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Effect of a Bone Morphogenetic Protein-2-derived peptide on the expression of tumor marker ZNF217 in osteoblasts and MCF-7 cells

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Abstract

Zinc Finger Protein 217 (ZNF217), a transcription factor and oncogene product, has been found to dysregulate Bone Morphogenetic Protein (BMP) signaling and induce invasion in breast tumors. In this study, the effect of BMP-2 or an active BMP-2 peptide, AISMLYLDEN, on the expression of *ZNF217, BMP4* and CDK-inhibitor p21 gene, *CDKN1A*, was investigated in DPSCs during osteogenic differentiation and in MCF-7 breast cancer cells. BMP-2 peptide reduced the expression of *ZNF217* during the first two weeks of osteogenesis and increased the expression of *CDKN1A* after three weeks. BMP-2 and BMP-2 peptide increased the expression of *BMP4* during the first week. The same genes were monitored in MCF-7 after treatment with BMP-2 or different concentrations of BMP-2 peptide. *CDKN1A* mRNA levels were 10-, 8- and 6-fold higher respectively in MCF-7 cells treated with BMP-2 (100 ng/ml) or BMP-2 peptide (45.2 and 22.6 ng/ml) than in untreated MCF-7. BMP-2 peptide, at a concentration of 22.6 ng/ml reduced *ZNF217* expression after 6 h and 12 h. BMP-2 reduced *BMP4* expression to undetected levels within 24 h. At appropriate concentrations, BMP-2 and the peptide AISMLYLDEN can be considered as a possible novel therapeutic method in breast tumors with a metastatic tendency to the bones.

1. Introduction

Bone morphogenetic proteins, BMPs, are growth factors that belong to the TGF-β superfamily. Initially, they were found to induce the formation of bone and cartilage in animal models. However, in recent years, it has been discovered that they regulate growth, differentiation and apoptosis of mesenchymal, epithelial and neuronal cells as well as monocytes. In addition, it has been found that they are involved in the morphogenesis of several tissue types and organs [1]. BMPs bind to surface receptors of target cells, BMPRs, and activate multiple signaling pathways, mainly the pathway of SMAD-1, -5 and – 8 transcription factors [2].

BMP-2 can trigger the differentiation of human mesenchymal stem cells (hMSCs) into osteoblasts [3]. In addition, it has been found to be involved in the proliferation and apoptosis of tumor cells [4–7]. Through its interaction with receptors BMPRIa/BMPRII, BMP-2 can trigger the SMAD pathway, which is the main pathway that leads to pre-osteoblast and osteoblast differentiation of hMCSs, as well as SMAD-independent pathways that involve MAP kinases. In the SMAD-dependent pathway, when BMP-2 forms a complex with its receptors, the constitutively active tyrosine kinase BMPRII phosphorylates and activates BMPRIa. The activated receptor BMPRIa phosphorylates SMAD-1, -5 or -8, which then forms a complex with co-SMAD, SMAD-4. The complex is transported to the nucleus where it activates the expression of several transcription factors that promote osteogenesis [2, 8]. Subsequently, genes necessary for osteogenesis, such as *ALPL* (alkaline phosphatase) are expressed and, a few weeks later, the mineralization of the extracellular matrix commences. BMP-2 also activates MAPK pathways, which lead to the expression of *ALPL*, *SPP1* (osteopontin) and *COL1A1/2* (type I collagen) [8–10].

ZNF217 is a transcription factor that belongs to the C_2H_2-type zinc-finger protein family. The *ZNF217* gene has been recognized as a proto-oncogene with important role in the occurrence and progression of

breast, colorectal, stomach, cervical, prostate and lung cancer [11, 12]. It is already known that ZNF217 is a component of a complex that contains the co-repressor CtBP1. CtBP1 is a co-repressor for many transcriptional repressors in histone deacetylase complexes (HDACs) or deacetylate-independent histone modifying complexes [13, 14]. Although ZNF217 was initially recognized as a transcriptional suppressor, recent studies also indicate its role in transcriptional activation. It has been found that in mouse embryonic stem cells, ZFP217, the murine homologue of ZNF217, binds to promoters and enhancers of pluripotency genes, such as *NANOG* and *SOX2*, and activates their expression, thus maintaining the cells in their un-differentiated state [15].

In recent studies, it was demonstrated that ZNF217 triggers BMP signaling leading to metastasis of breast cancer cells to bone. Increased *ZNF217* expression was observed in breast tumors that metastasized to the bones, in comparison to non-metastatic tumors and tumors that metastasized to other tissues. In a breast cancer cell line, high levels of *ZNF217* mRNA were also found to be correlated with the deregulation of several genes that are involved in osteogenesis. Among them, *BMP-2* and repressors of BMP pathway were downregulated while *BMP-4* and *BMPRIa*, *BMPRIb* and *BMPRII* were upregulated [16]. These observations exhibit a possible positive feedback loop between *ZNF217* and *BMP-4* expression, and a negative feedback loop between *ZNF217* and *BMP-2* expression. BMP-4 is overexpressed in a variety of human tumors and cancer cells lines. Interestingly, the addition of BMP-4 in breast cancer cell lines or the overexpression of its gene, has been shown to inhibit their proliferation, but increase their invasive and metastatic potential [17]. Treatment of MCF-7 breast cancer cells with BMP-2 has also been found to cause cell cycle arrest in G_1 phase by inducing the expression of CDK-inhibitor p21 [4].

The aim of this study was to investigate the effect of active dimeric BMP-2 or a short active decapeptide, derived from its carboxyterminal domain, on the expression of ZNF217 in dental pulp stem cells (DPSCs) during induced osteogenesis and in MCF-7 breast cancer cells. The BMP-2-derived peptide, AISMLYLDEN, is a short part of the carboxyterminal region of human BMP-2 that has been previously found to induce differentiation of hMSCs more effectively than the active dimeric BMP-2 (personal communication with Dr. S. Karoulias). It was determined how BMP-2 and different concentrations of the active peptide affect the expression of ZNF217, cell-cycle inhibitor p21 and BMP-4 in DPSCs, osteoblasts and MCF-7. In addition, an effort was made to find if there is an optimal range of BMP-2 peptide concentration that can reduce both the proliferative and the invasive potential of breast cancer cells.

2. Materials And Methods

2.1 Materials

The reagents that were used in this project are listed in Table 1. Disposable supplies were purchased from ThermoFisher Scientific (USA), Kisker Biotech (Germany) and Sarstedt (Germany).

Table 1

Cell culture, differentiation and staining assays	
Dulbecco's Modified Eagle's Medium (DMEM)	(ThermoFisher Scientific, USA)
StemPro® Osteogenesis Kit	
Fetal Bovine Serum (FBS)	
Penicillin/streptomycin solution	
Phosphate-Buffered Saline (PBS)	
Trypsin /EDTA solution (Biosera, France)	
Recombinant active human Bone Morphogenetic Protein-2, BMP-2 (Cusabio, USA)	
BMP-2 peptide, AISMLYLDEN (Biomatik, Canada)	
BCIP, C8H6BrClNOP4	
NBT, C40H30N10O • 2Cl6	
Alizarin Red S, $C_{14}H_8O_4$	
Formaldehyde, CH_2O	
Tris, $C_4H_{11}NO_3$	
Hydrogen chloride, HCl	
Sodium chloride, NaCl	
Sodium hydrogen phosphate, Na_2HPO_4	
Sodium dihydrogen phosphate dihydrate, $NaH_2PO_4 \cdot 2H_2O$	
Gene expression analysis	
NucleoSpin® RNA Kit (Macherey-Nagel, Germany)	
M-MuLV Reverse Transcriptase (Minotech Biotechnology (Enzyquest), Greece)	
$Oligo(dT)_{20}$	
dNTP mix	
Dithiothreitol (DTT)	
KAPA SYBR® FAST qPCR Kit Master Mix (2 X) ABI Prism™ (Sigma-Aldrich, USA)	

2.2. Culture and differentiation of DPSCs

Dental Pulp Stem Cells were kindly provided by Assistant Professor A. Bakopoulou from the School of Dentistry, Aristotle University of Thessaloniki. The cells had been established from third molars of young healthy donors, aged 18–24, with the enzymatic dissociation method described in *Bakopoulou et al*. (2016) [18]. The samples had been collected in accordance to all the relevant guidelines and regulations and had been approved by the Institutional Review Board of the Aristotle University of Thessaloniki (Nr. 66/18-06-2018). All the donors signed an informed consent form.

The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 ^{0}C and 5% CO_2. When the cells reached around 80% confluency, they were detached from the culture dish with 0.05% w/v Trypsin/EDTA solution, counted and seeded in 6-well and 12-well plates at a density of 20,000 cells per well. The cells were incubated overnight until they formed monolayers, then two groups of cells were treated with either 50 ng/ml active dimeric human BMP-2 or 50 ng/ml BMP2 peptide in the culture medium (Day 0). Three days later, DMEM was substituted by StemPro® Ostegenesis Complete Medium in all groups except for a negative control group which was cultured in DMEM. The medium was refreshed every three days. RNA was extracted on days 7, 14 and 21.

2.3 Culture and treatment of MCF-7 with BMP-2 and BMP-2 peptide

MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 ^{0}C and 5% CO_2 until they reached around 70–80% confluency. Then they were seeded in 6-well plates at a density of 10^5 cells per well and were incubated overnight to form monolayers. Once attached, the cells were treated with 100 ng/ml active dimeric BMP-2 or different concentrations of BMP-2 peptide (45.2 ng/ml, 22.6 ng/ml and 4.52 ng/ml). RNA was extracted 6 h, 12 h, 24 h and 48 h after treatment. For each time point, there was also a negative control group, which was not treated with BMP-2 or BMP-2 peptide (untreated).

2.4 Total RNA extraction

Total RNA was extracted from DPSCs, osteoblasts and MCF-7 using the NucleoSpin® RNA kit. The concentration of RNA in each sample was determined by measuring optical density at 260 nm.

2.5 Detection of Alkaline Phosphatase activity (Alkaline Phosphatase Assay)

The Alkaline phosphatase assay was performed on the 14th day of osteogenesis, on DPSCs that had been seeded on 12-well plates at a density of 20,000 cells/well and treated with BMP-2 or BMP2 peptide. The medium was removed and the cell monolayers were washed with 1 X PBS and then fixed in 10% formalin solution (10% v/v formaldehyde, 0.4% w/v $NaH_2PO_4.H_2O$, 0.65% w/v Na_2HPO_4) at room temperature for a maximum of 60 seconds (to avoid irreversible inactivation of alkaline phosphatase). Immediately afterwards, formalin was removed and the cells were washed twice with TWEEN® 20-PBS solution (1 X PBS, 0.05% v/v Tween-20). TWEEN® 20-PBS was removed and the monolayers were incubated in 1X Alkaline Phosphatase Solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$)

that contained 0.45% v/v of each of the substrates NBT and BCIP, at 37 ^{0}C until areas with blue NBT precipitate started to appear on the monolayers. The enzymatic activity was terminated by washing the cells with TWEEN® 20-PBS solution. Finally, TWEEN® 20-PBS was removed and 1 X PBS was added in all the wells. The cells were observed with a Nikon Eclipse TS-100 inverted optical microscope (objective lenses: 10 X and 40 X, ocular lens: 10 X) and photographs of the stained monolayers were taken at 100 X and 400 X magnification using a Nikon Coolpix P5100 camera.

2.6 Staining of extracellular calcium deposits (Alizarin Red S Assay)

The Alizarin Red staining assay was performed on the 21st day of differentiation, on DPSCs that had been seeded on 12-well plates at a density of 20000 cells/well and treated with BMP-2 or BMP2 peptide. The medium was removed and the cells were washed with 1 X PBS and then fixed in 10% formalin solution (10% v/v formaldehyde, 0.4% w/v $NaH_2PO_4.H_2O$, 0.65% w/v Na_2HPO_4) at room temperature for 30 minutes. Afterwards, formalin was removed, the monolayers were washed twice with sterile distilled H_2O and incubated in 2% w/v Alizarin Red S solution at room temperature for 25–45 minutes. Then, the cells were washed multiple times with sterile distilled water to remove excess dye. The cells were observed with a Nikon Eclipse TS-100 inverted optical microscope (objective lenses: 10 X and 40 X, ocular lens: 10 X) and photographs of the stained monolayers were taken at 100 X and 400 X magnification using a Nikon Coolpix P5100 camera.

2.7 cDNA synthesis

First-strand cDNA was synthesized from each total RNA sample with M-MuLV Reverse Transcriptase. Each reaction was set up with 0.5 ng/ml RNA, 5 μM Oligo$(dT)_{20}$ and 500 μM of each dNTP. The mixture was heated to 65 ^{0}C for 5 min to denature secondary structures of the RNA and the Oligo$(dT)_{20}$ and, immediately, cooled on ice for 1 min. 200 U of Reverse transcriptase (RT), 1 X RT Buffer and 5 μM DTT were added and the mixture was incubated at 25 ^{0}C for 5 min for efficient annealing of Oligo$(dT)_{20}$ on the RNA templates. First-strand cDNA synthesis was performed at 37 ^{0}C for 1 h, then the enzyme was inactivated at 70 ^{0}C for 15 min.

2.8 Real time qPCR

Relative quantification of gene expression against reference genes was performed on a StepOne™ Real time PCR System (ThermoFisher Scientific, USA), using KAPA SYBR® FAST qPCR Kit Master Mix (2 X) ABI PRISM™. Primers for *ZNF217*, *BMP4*, *CDKN1A*, *BGLAP* and for reference gene *RPLPO* were purchased from Eurofins Genomics, Germany, and ThermoFisher Scientific, USA. The sequences are listed in Table 2. Each 10 μl-reaction was prepared with cDNA (1 μl), gene-specific forward and reverse primers (1 pmol of each) and 1 X Master Mix. A Fast Program was selected for all reactions, with a universal primer annealing temperature of 60 ^{0}C. The Ct values were analyzed on Microsoft Excel and the charts were constructed on GraphPad Prism 8.

Table 2

Primer	Sequence (5' to 3')
ZNF217 forward	CAGCGAGGTCGATTCTCCAA
ZNF217 reverse	GGCCTTTTTCCTTCTAACGTCG
CDKN1A forward	GCAGACCAGCATGACAGATTTC
CDKN1A reverse	ATGTAGAGCGGGCCTTTGAG
BMP4 forward	GGAGGAGGAGGAAGAGCAGA
BMP4 reverse	CCAGATGTTCTTCGTGGTGGA
BGLAP forward	CCTCACACTCCTCGCCCTAT
BGLAP reverse	TGCTTGGACACAAAGGCTGC
RPLPO forward	AATGTGGGCTCCAAGCAGAT
RPLPO reverse	TGAGGCAGCAGTTTCTCCAG

3. Results

3.1 BMP-2 peptide promotes the differentiation of DPSCs to osteoblasts more effectively than full-length BMP-2

In this study, DSPCs (Dental Pulp Stem Cells) were induced to differentiate into osteoblasts with active dimeric human BMP-2 or BMP-2 peptide, a short peptide derived from its carboxyterminal region, that has been shown to promote osteogenesis more effectively than the full-length protein (personal communication with Dr. S. Karoulias). Four groups were tested: a) a negative control group, consisting of untreated DPSCs cultured in DMEM, b) a positive control group, consisting of DPSCs that were induced to differentiate with StemPro® Osteogenesis Complete Medium, c) DPSCs that were induced to differentiate with Osteogenesis Medium and were also treated with 50 ng/ml active dimeric human BMP-2, and d) DPSCs that were induced to differentiate with Osteogenesis Medium and were also treated with 50 ng/ml BMP2 peptide.

During the formation of their extracellular matrix (approximately 7th to 14th day of osteogenesis), proliferating osteoblasts exhibit high expression of the enzyme Alkaline Phosphatase (ALP). The detection of its activity is used frequently as a marker of successful differentiation of hMSCs into osteoblasts. ALP activity in the extracellular matrix of proliferating osteoblasts can be detected by chromogenic reaction (NBT-BCIP staining). Fourteen days after the addition of growth factor BMP-2 or BMP-2 peptide in the culture medium, ALP activity was detectable in all groups (Fig. 1a-d). However,

increased activity was observed in cells that were induced with either BMP2 or peptide 3 (Fig. 1c,d) compared to positive control cells (Fig. 1b), as indicated by multiple blue areas of reduced NBT. Negative control cells exhibited the highest level of alkaline phosphatase activity, which was comparable to the BMP2-treated and BMP-2 peptide-treated groups. The morphology of the positive control and the treated groups was indicative of proliferating osteoblasts (oval-shaped), while negative control cells maintained the elongated, fibroblast-like morphology that is typical of DPSCs.

At the final stage of osteogenic differentiation, osteoblasts form hydroxyapatite deposits in the extracellular matrix (mineralization). On the 21st day of osteogenesis, when mineralization is complete, successful bone tissue formation can be verified by staining the extracellular hydroxyapatite deposits with Alizarin Red S, a dye that binds to calcium cations. The Alizarin Red Assay showed that both the BMP-2-treated and BMP-2 peptide-treated cells exhibited higher concentration of calcium deposits compared to positive control cells. The highest concentration was observed in cells treated with BMP-2 peptide (Fig. 1e-h).

The successful induction of osteogenesis was also verified by monitoring with qPCR the marker gene *BGLAP* that codes for bone ECM glycoprotein, osteocalcin (Fig. 2). The mRNA levels of *BGLAP* in untreated DPSCs on the 7th Day of culture were set as the "reference levels". In untreated cells, *BGLAP* expression was diminished towards Day 21, by 33-fold, after a small rise (7-fold). Treatment with factors included in the Osteogenesis medium increased the levels by 280-fold on Day 7, but a decline to reference levels followed in the course of the weeks. However, treatment with BMP-2 increased the expression of osteocalcin by 50-fold on Day 21 and BMP2 peptide by almost 500-fold, compared to reference levels.

3.2 Effect of BMP-2 and BMP-2 peptide on the expression of ZNF217, BMP4 and cell-cycle inhibitor p21 gene, CDKN1A, in DPSCs and osteoblasts

The mRNA levels of *ZNF217*, *BMP4* and cell-cycle inhibitor gene *CDKN1A* (p21) were monitored by real-time qPCR on the 7th, 14th and 21st day after induction of osteogenesis with BMP2 or BMP-2 peptide. Relative quantification against reference gene *RPLP0* was performed. In untreated DPSCs (negative control), *ZNF217* expression declined 2.5-fold by Day 14 and 6-fold by Day 21, compared to Day 7 (Fig. 3a). Induction of differentiation with Osteogenesis Medium caused a temporary 2-fold decrease on Day 7, followed by a 4-fold increase on Day 14 and by a 4-fold decrease on the gene's mRNA levels. However, treatment with BMP-2 diminished the expression of the gene in the proliferating osteoblasts during the second week of differentiation. Treatment with BMP-2 peptide had almost immediate effect, reducing the expression on Day 7 by almost 6-fold compared to reference levels. The expression returned to almost reference levels upon Day 21. These results suggest that initial treatment of DPSCs with 50 ng/ml exogenous BMP-2 or BMP-2 peptide can profoundly impact the oncogene's expression during the first two weeks of osteogenesis.

In untreated DPSCs, BMP-4 expression remained unchanged during the weeks. (Fig. 3b). Induction of differentiation with osteogenesis medium was accompanied by an increase by 3- up to 6.5-fold in the gene's expression. Treatment with BMP-2 or BMP-2 peptide had an almost immediate and profound

effect on BMP-4 mRNA levels. BMP-2-treated proliferating osteoblasts exhibited 57-fold increase in the gene's expression on Day 7, compared to Day 7 untreated cells. BMP-2 peptide-treated proliferating osteoblasts had an almost 1800-fold increase on the same day. In the BMP-2-treated group, the expression dropped to the positive control group's levels after three weeks (Day 21). However, the BMP-2 peptide-treated group seemed to regain a high BMP-4 expression level by Day 21 (24-fold). These results suggest that initial treatment of DPSCs with exogenous BMP-2 may enhance the production of BMP-4 during the first week of osteogenic differentiation. However, similar treatment with 50 ng/ml BMP-2 peptide seems to impact the gene's expression almost 17 times as much as BMP-2 and to cause a second increase at the last stage of differentiation.

p21 is a cell-cycle inhibitor belonging to the Cip/Kip family of Cyclin-dependent kinase inhibitors. The expression of its gene, *CDKN1A*, was quantified and correlated with the expression pattern of *ZNF217* and *BMP-4*. The mRNA levels of *CDKN1A* in untreated DPSCs on the 7th day of culture were set as "reference levels" (Fig. 3c). In untreated cells, *CDKN1A* expression declined over the weeks, in a manner that resembled *ZNF217*, 15-fold by Day 14 and 24-fold by Day 21, compared to reference levels (Day 7). This suggests that the DPSCs continued to multiply until Day 21, retaining their undifferentiated state. During differentiation induced by the osteogenesis medium, *CDKN1A* mRNA levels decreased 3.3-fold compared to reference levels on Day 7, increased up to reference level by Day 14 followed by a second, 6-fold, decrease by Day 21. The evident increase in CDKN1A mRNA levels compared to untreated DPSCs in expected of the gradual entry of osteoblasts into a quiescent, differentiated state. Unexpectedly, in BMP-2-treated cells, *CDK1NA* expression dropped up to 86-fold by Day 21. In BMP-2 peptide-treated cells, *CDKN1A* expression dropped 14-fold on the 14th day but returned to the levels of the positive control in the late stage of differentiation.

3.3 Effect of BMP-2 and BMP-2 peptide on the expression of ZNF217, BMP4 and cell-cycle inhibitor p21 gene, CDKN1A, in MCF-7 breast cancer cells

The mRNA levels of *ZNF217*, *BMP4* and cell-cycle inhibitor gene *CDKN1A* (p21) were monitored by real-time qPCR 6, 12, 24 and 48 hours after treatment with 100 ng/ml BMP2 or different concentrations of BMP-2 peptide (45.2 ng/ml, 22.6 ng/ml and 4.52 ng/ml). The mRNA levels of *ZNF217* in untreated MCF-7 at 6 h of culture were set as the "reference levels" for *ZNF217*. Similarly, the mRNA levels of *BMP4* and *CDKN1A* in untreated MCF-7 at 6 h of culture were set as the reference levels for each of these genes, respectively.

In untreated MCF-7 cells, *ZNF217* mRNA levels quadrupled at 12 h compared to 6-h reference control (Fig. 4a), then decreased at 24 h and 48 h. Treatment with BMP-2 caused only a 2-fold increase at 12 h, but a 5-fold increase at 24 h, compared to reference levels. Therefore, BMP-2-treated MCF-7 seemed to display induction of the oncogene's expression later than the untreated group. Treatment with 45.2 ng/ml BMP-2 peptide caused an early 2-fold increase at 6 h, followed by 4-fold increase at 12 h before returning to reference levels at 24 h. Finally, at 48 h, an increase was observed, compared to 48 h negative control. The lowest concentration of BMP-2 peptide also seemed to have an upregulatory effect on *ZNF217* that

lasted until 48 hours later. However, an intermediate concentration of 22.6 ng/ml BMP-2 peptide had a different effect. It slowed the upregulation that was observed in the untreated group at 12 h, causing only 3.5-fold increase at this time point. At 24 h, *ZNF217* mRNA levels, decreased almost to reference level, while the untreated group had a 2-fold increase at the same time point. Finally, at 48 h, the group treated with 22.6 ng/ml BMP-2 peptide had approximately the same ZNF217 mRNA level as the untreated group.

In untreated MCF-7, BMP-4 was downregulated 3-fold at 24 h compared to the 6-h reference level, but then increased almost 5-fold between 24 h and 48 h. (Fig. 4b). Treatment with BMP-2 caused severe downregulation of the *BMP-4* gene at 24 h, when its mRNA levels were undetectable. However, between 24 h and 48 h, the expression increased, reaching the same levels as 48-h untreated cells. MCF-7 cells that were treated with 45.2 ng/ml or 22.6 ng/ml BMP-2 peptide seemed to retain *BMP-4* expression approximately at reference levels during the first 24 hours and showed a 2.5-fold increase at 48 h. On the contrary, MCF-7 treated with the lowest concentration of BMP-2 peptide, 4.52 ng/l (equimolar to 100 ng/ml BMP-2), showed 2-fold reduction in *BMP-4* mRNA levels at 6 h, followed by gradual increase that reached 4-fold the reference level at 48 h.

CDKN1A expression was monitored at 6 h, 12 h, 24 h and 48 h and was correlated with the expression of ZNF217. In untreated MCF-7, *CDKN1A* mRNA levels dropped 334-fold at 24 h and returned to reference levels at 48 h (Fig. 4c). However, treatment with 100 ng/ml BMP-2 or the higher concentrations of BMP-2 peptide seemed to lessen this downregulatory phenomenon. BMP-2-treated MCF-7 showed only 36-fold reduction in *CDKN1A* expression at 24 h, MCF-7 treated with 45.2 ng/ml BMP-2 peptide showed 42-fold and, those treated with 22.6 ng/ml Peptide 3, 54-fold reduction in *CDKN1A* mRNA levels at 24 h. Cells treated with the lowest concentration of BMP-2 peptide (4.52 ng/ml) showed approximately 220-fold downregulation of *CDKN1A* expression at 24 h. The MCF-7 groups that had been treated with BMP-2 peptide also showed a small reduction at 6 h (2-fold up to 7-fold), which was not observed in the BMP-2 treated group. It was also observed that the cells that had been treated with 4.52 ng/ml or 22.6 ng/ml had a 3-fold increase in *CDKN1A* expression at 48 h, compared to reference levels, which was not observed in the rest of the groups.

4. Discussion

In this study, we tried to determine how a key regulator of osteogenic differentiation, Bone Morphogenetic Protein-2, and a short functional peptide from its carboxyterminal region, BMP-2 peptide, may affect the expression of *ZNF217* in DPSCs that undergo osteogenic differentiation and in a breast cancer cell line, MCF-7.

4.1 Osteogenic differentiation with BMP-2 and BMP-2 peptide

DPSCs were incubated in osteogenesis medium in the presence of either exogenous BMP-2 or BMP-2 peptide, an active decapeptide derived from the carboxyterminal region of BMP-2. Alkaline Phosphatase and Alizarin Red Staining assays were performed on the 14th and the 21st day of osteogenesis respectively, to verify the differentiation of DPSCs to osteoblasts and the formation of mineralized ECM.

Additionally, the mRNA levels of osteocalcin-coding gene *BGLAP*, a marker of successful bone ECM formation, were monitored by real-time qPCR in DPSCs and osteoblasts at three stages of differentiation, on the 7th, 14th and 21st day. Alkaline phosphatase activity was detected (Fig. 1), which indicates that differentiation of DPSCs was successful. The blue regions were more abundant in DPSCs that had been treated with BMP-2 or BMP-2 peptide, compared to DPSCs that differentiated only in the presence of osteogenesis medium. Abundant blue staining observed in the negative control DPSCs is possibly caused by the presence of agents in the medium (DMEM) that reduced NBT to blue precipitate. The shape of the cells that differentiated in osteogenesis medium and those that had been treated with BMP-2 or BMP-2 peptide became oblate in the course of osteogenesis, in contrast to undifferentiated DPSCs whose shape remained elongated and spindle-shaped (Fig. 1).

The staining of calcium deposits in the extracellular matrix of osteoblasts with Alizarin Red S indicated the successful mineralization of the excreted ECM on the 21st day of osteogenesis (Fig. 1). The cells that were cultured in osteogenesis medium without exogenous factors exhibited few and spread red-stained regions (hydroxyapatite deposits) in the extracellular matrix. On the contrary, the red regions were more abundant in wells with DPSCs that had been differentiated under the effect of BMP-2 and the most abundant in wells with DPSCs that had been treated with BMP-2 peptide. Additionally, the monitoring of *BGLAP* expression in DPSC and osteoblasts indicated high production of osteocalcin in all the groups that had been induced to differentiate, while negative control DPSCs maintained the lowest expression throughout the 21 days (Fig. 2). The cells that had been treated with BMP-2 peptide showed the highest expression of *BGLAP*, especially on the 7th and 21st day, which supports the findings that the short peptide is a more efficient inducer of differentiation of DPSCs into osteoblasts than the full-length growth factor.

4.2 *ZNF217* is downregulated after treatment of DPSCs with BMP-2 peptide

The expression of transcription factor gene *ZNF217* was monitored during the differentiation of DPSCs to osteoblasts (Fig. 3a). Untreated DPSCs showed gradual downregulation of *ZNF217* expression over the weeks (2.5-fold down on Day 14 and 6-fold down on Day 21). Positive control cells (DPSCs that differentiated only under the effect of factors contained on the osteogenesis medium) exhibited 4-fold upregulation of *ZNF217* on Day 14, during their proliferative stage. This was followed by 8-fold decline by Day 21, when mineralization of the ECM was complete. Interestingly, treatment of DPSCs with 50 ng/ml dimeric human BMP-2 at the beginning of their differentiation process, had an upregulatory effect on *ZNF217* on Day 7, which was followed by gradual downregulation, up to 3-fold by Day 21. On the contrary, treatment with 50 ng/ml BMP-2 peptide seemed to have an early and strong downregulatory effect on the gene's expression (6-fold down on Day 7). Considering the observations about the different signaling induced by BMP-2 and BMP-2 peptide, it is possible that the short peptide avoids and/or inhibits pathways that would otherwise lead to upregulation of *ZNF217*, as observed in positive control and BMP-2-treated cells, or activates pathways that suppress the gene's transcription.

CDKN1A mRNA levels were quantified and studied alongside *ZNF217*, to assess the effect that BMP-2 and BMP-2 peptide may have on cell division during osteogenesis. The product of *CDKN1A*, p21, known

to bind to and inhibit E-CDK2 and A-CDK2 during G_1 and S phases, causing cell cycle arrest in G_1, in case of DNA damage and other types of cellular stress. However, it has also been shown that p21 increases normally in response to mitogenic signaling, through the ERK pathway, to form complexes with D-CDK-4/6. The binding activates the D-CDKs and promotes G_1 progression, while at the same time it alleviates the negative control on CDK2 complexes, which are responsible for the progression of S phase. In response to cellular stress, *CDKN1A* expression is induced in a p53-dependent manner and high levels of the protein accumulate, thus inhibiting CDK2 complexes and preventing the progression of S-phase and DNA replication [19–21]. Several studies, which will be discussed in the following section, have shown that BMP-2 reduces the production of p21 in breast cancer cells and causes cell cycle arrest. However, the effect of BMP-2 on this critical cell cycle regulator has not yet been studied in healthy mesenchymal cells during osteoblastic differentiation. In the investigation presented herein, *CDKN1A* expression declined over the weeks in untreated DPSCs, up to 24-fold compared to reference levels (Fig. 3c), indicating that the cells continued to proliferate. The induction of osteogenesis in positive control cells was accompanied by evident increase in p21 mRNA levels after the first week, as expected, indicating the gradual entry into a differentiated state. Interestingly, treatment with BMP-2 was associated with dramatic drop in *CDKN1A* mRNA levels, especially towards the final stage of osteogenesis (Day 21), when they were 14 times lower than those of the positive control on Day 21. This is in contrast with what has been observed in cancer cell lines, both in published studies and in this investigation, as will be discussed later on [4, 16, 22, 23]. On the other hand, BMP-2 peptide-treated cells exhibited downregulation of *CDKN1A* during the first weeks, but, by Day 21, the mRNA levels increased and became equal to Day 21-positive-control levels. Therefore, it seems that BMP-2 and the peptide act similarly during the first 7 days of osteogenesis, but different signaling networks probably begin to affect the expression of p21 in the weeks that follow.

It is interesting, that in BMP-2-treated DPSCs, upregulation of *ZNF217* on Day 7 preceded the downregulation of *CDKN1A* on Days 14 and 21. Similarly, *ZNF217* in BMP-2 peptide-treated cells, downregulation of *ZNF217* in BMP-2 peptide-treated cells preceded the increase in *CDKN1A* mRNA levels on Day 21. ZNF217 has been found to form a complex with the ubiquitin-ligase that regulates the stability of p53, Mouse Double Minute homolog-2 (MDM2), and, as a result, the transcription of the *CDKN1A* gene. Possible ZNF217 binding sites have also been found *in silico* in the promoter of *CDKN1A* [24]. Therefore, ZNF217 may act directly and/or indirectly as a negative regulator of CDKN1A expression in osteoblasts. Treatment with BMP-2 or BMP-2 peptide at the beginning of the differentiation process seems to affect the interplay between ZNF217 and p21 profoundly.

BMP-4 is a major regulator of osteoblastic differentiation and is known to act through the same receptors as BMP-2 [1, 25]. While, in untreated DPSCs and positive control cells, BMP-4 expression remained relatively low, in BMP-2-treated and BMP-2 peptide-treated cells there was significant upregulation of the *BMP4* gene during the first week of differentiation (Fig. 3b). Especially, in the case of BMP-2 peptide, *BMP4* mRNA levels increased almost 1800-fold by Day 7 and dropped to reference levels by Day 14, before increase again 24-fold by Day 21. Evidently, BMP-2 peptide is a potent promoter of osteogenesis,

increasing the production of another Bone Morphogenetic factor early in the differentiation process and keeping it at relatively high levels even after the mineralization process has begun.

4.3 BMP-2 or BMP-2 peptide can increase CDKN1A expression and maintain low ZNF217 and BMP4 mRNA levels in MCF-7 breast cancer cells

The results of several studies on breast cancer cell lines and breast tumors with metastatic potential to the bone suggest an interplay among BMP signaling, ZNF217 and p21, which affects the proliferation and metastasis of the cells. Overexpression of *BMP4* or exogenous addition of the protein in breast cancer cell lines, including MCF-7 and MDA-MB-231, has been shown to cause growth arrest and increase migration and invasion. Inhibition of BMP-4 signaling via *BMP4* siRNA or noggin, reduces migration and invasion of these cells [17]. Overexpression of *BMP4* has been associated with high probability of relapse in patients with breast tumors [26]. It has also been found that administration of BMP-4 to mice increases the probability of the emergence of metastatic tumors to the bone [27]. On the other hand, BMP-2 has been associated with cell cycle arrest and inhibition of invasion of breast cancer cells. It has been found that *BMP2* is downregulated during the first stages of malignant transformation of stem cells and downregulation of *BMP2* in patients with primary breast tumors has associated with poor prognosis [22, 23]. MTT and immunoblotting assays have demonstrated that BMP-2 inhibits estradiol-induced proliferation of MCF-7 cells, by inducing the production of p21, which leads to inactivation of CDK-2 and hypophosphorylation of Rb [4]. A recent study has shown that MDA-MB-231 breast cancer cells that overexpress *ZNF217* show significant downregulation of *BMP2* and upregulation of *BMP4*, of BMP-receptor genes *BMPR1A*, *BMPR1B* and *BMPRII* and of the target genes of BMP-SMAD signaling, *ID* and *RUNX*, compared to MDA-MB-231 that do not overexpress *ZNF217* [16].

In this study, it was attempted to determine how BMP-2 and BMP-2 peptide affect the expression of *ZNF217*, *CDKN1A* and *BMP4* in MCF-7 at different time points and if there is an optimal range of BMP-2 peptide concentration that can reduce both the proliferative and the invasive potential of breast cancer cells. In this investigation, MCF-7 that had been treated with BMP-2 or different concentrations of BMP-2 peptide in the culture medium exhibited increased *CDKN1A* expression compared to untreated MCF-7 (Fig. 4c). The expression pattern was similar; *CDKN1A* mRNA levels decreased at 24 h and increased again up to reference levels at 48 h. The population doubling time for MCF-7 cultures is approximately 29 h [28]. Therefore, there seems to be a fluctuation in p21 levels along the phases of the cell cycle, which is affected by the signaling induced by BMP-2 and BMP-2 peptide. MCF-7 treated with 100 ng/ml BMP-2 had almost 10 times higher *CDKN1A* mRNA levels at 24 h than untreated cells. Similarly, MCF-7 treated with 45.2 ng/ml or 22.6 ng/ml BMP-2 peptide had 8 times higher and 6 times higher *CDKN1A* mRNA levels than untreated cells at 24 h, respectively. Treatment with low concentration of BMP-2 peptide (4.52 ng/ml, equimolar to 100 ng/ml BMP-2) had a small upregulatory effect on *CDKN1A* at 24 h, but caused 3-fold upregulation of *CDKN1A* at 48 h compared to 48 h-untreated cells. 3-fold upregulation at 48 h was also observed in MCF-7 treated with 22.6 n/ml BMP-2 peptide. It seems that BMP-2 at a concentration of 100 ng/ml and BMP-2 peptide concentrations that are 5 times (22.6 ng/ml) or 10 times

(45.2 ng/ml) the molarity of 100 ng/ml BMP-2 upregulate the expression of *CDKN1A*, 24 h after their addition to the *in vitro* culture. At a concentration of 22.6 ng/ml, BMP-2 peptide can promote further upregulation of the CDK inhibitor after the first 24 h.

The expression of oncogene *ZNF217* in untreated cells increased 4-fold at 12 h, then began to decrease until it reached 1.6-fold the reference level at 48 h (Fig. 4a). Treatment with BMP-2 was associated with a later increase in *ZNF217* expression (5-fold upregulation at 24 h). However, BMP-2 peptide, at the concentration of 22.6 ng/ml, reduced the mRNA levels at 12 h, 24 h and 48 h, while keeping p21 levels higher, as discussed previously.

BMP4 mRNA levels in untreated MCF-7 decreased 3-fold at 24 h, then increased up to 2-fold the reference level at 48 h (Fig. 4b). In BMP-2 treated MCF-7, *BMP4* expression followed a similar pattern, but decreased to undetectable levels at 24 h. On the other hand, BMP-2 peptide altered the expression pattern of *BMP4*, in a concentration-dependent manner. The cells that had been treated with 45.2 ng/ml or 22.6 ng/ml BMP-2 peptide did not exhibit the downregulatory effect at 24 h which was observed in untreated and in BMP-2-treated MCF-7. Moreover, at 48 h, they exhibited 50% higher *BMP4* mRNA levels than untreated and in BMP-2-treated MCF-7. Treatment with low concentration of BMP-2 peptide caused an early downregulation at 6 h, followed by increase to reference levels at 12 h, 50% decrease at 24 h and 4-fold increase at 48 h.

Therefore, it is apparent that equimolar concentrations of BMP-2 and of BMP-2 peptide do not affect key genes of cell proliferation and BMP signaling similarly in MCF-7 cells. However, both BMP-2 and BMP-2 peptide, at higher concentrations, can alter the expression of certain genes, to slow cell division and possibly the BMP-4-dependent invasive potential. The results of this study show that BMP-2 can increase the expression of CDK inhibitor p21 gene, *CDKN1A*, and decrease the expression of *BMP4*, whose product is associated with increased invasive potential and metastasis of breast cancer cells to the bone. However, it is not as efficient in reducing *ZNF217* expression. BMP-2 peptide, at a concentration of 22.6 ng/ml, can increase the expression of *CDKN1A*, while reducing the expression of oncogene *ZNF217*. Contrary to BMP-2, it has a small upregulatory effect on *BMP4.*

5. Conclusions

Altogether, these results show that BMP-2 and the active peptide AISMLYLDEN can affect key genes of cell-cycle regulation and of BMP signaling when administered to healthy stem cells undergoing osteogenic differentiation and to MCF-7 cancer cells. The BMP-2 peptide downregulates the expression of oncogene *ZNF217* during the first two weeks of osteogenic differentiation, while successfully promoting the differentiation of DPSCs and the formation of mineralized bone ECM. Also, both BMP-2 and the peptide, in a concentration-dependent manner, downregulate *ZNF217* and upregulate the cell-cycle inhibitor p21 gene in less than 48 hours after being added to MCF-7 cultures. BMP-2 reduces to undetected levels the expression of invasion-associated factor BMP4 in MCF-7 cultures within 24 h. Administration of the BMP-2 peptide at the appropriate concentration or of a combination of BMP-2 and

BMP-2 peptide could be considered and studied further as a therapeutic method in breast tumors with a metastatic tendency to the bones.

Declarations

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Author contributions statement

AM performed most of the experimental procedures and participated in the writing of the manuscript. PL contributed to the interpretation of the staining assays' and the gene analyses' results and participated in the writing of the manuscript. SK tested various short regions of the C-terminal region of BMP-2 and found the active decapeptide that was used in this project. RP contributed to the qPCR experimental procedures and participated in writing the manuscript. TC-P conceived, designed and coordinated the project and participated in writing the manuscript. All authors have read the manuscript and approved the submitted version.

Additional information

The authors declare no competing interests.

References

1. Kawabata, M., Imamura, T. & Miyazono, K. Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev.* **9**, 49–61 (1998).
2. Bragdon, B. *et al.* Bone Morphogenetic Proteins: A critical review. *Cell. Signal.* **23**, 609–620 (2011).
3. Beederman, M. *et al.* BMP signaling in mesenchymal stem cell differentiation and bone formation. *J. Biomed. Sci. Eng.* **6**, 32–52 (2013).
4. Ghosh-Choudhury, N. *et al.* Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells. *Biochim. Biophys. Acta* **1497**, 186–196 (2000).

5. Arnold, S. F., Tims, E. & Mcgrath, B. E. Identification of bone morphogenetic proteins and their receptors in human breast cancer cell lines: importance of BMP-2. *Cytokine* **11**, 1031–1037 (1999).
6. Wang, L. *et al.* BMP-2 inhibits tumor growth of human renal cell carcinoma and induces bone formation. *Int. J. Cancer* **131**, 1941–1950 (2012).
7. Wang, L. *et al.* BMP-2 inhibits the tumorigenicity of cancer stem cells in human osteosarcoma OS99-1 cell line. *Cancer Biol. Ther.* **11**, 457–463 (2011).
8. Sieber, C., Kopf, J., Hiepen, C. & Knaus, P. Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev.* **20**, 343–355 (2009).
9. Katagiri, T. *et al.* Identification of a BMP-responsive element in Id1 , the gene for inhibition of myogenesis. *Genes to Cells* **7**, 949–960 (2002).
10. Ryoo, H. M., Lee, M. H. & Kim, Y. J. Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. *Gene* **366**, 51–57 (2006).
11. Krig, S. R. *et al.* Identification of genes directly regulated by the oncogene ZNF217 using chromatin immunoprecipitation (ChIP)-chip assays. *J. Biol. Chem.* **282**, 9703–9712 (2007).
12. Collins, C. *et al.* Comprehensive Genome Sequence Analysis of a Breast Cancer Amplicon. *Genome Res.* **11**, 1034–1042 (2001).
13. Cowger, J. J. M., Zhao, Q., Isovic, M. & Torchia, J. Biochemical characterization of the zinc-finger protein 217 transcriptional repressor complex: Identification of a ZNF217 consensus recognition sequence. *Oncogene* **26**, 3378–3386 (2007).
14. Shi, Y. *et al.* Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422**, pages735–738 (2003).
15. Lee, D.-F., Walsh, M. J. & Aguiló, F. ZNF217/ZFP217 Meets Chromatin and RNA. *Trends Biochem. Sci.* **41**, 986–988 (2016).
16. Bellanger, A. *et al.* The critical role of the ZNF217 oncogene in promoting breast cancer metastasis to the bone. *J. Pathol.* **242**, 73–89 (2017).
17. Kallioniemi, A. Bone morphogenetic protein 4-a fascinating regulator of cancer cell behavior. *Cancer Genet.* **205**, 267–277 (2012).
18. Bakopoulou, A. *et al.* Wnt/β-catenin signaling regulates Dental Pulp Stem Cells' responses to pulp injury by resinous monomers. *Dent. Mater.* **31**, 542–555 (2015).
19. Vermeulen, K., Van Bockstaele, D. R. & Berneman, Z. N. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.* **36**, 131–149 (2003).
20. Karimian, A., Ahmadi, Y. & Yousefi, B. Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. *DNA Repair (Amst).* **42**, 63–71 (2016).
21. LaBaer, J. *et al.* New functional activities for the p21 family of CDK inhibitors. *Genes Dev.* **11**, 847–862 (1997).
22. Chapellier, M. *et al.* Disequilibrium of BMP2 levels in the breast stem cell niche launches epithelial transformation by overamplifying BMPR1B cell response. *Stem Cell Reports* **4**, 239–254 (2015).

23. Davis, S. R., Watkins, G., Douglas-Jones, A., Mansel, R. & Jiang, W. G. Bone morphogenetic proteins 1 to 7 in human breast cancer, expression pattern and clinical/prognostic relevance. *J. Exp. Ther. Oncol.* **7**, 327–338 (2008).
24. Mantsou, A., Koutsogiannouli, E., Haitoglou, C., Papavassiliou, A. G. & Papanikolaou, N. A. Regulation of expression of the p21CIP1 gene by the transcription factor ZNF217 and MDM2. *Biochem. Cell Biol.* **94**, 560–568 (2016).
25. Miyazono, K., Kamiya, Y. & Morikawa, M. Bone morphogenetic protein receptors and signal transduction. *J. Biochem.* **147**, 35–51 (2010).
26. Alarmo, E. L. *et al.* Bone morphogenetic protein 4 expression in multiple normal and tumor tissues reveals its importance beyond development. *Mod. Pathol.* **26**, 10–21 (2013).
27. Ampuja, M. *et al.* The impact of bone morphogenetic protein 4 (BMP4) on breast cancer metastasis in a mouse xenograft model. *Cancer Lett.* **375**, 238–244 (2016).
28. American Type Culture Association (ATCC). MCF-7 (ATCC HTB-22). (2016). Available at: https://www.lgcstandards-atcc.org/products/all/HTB-22.aspx.

Figures

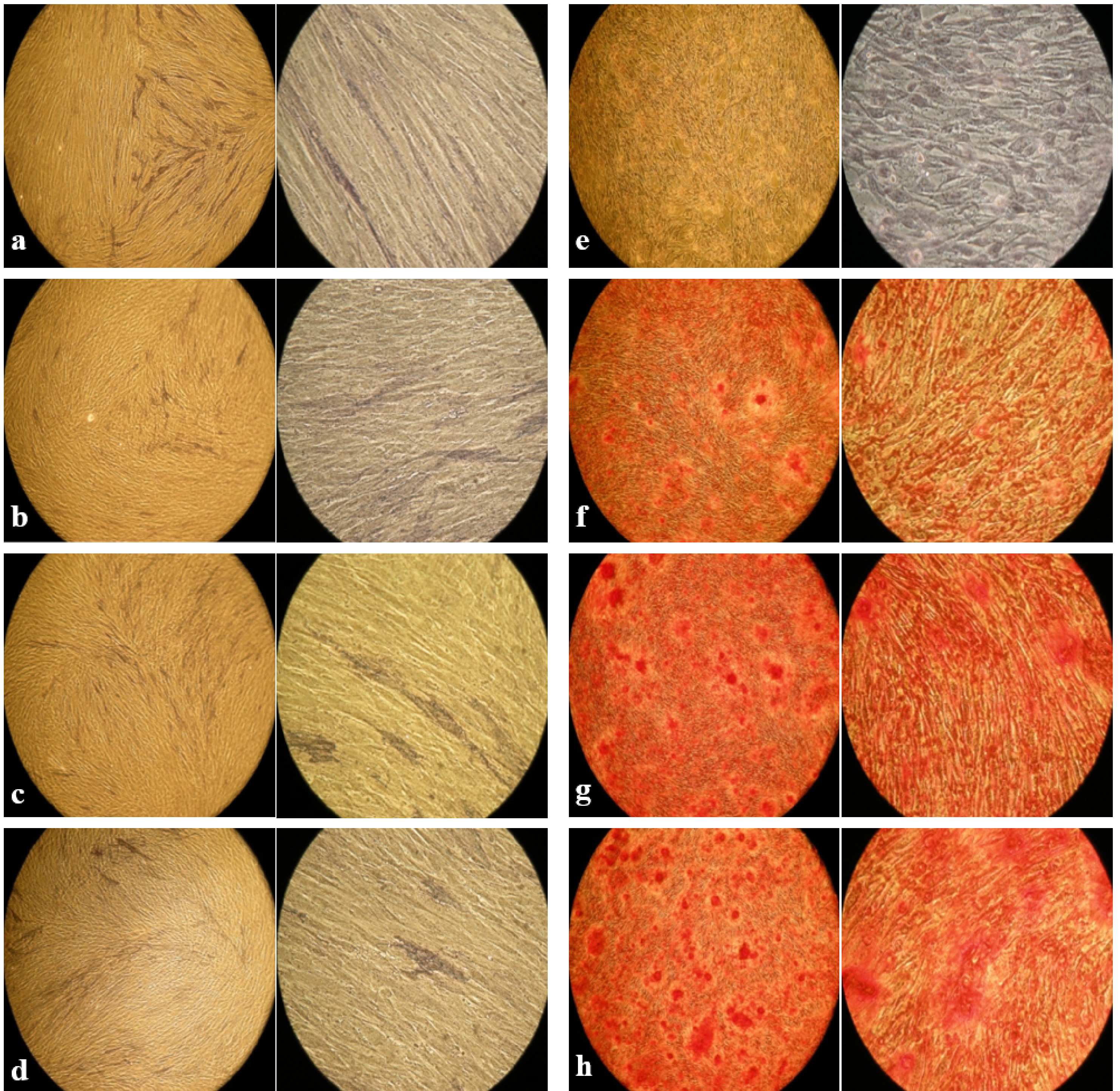
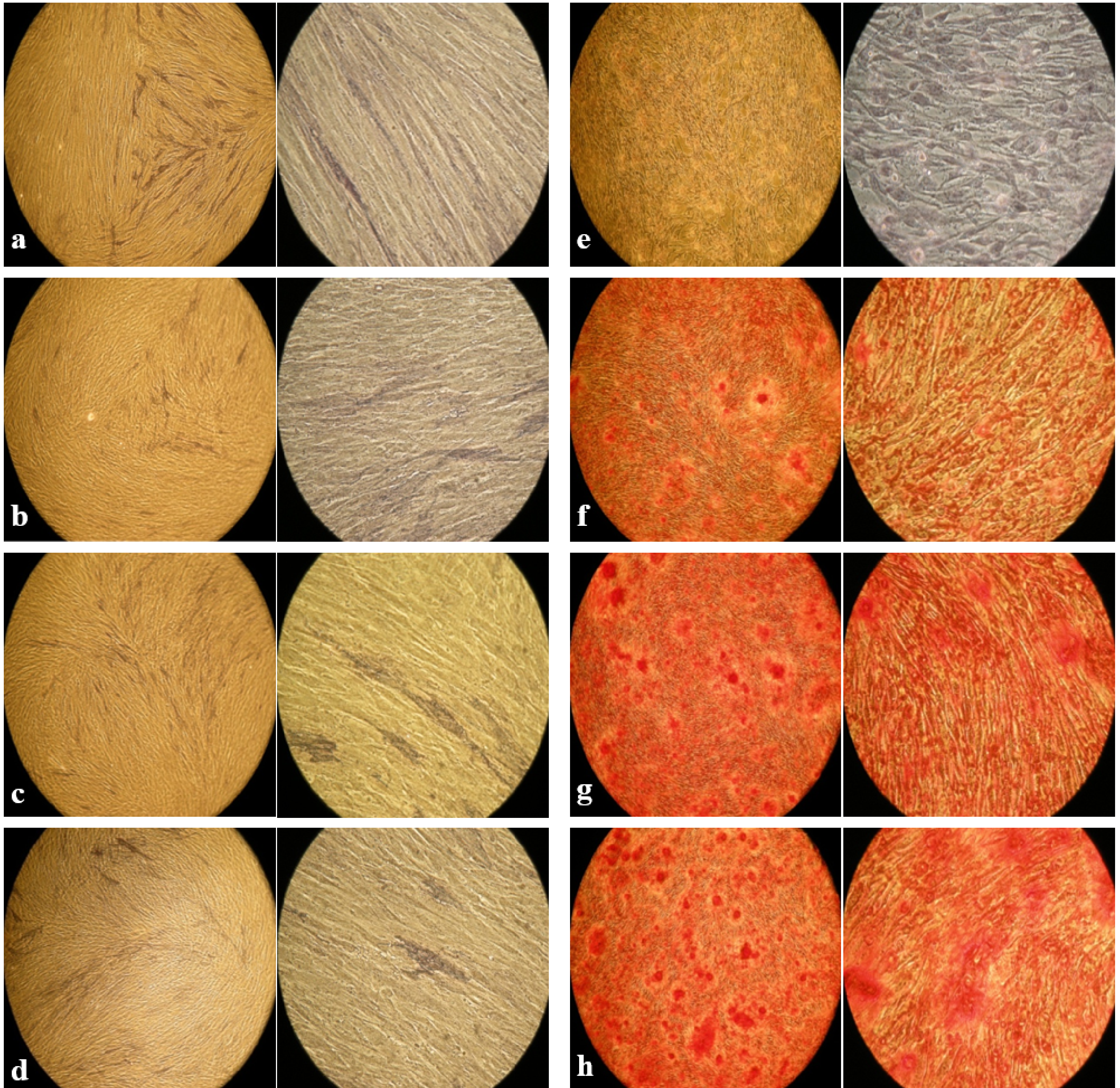


Figure 1

Staining assays for the verification of osteogenesis. (a-d): Alkaline Phosphatase Assay performed on the 14th day of osteogenesis, for the verification of successful differentiation of DPSCs into osteoblasts. a) Untreated DPSCs in DMEM (negative control), b) DPSCs in Osteogenesis Medium (positive control), c) BMP-2-treated DPSCs in Osteogenesis Medium, d) BMP-2 peptide-treated DPSCs in Osteogenesis Medium. Reduced NBT precipitate was observed in all groups. Left: 100 X magnification, right: 400 X magnification. (e-h): Alizarin Red Assay performed on the 21st day of osteogenesis, for the verification of successful mineralization of the extracellular matrix. e) Untreated DPSCs in DMEM (negative control), f)

DPSCs in Osteogenesis Medium (positive control), g) BMP-2-treated DPSCs in Osteogenesis Medium, h) Peptide 3-treated DPSCs in Osteogenesis Medium. Positive control DPSCs, BMP2-treated and BMP-2 peptide-treated DPSCs differentiated successfully into osteoblasts. Left: 100 X magnification, right: 400 X magnification.



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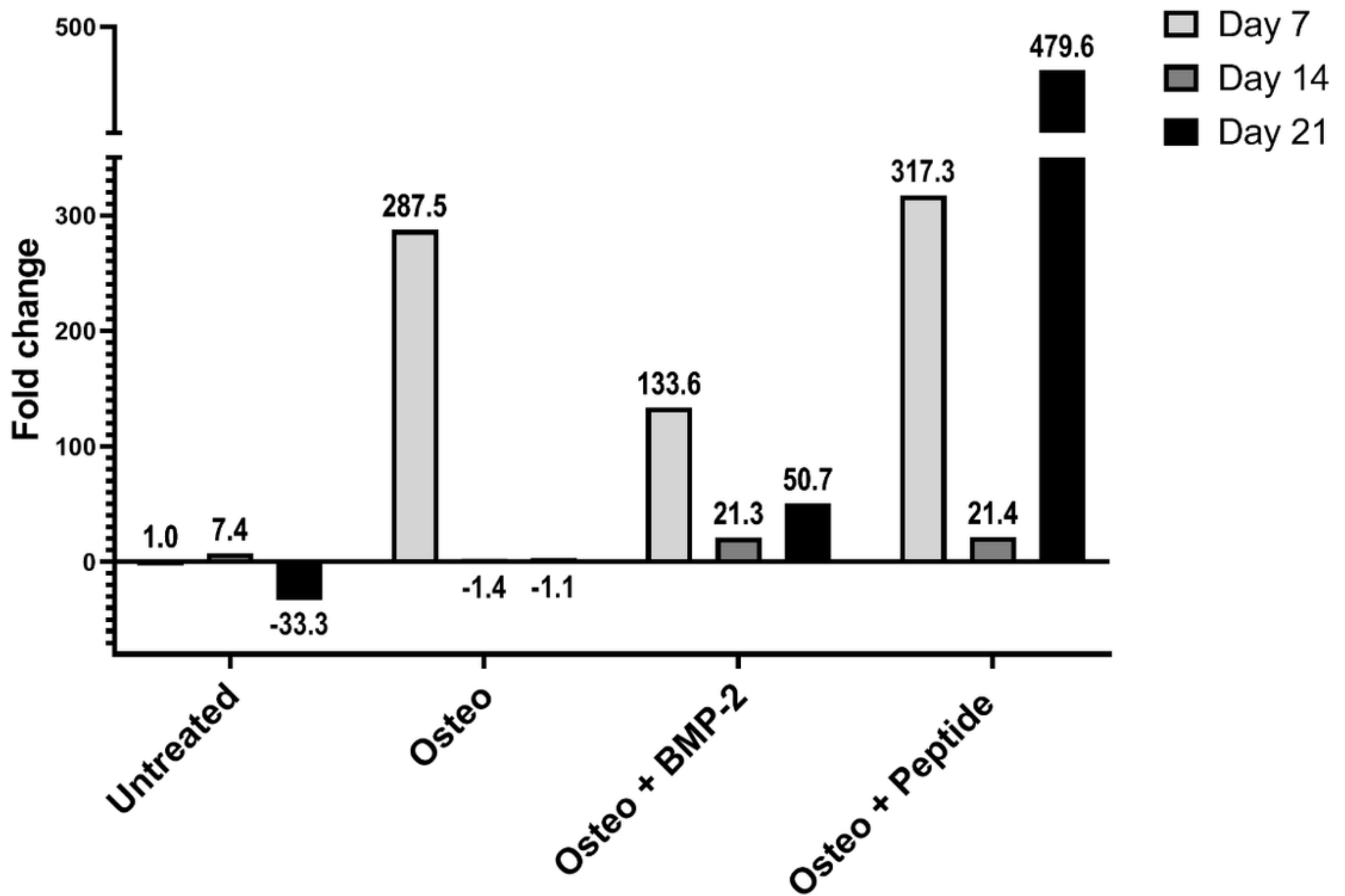

Figure 2

Fold change (FC) of BGLAP mRNA levels of osteoblasts compared to Day 7 Untreated DPSCs. Untreated: DPSCs cultured in DMEM, without factors that promote differentiation, Osteo: DPSCs treated with Stem Pro Osteogenesis Medium, Osteo + BMP-2: DPSCs treated with Stem Pro Osteogenesis Medium and 50

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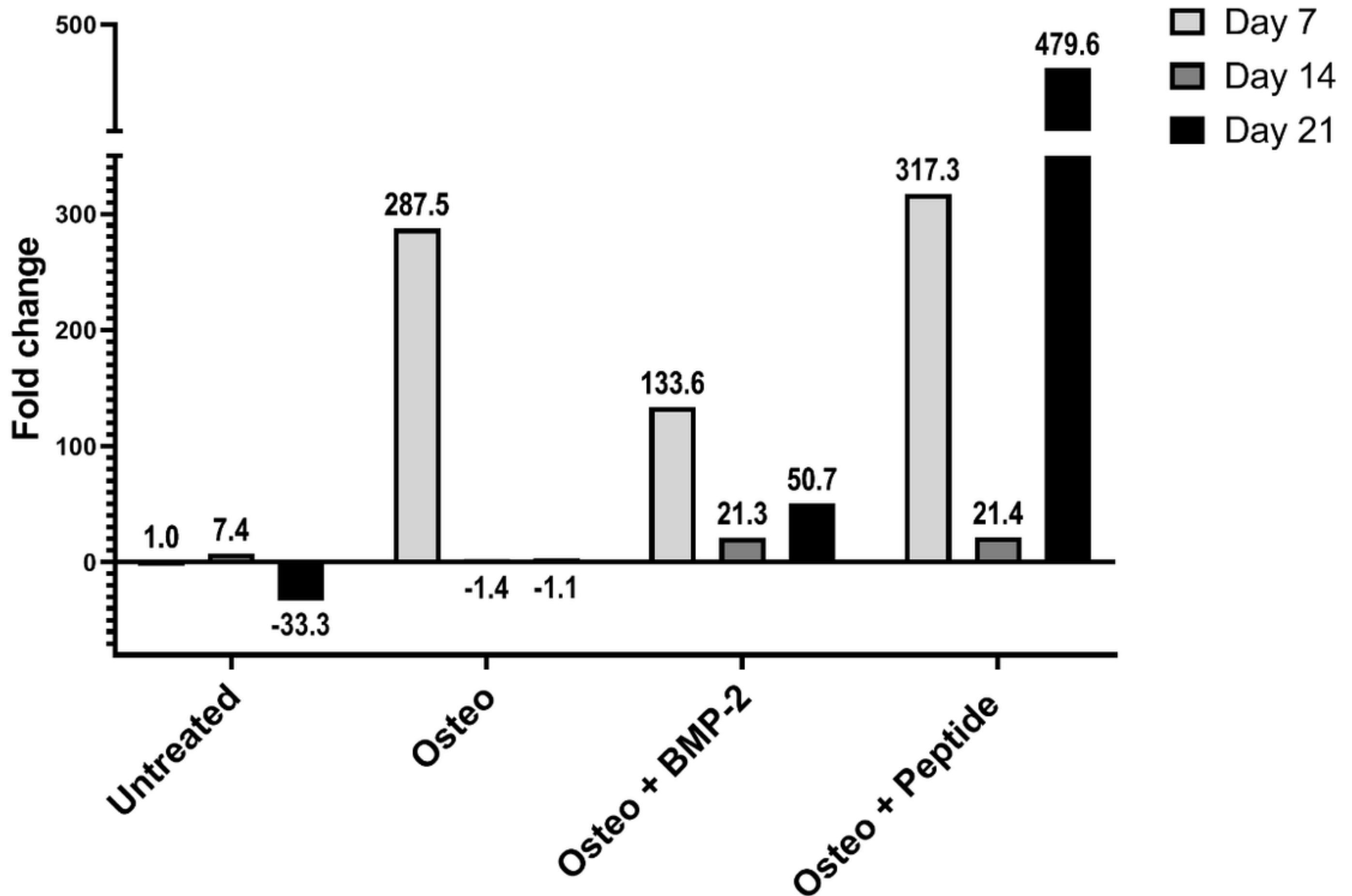

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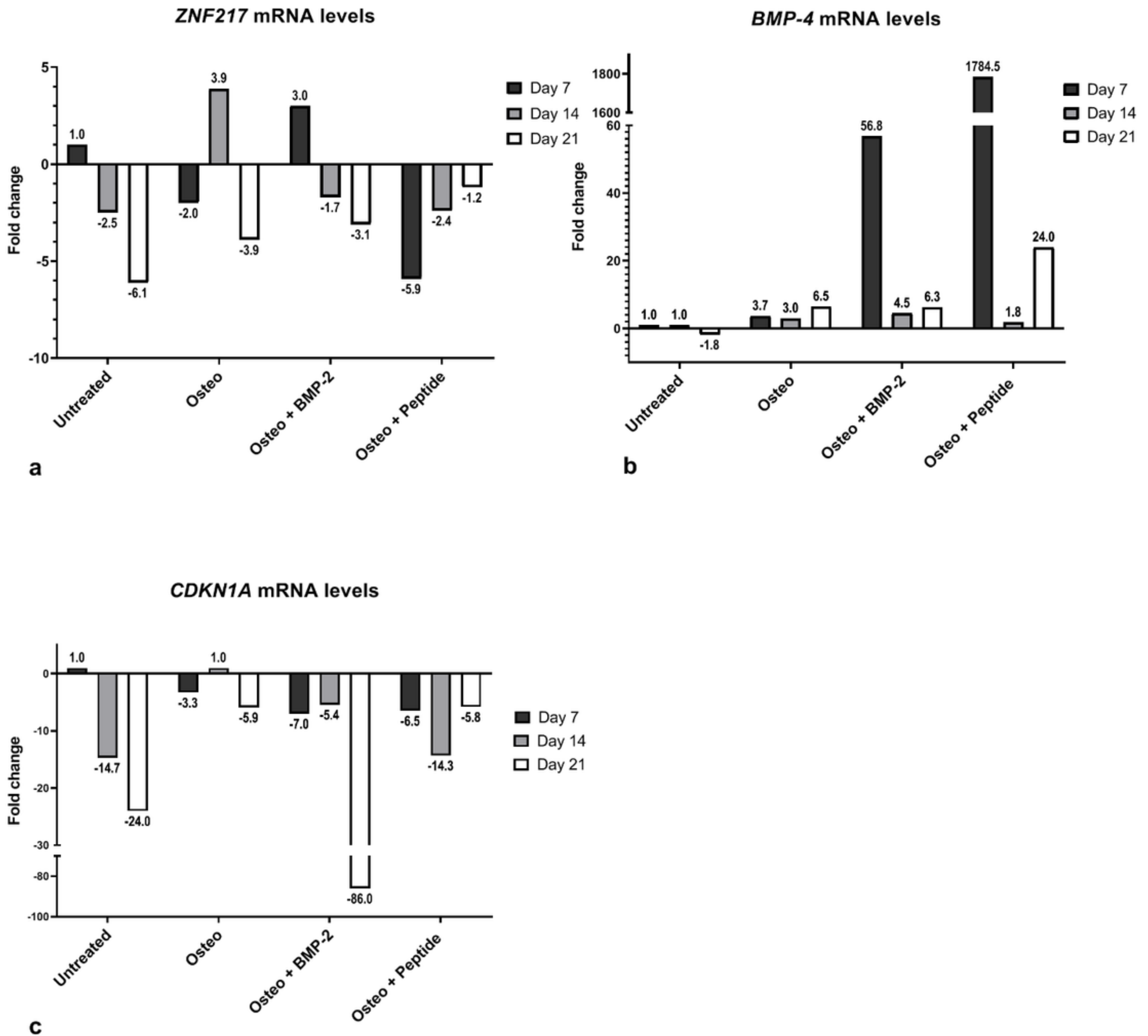
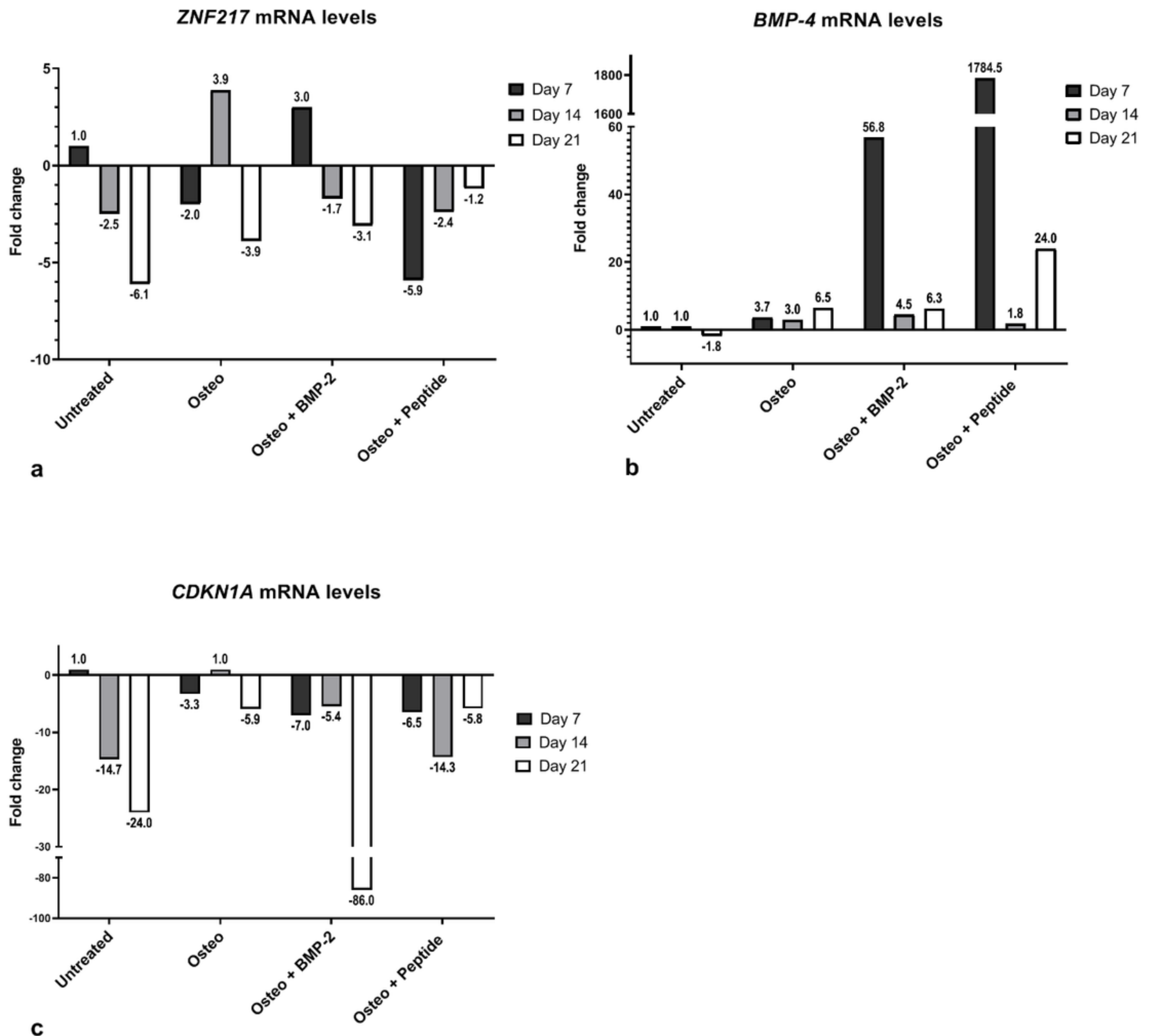


Figure 3

Fold change of ZNF217 (a), BMP-4 (b) and CDKN1A (c) mRNA levels in DPSCs and osteoblasts at 7, 14 and 21 days after induction of osteogenesis. Untreated: DPSCs cultured in DMEM, without factors that promote differentiation, Osteo: DPSCs treated with Stem Pro Osteogenesis Medium, Osteo + BMP-2: DPSCs treated with Stem Pro Osteogenesis Medium and 50 ng/ml BMP-2, Osteo + peptide: DPSCs treated with Stem Pro Osteogenesis Medium and 50 ng/ml BMP-2 peptide. The mRNA levels of untreated DPSCs at 7 days were determined as the reference levels in all of the cases, a, b and c.



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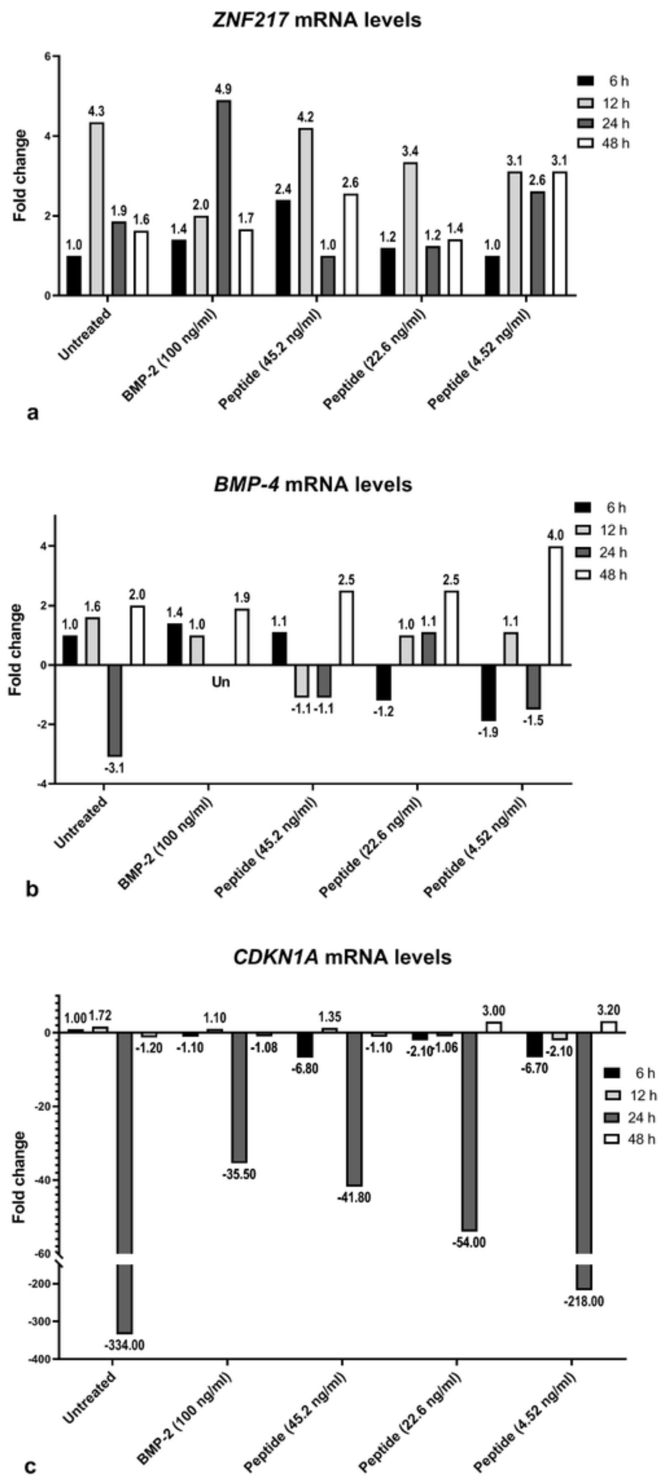

Figure 4

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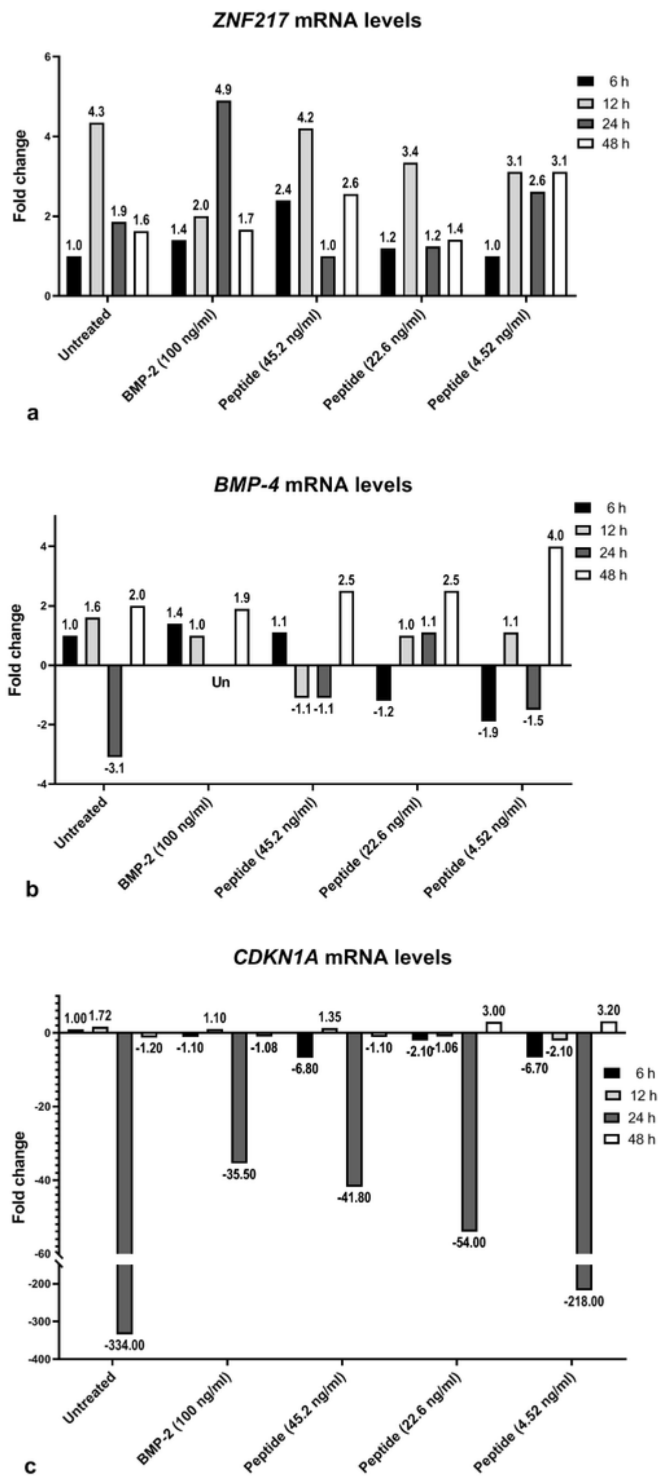

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