ORIGINAL ARTICLE

Osteogenic differentiation of 3D cultured mesenchymal stem cells induced by bioactive peptides

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Abstract

Objectives: Bioactive peptides derived from receptor binding motifs of native proteins are a potent source of bioactive molecules that can induce signalling pathways. These peptides could substitute for osteogenesis promoting supplements. The work presented here compares three kinds of bioactive peptides derived from collagen III, bone morphogenetic protein 7 (BMP-7) and BMP-2 with their potential osteogenic activity on the model of porcine mesenchymal stem cells (pMSCs).

Materials and methods: pMSCs were cultured on electrospun polycaprolactone nanofibrous scaffolds with different concentrations of the bioactive peptides without addition of any osteogenic supplement. Analysis of pMSCs cultures included measurement of the metabolic activity and proliferation, immunofluorescence staining and also qPCR.

Results: Results showed no detrimental effect of the bioactive peptides to cultured pMSCs. Based on qPCR analysis, the bioactive peptides are specific for osteogenic differentiation with no detectable expression of collagen II. Our results further indicate that peptide derived from BMP-2 protein promoted the expression of mRNA for osteocalcin (OCN) and collagen I significantly compared to control groups and also supported deposition of OCN as observed by immunostaining method.

Conclusion: The data suggest that bioactive peptide with an amino acid sequence of KIPKASSVPTLSAISTLYL derived from BMP-2 protein was the most potent for triggering osteogenic differentiation of pMSCs.

1 | INTRODUCTION

The process of bone healing in critical size defects often fails and origins in non-unions. Such critical defects must be temporally filled with a material to induce the bone healing process. Cell-based therapies of healing bone defects are centred on implantation of scaffolds with seeded cells into the site of the defect. This cell-based strategy is connected with the harvesting of mesenchymal stem cells (MSCs) and subsequently with *ex vivo* manipulation, however, these steps pose several limitations.¹ The biggest concerns taken into account are heterogeneity in the expandability of MSCs after aspiration.² Long-term cultivation MSCs are also susceptible to malignant transformation³

and do not maintain the multilineage differentiation potential.⁴ On the other hand, non-cellular-based scaffolds could avoid the disadvantages relating to *in vitro* cultivation.⁵ Non-cellular-based scaffolds specifically control a given type of tissue regeneration at the site of defect due to proper physico-chemical properties and also by mimicking the natural chemical gradients (i.e., regulated drug delivery).

In an appropriate environment, it is possible to induce osteogenic differentiation of MSCs. Nowadays, there are diverse conditions for induction of osteogenic differentiation *in vitro*. Widely used differentiation agents are β -glycerol phosphate, dexamethasone, ascorbate-2-phosphate or vitamin D3.^{6–9} Outside the use of classic differentiation agents, it is also possible to add growth factors or

hormones. Commonly used agents are bone morphogenetic protein 2 (BMP-2), BMP-7, or basic fibroblast growth factor.^{10–12} Furthermore, BMP-2 and BMP-7 are already approved for clinical use.^{13,14} Therefore, developing an efficient protocol is a precondition for induction of osteogenic differentiation.

The present study focuses on the comparison of three types of bioactive peptides as osteogenesis-promoting factors. Bioactive peptides are used as an alternative for full-length native proteins, being derived from the receptor binding sequences. Thus, only the sequence necessary for receptor binding and activation is left. The bioactive peptides provide numerous advantages including improved shelf life, a lower price, and resistance to degradation by proteases.

According to current literature, we chose three promising types of bioactive peptides. The chosen peptides are derived from receptor-binding sequences of proteins that promote osteogenic differentiation. Peptide with amino acid sequence of IAGVGGEKSGGF (I) is a cryptic peptide derived from telopeptide of collagen III α .^{15,16} Peptide with amino acid sequence GQGFSYPYKAVFSTQ (G) was derived from BMP-7.^{17,18} Peptide with amino acid sequence KIPKASSVPTLSAISTLYL (K) was derived from BMP-2.^{19–21} These peptides have never been tested in a single study to compare their differential potential. Porcine mesenchymal stem cells (pMSCs) were cultured on electrospun polycaprolactone (PCL) scaffolds, which should mimic 3D environment for cells. Bioactive peptides were freely added into growth medium without any osteogenic supplements. Furthermore, we tested the dose dependence of bioactive peptides to induce osteogenic differentiation of pMSCs. The data suggest that differentiation potential of bioactive peptides is dose dependent and that the peptide derived from BMP-2 has the biggest potential for osteogenic differentiation. Moreover, none of peptides presented here induced chondrogenic differentiation.

2 | MATERIALS AND METHODS

2.1 | Scaffold preparation

PCL fibre materials were prepared using an electrospinning method. The molecular weight of PCL delivered by Sigma-Aldrich (St Louis, MO, USA) was 45 000 Da. Electrospinning was performed using 24% (w/v) PCL solution dissolved in chloroform:ethanol at a ratio of 9:1 (v/v). Electrospinning was carried out on a Nanospider NS500 (Elmarco, Liberec, Czech Republic) electrospinning unit with a wire needleless

electrode. The applied voltage range was 80–100 kV. The temperature was 24 $\pm$ 2°C and humidity 55 $\pm$ 10%. Electrospun fibres were deposited on an electrode spunbond collecting textile. The distance between the wire electrode and the collector was 20 cm. Nanofibres were stored in a desiccator until used.

2.2 | Scaffold characterization

The morphology of electrospun nanofibres was evaluated using scanning electron microscopy (SEM) performed using a Vega 3 Tescan instrument (Tescan, Brno, Czech Republic). Pieces of PCL fibre sheets were coated with a thin layer of gold in a Quorum Q150R apparatus (Quorum Technologies). The images were collected at accelerator voltage 10–30 kV in high-vacuum mode. Nanofibre diameters and pore sizes were measured from arbitrarily selected sections of SEM images of five randomly chosen pieces of PCL. The average fibre diameter was calculated from 280 measurements in ImageJ software and pore size was estimated from 840 measurements. In order to evaluate wettability of the scaffold, the contact angle of distilled water was measured with a See System E instrument on 10 randomly chosen pieces of PCL scaffold at ambient temperature. Of distilled water, 10 μ L was placed on the scaffold and the contact angle was captured with a built-in camera.

2.3 | Peptide preparation

All bioactive peptides were purchased from Vidia (Czech Republic). Peptides were dissolved in phosphate-buffered saline (PBS; pH 7.4) or in PBS supplemented with dimethylsulfoxid in the case of peptide K, as this sequence was hard to dissolve. Aliquots were stored in –20°C until use. Selected peptide concentrations and their indications are presented in Table 1.

2.4 | Isolation, separation and cultivation of MSCs

Blood marrow aspirates were obtained from the os illium (tuber coxae ala osis illii) of anesthetized miniature pigs (Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic, Libechov, Czech Republic). The bone marrow blood was aspirated into a 10 mL syringe with 5 mL Dulbecco's phosphate-buffered saline with 2% foetal bovine serum (FBS; StemCell

Amino acid sequences	Peptide concentrations and their indications			
	0 μ g/mL	1 μ g/mL	5 μ g/mL	10 μ g/mL
IAGVGGEKSGGF (I)	–	I1	I5	I10
GQGFSYPYKAVFSTQ (G)	–	G1	G5	G10
KIPKASSVPTLSAISTLYL (K)	–	K1	K5	K10
Control group cultivated in growth medium (Cn)	Cn	–	–	–
Control group cultivated in osteogenic medium (Cd)	Cd	–	–	–

TABLE 1 List of used peptide concentrations and their indications

Technologies, Cologne, Germany), and 25 IU heparin/mL, connected to a bioptic needle (15G/70 mm). Under sterile conditions, the bone marrow blood (about 20 mL) was placed into 50 mL centrifuge tubes and 5 mL of 6% Tetraspan (B. Braun Medical) was added. After 30 minutes of incubation at room temperature, the blood was centrifuged at 400 *g* for 15 minutes. Subsequently, the layer of mononuclear cells was removed and seeded into a culture flask, then cultured at 37°C in a humidified atmosphere with 5% CO₂. α -Minimum essential medium with Earle's Salt (α -MEM) and L-glutamine (supplemented with 10% FBS and 1% penicillin/streptomycin; 100 IU/mL and 100 μ g/mL respectively) was used as the culture medium. The cells were passaged using the trypsin-EDTA method before confluence was reached. The cells from the second or third passage were used for the cell culture study.

2.5 | MSC seeding on scaffolds

Round-shaped scaffolds with a diameter of 6 mm were sterilized in 70% ethanol (v/v) for 30 minutes and then washed three times in PBS. Cells were seeded on scaffolds at a density of $124 \times 10^3/\text{cm}^2$ in 96-well plates corresponding approximately to 35×10^3 cells/scaffold. Scaffolds with seeded MSCs were cultured in 250 μ L growth medium: α -MEM supplemented with 10% FBS, penicillin/streptomycin (100 IU/mL and 100 μ g/mL respectively). Medium was supplemented with freely added peptides of concentrations 1, 5 or 10 μ g/mL of peptides. Control sample marked as Cn were cells incubated without peptides in growth medium, control sample marked as Cd were cells incubated in osteogenic medium consisted of growth medium supplemented with osteogenic supplements, namely 100 nmol/L dexamethasone, 40 μ g/mL ascorbic acid-2-phosphate and 10 mmol/L β -glycerol phosphate disodium salt hydrate. The medium was changed every 7 days. Tests for cell metabolic activity and proliferation were always performed on the same sample.

2.6 | Detection of cell metabolic activity using MTS assay

The metabolic activity of MSCs was determined using the 3-(4,5-di methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) assay (CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay; Promega Corporation, Fitchburg, WI, USA). At 1, 7, 14 and 21 days of cultivation, the scaffolds were transferred to a new 96-well plate containing 100 μ L of fresh medium and 20 μ L of MTS reagent per well. The formazan absorbance in 100 μ L of the solution was measured ($\lambda_{\text{sample}}=490$ nm, $\lambda_{\text{reference}}=690$ nm) after 2-hour incubation at 37°C and 5% CO₂ on a multiplate fluorescence reader (Synergy HT). To avoid the influence of medium to the measured results, the absorbance without cells was deducted from the cell-seeded samples. All results are from three independent measurements.

2.7 | Cell proliferation analysis

A PicoGreen assay kit was performed using the Quant-iT[™] PicoGreen[®] dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and

proliferation of MSCs on scaffolds was tested on days 1, 7, 14 and 21. To process material for analysis of DNA content, 500 μ L of cell lysis solution (150 mmol/L NaCl, 10 mmol/L Tris, 0.1% w/v sodium dodecyl sulphate, 0.1% v/v Triton X-100, 24 mmol/L sodium deoxycholate, 50 mmol/L ethylenediaminetetraacetic acid) was added to each well containing a scaffold sample. To prepare cell lysate, samples were processed through three freeze/thaw cycles. Between each freeze/thaw cycle, scaffolds were roughly vortexed. Prepared samples were stored at -80°C until analysis. DNA content was determined by mixing 200 μ L of PicoGreen reagent and 20 μ L of DNA sample. Specimens were loaded in triplicates and fluorescence intensity was measured on a multiplate fluorescence reader (Synergy HT, $\lambda_{\text{ex}}=480$ -500 nm, $\lambda_{\text{em}}=520$ -540 nm). A calibration curve based on standards was created to evaluate the amount of DNA.

2.8 | Cell visualization on scaffolds using confocal microscopy

Staining with the fluorescent probe DiOC6 (3,3'-diethyloxacarbo cyanine iodide) was used to detect the adhesion of cells on the scaffolds. Every 1, 3, 7, 14 and 21 days samples were fixed with frozen methylalcohol (-20°C) for 10 minutes and rinsed with PBS. Subsequently, DiOC6 (0.1-1 μ g/mL in PBS, pH 7.4) was added and incubated with the samples for 45 minutes at room temperature. The samples were rinsed with PBS (pH 7.4), and propidium iodide (5 μ g/mL in PBS, pH 7.4) was added for 10 minutes. Samples were rinsed three times with PBS (pH 7.4). The stain was visualized using a Zeiss LSM 5 DUO confocal microscope (Zeiss, Oberkochen, Germany). $\lambda_{\text{ex}}=488$ and 560 nm and $\lambda_{\text{em}}=520$ and 580 nm were used for DiOC6 and propidium iodide detection respectively.

2.9 | Cell visualization on scaffolds using scanning electron microscopy

The cell's morphology was evaluated by SEM on days 1 and 21. Scaffolds with pMSCs were washed in PBS and fixed in 2.5% glutaraldehyde for 2 hours at 4°C. Then the samples were dehydrated in ethanol ranging from 35%-100%. To dry the scaffolds, hexamethyldisilazane (Sigma-Aldrich) was added. Scaffolds were analysed using Vega 3 Tescan as described previously.

2.10 | Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted on days 7 and 14 using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Synthesis of cDNA was performed using a RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the standard manufacturer's protocol. Osteocalcin (OCN), collagen I, collagen II and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression levels were quantified by means of LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using TaqMan probes (Roche Diagnostics), according to the manufacturer's protocol.

Primers used were as follows: OCN, sense 5'-GAG CTG GCT GAT CAC ATC G-3', antisense 5'-CTG CGA GGT CTA GGC TAT GC-3' (69 bp); Collagen I, sense 5'-ATG TTC AGC TTT GTG GAC CTC-3', antisense 5'-CTT CTT CTT GGC CCT CCT CT-3' (59 bp); Collagen II, sense 5'-CCC AGG TCT AGA TGG TGC TAA-3, antisense 5'-GGA ACC ACT CTC ACC CTT CA-3' (64 bp); and GAPDH, sense 5'-ACA GAC AGC CGT GTG TTC C-3', antisense 5'-ACC TTC ACC ATC GT GT CTC A-3' (60 bp). Polymerase chain reaction conditions were initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 54°C for 10 seconds, and extension at 72°C for 20 seconds. Expression levels of OCN and collagen I mRNA were adjusted, using the level of GAPDH mRNA as a housekeeping gene. Gene expression data were analysed by the $2^{-\Delta\Delta Ct}$ method (relative quantification).

2.11 | Detection of osteogenic markers using indirect immunofluorescence staining

The presence of OCN, as a marker of osteogenic differentiation, was confirmed using indirect immunofluorescence staining as described previously²² every 14 and 21 days. Briefly, samples were fixed with 10% formaldehyde/PBS for 10 minutes and permeabilized by PBS with 1% BSA/0.1% Triton X-100 for 30 minutes at room temperature. The samples were incubated with the primary antibody against OCN (1:20 dilution, mouse anti-OC, diluted 1:20, Abcam, Cambridge, USA) incubation over night at 4°C. Following three washes with PBS/0.05% Tween 20 after 3, 10 and 15 minutes, samples were incubated with a secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG [H+L]; Invitrogen, Carlsbad, CA, USA), diluted 1:300 and incubated for 45 minutes at room temperature. The cell nuclei were counterstained by incubating with propidium iodide stain for 5 minutes and subsequently washed three times with PBS/0.05% Tween 20. The stain was visualized using an Olympus FV10i confocal microscope. $\lambda_{ex}=495$ and 560 nm and $\lambda_{em}=520$ and 580 nm were used for Alexa Fluor 488 and propidium iodide (5 μ g/mL in PBS, pH 7.4) detection respectively. The average value of the intensity of non-specifically bound secondary antibody was subtracted from all images in the ImageJ program.

2.12 | Statistical analysis

Quantitative data are presented as mean $\pm$ standard deviation. For *in vitro* tests, average values were determined from at least three independently prepared samples. The results were evaluated statistically using SigmaStat software (Systat Software Inc., Chicago, IL, USA). The level of significance was set at .05.

3 | RESULTS

3.1 | Scaffold characterization

In order to mimic the natural 3D environment, cells were cultured on fibrous scaffolds of electrospun PCL, instead of traditionally used 2D cultivation plastic. Measurement of pore size revealed that its

mode, i.e., the value of the most likely sampled pore radius, is 319 nm (Figure 1A). The electrospun PCL scaffolds had fibre diameters ranging from 100-1410 nm (Figure 1B), with majority of fibres having a diameter of 200 nm. The density probability of fibres decreased as the fibre diameter increased. Furthermore, there were no detectable fibres in the interval from diameters of 1000-1150 nm. There are a number of diverse possible explanations for this observed phenomenon. As PCL was dissolved in ethanol and chloroform, the ethanol phase of the fibres form filaments with a smaller diameter, while fibres in the chloroform phase form filaments with a larger diameter. Contact angle measurement demonstrated that the PCL scaffold was largely hydrophobic, with a contact angle of $82.61 \pm 21.35^\circ$.

3.2 | Metabolic activity and proliferation of pMSCs

The effect of the peptides on metabolic activity and proliferation of pMSCs was examined on days 1, 7, 14 and 21. In this study, we tested three kinds of peptides derived from collagen III (peptide I), from BMP-7 (peptide G) and BMP-2 (peptide K) at concentrations of 1, 5 and 10 ng/mL, indications of samples are listed in Table 1. Cell proliferation was evaluated by measuring DNA content with a PicoGreen assay (Figure 2A). Analysis of DNA content on the first day showed that cell seeding was homogenous across all samples, apparent by all containing ~ 6 ng/sample DNA on day 1. The concentration of DNA, used as an indicator of cell proliferation, increased in all tested groups over the measured time period. On groups I1-10 and G1-5, there were no statistic differences. On the final day (day 21), the concentration of DNA was statistically ($P < .05$) highest on groups G10 and K10.

Metabolic activity was quantified using an MTS metabolic assay (Figure 2B). The rate of metabolic activity was comparable between samples treated with peptides and control samples. On day 1, there were no statistical differences ($P < .05$) with the exception of groups K5 and Cn where pMSCs were the most metabolic active. As the cells were cultured, the differences united and the absorbance rose steadily over the 3-week period. The only exceptions were groups K1 and Cn where metabolic activity of cells did not rise since day 14. The results showed that with increasing culture time, the viability and proliferation rate of cells on each sample increased. Therefore, we assumed that no detrimental effect of peptides was demonstrated and these peptides are suitable for cell cultivation.

3.3 | Cell morphology

Confocal microscopy observations were undertaken to investigate pMSCs morphological characteristics on scaffolds throughout the 3 weeks of incubation. Membranes of pMSCs were stained using DiOC6 with the use of propidium iodide for staining the nuclei at days 1, 7, 14 and 21. pMSCs were present in aggregates for the first 7 days, which then spread on the scaffold's surface with no observed differences between groups, with continuous cell growth on the scaffolds seen for all samples (Figure 3). Confocal microscopy observations clearly supported results obtained from the PicoGreen assay showing

FIGURE 1 PCL scaffolds characterization. A, Probability density of pore radius. B, Diameter of fibres. Abbreviations: f , probability density of pore radius

that the addition of peptides did not have negative or positive effects on pMSC cultivation.

The results from the PicoGreen assay and confocal microscopy were in agreement with SEM images taken on days 1 and 21 (Figure 3). On day 1, we observed that cell adhesion was uniform and cell spreading on the scaffolds for all samples had occurred from day 1. After 21 days of incubation, we observed confluent layers of cells which had spread over the scaffold.

3.4 | Detection of osteogenic and chondrogenic differentiation using qPCR analysis

qPCR analysis of collagen II was undertaken to detect chondrogenic differentiation. No mRNA expression of collagen II was observed through 45 cycles of qPCR for any group cultivated with peptides on day 14 (data not shown). These results indicate that selected peptides did not induce chondrogenic differentiation. The expression levels of OCN and collagen I, which are markers of osteogenic differentiation, were detected on days 7 and 14.

The expression level of mRNA for OCN (Figure 4A) decreased since day 7. There was a slight difference between I and G. The incubation of cells in the presence of peptide G had no statistical influence on mRNA production in comparison to Cn group. The most significant differences ($P < .05$) were observed on groups K1-10 that supported the expression of OCN the most. The results further imply

on concentration dependence of used peptide I and K. With higher concentration of peptides used, we observed significantly ($P < .05$) higher amount of expressed mRNA for OCN with the highest amount on K5 and K10 groups on both tested days.

The expression of collagen I was undertaken also in MSCs cultivated in osteogenic medium. On day 7, the highest expression level of mRNA for collagen I (Figure 4B) was detected on samples I10 and G1 ($P < .05$). However, on day 14, the highest expression ($P < .05$) was measured on sample K10 followed by samples K1, I5 and I10. Based on this qPCR analysis, we interpret that the influence of peptides I and K on the expression of OCN and collagen I was dose-dependent. The expression of collagen I was promoted even more compared to control group cultivated in osteogenic medium.

3.5 | Detection of osteocalcin by indirect immunofluorescent staining

Immunofluorescent staining of OCN was undertaken to verify the results obtained from qPCR (shown previously), to investigate the presence of OCN. Therefore, OCN was detected using confocal microscopy on days 14 and 21, with OCN present in all samples (Figure 5). Clearly, the addition of peptide K and G, at all concentrations presented here, supported the production of OCN at both days 14 and 21. The concentration of peptide I used influenced the production of OCN in comparable manner as Cn and Cd group with group

FIGURE 2 Proliferation of porcine mesenchymal stem cells (pMSCs) determined using the PicoGreen assay and metabolic activity of pMSCs measured by MTS assay. A, Proliferation of cells; B, metabolic activity of cells; the abbreviations above the bars denote to statistical difference with $P < .05$. Abbreviations: MSCs, mesenchymal stem cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; I, IAGVGGEKSGGF; G, GQGFSYPYKAVFSTQ; K, KIPKASSVPTELSAISTLYL; 1, 1 $\mu\text{g/mL}$; 5, 5 $\mu\text{g/mL}$; 10, 10 $\mu\text{g/mL}$; Cn, control group

I10, containing the highest amount of OCN on day 21. From these results, we concluded that the addition of all peptides had a positive effect on the production of OCN as the amount of OCN was low in the control groups with all samples containing peptides displaying a comparable or higher amount of OCN.

4 | DISCUSSION

Non-cellular-based therapy is based on selective regulation of cell behaviour using scaffolds and specific active molecules. In order to fulfil this demand, selective molecules can be immobilized onto a non-cellular scaffold. The interactions between specific binding sequences of proteins with cell receptors result in specific cellular responses. The use of native proteins is connected with a number of shortcomings. Protein-based signalling molecules have a complex secondary and tertiary structure. The covalent immobilization of full-size native proteins is connected with risk of steric hindrance of the receptor-binding motifs.²³ Moreover, native proteins are susceptible to degradation in aqueous solutions and thereby lose their bioactivity.²⁴ Furthermore, the method for production of recombinant proteins is often very expensive and difficult.²⁵

The shortcomings of using native proteins for therapies can be eliminated by the use of short peptides. Short peptide sequences derived from the active area of native proteins has been shown to have

the same or greater influence to induce osteogenesis^{18,19} compared to whole native proteins.

Use of short peptide sequences has several advantages. The use of peptides avoids possible immunogenicity, and issues surrounding the short half-life of native proteins.²⁶ Furthermore, manufacturing short peptides is a simple method compared to the production of recombinant proteins. Importantly, peptides can be easily modified and covalently immobilized onto the scaffolds. Moreover, after the immobilization of peptides onto scaffolds, receptor-binding sequences are available for interaction with cell receptors, as the peptides are only several amino acids long in comparison to whole native proteins being thousands of amino acids long. For these reasons, it is believed that biomaterials with suitable combinations of peptides are eligible for the use in regenerative therapy as non-cellular scaffolds. The negatives of bioactive peptides compared to full-length proteins are mainly in the absence of adjacent regulatory sequences presented in native proteins. In addition, growth factors could be easily inactivated, whereas bioactive peptides are active for longer time period even though the signalling is not further benefit.

The aim of this study was to test the selectivity of peptides to induce osteogenic differentiation. The onset of chondrogenic differentiation was not detected, evident by qPCR analysis. Bioactive peptides were derived from the receptor-binding sequences^{15,17–21} of collagen III, BMP-7 and BMP-2 and they are denoted as peptide I, G and K respectively. Integrin receptors are possible targets of peptide I derived

FIGURE 3 Visualization of cells by confocal microscopy and SEM. Notes: DNA was stained by propidium iodide (red) and membrane structures were stained by DiOC6 (green). (Aa) MSCs on day 1, group I1 (Ab) MSCs on day 1, group I1 (Ac) MSCs on day 21, group I1 (Ad) MSCs on day 21, group I1 (Ba) MSCs on day 1, group I5 (Bb) MSCs on day 1, group I5 (Bc) MSCs on day 21, group I5 (Bd) MSCs on day 21, group I5 (Ca) MSCs on day 1, group I10 (Cb) MSCs on day 1, group I10 (Cc) MSCs on day 21, group I10 (Cd) MSCs on day 21, group I10 (Da) MSCs on day 1, group G1 (Db) MSCs on day 1, group G1 (Dc) MSCs on day 21, group G1 (Dd) MSCs on day 21, group G1 (Ea) MSCs on day 1, group G5 (Eb) MSCs on day 1, group G5 (Ec) MSCs on day 21, group G5 (Ed) MSCs on day 21, group G5 (Fa) MSCs on day 1, group G10 (Fb) MSCs on day 1, group G10 (Fc) MSCs on day 21, group G10 (Fd) MSCs on day 21, group G10 (Ga) MSCs on day 1, group K1 (Gb) MSCs on day 1, group K1 (Gc) MSCs on day 21, group K1 (Gd) MSCs on day 21, group K1 (Ha) MSCs on day 1, group K5 (Hb) MSCs on day 1, group K5 (Hc) MSCs on day 21, group K5 (Hd) MSCs on day 21, group K5 (Ia) MSCs on day 1, group K10 (Ib) MSCs on day 1, group K10 (Ic) MSCs on day 21, group K10 (Id) MSCs on day 21, group K10 (Ja) MSCs on day 1, group Cn (Jb) MSCs on day 1, group Cn (Jc) MSCs on day 21, group Cn (Jd) MSCs on day 21, group Cn. Abbreviations: MSCs, mesenchymal stem cells; I, IAGVGGEKSGGF; G, GQGFSYPYKAVFSTQ; K, KIPKASSVPTLSAISTLYL; 1, 1 µg/mL; 5, 5 µg/mL; 10, 10 µg/mL; Cn, control group; DiOC6, 3,3'-diethyloxacarbocyanine iodide

FIGURE 4 Expression of osteogenic gene markers. A, Expression of osteocalcin gene. B, Expression of collagen I gene. The abbreviations above the bars denote to statistical difference with $P < .05$; * means significant difference of K10 group compared to all other tested samples. Abbreviations: MSCs, mesenchymal stem cells; OCN, osteocalcin; Col I, collagen I; I, IAGVGGEKSGGF; G, GQGFSPYKAVFSTQ; K, KIPKASSVPTLSAISTLYL; 1, 1 $\mu\text{g/mL}$; 5, 5 $\mu\text{g/mL}$; 10, 10 $\mu\text{g/mL}$; Cn, control group cultivated in growth medium; Cd, control group cultivated in osteogenic medium

from collagen III α .¹⁵ Integrins are heterodimeric cell receptors that upon binding to certain ligands trigger signalling pathways that can contribute to osteogenesis.^{27,28} Peptide G is derived from BMP-7 protein that binds to BMP receptors.²⁹ Peptide K is derived from BMP-2 protein, specifically from the knuckle epitope, which is responsible for binding to BMP receptor type II.¹⁹ Signalling from BMP receptors results in forming of heteromeric complexes that translocate to the nuclei where induce expression of BMP responsive genes associated with osteogenic differentiation.^{30,31}

Plenty of reports which look into the effect of these peptides on osteogenesis have been published.^{17,18,20,32–35} However, culture conditions of cells used in this work differ significantly. Other reports looked only at the synergistic effect of peptides together with added osteogenic supplements.^{17,18,32} There are also plenty of studies that are interested in the enhanced effect of peptides together

with hydroxyapatite matrices,^{20,33} with further studies dealing with the effect of peptides on the differentiation of osteoblasts which are already determined for osteogenic line.^{34,35} In contrary, we selected pMSCs as a cell type, and PCL nanofibres as a scaffold for MSC cultivation with no addition of osteogenic supplements. We used three different concentrations ranging from 1–10 $\mu\text{g/mL}$ of each peptide to verify how the pMSCs proliferation, metabolic activity, and expression of OCN and collagen I are all influenced by peptide concentration.

PCL scaffold characterization revealed that most abundant pore radius was 319 nm. Pores of this size do not allow cells to penetrate into the scaffolds,³⁶ thus the cells remained on the surface of the scaffold. Diameter of fibres ranged from 100–1410 nm, which closely mimics the physical characteristics of extracellular matrix which favours cell spreading.³⁷

FIGURE 5 Immunofluorescent detection of osteocalcin. Osteocalcin was indirectly stained by Alexa Fluor 488 (green) and DNA was stained by propidium iodide (red). (A) OCN on day 14, group I1 (B) OCN on day 21, group I1 (C) OCN on day 14, group I5 (D) OCN on day 21, group I5 (E) OCN on day 14, group I10 (F) OCN on day 21, group I10 (G) OCN on day 14, group G1 (H) OCN on day 21, group G1 (I) OCN on day 14, group G5 (J) OCN on day 21, group G5 (K) OCN on day 14, group G10 (L) OCN on day 21, group G10 (M) OCN on day 14, group K1 (N) OCN on day 21, group K1 (O) OCN on day 14, group K5 (P) OCN on day 21, group K5 (Q) OCN on day 14, group K10 (R) OCN on day 21, group K10 (S) OCN on day 14, group Cn (T) OCN on day 21, group Cn (U) OCN on day 14, group Cd (V) OCN on day 21, group Cd. Abbreviations: MSCs, mesenchymal stem cells; I, IAGVGGEKSGGF; G, GQGFSPYKAVFSTQ; K, KIPKASSVPTLSAISTLYL; 1, 1 $\mu\text{g/mL}$; 5, 5 $\mu\text{g/mL}$; 10, 10 $\mu\text{g/mL}$; Cn, control group cultivated in growth medium; Cd, control group cultivated in osteogenic medium

One important finding was that none of the peptides (I, G or K) had a detrimental effect on cell proliferation or metabolic activity, with this being consistent with those published previously.^{15,17,32,38–40} However, in contrast to these findings, there are similar studies that showed a decrease in cell numbers after treatment cells with peptide K in growth medium.^{41,42} In our study, we were concerned only with short peptide sequences derived from native proteins, as opposed to whole native proteins having a diverse number of effects, as has been demonstrated. For example, the treatment of cells with BMP-7 resulted in a decrease in the number of cells in both growth and osteogenic medium.^{42–44} Nonetheless, research has shown that the addition of BMP-7 to osteogenic medium is dose-dependent and in higher concentrations leads to an increase in cell proliferation.^{31,45} An increase in metabolic activity was also shown in studies where cells were treated with BMP-2 protein.^{46–48} In the current study, confocal microscopy and SEM showed that cells formed confluent cell layers on the fibrous scaffolds. The morphology of spread cells was typical for MSCs.

As revealed by qPCR, in the case of peptide I and K, we observed a dependence of osteogenic gene expression on peptide concentrations. Few studies show that the addition of peptide K into growth or osteogenic medium led to an increase in the level of mRNA for OCN.^{19,20} Using peptide G, we detected no differences compared to Cn group when mRNA expression for OCN was analysed. However, expression of collagen I was highest on day 7 on sample G1. However, the study of Kim et al.¹⁸ showed that the treatment of MSCs with peptide G significantly increased the production of OCN in osteogenic medium.

qPCR was performed on days 7 and 14, however, immunostaining of OCN was undertaken on days 14 and 21 as translation of proteins occurs afterwards the gene expression. By immunostaining of OCN, we observed that the addition of all tested peptides, including peptide G, led to an equal or higher production of OCN compared to Cn and Cd groups. This inconsistency from qPCR could be the consequence of different mRNA stability and also of different regulation of protein translation and possible wash away of OCN into the cultivation medium during the cultivation period.

From all the results presented, we found that peptide K was the most potent to induce the osteogenic differentiation of pMSCs. However, both peptides K and G are derived from BMPs which activate BMP signalling pathway,^{19,49} whereby peptide I activates an integrin signalling pathway.¹⁵ Lavery et al.²⁹ proposed a model of utilizing different BMP receptors by diverse BMP proteins. BMP-2 (where peptide K is derived) binds to three kinds of BMP receptors, whereas BMP-7 (where peptide G is derived) binds to only two kinds.²⁹ Thus, differences in the receptor utilization might be the reason why peptide K was more potent for inducing osteogenesis compared to peptide G.

The finding that peptide K-induced osteogenesis in pMSCs will be further evaluated in future studies. We will specifically focus on comparing the efficacy compared to the full-length BMP-2 protein. The future study should focus on more complex molecular characterization of osteoinductive properties both *in vitro* and *in vivo*. The comparison will also include an analysis of protein/peptide stability and the ability to induce differentiation under long-term cultivation.

5 | CONCLUSION

From this comparative study concerning the ability of diverse peptides to induce osteogenic differentiation of pMSCs, we conclude that peptide K derived from BMP-2 protein had the greatest potential for osteogenic differentiation of pMSCs incubated in growth medium. Peptide K led to an increase in production of mRNA for OCN and collagen I. Moreover, we did not detect any detrimental effect of peptides on pMSCs behaviour. The osteogenic activity of the BMP-2-derived peptide holds great potential for future use in the field of tissue engineering and regenerative medicine.

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