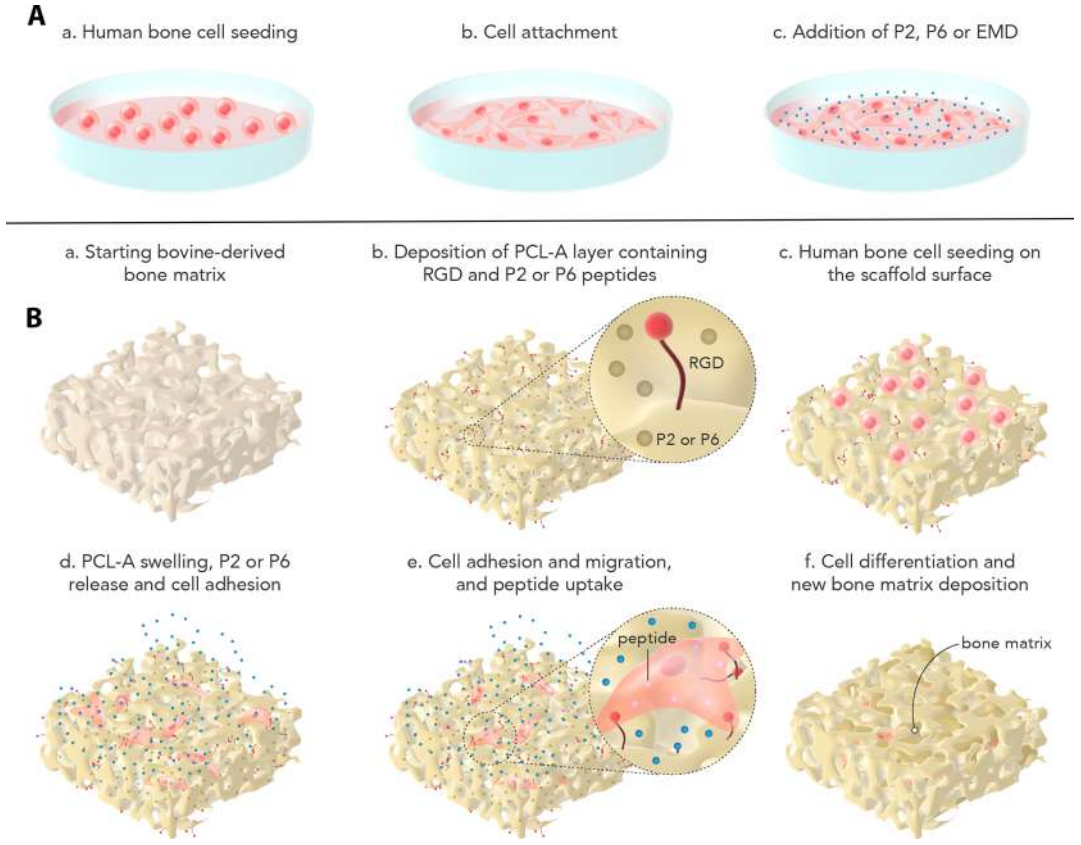

Figure 1. Graphical overview of the experimental part. (A) Top part: the direct method where EMD, P2, and P6 were added to cell culture media. (B) Bottom part: physical entrapment of P2 and P6 in a polymer matrix and osteoblasts seeded onto the BG.

Table 3. Sequences of q-PCR Primers

genes	forward primer	reverse primer
OPN	GCCGAGGTGATAGTGTGGTT	TGAGGTGATGTCCTCGTCTG
OC	GAAGCCCAGCGGTGCA	CACTACCTCGCTGCCCTCC
COL1A1	CCTGACGCACGGCCAAGAGG	GGCAGGGCTCGGGTTTCCAC
ALP	CCAAGGACGCTGGGAAATCT	TATGCATGAGCTGGTAGGCG
VEGFA	TCTTCAAGCCATCCTGTGTG	ATCTGCATGGTGATGTTGGA
GADPH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
BACT	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAA

Germany) following the manufacturer's protocol. Cells treated with P2, P6, and EMD were stained with the OsteoImage Assay after 21 days of culturing and read on a plate reader.

2.1.6. Quantification of Gene Expression Levels. Total RNA was isolated using a Dynabeads mRNA DIRECT purification kit (Thermo Fisher Scientific, Oslo, Norway) according to the manufacturer's protocol. Total RNA was quantified at 260 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The same amount of RNA (350 ng) was reverse-transcribed to cDNA using a high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), according to the protocol of the supplier. Aliquots of each cDNA were frozen (−20 °C) until the PCR reactions were carried out. Real-time PCR was performed in a CFX384 Touch real-time PCR system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Real-time PCR was performed for target genes osteopontin (*OPN*), osteocalcin (*OC*), collagen1A1 (*COL1A1*), alkaline phosphatase (*ALP*), and vascular endothelial growth factor A (*VEGFA*) and for reference genes glyceraldehyde phosphate dehydrogenase (*GADPH*) and β-actin (*BACT*) (Table 3). The amplification program was set according to the manufacturer's recommendation, which included 2 min of initial denaturation and enzyme activation at 95 °C, 15 s of denaturation at 95 °C, 30 s of annealing at 55 °C, and 30 s of extension at 72 °C. A total of 40 cycles were performed.

2.2. Physical Entrapment of the Biomimetic Synthetic Peptides in a BG. *2.2.1. BG Preparations.* The xenohybrid BG SmartBone (SBN) is manufactured by I.B.I. SA (Industrie Biomediche Insubri SA, Mezzovico-Vira, Ticino, Switzerland) and it consists of a bovine bone-derived mineral matrix which is improved by reinforcement with the copolymer coating poly(L-lactide-*co*-ε-caprolactone) and the addition of RGD-exposing collagen fragments from animal-derived gelatine.[43] During the standard manufacturing process of SBN, the biomimetic peptides P2 and P6 (Table 1) were embedded into the polymer coating of SBN, producing SmartBone-Pep (SBP), as described by Perale et al.,[44] with a nominal concentration capable of providing a release rate equivalent to 1 μg/cc per day over a two weeks' time (Figure 1). These two novel BGs were called SBP2 and SBP6, respectively. A third group was also tested which had both P2 and P6 added together, this was called SBP2 + P6. The release profiles, cytotoxicity, scanning electron microscopy results, and mechanical strength of both SBN and SBP are already described by Perale et al.[45] and thus not repeated here.

2.2.2. Bone Cell Seeding on BGs with Physically Entrapped Peptides. The same bone cell donors as those used in Section 2.2

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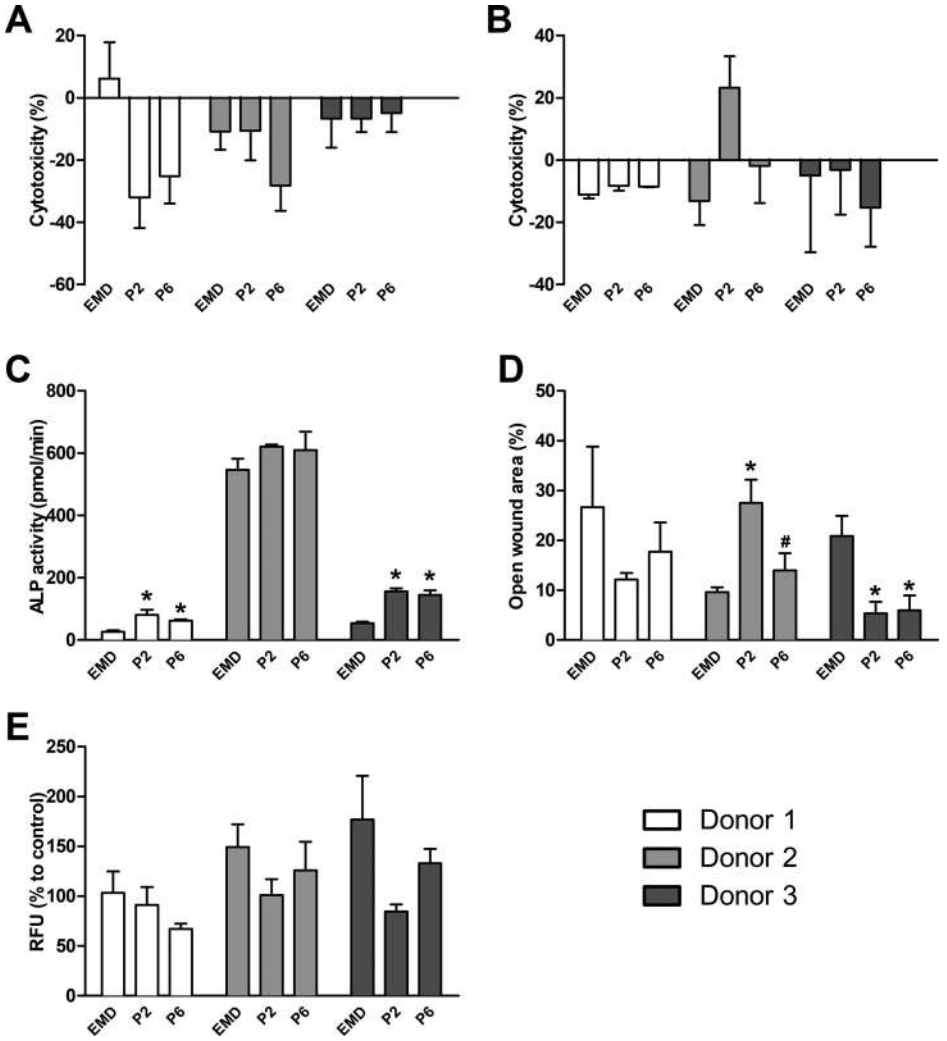

Figure 2. (A,B) Cytotoxicity of NuPep analyzed via LDH activity on days 1 and 3. (C) *ALP* activity. (D) Wound healing test. (E) Mineralization. All the experiments were run with three different donors.

(normal human osteoblasts, Lonza, Germany) were used in direct seeding onto the BGs SBN, SBP2, SBP6, and SBP2 + 6. These BGs were placed in a 24-well plate and a bone cell suspension of 8×10^4 cells was seeded on the surface of each BG (Figure 1). The medium was changed on days 2, 5, and 7 of every week, and cells were cultured until day 28. On days 2, 7, 14, 21, and 28, medium was collected for protein quantification.

2.2.3. Cytotoxicity. LDH activity on bone cells attached to different BG materials was used to indicate the cytotoxicity as described in Section 2.2.2. Cells cultured in growing media and treated with PBS were set as the negative control, while cells treated with 1% Triton X-100 were set as the positive control.

2.2.4. Micro-Computed Tomography. Samples from all BGs types (SBN, SBP2, SBP6, and SBP2 + 6) were scanned using micro-computed tomography (CT) (Bruker microCT 1172, Kontich, Belgium) with the scanning parameters set at 104 μA, 95 kV, image rotation of 0.400°, and a resolution of 7.9 μm with a aluminum copper filter. Three-dimensional models were reconstructed in the software CTVox (Bruker, Kontich, Belgium) and snapshots were obtained therefrom. The structure parameters were calculated using the software CT Analysis (Bruker, Kontich, Belgium).

2.2.5. Confocal Microscopy. Confocal microscopy was performed for the BGs on days 2, 7, 14, and 28 to observe the cell attachment. BGs were first washed with PBS three times and then fixed with 4% paraformaldehyde for 12 min. After washing with PBS, BGs were stained with Alexa568-labeled phalloidin and 4′,6-diamidino-2-phenylindole. Images were taken using laser scanning confocal microscopy (Leica TCS SPE Microsystems Wetzlar GmbH, Wetzlar, Germany).

2.2.6. Quantification of Specific Extracellular Proteins. On days 2, 7, 14, 21, and 28, medium was collected for each group. Medium samples were stored in a −80 °C freezer until use. Multi-analyte profiling of protein levels in the culture medium was performed on the Luminex 200 system (Luminex, Austin, TX, USA), employing xMAP technology. Bone Metabolism Multiplex Assay was used (Human Bone Magnetic Bead Panel, MILLIPLEX, Germany). Acquired fluorescence data were analyzed using xPONENT 3.1 software (Luminex, Austin, TX, USA). The amount of osteocalcin (*OC*), osteopontin (*OPN*), osteoprotegerin (OPG), dickkopf-related protein 1 (DKK-1), and sclerostin (SOST) in the culture medium of different time points was selected and analyzed. All processes were performed, according to the manufacturer's protocols.

2.3. Statistical Analysis. Datasets were run for normality test first. Normally distributed results therefore were expressed as means ± standard deviation. One-way ANOVA and Tukey's tests were utilized for multiple comparisons among groups, while two-way ANOVA and Bonferroni post-tests were applied when different time points were included. Statistical analysis was run in SPSS12 (IBM SPSS. Armonk, NY 10540, U.S.A), and significant differences were considered at $p < 0.05$.

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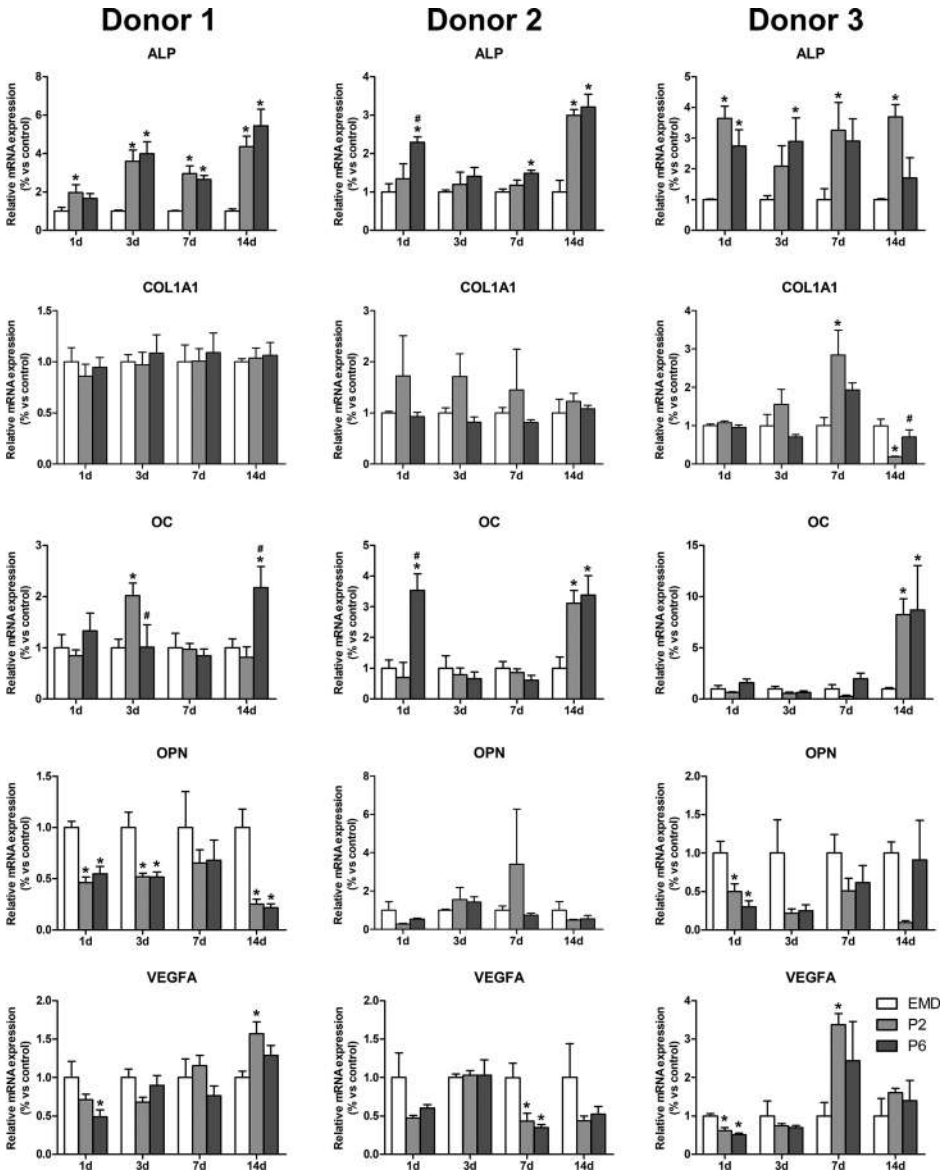

Figure 3. Osteogenic-related gene expression of human osteoblasts after treatment of EMD, P2, and P6 analyzed with qPCR.

3. RESULTS

3.1. Direct Effects of P2 or P6 on Human Osteoblasts in Culture. *3.1.1. Cytotoxicity.* No cytotoxicity of the peptides was detected for all the donors at different time points and no significant difference was found by comparing different groups at the same time point (Figure 2A,B).

3.1.2. ALP Activity. *ALP* activity was tested with all donors after 21 days. Cells treated with EMD showed significantly lower *ALP* activity compared to P2 and P6, specifically in donor 1 and donor 3. No other significant differences were found (Figure 2C).

3.1.3. Wound Healing. Significantly decreased area of wound was observed by comparing P2 and P6 to EMD donor 3, while in donor 2, cells treated with P2 exhibited the largest wound area. In donor 1, no difference was observed. The proliferation effect of P2 and P6 displayed strong individual variance (Figure 2D).

3.1.4. Mineralization. No difference of RFU was observed when comparing P2 and P6 to EMD in all the donors. The mineralization effects were not changed in osteoblasts when stimulated with P2 or P6 (Figure 2E).

3.1.5. Quantification of mRNA Levels. Five genes were selected for mRNA expression quantification (Figure 3). For *ALP*, cells treated with EMD generally exhibited lower expression, while cells treated with P2 or P6 were generally highly expressed. Significant differences were found by comparing P2 and P6 to EMD at all time points in donor 1, except the first day. In donor 2, *ALP* expression in P6 was significantly higher than EMD on days 1, 7, and 14, while P2 was only statistically higher on day 14. In donor 3, cells treated with P2 and P6 were generally highly expressed but a significant difference was not shown on day 3 in P2 and on day 7 and 14 in P6.

For *COL1A1*, cells treated with P2 exhibited higher expressions in donor 2 but a significant difference was not observed. In donor 3, the expression of *COL1A1* was higher in P2 than EMD on day 7. No other difference could be observed.

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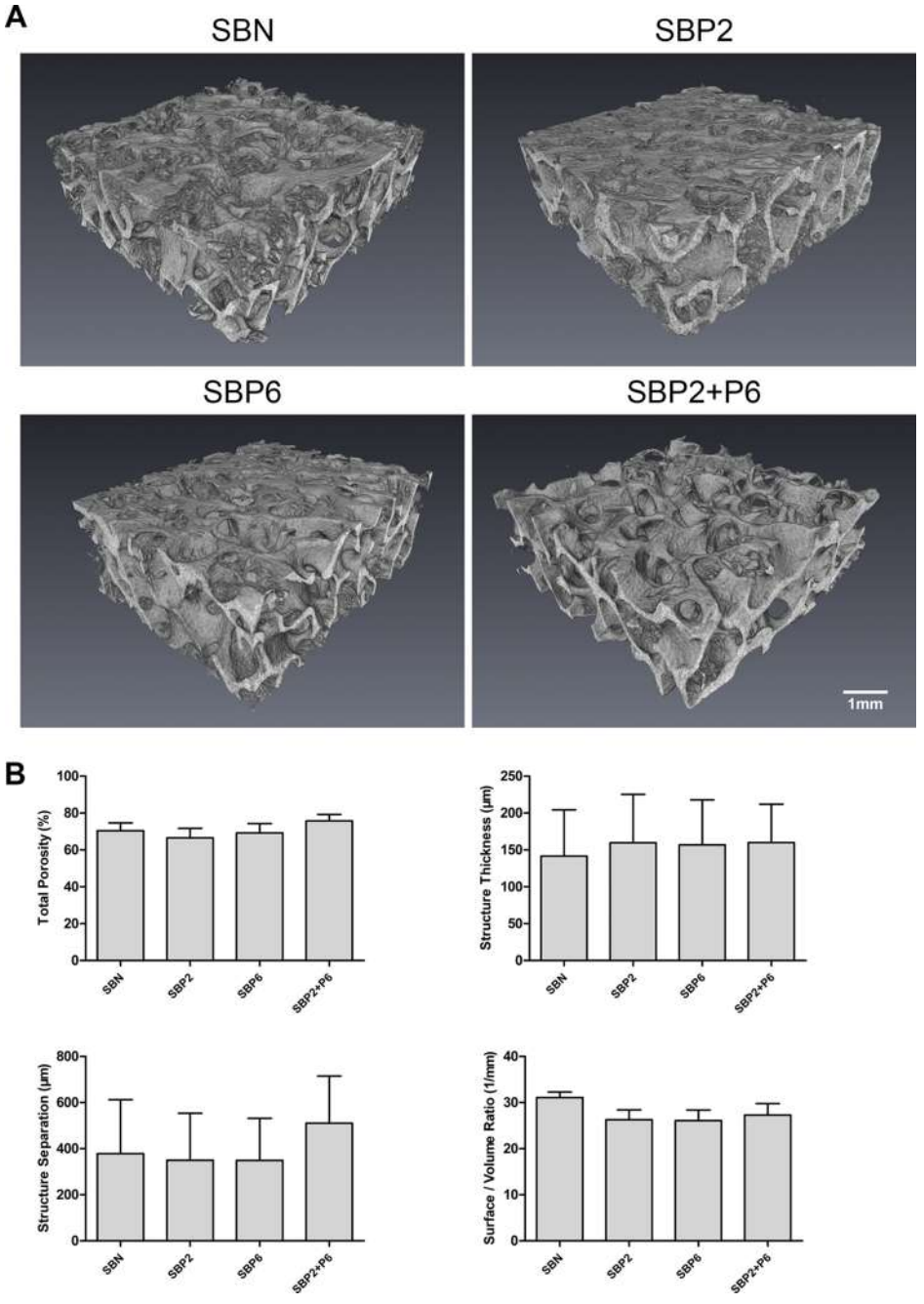

Figure 4. Micro-CT results of SBN and SBP. (A) 3D images of SBN and SBP displaying similar pore morphology and (B) calculated morphology parameters of SBN and SBP showing no significance differences.

For *OC*, P2 showed the highest expression on day 3, while P6 exhibited the highest expression on day 14 in donor 1. However, in donor 2, expression of *OC* in P6 was the highest on days 1 and 14. Significant differences could be found on the 14th day when compared P2 and P6 to EMD in donor 3.

For *OPN*, EMD generally showed higher expression at all different time points except day 7 in donor 1. This phenomenon was also observed in donor 3 on day 1.

For *VEGFA*, expression was lower in P6 on the first day while higher in P2 on the 14th day compared to EMD in donor 1. In donor 2, a significant difference was observed on day 7 in P2 and P6. In donor 3, P2 and P6 exhibited low expression on day 1 but high expression on day 7.

3.2. Physical Entrapment of P2 or P6 in SBN BG.

3.2.1. Cytotoxicity. No cytotoxicity was observed in all groups with three different donors. Results that are preferable were shown in SBP2 compared to other groups in all donors (Figure S1).

3.2.2. Micro-Computed Tomography. Micro-CT results demonstrated that there was no structural difference before and after SmartBone was added to P2 or P6, including total porosity, structural thickness, structural separation, and surface/volume ratio. The pores were accessible through each other, and no closed pores were detected (Figure 4).

3.2.3. Confocal Microscopy. In the initial stage, enhanced cell attachment could be observed in SBP2 + P6 on day 2 in all donors, while cells on SBN remained round-shaped. Cells on

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SBP2 and SBP6 also exhibited better attachment compared to SBN, in which the cells on the P6 showed more extension. On day 7, more cells could be observed on the BGs treated with P2 or P6. Strong proliferation effects were observed on day 14 in P2 compared to P6 and multilayers of cells proliferated could be observed on day 28. The three donors exhibited similar trends but were different in temporal aspect. For donor 1, generally, the attachment, spreading, and proliferation phenomena were poorer and slower compared to donor 2 and donor 3 (Figure 5).

3.2.4. Quantification of Specific Extracellular Proteins. Five specific extracellular proteins were selected for evaluating the osteogenic properties of the BGs. Generally, the three donors exhibited the similar trends along the time line (Figure 6). The donor variance was more significant concerning the secretion of *OC* where donor 3 gave a more fluctuant expression pattern. After normalizing the results to the control, detailed statistic results were elucidated as follows (Figure S2).

For *OC*, generally, not much difference was observed among the four groups at different time points. SBP6 exhibited higher expression on day 14 in donor 3.

For OPG, SBP2, SBP6, and SBP2 + P6 were significantly higher than SBN on days 7, 14, and 21 in donor 1, while in donor 2, significant higher expression was observed in SBP2 on day 14 in SBP2 and SBP2 + P6. In donor 3, SBP2 exhibited high expression on day 14 and 21, while SBP2 + P6 exhibited high expression on days 21 and 28.

For *OPN*, generally, the expression was promoted in SBP. After 21 days, SBP6 was significantly different from SBN in donor 1, while protein concentration in SBP2 and SBP2 + P6 was significantly higher than SBN in donor 2 on days 14 and 21. For donor 3, significant differences were found when comparing SBP2 with SBN after day 14.

For DKK-1, high expression could be found in SBP2 and SBP6 on day 7 in donor 1, while no other differences could be observed at other time points in different donors.

For SOST, high expression could be found in SBP2 + P6 on the time points of day 2 and day 7 in donor 1. Besides, in donor 2, SBP6 also displayed high secretion on day 2. No other differences were found among the groups and different time points.

4. DISCUSSION

In this study, the effect of biomimetic synthetic peptides was assessed on human osteoblasts with a direct (addition of peptides to cell culture media) and indirect method (physical entrapment into a polymer matrix coating a bovine-derived bone scaffold). Human osteoblast is one of the most representative cells that could simulate bone behaviors in vitro.[46,47] In order to make the results more representative, human bone cells from three different donors were included, which differ in several aspects such as gender, age, and site of donation.[48]

The peptides used in this study belong to a family of proteins called IDPs. The IDPs are a newly discovered class of proteins, which have no definite three-dimensional structure under natural conditions.[25] This special category of protein possesses unique advantages in combination, spatial transformation, and coordination. IDPs participate in plenty of biological activities and are regarded as "one-to-many signaling" proteins. In particular, biomineralization is closely associated with IDPs, where almost all proteins closely related to hydroxyapatite (HAp) crystal formation, growth control,

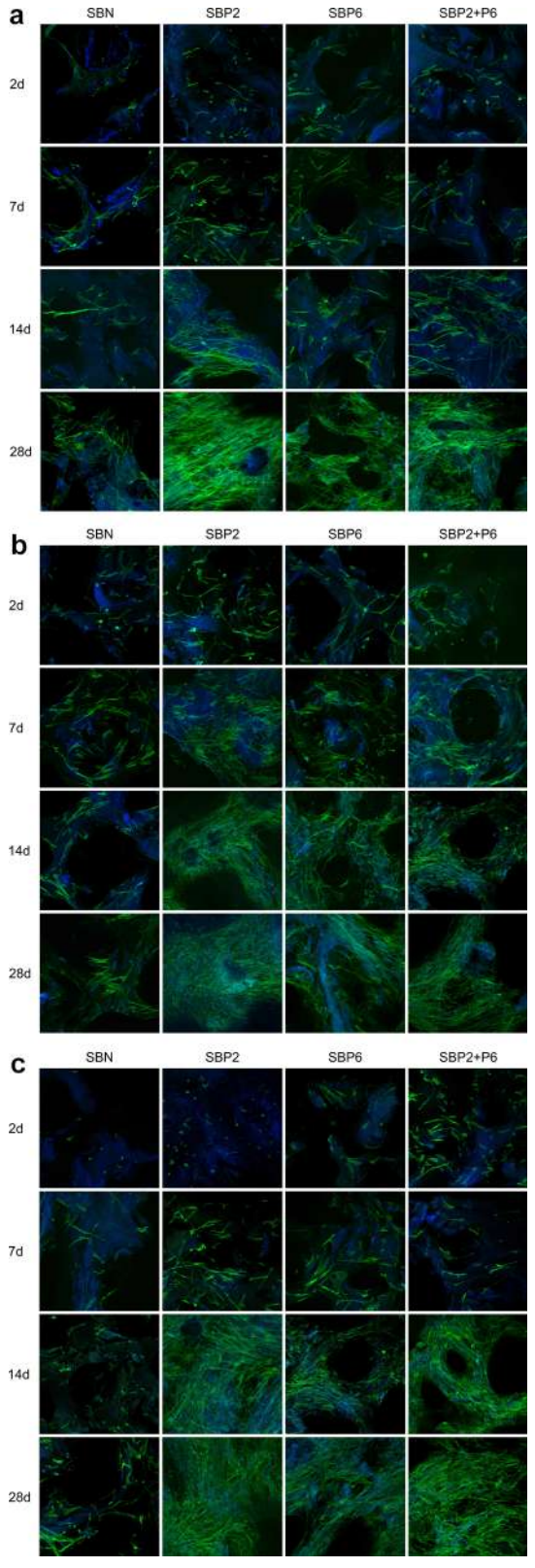

Figure 5. (a) Cell behavior of osteoblasts from donor 1 observed under laser scanning confocal microscopy. (b) Cell behavior of osteoblasts from donor 2 observed under laser scanning confocal microscopy. (c) Cell behavior of osteoblasts from donor 3 observed under laser scanning confocal microscopy.

and orientation are mostly or fully disordered.[23] P2 and P6 were designed based on the common characteristics of the

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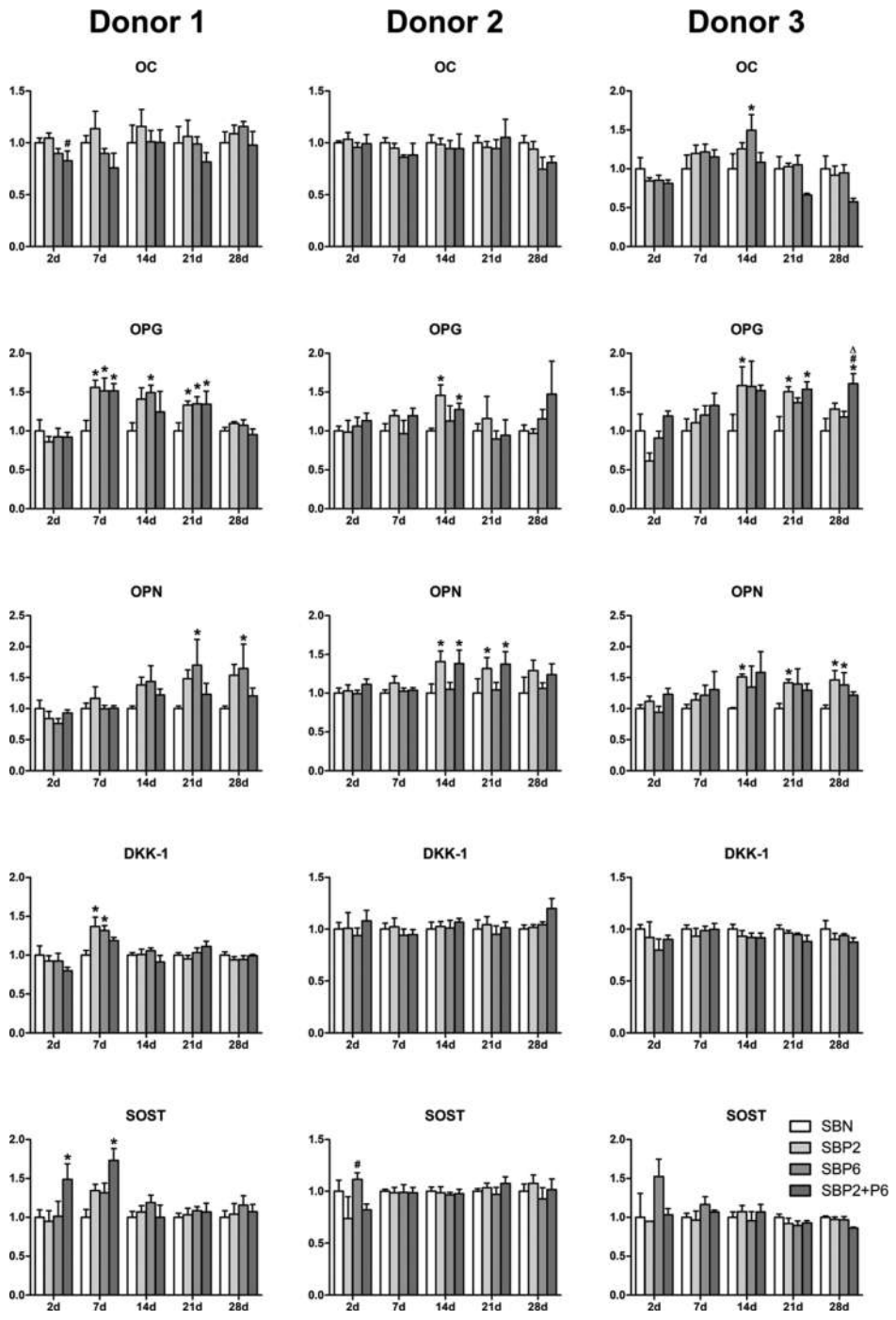

Figure 6. Quantification results of specific protein secretion. Results were shown normalized to SBN at different time points.

proline-rich region in hard tissue extracellular matrix proteins AMEL and ameloblastin, which did not provide any secondary structure elements because of the rich content of prolines, thus exhibiting disorganized characters. Considering this similar structure to human proteins, both P2 and P6 did not show any cytotoxic effects on the osteoblasts after 1 and 3 days of culture. The osteogenic activity of both P2 and P6 was affirmed in our previous in vitro experiments;[36,44] however, these studies were done on cell lines. In this study, primary bone cells treated with P2 or P6 also exhibited preferable biological reactions. From the results of *ALP* activity, P2 and P6 generally were better than EMD. EMD is the active protein in the product Emdogain which is currently available on the market by Straumann AG and has been proved to regenerate bone tissues around teeth.[49−51] The most abundant protein in EMD is AMEL, which has been shown to be an IDP.[52−54] As to the selected peptides, P2 was designed as the consensus peptide based on the common characteristics of the proline-rich regions in hard tissue extracellular matrix proteins, while P6 was the analogous peptide to human AMEL. These two peptides promoted *ALP* activity in all the three donors, which was also proved in qPCR results, a hallmark of functional osteoblasts. In addition, the gene expression of *OC*, another characteristic osteoblast marker, was also upregulated with P2 and P6. For the wound closure test using a chamber migration assay, the influence of donor variability was demonstrated,

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where P2 promoted migration in donor 3 but suppressed this in donor 1 and 2. Nevertheless, the gene expression of *OPN*, which has been related to immune modulation and wound healing,[55] showed a similar pattern for the three donors, thus indicating that further studies are needed to confirm these findings. All these results indicate that both P2 and P6 are bio-active and that can maintain and promote the osteoblast phenotype more efficiently than EMD. Considering this, EMD was not included in the further studies.

Based on the results of the current study, the idea of the BG substitute introduced in this study was to combine the commercially available polymer-reinforced xenograft SmartBone with a bio-active and biomimetic peptide which incorporates the functions of IDPs. The demand of advanced BG that possesses a higher degree of bioactivity and biomimetic properties is increasing nowadays among the clinicians not only for the normal patient but also for the more challenging cases.[56,57] Although multiple recombinant growth factors were loaded on BGs and implants for the purpose of promoting osteogenic differentiation or enhancing osseointegration, the high cost of recombinant proteins is unneglectable, where clinically, the dose of usage is lacking enough evidence of safety and efficacy. However, the synthetic peptides are cost-effective products. As small molecules with lower molecular weight, more active motifs could be loaded to the material at the same weight. Additionally, recombinant proteins still exhibited the potential risk of immunoreactions,[58,59] while synthetic peptides are small molecules, thus the aforementioned risk is relatively reduced. The chemical and biological properties could also be altered via modifying the sequence, which makes synthetic peptides easy to manipulate. Indeed, in some cases, recombinant growth factor-loaded biomaterials did not show preferable outcome compared to autograft transplantation, for example, osteogenic protein-1 (OP-1), which was applied in spine surgery.[60]

As a polymer-reinforced xenograft, SBN was proved to be a good choice for substituting autograft and allograft. Previous studies had shown the high porosity, interconnectivity, and surface area-to-volume ratio properties of the SBN BG substitute. It was demonstrated that the SBN BG substitutes outperformed other commercial BG substitutes with respect to interconnectivity, particularly, when the minimum connection size increased and possessing a large extent of the open pore structure.[61−65] As shown earlier,[44] the addition of P2 or P6 or both did not alter the microstructure of the porous structure of the BG, still mimicking bone. The intended use of this new composite bone substitute is for the regeneration of bone defects and losses in pediatric skull defects, including oncological ones.

The direct method studied the functions of the IDPs, while osteoblasts are in direct contact with entrapped P2 and P6 and thus more resembles a clinical situation where cells would grow into the porous material and get direct exposure to the IDPs. Nevertheless, the direct method is a static method and an in vivo situation would have flow through the porous material. Thus, one would also expect that some cells, particularly, in proximity of the graft material would be indirectly exposed to P2 and P6. The indirect method somehow resembles such a scenario. Therefore, we utilize both direct and indirect methods for testing the effect of P2 and P6.

The entrapment of the synthetic biomimetic peptides P2 and P6 promoted cell attachment to the BG and increased cell proliferation. This was confirmed in confocal microscopy where SBP showed higher proliferation properties compared to SBN at all different time points. Especially, for SBP2, strong proliferation effects could be observed at the early time points when compared to SBP6. Although different cell behaviors were observed at the same time point comparing different donors, all the three donors exhibited similar trends during the whole timeframe among all the groups. One reason for this behavior could be the multifunctional properties of both P2 and P6, which has also been seen by other types of IDPs.[66−68] The cells' origin used for the study, that is, different bones, might also play a role, influencing their characteristics in cell culture.

To test whether SBP showed better osteogenic properties compared to SBN, Luminex analysis was performed using a bone panel containing several bone markers. *OC* and *OPN* are recognized as important osteogenesis markers. During the whole experiment of 28 days, a significant difference was barely observed in *OC*, indicating that this marker was not influenced with the addition of P2 or P6 in SBN. However, the expression line of *OPN* in SBP was generally above SBN, which indicated that the process of osteogenesis was promoted. Probably, the controlled release of the peptides from SBP is different from the media changes, which changes the expression of bone markers. One must also bear in mind that human osteoblasts will behave different in 2D and 3D cultures, and that, the cells grown in 3D culture are more likely to retain their expressed proteins.[69,70]

It is interesting to notice that in donor 2, SBP2 and SBP2 + P6 gave the similar effects. An explanation could be that P2 contributed more stimulating effects because *OPN* secretion in SBP6 did not show a significant change. Meanwhile, as a protecting marker, OPG was also higher expressed in SBP, which further confirmed the effects. It was intriguing to notice that donor 1 showed preferable results in all SBPs in OPG, while donor 2 and donor 3 exhibited better results in SBP2 and SBP2 + P6 for both OPG and *OPN*, where SBP6 did not give satisfying outcomes, indicating that P2 possibly mainly contributed to the effects on these 2 donors. As to DKK-1 and SOST, which are involved in the downregulating of osteogenesis, few differences were detected among the groups, showing that there was no suppressing effect on these two inhibitors. It is evident that the addition of P2 and P6 into SmartBone had multistimulating effect on the human osteoclasts for cell attachment, proliferation, and differentiation. Future work will include in vitro testing with stem cells and appropriate animal models to verify the current findings.

5. CONCLUSIONS

Two synthetic candidate biomolecules peptides, P2 and P6, were successfully proven to have multimodal biological effects on primary human osteoblasts, both for 2D and 3D cultures. The successful physical entrapment of P2 into a composite xenohybrid BG (SmartBone) increased the biomineralization and cell attachment and proliferation. It has been shown that P2 and P6 are vigorous and bio-active biomolecules and may be an improved in vivo bone formation-enhancing candidate for clinical successful next-generation BGs. Thus, these synthetic IDPs have shown in vitro to be promising supplements to enhance performance of the BG.

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■ ASSOCIATED CONTENT

Supporting Information

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Cytotoxicity of SBP and quantification results of specific protein secretion (PDF)

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Notes

The authors declare the following competing financial interest(s): Lyngstadaas is inventor of patent behind consensus peptides. Lyngstadaas hold patents for the NuPep technology as cited in the reference list (US Patent US8367602B2, PCT/IB2007/004068). The rights for this patent belong to of Industrie Biomediche Insubri S.A. (Switzerland). Giuseppe Perale is a founding shareholder and the executive vice president of Industrie Biomediche Insubri S.A. (Switzerland), the company that fully owns all IPRs (EP Patent EP2358407B1 and related bundle) on SmartBone and SmartBonePep.

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