

Specific amino acids from the broad C-terminal region of BMP-2 are crucial for osteogenesis

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ABSTRACT

The shortest functional domains of growth factor **B**one **M**orphogenetic **P**rotein 2 (BMP-2) that are dynamical implicated in osteogenesis have been investigated and well characterized. In particular, the broad C-terminal region expanding from Val63 to Arg114 as well as its shorter sequence 86-AISMLYLDEN-95 exhibited the highest osteogenic ability for regeneration and reconstruction of bone tissue. In addition, the amino acids Ser88 and Leu90 have been identified as crucial for receptor binding and osteogenic efficacy. Furthermore, the above-mentioned domains in contrary to full length BMP-2 protein signal mainly through the Smad pathway as it is evidenced by phosphorylation decrease of Extracellular-signal-Regulated Kinase (ERK1/2). Taking together, our results are significant for clinical applications regarding the generation of biomaterials and healing of orthopedic fractures.

1. Introduction

Bone morphogenetic proteins (BMPs) that are also known as cytokines and metabologens belong to the TGF- β superfamily of growth factors (Transforming Growth Factor Beta Proteins) (Chen et al., 2004). This superfamily is consisted of proteins that regulate structural cell features (Dhillon et al., 2007). Full length BMP-2 protein is a vital morphogenetic agent that organizes the architecture of tissue throughout the body (Wagner et al., 2010) and induces osteogenic differentiation and endochondral ossification in mesenchymal stem cells (Tsumaki and Yoshikawa, 2005; Rosen, 2009; Bais et al., 2009).

BMP-2 signals through type II receptors (BMPRII) that are located at cell surface as preformed heterocomplexes or monomers (Heining et al., 2011). Growth factors bind and activate BMPRII receptors, which in turn activate type I receptors (BMPRI) initiating two signal transduction pathways (Heldin et al., 1997; Hassel et al., 2003). The canonical pathway signals through Smad proteins and the non-canonical pathway through mitogen activated protein kinases (MAPKs) (Massagué et al., 2005; Derynck et al., 2003; Sieber et al., 2009). BMP-2 binds via the C-

terminus to heterocomplexes formed by BMPRII receptors and initiates the Smad signaling pathway. In this canonical pathway R-Smads (1,5,8) are phosphorylated by BMPRI and interact with Co-Smad (Smad 4) (Yamamoto et al., 1997). The formed complex is transferred to the nucleus where Smads initiate their transcriptional activity (Miyazono, 2000; Lee et al., 2000; Banerjee et al., 2015). The non-canonical pathway is initiated in the absence of preformed receptor heterocomplexes (Yang et al., 2013). In these conditions BMP-2 bridges the two receptors through binding to BMPRII via C-terminus and BMPRI via N-terminus. BMPRII phosphorylates BMPRI, which in turn activates MAPK pathway (Iwasaki et al., 1999; Nohe et al., 2002; Gilboa et al., 2000). Finally, kinases translocate to the nucleus and activate transcriptional factors (Lai, 2002). Deregulation of signaling of BMP-2 leads to pathological conditions towards skeletal formation and fetal development and is involved in the emergence and progression of various types of cancers (Zhao, 2003; Singh and Morris, 2010).

This work is mainly focused on the ability of distinct protein regions quite shorter than its entire origin, to effectively stimulate osteogenesis. In particular, the C-terminal domain of BMP-2 (Val63-Arg114) was

Abbreviations: BMP, Bone Morphogenetic Protein; BMPR, Bone Morphogenetic Protein receptor; TGF, Transforming Growth Factor; MAPK, Mitogen-Activated Protein Kinase; ERK, Extracellular signal-Regulated Kinase; AP, Alkaline Phosphatase; B2M, beta-2 microglobulin.

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overproduced, purified and its activity on osteogenesis in comparison to full-length BMP-2 protein has been studied.

Based on previously published data from molecular simulation's approaches regarding the direct implication of residues Ser88 and Leu90 on the interaction with BMPRII receptor, the decapeptide 86-AISMLYLDEN-95 and its mutated form on amino acids Ser88 and Leu90 were synthesized, and their ability to stimulate osteogenesis was experimentally verified. In addition, signal transduction experiments clearly indicated a promotion of Smad signaling opposed to full length protein.

Taken into account the above summarized information, our data indicate that the peptide attached by bio-functionalization methodologies on scaffolds, thin layer surfaces or nanoparticles would be applied for several orthopedic problems regarding healing fractures.

2. Materials and methods

2.1. Amplification and cloning of C-terminal BMP-2

C-terminal domain of BMP-2 was amplified using as template the vector pENTR211 that expresses the mature BMP-2 gene of human origin and was purchased from Labome (USA). Specific primers were designed for amplification and cloning of C-terminal BMP-2 into pAN5 vector:

Forward 5'-CCCCTCGAGGTTTCAGACGTTGGTCAACTCTG-3',
Reverse 5'-CCGCAAGCTTCTAGCGACACCCACAACCTCC-3'.

Also, specific primers were designed for the amplification and cloning of C-terminal BMP-2 into pET29c vector (Novagen, USA) from pAN5 (Avidity, USA) recombinant vector:

Forward 5'-CCGCCATATGTCCGGCCTGAACGACATCTTCG-3',
Reverse 5'-CCGCGGATCCCTAGTGATGGTGATGGTGATGGCGACACCCACAACCTCC-3'.

Each sequence was amplified with PCR using Taq DNA polymerase kit (Finnzymes, Finland): DNA template 10–20 ng, Forward Primer 0.5 M, Reverse primer 0.5 M, Polymerase Buffer 1×, DNA polymerase 1 U/L, dNTPs 200 mM each, ddH₂O up to 50 µL. The conditions of each reaction were: a) Initial denaturation at 94 °C for 5 min, 25 cycles, b) denaturation at 94 °C for 30 s, c) annealing at 58 °C for 30 s, d) extension at 72 °C for 30 s, e) final extension at 72 °C for 7 min and storage at 4 °C. The C-terminal BMP-2 PCR product and pAN5 vector were digested with the restriction enzymes *Xho*I and *Hind*III (New England Biolabs, NEB, USA) at 37 °C for 1 h. Digested PCR products and vectors were isolated and purified with the "Gel and PCR clean up" kit (Macherey–Nagel, Germany). The ligation reaction was performed at 16 °C for 16 h using T4 DNA ligase (Takara, JAPAN) and *E. coli* TOP10 competent cells (Invitrogen, USA) were transformed with the ligation product. Positive colonies were selected after digestion with the restriction enzymes (*Xho*I

and *Hind*III). DNA sequencing results confirmed the successful cloning of C-terminal BMP-2 into pAN5 vector. The recombinant pAN5 vector including the BMP-2 C-terminal was used as a template for the amplification of the sequence of BMP-2 C-terminal together with the biotinylation sequence. The PCR product and pET29c vector were digested with *Nde*I and *Bam*HI (NEB, USA) restriction enzymes at 37 °C for 1 h. The ligation reaction was performed with T4 DNA ligase (Takara, Japan) at 16 °C for 16 h. *E. coli* TOP10 competent cells (Invitrogen, USA) were transformed with the ligation product and positive colonies were selected after restriction enzymes (*Nde*I and *Bam*HI) digestion.

2.2. Mutagenesis

Mutations were performed on Ser88Gly and Leu90Gly of BMP-2 C-terminal with Site-Directed Mutagenesis Kit (Cat. No. 200521) (Agilent Technologies, USA). The following specific primers were designed according to manufacture instructions:

For (Ser88Gly) mutation:

Forward 5'-GACAGAACTCAGTGCTATCGGGATGCTGTACCTTGA
CGAG-3',
Reverse 5'-CTCGTCAAGGTACAGCATCCCGATAGCACTGAGTTC
TGTC-3',

For (Leu90Gly) mutation:

Forward 5'-GAACTCAGTGCTATCGGGATGGGGTACCTTGACGA
GAATGAAAAG-3',
Reverse 5'-CTTTTCATTCTCGTCAAGGTACCCCATCCCGATAGCA
CTGAGTTC-3'.

2.3. Peptide design and synthesis

Three peptides of the C-terminal BMP-2 protein that according to Zouani et al. (2010) include critical amino acids for protein's/receptor interaction were synthesized by BIOMATIK (Canada). Protein and peptide sequences are indicated at Table 1. Bold letters indicate the C-terminal sequence of the entire protein.

2.4. Cloning and over expression of C-terminal wild type or mutant BMP-2 into *E. coli* BL21 (DE3) cells

The over expression of C-terminal wild-type or mutant BMP-2 was performed in *E. coli* BL21 (DE3) competent cells (NEB, USA). *E. coli* BL21 (DE3) competent cells were transformed simultaneously with recombinant pET29c vectors and pBIRAcM and cultured in LB (Luria-Bertani) agar (AppliChem, Germany) plates resistant to both kanamycin (Sigma-Aldrich) (50 µg/mL) and chloramphenicol (Sigma-Aldrich, USA) (10 µg/mL) at 37 °C for 16 h. Selected positive colonies were initially inoculated and grown in LB medium, with the appropriate antibiotic selection, at 37 °C for 16 h. Following, 10 mL of the overnight culture were inoculated in 500 mL of LB medium, containing the appropriate selective

Table 1

Amino acid sequences of wild type protein, wild type C-terminal domain, peptides and mutated C-terminal domains.

Protein/peptide	Amino acid sequence
BMP-2 wild type	MVAGTRCLLALLLPQVLLGGAAGLVPELGRKFAAASSGRPSSQPSDEVLSFEFLRLLSMFLGKQRPTPSRDA VPPPYMLDLYRRHSGQPGSPADHRRLERAASRANTVRSFHHESLEELPETS SGKTRRRFFNLSSIPTTEEFITSA ELQVRFREQMDALGNSSFFHRRINIYEIKPATANSKFPVTRLLDTRLVNQNASRWESFDVTPA VMRWTAQGHANHFVVEVAHLEEKQGVSKRHVRISRSLHQDEHSWSQIRPLLVTFGHDGKGHPLHK REKRQAKHKQRKRLKSSCKRHPLYVDFSDVGVNDWIVAPPYGHAFYCHGECFPFLADHLNSTNHA VQTLVNSVNSKIPKACCVPTLSAISMLYLDENEKVVLNKYQDMVVEGCGR
C-terminal BMP-2 wild type	VQTLVNSVNSKIPKACCVPTLSAISMLYLDENEKVVLNKYQDMVVEGCGR
Peptide 1	AISMLYLDENEKVVLNKYQD
Peptide 2	AISMGYLDENEKVVLNKYQD
Peptide 3	AISMGYLD
Mutation 1	VQTLVNSVNSKIPKACCVPTLSAISMLYLDENEKVVLNKYQDMVVEGCGR
Mutation 2	VQTLVNSVNSKIPKACCVPTLSAIGMGYLDENEKVVLNKYQDMVVEGCGR

antibiotic. Bacterial cell culture was grown with vigorous stirring at 37 °C until the optical density (OD) at 600 nm reached the value of 0.6. Induction was conducted with 1 mM IPTG (IsoPropyl β -D-1-Thio-Galactopyranoside) (Sigma-Aldrich, USA) at 37 °C for 3 h in biotin solution (50 mM in Bicine Buffer, 10 mM, pH 8.3). Cell pellet was collected after centrifugation at 6000 rpm (4500 \times g) for 20 min for further protein isolation and purification. Proteins overexpressed in pET29c vector carry the His-tag sequence at the C-terminus, that enable their purification with Ni-NTA beads (Qiagen, Germany).

2.5. Protein purification

The C-terminal region was purified by following a protocol developed as described by [Tsitouroudi et al. \(2017\)](#). Cell pellets carrying C-terminal wild-type or mutant BMP-2 were lysed in 50 mM Tris-HCl [Tris (hydroxymethyl) aminomethane Hydrochloride] (Sigma-Aldrich, USA) pH 7.5, 150 mM NaCl (Sigma-Aldrich, USA), 10% v/v Glycerol, 5 mM DTT (Sigma-Aldrich, USA) and 0.1% v/v Triton X-100 (Sigma-Aldrich, USA). Re-suspended cells were treated with low frequency and low energy ultrasound for 20 s pulse on and 40 s pulse off (6 cycles) followed by centrifugation of lysates for 15 min at 15,000 rpm and the supernatant was collected. Overexpressed proteins were tagged with His₆-tag at the Carboxyl terminal region and were purified using nickel bridging column (Ni-NTA column, agarose beads). The equilibration of the column beads was performed by 10 min rotation with equilibration buffer (50 mM Tris-HCl pH 7.5 and 150 mM NaCl) at 4 °C followed by centrifugation at 4000 rpm for 5 min (3 times). The protein extract was mixed with equilibrated column beads at 4 °C for 2 h by rotation. Three column washes were performed with 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 5 mM Imidazole (Sigma-Aldrich, USA) by rotation at 4 °C for 10 min. After every wash, beads were collected by centrifugation of the mixture at 4000 rpm for 1 min and the supernatant was discarded. Elutions were carried out by rotation at 4 °C for 15 min with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% v/v Glycerol, 5 mM DTT and 250 mM Imidazole (3 times). Buffer was exchanged by dialysis against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% v/v Glycerol and 5 mM DTT overnight at 4 °C.

Protein was identified by using the anti-His antibody or the streptavidin antibody conjugated to alkaline phosphatase because the proteins were *in vitro* biotinylated too. For the Dot Blot Assay, proteins were detected using streptavidin antibody conjugated to alkaline phosphatase. Purified proteins were transferred dropwise to different points of nitrocellulose membrane until absorbed. As positive and negative control, biotinylated GFP (Green Fluorescent Protein) and SRPK1 (Serine/Threonine-Protein Kinase 1) were used, respectively. Membrane was incubated in saturation buffer (10% w/v milk powder in TBS) for 1 h at room temperature and was then incubated with streptavidin alkaline phosphatase buffer (5% w/v milk powder dissolved in TBS) for 2.5 h at room temperature. After three 5 min washes with PBS-Tween-20 (Sigma-Aldrich) (0.04% v/v), the detection of alkaline phosphatase was performed by incubating the membrane in BCIP/NBT (5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium) for 10 min.

2.6. Cell differentiation

Human dental pulp stem cells (DPSCs) constitute a valuable source of multipotent stem cells and many studies highlight their capacity to *in vitro* differentiate in active osteoblasts ([Mortada and Mortada, 2018](#)). DPSCs at passage 2 were isolated and provided by the Greek stem cells bank "Biohellenica" and the Dental School of the Aristotle University of Thessaloniki. Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, USA) containing 10% v/v Fetal Bovine Serum (FBS, Gibco, USA) and 1% v/v antibiotic-antimycotic mixture (Gibco, USA) at 37 °C and 5% v/v CO₂. Cells were collected with cell lifters in PBS and were centrifuged at 1100 rpm for 3 min. The pellet was re-suspended in DMEM (10% v/v FBS and 1% v/v antibiotic-antimycotic mixture) and

the cells were counted prior to the deposition in multi-position plates (12-well plates, 20,000 cells/well). The next day, DMEM nutrient medium was changed to osteogenesis-specific growth medium (StemPro Osteogenesis Differentiation Kit, Invitrogen, USA). Proteins and peptides were added to the plates at the same concentrations (50 ng/mL).

2.7. Alkaline phosphatase assay

This assay was developed to detect osteogenesis at the 14th day of differentiation since during bone formation *in vitro*, proliferating osteoblasts exhibit enhanced alkaline phosphatase (AP) activity. BCIP/NBT (5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium) (Sigma-Aldrich, USA) were used to stain the cells in the presence of the alkaline phosphatase enzyme. After 14th day of differentiation osteogenic medium was aspirated, cells were washed with PBS and fixed with 10% v/v formalin buffer (Sigma-Aldrich, USA) not longer than 1 min, to avoid inactivation of alkaline phosphatase. Formalin buffer was aspirated, and cells were washed with wash buffer (0.05% v/v Tween-20 in PBS) (Sigma-Aldrich, USA). Finally, BCIP/NBT substrate was added to cells followed by 5–10 min incubation at room temperature in the dark. Differentiated cells were detected in blue-purple color and the reaction was stopped with wash buffer.

2.8. Alizarin Red staining assay and quantification

Alizarin Red staining of extracellular calcium stores was done after the 21st day of differentiation as it is described in the literature. After 21st day of differentiation, osteogenic medium was discarded and cells were washed with PBS (Gibco, USA) and fixed with 10% v/v formalin buffer (Sigma-Aldrich, USA) for 30 min. Formalin was discarded and cells were washed with bis-distilled water before addition of Alizarin Red Staining solution containing 2 g of Alizarin Red (Sigma-Aldrich, USA) in 100 mL distilled water at pH 4.1–4.3. Cells were incubated at room temperature for 20 min in the dark. After Alizarin Red staining was removed, cells were washed with distilled water followed by PBS. Calcium deposits were detected in red color.

Quantification was conducted upon removal of staining and incubation of cells with 10% w/v Cetylpyridinium Chloride ([Roberts and Der, 2007](#)) (CPC) (Sigma, Aldrich, USA) buffer in 10 mM Na₂HPO₄ (pH 7.0) at 37 °C for 12 h. The Optical Density (OD) was measured at 550 nm by microplate reader (Biotek Plate Reader).

2.9. Western blot analysis

For western blot analysis, samples that were extracted from cultured cells were analyzed for total protein content with Bradford assay and underwent SDS-PAGE electrophoresis on polyacrylamide gel (12.5% w/v). Proteins were transported on PVDF (PolyVinylidene Difluoride) membrane (Bio-Rad) for 3 h under 20 mA with the Bio-Rad Blot device (Kisker, SD10, 172). Membranes were blocked with 5% w/v milk in PBS for 45 min. They were then incubated overnight at 4 °C with 5% w/v milk in PBS containing Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit mAb (Cell signaling technology). The used antibody Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) detects endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) when dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2) and does not react with non-phosphorylated ERK. In addition, the p44/42 MAPK (Erk1/2) signaling pathway can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors, and cytokines and research investigators consider it an important target in the diagnosis and treatment of cancer ([Roberts and Der, 2007](#)).

The membranes were washed three times for 10 min each with PBS-Tween (0.1% v/v) and after were incubated with secondary anti-rabbit antibody in 5% w/v milk in PBS. After three 10 min washes with PBS-Tween (0.1% v/v) they were detected with alkaline phosphatase

buffer including NBT/BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate/ Nitro Blue Tetrazolium). Reaction was stopped with deionized water.

2.10. RNA isolation, cDNA synthesis and Real Time PCR

The expression of osteocalcin (BGLAP), osterix and alkaline phosphatase (ALP) upon treatment of DPSCs with differentiation factors (C-terminal BMP-2 protein and peptides) was estimated by RT-PCR. Total mRNA was isolated from DPSCs using Nucleospin RNA isolation kit (Machery-Nagel, Germany) after treatment with C-terminal BMP-2 proteins or peptides. cDNA synthesis was performed with the Finnzymes RobusTTMI kit following the manufacturer's instructions. Real time PCR reactions were performed using a SYBR-Select PCR Master Mix (Applied Biosystems, USA) in a Step One Plus thermal cycler (Applied Biosystems, USA). The primers for the *BGLAP*, *OSTERIX* and *ALP* genes were designed with the Primer-Blast software. The housekeeping gene *B2M* was used to analyze the results. The primers used were the following: *BGLAP*, Forward primer 5'-ACACTCCTCGCCCTATTG-3', Reverse primer 5'-GATGTGGT-CAGCCAACTC-3'; *OSTERIX* Forward primer 5'-CCAGCAACCCAGAGAAAG-3', Reverse primer 5'-TGGCAAGCAGTGGTCTAG-3'; *ALP* Forward primer 5'-GCTGTAAGGACATCGCCTACCA-3', Reverse primer 5'-CCTGGCTTCTCTGCTACTCTCA-3'; *B2M* Forward primer 5'-GGCATTCTGAAGCTGACAG-3', Reverse primer 5'-TGGATGACGTGAGTAACTG-3'.

2.11. Statistical analysis

Real time PCR experiments were performed as 3 independent repeats. The expression of each gene is presented as mean \pm standard deviation (SD) and was normalized using the housekeeping gene *B2M*.

Student's *t*-test, was used for statistical evaluations of two group comparisons and $p < 0.05$ was considered to be statistically significant. Graphic data were prepared with GraphPad Prism 8.2.1 (GraphPad Software, La Jolla CA, USA).

3. Results and discussion

3.1. Protein overproduction and purification

The C-terminal BMP-2 (wild type and mutated forms) were overproduced and purified as it is described in details under materials and methods. In particular, in Fig. 1 are indicated both after running onto agarose gel 1% w/v, A: the amplified gene (157 bp) and B: its insertion into pET29c plasmid.

The protein was overproduced, fused with biotin and His₆-tag in order to enable its functional immobilization on scaffolds/specific surfaces and its purification as well, respectively. It has to be mentioned that both the biotinylated or non-biotinylated form were studied regarding their ability to induce osteogenesis but no differences were detected for both cases (data not shown).

Fig. 2 shows the steps of protein purification that have been followed. Thus, IPTG induction/overproduction as well as their purification by using affinity chromatography namely nickel column, are clearly indicated on Fig. 2, A and B, respectively.

3.2. Purified C-terminal BMP-2 protein is functional and more active than purified full-length BMP-2 when are added to the growth medium at the same concentration

Fig. 3A shows the alkaline phosphatase activity (AP) of cells that are

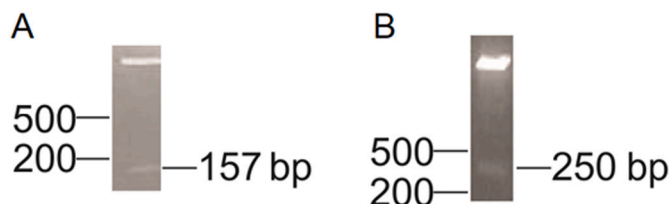


Fig. 1. Amplification and cloning of C-terminal BMP-2 gene. A) PCR amplified gene (157 bp) was detected as it is shown in 1% w/v agarose gel. B) Diagnostic restriction enzyme digestion in agarose gel 1% w/v. Cloned C-terminal BMP-2 carrying biotinylation sequence into pET29c plasmid was detected at 250 bp.

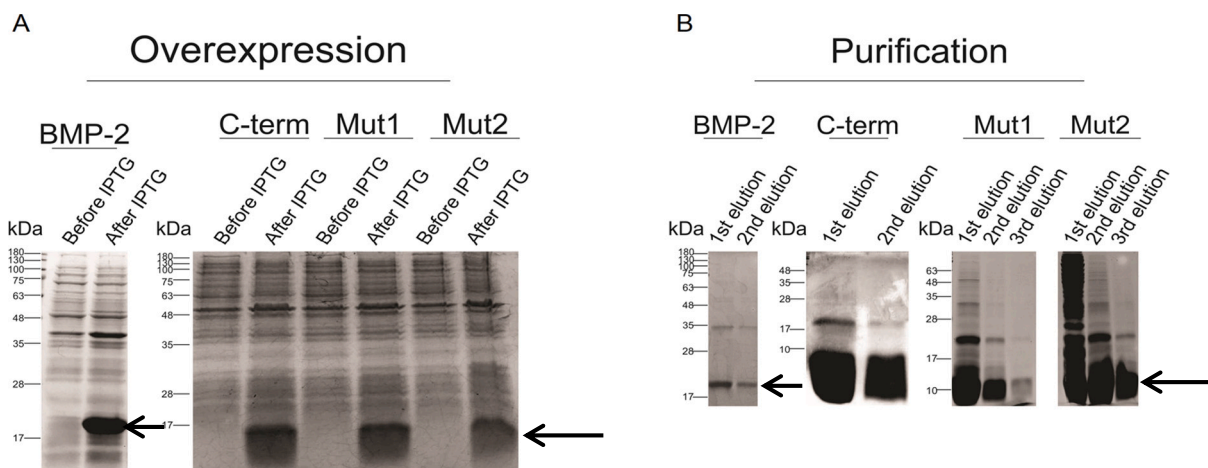


Fig. 2. SDS-PAGE electrophoresis for overexpression and purification of BMP-2 full length and its C-terminal region (wild type and mutants). A: The arrows show the entire protein or its C-terminal region (wild type and mutants) before or after IPTG. B: SDS-PAGE electrophoresis for purification of monomeric form of full-length BMP-2 protein, and its C-terminal region (wild type and mutants). The arrows show the purified entire BMP-2 close to 18 kDa or its C-terminal region at 9 kDa, respectively. Mut1 and mut2 represents the C-terminal mutated at S88 and Leu90 into glycine, respectively.

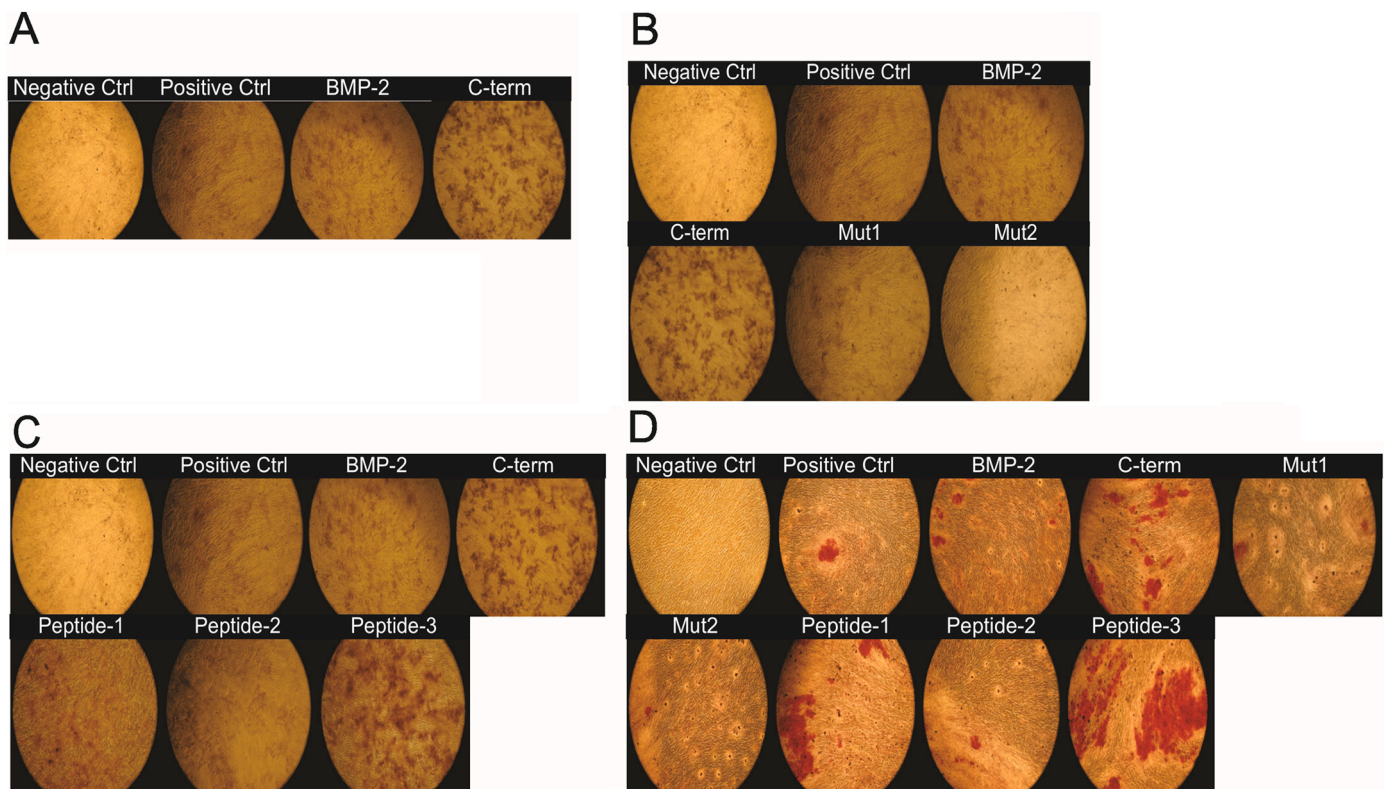


Fig. 3. A) Alkaline Phosphatase assay in Dental Pulp Stem Cells. DPSCs were treated with purified full-length BMP-2 or BMP-2 C-terminal protein at the same concentration (50 ng/mL). As negative control and positive control uninduced cells and cells in osteogenesis medium were used, respectively. C-terminal BMP-2 induced higher levels of osteogenesis than full length BMP-2 protein. Uninduced cells in DMEM medium and cells in osteogenesis medium were used as negative and positive controls, respectively to determine the assay efficiency. Images were taken under the microscope at 10× magnification.

B) Comparison of the activity of full length, C-terminal wild type or mutant forms of BMP-2 in alkaline phosphatase assay. DPSCs were treated with same concentrations (50 ng/mL) of either full-length BMP-2 protein, BMP-2 C-terminal wild type or mutant BMP-2 forms (mutant 1 and mutant 2) and were subjected to alkaline phosphatase assay at 14th day of differentiation. BMP-2 C-terminal protein was the most active towards osteogenesis followed by full-length BMP-2 protein. Mutant form 1 induced impaired osteogenesis in contraction to the mutant form 2 which abolished its activity. As negative and positive controls were used uninduced and induced DPSCs. Images were taken under the microscope at 10× magnification.

C) DPSCs were treated with either purified full-length BMP-2 protein, BMP-2 C-terminal or each peptide 1, 2 and 3 at the same concentrations (50 ng/mL) and were subjected to Alkaline Phosphatase assay at the 14th day of differentiation. Full length BMP-2 protein induced equivalent levels of AP activity with Peptide 1, but lower than C-terminal BMP-2, while Peptide 3 was the most active. Peptide 2 did not induce any differentiation. As negative and positive controls were used uninduced and induced DPSCs. Images were taken under the microscope at 10× magnification.

D) Alizarin Red staining assay in DPSCs. DPSCs were treated with full-length BMP-2 protein, C-terminal BMP-2, mutant form 1 mutant form 2, Peptides 1, Peptide 2 and Peptide 3 at equimolar concentrations (50 ng/mL). Alizarin Red assay was performed at 21st day of differentiation. Peptide 3 performed highest induction of differentiation towards osteogenesis, followed by C-terminal BMP-2 and then by full-length BMP-2 and Peptide 1 that induced similar levels of osteogenesis. Peptide 2 was not active, similar to mutant form 2, while mutant form 1 shown reduced levels of osteogenesis. As negative control were used DPSCs in DMEM medium and as positive control DPSCs in osteogenesis.

E) Spectrophotometric quantification of Alizarin Red stain. Alizarin Red staining was extracted from DPSCs after treatment with either full-length BMP-2 protein or C-terminal BMP-2 or Mutants 1, 2, or Peptides 1, 2, 3 once in day 0 at the same concentrations. Measurement and analysis were performed at 21st day after of differentiation at 550 nm. Uninduced and induced cultures were used as negative and positive controls. Error bars represent the value of standard deviation after 3 repeats of the experiment.

F) Expression levels of osteocalcin, osterix and alkaline phosphatase. DPSCs cultures were treated once with either full-length BMP-2 or BMP-2 C-terminal or Peptide 3 at the same concentrations (50 ng/mL). To quantify the osteocalcin mRNA levels, from each cell culture, total RNA was isolated at the 7th day of differentiation and was used as a template to generate complementary DNA (cDNA) and perform a PCR reaction (RT-PCR). Real-time PCR analysis of OSTERIX gene at 1st day of differentiation and Real-time PCR analysis of alkaline phosphatase the 14th day of differentiation. In all cases peptide 3 induced higher expression of alkaline phosphatase than C-terminal BMP-2 or full-length BMP-2 protein. Uninduced and induced cultures were used as negative and positive controls respectively and the results of both RT-PCR and Real time PCR were analyzed/evaluated taking into account the housekeeping gene, B2M. Error bars in all three graphs represent the value of standard deviation after 3 repeats of the experiments.

subjected under osteogenesis conditions after the addition of the entire or C-terminal BMP-2. It has been found that both C-terminal and full-length BMP-2 protein were functional and active towards osteogenesis. However, C-terminal BMP-2 demonstrated higher alkaline

phosphatase activity indicating increased osteogenesis compared to full-length BMP-2 protein. Specifically, at the 14th day of differentiation, purified C-terminal BMP-2 induced higher activity and levels of osteogenesis compared to full-length protein.

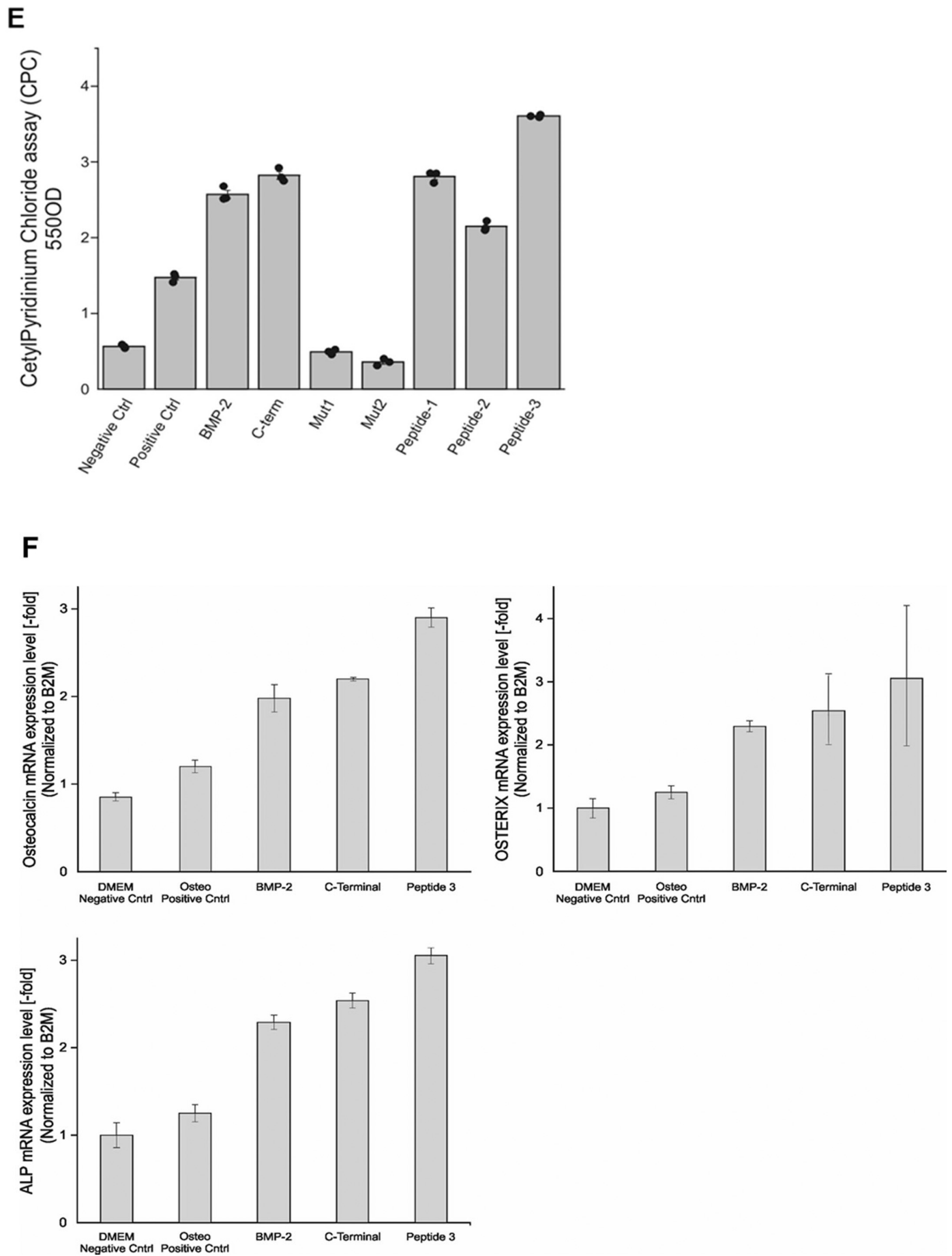


Fig. 3. (continued).

3.3. Introduction of Ser88Gly and Leu90Gly mutations result in impaired activity of C-terminal BMP-2 purified protein

In order to determine crucial amino acids of C-terminal BMP-2 that regulate protein activity, point mutations that affect receptor binding according to molecular dynamics analysis have been introduced. Thus, introduction of Ser88Gly into the C-terminal BMP-2 resulted in impaired levels of differentiation towards osteogenesis in alkaline phosphatase assay. Ser88Gly demonstrated lower levels of differentiation towards osteogenesis than the full-length BMP-2 protein at the 14th day of differentiation. Interestingly, the insertion of Leu90Gly did not promote any differentiation and osteogenesis indicating that this mutation blocks efficiently receptor binding and downstream pathway activation and is crucial for the activity of short C-terminal BMP2 (Fig. 3B).

3.4. Peptide 3 coding a shorter sequence of C-terminal BMP-2 protein is functional and more active

As it is mentioned before, three peptides (Peptide 1: AISMLYLDE-NEKVVVKNYQD, Peptide 2: AISMGYLDENEKVVVKNYQD and Peptide 3: AISMLYLDEN) containing the crucial amino acids S88 and L90 were synthesized and their activity regarding the promotion of osteogenesis was investigated with the Alkaline phosphatase (AP) assay.

Fig. 3C clearly indicates that peptide 3 is the most active since it induced the greatest AP activity at the 14th day of differentiation and subsequently the highest osteogenesis levels. Peptide 2, containing the mutation L90G did not induce osteogenesis, while Peptide 1 induced activity levels similar to full-length BMP-2 protein. The alkaline phosphatase assay demonstrates that the short Peptide 3 including the C-terminal amino acid sequence AISMLYLDEN would be more efficient (at the same concentration) than the C-terminal domain or the full-length BMP-2 protein. This peptide contains the most crucial amino acids S88 and L90 for BMP-2 protein function towards osteogenesis.

3.5. Amino acid sequence AISMLYLDEN of C-terminal BMP-2 is crucial for protein function and induces osteogenesis

To investigate further the activity of Peptides, C-terminal and full-length BMP-2 protein we performed one more assay, the Alizarin Red staining (Fig. 3D). At the 21st day of differentiation the shortest protein domain, Peptide 3, induced greater differentiation and demonstrated the highest activity compared to the C-terminal, full length BMP-2 protein and Peptide 1. Introduction of Ser88Gly mutation in peptide 2 resulted in reduced osteogenic activity. Thus, following our previous observations, the shortest protein domain, Peptide 3, coding the amino acid sequence AISMLYLDEN is the most active as it demonstrated greater differentiation than all other forms of BMP-2 tested. The Alizarin Red staining data were quantified with Cetylpyridinium Chloride assay (CPC) by extracting staining from the extracellular calcium deposits. Once more, CPC data demonstrated that Peptide 3 induced osteogenesis at higher levels followed by the C-terminal BMP-2 and Peptide 1, which are followed by full length BMP-2 (Fig. 3E). Furthermore, mutant form 1 induced osteogenesis, while mutant form 2 and Peptide 2 did not induce osteogenic activity confirming our previous data and observations.

3.6. Peptide 3 induces osteocalcin, osterix and alkaline phosphatase expression levels

The results regarding induction of osteogenesis were verified by studying the expression levels of the bone hormone osteocalcin by reverse transcriptase reaction (RT-PCR). Thus, the detected RNA levels at the 7th day of differentiation were further analyzed with GelPro software (Fig. 3F). The obtained data indicated that among all forms, Peptide 3 induced higher osteocalcin expression, followed by C-terminal and full-length BMP-2. Subsequently the results confirmed the higher functionality of peptide 3 regarding osteogenesis promotion.

To further study the function of Peptide 3, C-terminal and full-length

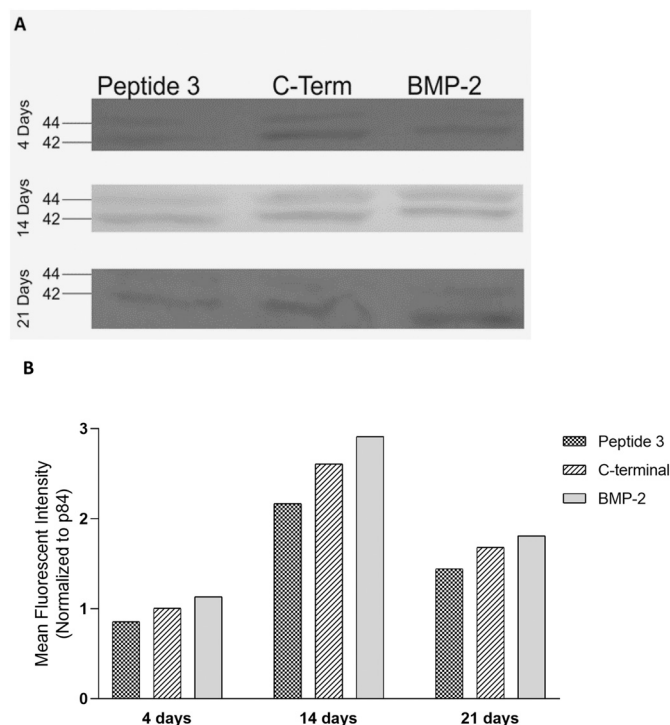


Fig. 4. Peptide 3 signals through the SMAD pathway. A) DPSCs were treated with either Peptide 3, or C-terminal or full-length BMP-2 at the same concentrations (50 ng/mL). To detect the phosphorylation of ERK protein, total proteins were isolated from DPSCs cultured at three different time points at 4th, 14th and 21st day of differentiation and western blot analysis was performed. At all time-points full-length BMP-2 induced higher phosphorylation of ERK and was followed by C-terminal BMP-2. Peptide 3 induced less pERK levels at any time point tested. B) Protein bands normalized to p84 and were analyzed with Fiji software.

BMP-2 protein the mRNA expression levels of the transcription factor of osteoblast differentiation, osterix, were compared at the 1st day of differentiation. The expression levels of alkaline phosphatase that mediates tissue formation at the 14th day of differentiation were studied too. Real Time PCR revealed that Peptide 3 induced in all cases the highest expression levels, followed by C-terminal BMP-2 and full-length BMP-2, accordingly (Fig. 3F).

Taken together the results, strengthen the obtained data that Peptide 3, encoding amino acid sequence AISMLYL DEN of the C-terminus of BMP-2, is active and induces differentiation towards osteogenesis more efficient than either C-terminal or full-length BMP-2 protein.

3.7. Peptide 3 signals preferably through the Smad pathway

To interpret signal transduction through either full-length or C-terminal BMP-2 or peptide 3, phosphorylation of ERK1/2 at the 4th day of differentiation was studied (Fig. 4A, B). It has been found that while full length BMP-2 induced ERK1/2 phosphorylation, Peptide 3 and C-terminal BMP-2 had the opposite effect and they reduced ERK1/2 phosphorylation. Similar results were obtained on 14th day and 21th day (Fig. 4A, B) where full-length BMP-2 protein induced higher levels of ERK1/2 phosphorylation than Peptide 3 and C-terminal BMP-2. Western blot zone analysis (Fig. 4A, B) confirmed that Peptide 3 reduced levels of ERK1/2 phosphorylation in all three time-points, followed by C-terminal BMP-2. Taken together our data propose that Peptide 3, coding sequence AISMLYL DEN and C-terminal BMP-2 bypass ERK1/2 signaling, and probably transduce downstream signaling through the SMAD pathway.

BMP-2 protein induces osteogenesis and bone tissue formation *in vitro* and *in vivo* of stem cells (Dhillon et al., 2007). Taken together, our data support the activation of the mentioned receptor by protein regions that include the crucial amino acids S88 and L90, at least. Whether some other protein regions of BMP-2 behave similar, it has to be elucidated. Our explanation regarding the C-terminal activity is that it might be a consequence of lack of the N-terminus that affects protein regulation through interaction with heparin of the extracellular matrix (Yamaguchi et al., 1991; Kanzaki et al., 2008; Mitsi et al., 2008).

Deregulation of BMP-2 signaling transduction is implicated in pathological conditions and BMP-2 is found highly expressed in various types of cancers (Cunha et al., 2017; Blanco Calvo et al., 2009). Deregulated signaling through MAPK pathway results in phosphorylation of kinases and tumor cell proliferation (Dhillon et al., 2007). In this study we report an alternative activation of osteogenic pathways through short and active peptides of BMP-2. While BMP-2 full-length signals through both canonical and non-canonical pathways, our data indicate that the short active peptide drives preferentially the canonical SMAD pathway over the noncanonical MAPK. According to our study, short BMP-2 protein doesn't signal through MAPK pathway and is predicted not to induce tumor proliferation though ERK phosphorylation. Thus, our model suggests the usage of short BMP-2 as a safer and more effective tool to be attached to biomaterial and applied in orthopedic healing fractures.

CRedit authorship contribution statement

Stylianos-Zapheirios Karoulias: Investigation, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Visualization. **Maria Pitou:** Investigation, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Visualization. **Rigini Papi:** Investigation, Validation, Writing – review & editing. **Paraskevas Lamprou:** Writing – review & editing, Visualization. **Theodora Choli-Papadopoulou:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

None.

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