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# Dose-dependence of PTH-related peptide-1 on the osteogenic induction of MC3T3-E1 cells in vitro

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**Abstract**

Parathyroid hormone (PTH), an 84-amino acid peptide, is an endocrine hormone that is secreted by parathyroid glands. PTH performs important functions in calcium regulation and bone remodeling. The PTH (1–34) named teriparatide, a 34-amino acid peptide derived from the N-terminus of PTH, conserves most of the functions of PTH, specifically the osteogenic capability. However, teriparatide is only used by injection and exhibits short duration. In addition, this PTH could not thoroughly expose active sites. In this study, a novel PTH-related peptide (designated PTHrP-1) derived from the N-terminus of PTH was added into the complete medium at different concentrations of PTHrP-1 (0, 50, 100, and 200 ng/mL) to induce the MC3T3-E1 cells. PTHrP-1 was detected by high-performance liquid chromatography and matrix-assisted laser desorption/ionization–time-of-flight mass spectroscopy. Cell morphology, cell proliferation, alkaline phosphatase (ALP), and ALP activity, osteocalcin concentration, and collagen type I (Col-I), osteopontin (OPN), and osteocalcin (OCN) mRNA expression by RT-PCR and protein expression by western blotting were observed and detected. The purity of the PTHrP-1 was 95.14%, and the PTHrP-1 can induce MC3T3-E1 cells into osteoblasts, thus improving ALP activity and OCN concentration, and increasing Col-I, OPN, and OCN mRNA expression and protein expression in MC3T3-E1 cell cultures. The PTHrP-1 proved to be an ideal active peptide. In addition, the osteogenic ability of PTHrP-1 at 200 and 100 ng/mL concentrations was not significantly different but significantly higher than 50 and 0 ng/mL groups. Results indicate that PTHrP-1 is a kind of active peptides that exhibits good biocompatibility with MC3T3-E1 cells and could improve cell proliferation and osteogenic differentiation. Moreover, PTHrP-1, at the preferable concentration of 100 ng/mL, could effectively promote MC3T3-E1 cells into osteoblasts.

**Abbreviations:** ALP = alkaline phosphatase, BMPs = bone morphogenetic proteins, Col-I = collagen type I, HPLC = high-performance liquid chromatography, OCN = osteocalcin, OPN = osteopontin, PRC = polymerase chain reaction, PTH = parathyroid hormone, PTHrP-1 = PTH-related peptide.

**Keywords:** dose-dependence, MC3T3-E1 cells, osteogenic capability, parathyroid hormone, PTH-related peptide

## 1. Introduction

Patients with bone defects from trauma and a variety of bone disease exceed 6 million annually in China. Thus, abundant bone repair materials are urgently needed.[1] One of the most critical issues of the current study on bone repair materials is to determine a mechanism for obtaining analogous bone-inducing activity.[2] The interest in clinical research and applications of growth factors and other peptides to induce bone repair has stimulated several notable studies that has yet to result in definitive conclusions and ideal recommendations.[3,4] Over the last decade, different growth factors and osteogenic active peptides have been designed for new bone formation, including bone morphogenetic proteins (BMPs), vascular endothelial growth factors, basic fibroblast growth factors, insulin-like growth factors, platelet-derived growth factors, BMP-2-related peptides, RADA16-P24 peptide, parathyroid hormone (PTH) (1–34), collage-derived peptides, peptides derived from bone sialoprotein, and enamel matrix proteins.[5–11]

PTH, an 84-amino acid peptide, is an endocrine hormone secreted by parathyroid glands;[12] this peptide performs important functions in calcium regulation and bone remodeling.[13] In addition, PTH is receiving increasing attention over the recent years in the field of bone repair because of its various biological activities in inducing bone formation both orthotopically and ectopically in the body.[14,15] The PTH (1–34) named teriparatide, a 34-amino acid peptide derived from the *N*-terminus of PTH, conserves most of the functions of PTH, specifically osteogenic capability.[13,16] However, teriparatide is expensive, could only be used through injection, and possesses a short lasting duration, which could cause hypercalcemia and even the subsequent risk of osteosarcoma.[17] To overcome these drawbacks, we need to optimize the structure of teriparatide.

In our previous study, we successfully designed and synthesized a novel short peptide, BMP-2-related peptide P24, based on the

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residues of the knuckle epitope of BMP-2 using FMOC/tBu solid-phase synthesis.[6,18] Termination included a repeating Asp sequence. Related studies have shown that the P24 peptide could induce osteogenic differentiation of bone marrow stromal cells and could be administered as a combination to maintain effective peptide concentration.[19,20] Using the same approach, our group designed and synthesized a novel PTH-related peptide (S $^{[PO4]}$ VSEI–QLMHN–LGKHL–NSMER–VEWLR–KKLQD–VHNF DDD, PTHrP-1), including repetitive Asp (aspartic acid) and phosphorylated Ser (serine), which may mimic the function of organizing and accumulating mineralization of natural bone. In this study, we focused on studying the oriented osteogenic differentiation ability of different concentrations (0, 50, 100, and 200 ng/mL) of PTHrP-1 to induce MC3T3-E1 cells in vitro, as well as a preliminary study on the optimal osteogenic dose of PTHrP-1.

## 2. Materials and methods

### 2.1. Ethics statement

All experiments and the study protocol were approved by the Ethics Committee of the Zhongnan Hospital of Wuhan University.

### 2.2. Main reagents and instruments

MC3T3-E1 cells were purchased from the China Center for Type Culture Collection in Wuhan University (China). Fetal bovine serum, TRIzol RNA extraction kit, and first-strand cDNA synthesis kit were obtained from Gibco. All cell culture-related reagents were obtained from Nanjing Jiancheng Biological Products Co., Ltd. (China). The real-time PCR instrument (7900/Viia7), vacuum drying oven, lyophilizer, and high-performance liquid chromatography (HPLC) system (L-2000) used in the experiments were purchased from ABI7900/ Illumina Eco (USA), Shanghai Suopu Instrument Co., Ltd. (China), Beijing Boyikang Instrument Co., Ltd. (China), and Hitachi (Japan), respectively. The PTH-related peptide (PTHrP-1) was designed and prepared by our group.

### 2.3. Peptide synthesis

The PTH-related peptide (S$^{[PO4]}$ VSEI–QLMHN–LGKHL–NSMER–VEWLR– KKLQD–VHNF DDD, PTHrP-1) was derived from the N-terminus of PTH and obtained manually by solid-phase peptide synthesis using Fmoc chemistry (Fmoc–AA–OH/NMM/HBTU = 3/6/2.85). Crude peptide produced from this chemosynthetic method was then preliminarily purified by gel filtration. HPLC and matrix-assisted laser desorption/ ionization–time-of-flight (MALDI TOF) mass spectroscopy (molecular ion) were used to evaluate synthesis of PTHrP-1.

### 2.4. Cell culture

The MC3T3-E1 cells were plated at a density of $1 \times 10^5$ cells/cm$^2$ in 6-well plates and grown at 37°C in an atmosphere of 5% $CO_2$ and 95% humidity in Dulbecco's modified Eagle's medium. After 24 hours, 4 PTHrP-1 solutions with different concentrations (0, 50, 100, and 200 ng/mL) were added into the complete medium. The cells were cultured for a longer period, and the medium including PTHrP-1 was changed every 2 to 3 days. Finally, cell morphology was observed.

### 2.5. Evaluation of cell proliferation

Cell proliferation with the MTT assay (Sigma) was performed according to the manufacturer's protocol. The MC3T3-E1 cells ($1 \times 10^3$ per well) were seeded in 96-well plates with membranes of 4 groups (0, 50, 100, and 200 ng/mL). After incubation of 1, 3, 5, 7, 10, 12, or 15 days, 20 μL MTT (5 mg/mL, pH 7.4) were added to each well and the sample was maintained for another 4 hours. Then, the supernatant was replaced by 100 μL DMSO and quantification of the formazan dye formed was determined by using a spectrophotometer (Tianjin Tianguang Optical Instrument Co., China) at a wavelength of 570 nm.

### 2.6. Osteogenic induction of MC3T3-E1

#### 2.6.1. Alkaline phosphatase (ALP) and ALP activity assay.

The MC3T3-E1 cells were plated at a density of $1 \times 10^4$ cells/well in 24-well plates that contained 4 types of PTHrP-1 solutions (0, 50, 100, and 200 ng/mL) in complete medium at 37°C in an atmosphere of 5% $CO_2$ and 95% humidity. After 24 hours, the medium was replaced with 4 types of PTHrP-1-containing medium at different concentrations for every 2 to 3 days. After 14 days, ALP staining was measured as previously described.[21] ALP activity was spectrophotometrically determined by lysing the cells. After 5, 10, 15, and 20 days of incubation, the ALP activity in the supernatant of the cell lysate was assayed at 520 nm. The average absorbance levels in each group were then calculated. This quantity was normalized to a total protein quantity that was measured using the ALP protein assay kit.

**2.6.2. Osteocalcin assay.** After 1, 2, 3, and 4 weeks of incubation in the same method, osteocalcin (OCN) was tested by the ELISA assay kit to assess the differentiation of cells into osteoblasts. The absorbance of samples was measured at 450 nm. OCN concentration was calculated using a standard method.[22]

**2.6.3. RNA isolation and RT-PCR.** The plated MC3T3-E1 cells were collected at day 14 and were preserved in RNAlater (Genecopoeia). All cells were then pulverized, and total RNA was extracted using TRIzol reagent (Aidlab, China) following the instructions provided. RNA was used to synthesize first-strand cDNA by reverse transcriptase (Genecopoeia). For the polymerase chain reaction (PRC), aliquots of synthesized cDNA were added to PCR mixtures containing *Taq* polymerase (TAKARA, Japan) and cycled on a DNA thermal cycler. PCR primers were as follows: (1) collagen type I (Col-I) fwd 50 GGCAAAGATGGAGAAGCTGG 30, COL I rev 50 GGAAACCTCTCTCGCCTCTT 30; (2) osteocalcin (OCN) fwd 50 GGACCATCTTTCTGCTCACTC 30, OCN rev 50 CTGCTTGGACATGAAGGCTT 30; and (3) osteopontin (OPN) fwd 50 TCACTCCAATCGTCCCTACA 30, OPN rev 50 GACTCCTTAGACTCACCGCT 30. PCR products were then determined after calculating the optimal annealing temperature for each primer pair by using densitometry and OLIGO Primer Analysis Software.

**2.6.4. Protein assay by Western blot.** Cultured 14d MC3T3-E1 cells were lysed in M-Per protein extraction reagent containing Halt protease inhibitors (Pierce). Proteins were resolved in SDS-PAGE gels and transferred onto nitrocellulose (Pall). Blots were developed with Western Lightning chemiluminescence reagents from PerkinElmer. To detect the proteins of interest, the following antibodies: Col-I, OPN, and OCN from Sigma were used.

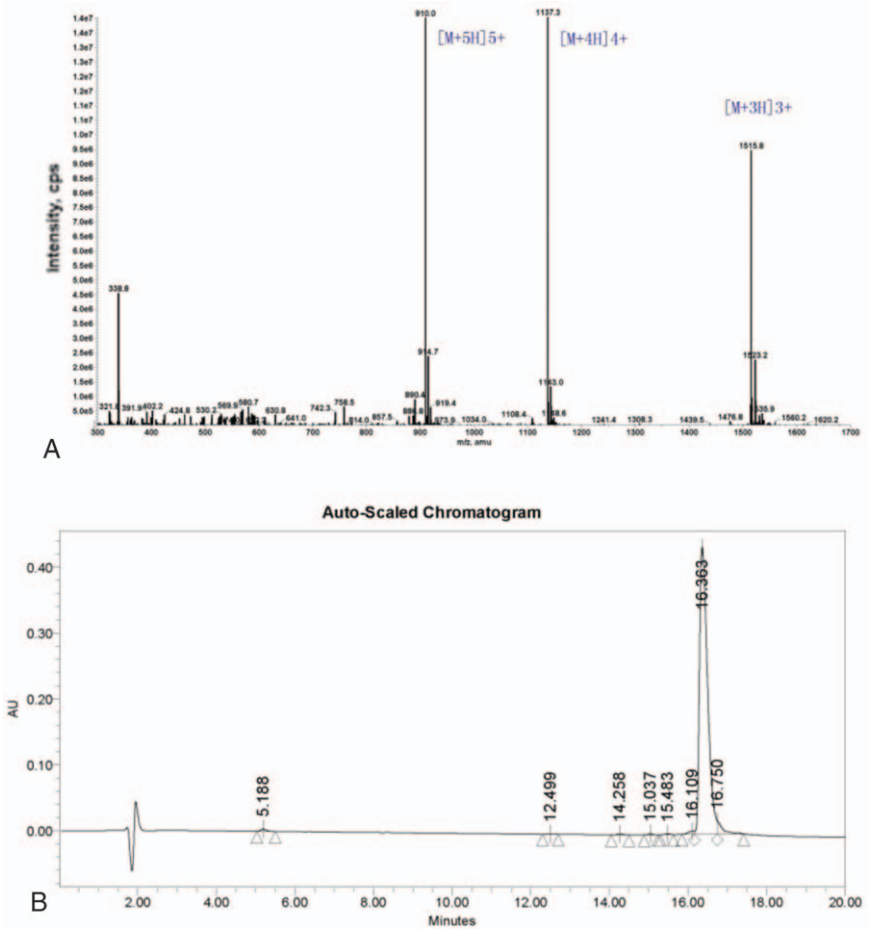


**Figure 1.** (A) High-performance liquid chromatogram of PTHrP-1. (B) Mass spectrum of PTHrP-1. PTHrP-1 = PTH-related peptide.

### 2.7. Statistical analysis

Data were expressed as the arithmetic mean ± standard deviation. Statistical significance was assessed by ANOVA using SPSS 19.0 (SPSS Inc., Chicago, IL). Tukey's post hoc test was performed to compare individual pairs of groups. Differences at $P < .05$ were considered statistically significant.

## 3. Results

### 3.1. Characterization of the peptide

In the MS spectrogram, the m/z values of molecular [M+nH]+n ions produced from PTHrP-1 components in the fractions analyzed were correlated to the theoretical masses of the PTHrP-1 fragments to identify their origin and the cleavage site. And MALDI TOF mass spectroscopy results showed the successful synthesis of PTHrP-1(S $^{[PO4]}$ VSEI–QLMHN–LGKHL–NSMER–VEWLR–KKLQD–VHNF- DDD) (Fig. 1A). The purity of PTHrP-1 was about 95.14% as determined by HPLC (Fig. 1B, Table 1). And the molecular weight of PTHrP-1 was about 4543.07.

### 3.2. Detection of cell morphology

The MC3T3-E1 cells were cultured after 4 hours, and then it was observed under the inverted-phase contrast microscope. First of all, the cells begin to adhere and appear round or oval.

**Table 1**

**Peak results.**

| | Retention time (min) | Int type | Area (μv* s) | Height (μv) | Width (s) | %Area |
|---|---|---|---|---|---|---|
| 1 | 5.188 | BB | 32058 | 3594 | 28.000 | 0.49 |
| 2 | 12.499 | BB | 7365 | 621 | 24.000 | 0.11 |
| 3 | 14.258 | BB | 15114 | 1467 | 27.000 | 0.23 |
| 4 | 15.037 | bb | 14488 | 1728 | 21.000 | 0.22 |
| 5 | 15.483 | bb | 10253 | 912 | 20.000 | 0.16 |
| 6 | 16.109 | bV | 47983 | 4638 | 19.000 | 0.74 |
| 7 | 16.363 | Vv | 6169989 | 435984 | 35.000 | 95.14 |
| 8 | 16.750 | vb | 188190 | 19346 | 40.000 | 2.90 |

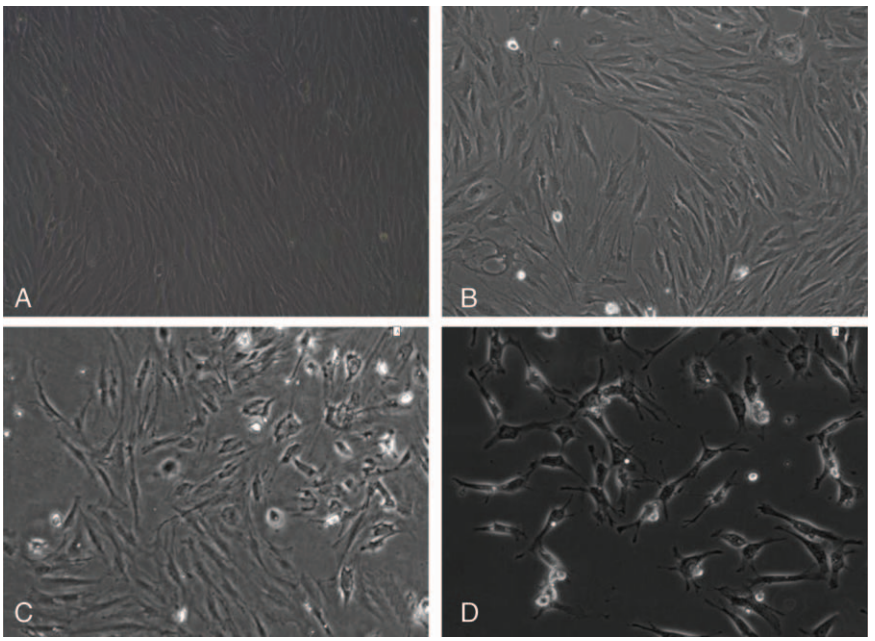


**Figure 2.** Inverted phase contrast microscope images of 4 groups after 3 days: PTHrP-1 200ng/mL group (A); 100 ng/mL group (B); 50 ng/mL group (C); 0 ng/mL group (D) (A: magnification: 100×, B, C, D: 200×). PTHrP-1 = PTH-related peptide.

Subsequently, the cells gradually increased in number and presented fusiform. From the first to third day, the number of cells increased significantly showed a monolayer structure and could cover the bottom. However, by inducing culture medium after 2 to 3 days, the cell morphology was evidently changed from the long fusiform into short spindle or polygonal shape. With increasing concentration of PTHrP-1 of the culture medium, the time of cells into bone-like changed in advance. The cells in 200 ng/mL group covered the bottom after 3 days, those in the 100 ng/mL group nearly covered the bottom after 3 days, those in the 50 ng/mL group covered the bottom after 5 days, and those of 0 ng/mL group required longer time to cover the bottom (Fig. 2).

### 3.3. Cell proliferation assay

With the time of incubation, the number of the MC3T3-E1 cells increased gradually. At each time-point, the number of MC3T3-E1 cells in groups of 200 ng/mL group and 100 ng/mL group were significantly greater than that of 50 ng/mL group and 0 ng/mL group. And there was no significant difference between 200 ng/mL group and 100 ng/mL group (Fig. 3).

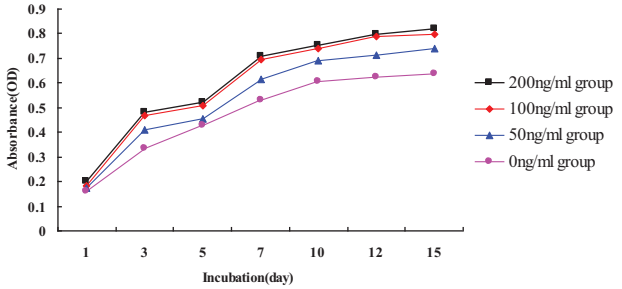


**Figure 3.** Proliferation of MC3T3-E1 cells in 4 groups. There was a linear relationship between cell proliferation activity and absorbance.

### 3.4. ALP staining and ALP activity assay

ALP staining demonstrated that the MC3T3-E1 cells in the PTHrP-1 200, 100, and 50 ng/mL groups accumulated to form multiple layers and were of spindle or polygonal shape after 14 days of culture. This staining also showed the increasing deposition of brown particles in the cytoplasm of ALP-positive cells, with increasing concentration of PTHrP-1 of the culture medium (Fig. 4).

The ALP activity in MC3T3-E1 cell cultures gradually increased with increasing concentration of PTHrP-1 from the 5th day to the 20th day. The groups with PTHrP-1 concentration of 200 and 100 ng/mL showed significantly increased ALP activity compared with the other 2 groups, but both 200 and 100 ng/mL groups did not show significant difference. ALP activity in the 200, 100, and 50 ng/mL groups were significantly higher than that in the 0 ng/mL group ($P < .05$) (Fig. 5).

### 3.5. Osteocalcin assay

At the 4 study time-points, the OCN concentrations of the 200 and 100 ng/mL groups were significantly higher than those of 50 and 0 ng/mL groups ($P < .05$). After culturing for 2 weeks, the OCN concentration of the 200, 100, and 50 ng/mL groups reached the peak value, and then the OCN concentration decreased to varying degrees. However, the OCN concentration of the 4 groups at 4 weeks was still higher than that at 1 week (Fig. 6).

### 3.6. RT-PCR and Western blotting assay

The MC3T3-E1 cells were induced by different concentrations of PTHrP-1 culture medium for 14 days. Col-I, OPN, and OCN mRNA expression levels in 200, 100, and 50 ng/mL groups were evidently higher than those in the 0 ng/mL group ($P < .05$). The Col-I, OPN, and OCN mRNA expression levels in MC3T3-E1 cell cultures gradually increased with increasing concentration of PTHrP-1 at 14 days. The mRNA for all osteoblast markers at 14

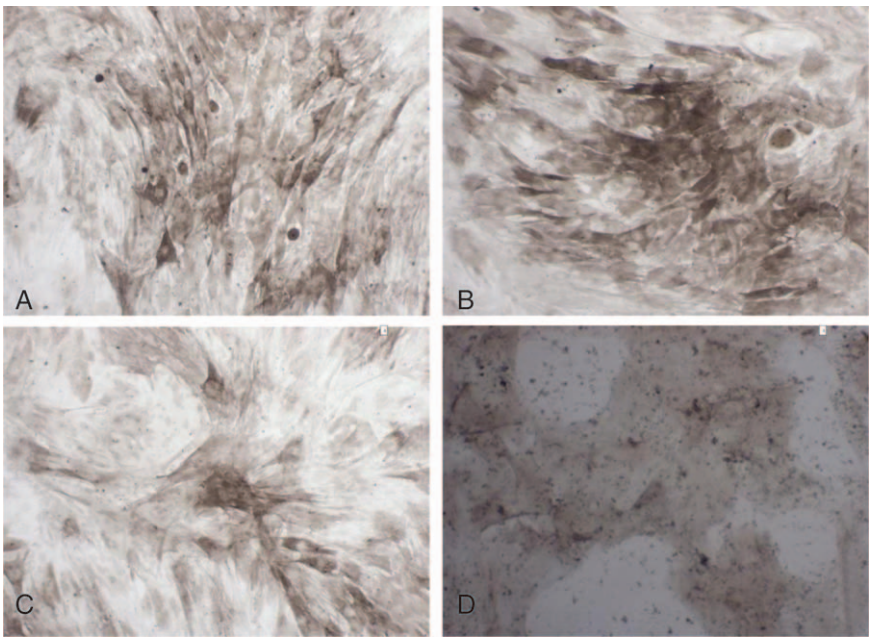


**Figure 4.** ALP staining: PTHrP-1 200 ng/mL group (A); 100 ng/mL group (B); 50 ng/mL group (C); 0 ng/mL group (D) indicated deposition of brown particles in the cytoplasm of ALP positive cells at 14 days of culture (magnification: 400 ×). ALP = alkaline phosphatase, PTHrP-1 = PTH-related peptide.

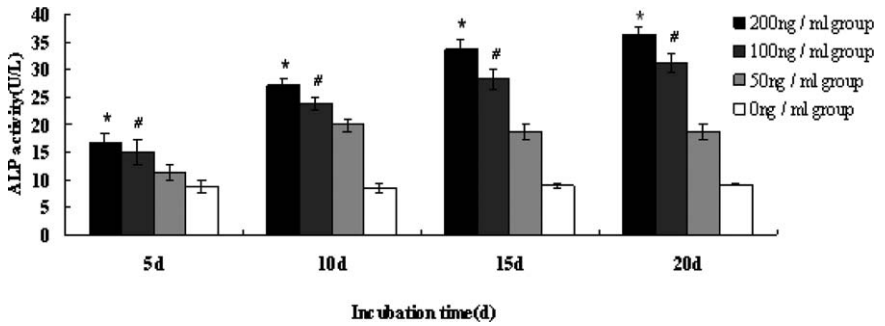


**Figure 5.** ALP activities in 4 groups (200 ng/mL group, 100 ng/mL group, 50 ng/mL group, and 0 ng/mL group). The ALP activity in MC3T3-E1 cell cultures increased from the 5th day to the 20th day. $^{*}P > .05$, 200 ng/mL group compared with 100 ng/mL group; $^{\#}P < .05$, 100 ng/mL group compared with 50 ng/mL group and 0 ng/mL group. ALP = alkaline phosphatase.

days was significantly higher in 200 and 100 ng/mL groups compared with that in 50 and 0 ng/mL groups ($P < .05$), but no significant difference was found between the 200 and 100 ng/mL groups (Fig. 7A). At the same time, Western blotting showed that the protein expression level of Col-I, OPN, and OCN in 200 and 100 ng/mL groups were significantly higher than that of 50 and 0 ng/mL groups ($P < .05$), but there was no significant difference between the 200 and 100 ng/mL groups (Fig. 7B).

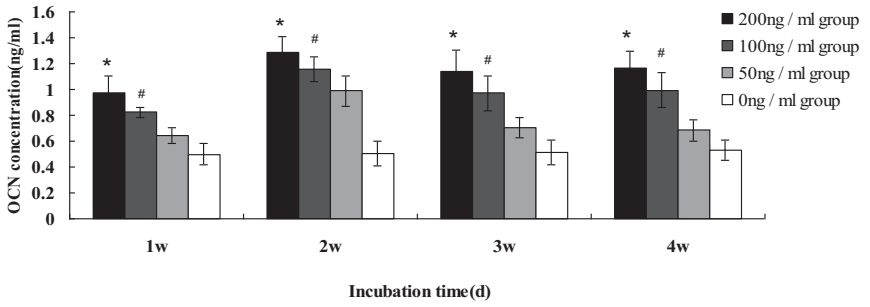


**Figure 6.** OCN concentration in 4 groups (200 ng/mL group, 100 ng/mL group, 50 ng/mL group, and 0 ng/mL group). $^{*}P > .05$, 200 ng/mL group compared with 100 ng/mL group; $^{\#}P < .05$,100 ng/mL group compared with 50 ng/mL group and 0 ng/mL group. OCN = osteocalcin,

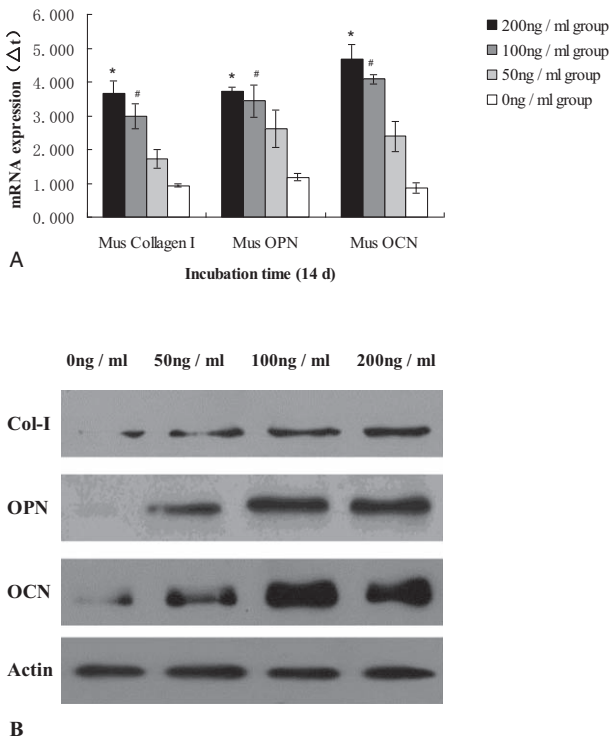


**Figure 7.** Col-I, OPN, and OCN mRNA expression (A) and protein expression (B) in 4 groups (200 ng/mL group, 100 ng/mL group, 50 ng/mL group, and 0 ng/mL group) at 14 days. $^{*}P > .05$, 200 ng/mL group compared with 100 ng/mL group; $^{\#}P < .05$,100 ng/mL group compared with 50 ng/mL group and 0 ng/mL group. Actin was used as the internal control. Col-I = collagen type I, OCN = osteocalcin, OPN = osteopontin.

## 4. Discussions

In the differentiation of MC3T3-E1 cells into osteoblasts, cell morphological changes are often detected, and Col-I, ALP, OCN, and OPN are commonly used as indicators.[23] Col-I is an important component of the extracellular matrix in bone tissue. In addition, Col-I is a characterized enzyme cultured osteoblast in vitro and performs an important function in bone tissue regeneration. Exogenous Col-I can promote cell attachment and stimulate cell differentiation.[23] ALP is one of the key enzymes in early differentiation of mature osteoblasts, playing an important function in the osteogenesis of MC3T3-E1 cells in vitro, which can increase the local content of phosphoric acid to promote matrix mineralization. In addition, ALP is a specific marker to assess osteoblast activity and tissue calcification capability, and the activity level can reflect the mature situation of osteoblasts.[24] OCN is an abundant non-collagenous protein secreted by osteoblasts, which is a functional state flag in bone metabolism on cell differentiation and maturation.[25] OPN is a kind of acid glycoprotein that contains an Arg-Gly-Asp (RGD) structure, which exists in mineralization and active deposition region. In addition, OPN is closely related to the active protein that induces mature osteoblast phenotype expression and mineralized bone matrix formation. OPN is considered as an osteogenic marker of the differentiation and maturation of osteoblast.[26] In our in vitro study, we synthesized a novel biomimetic peptide, PTHrP-1, and examined its osteogenic capability in vitro. The PTHrP-1 could induce MC3T3-E1 cells into osteoblasts, improving ALP activity and OCN concentration, as well as elevating Col-I, OPN, and OCN mRNA expression and protein expression levels in MC3T3-E1 cell cultures. The PTHrP-1 proved to be an ideal active peptide. The osteogenic ability of 200 and 100 ng/mL PTHrP-1 concentration groups did not show significant difference but were significantly higher than those of 50 and 0 ng/mL groups. Therefore, we opine that PTHrP-1 at the concentration of 100 ng/mL could effectively promote the osteogenic differentiation of MC3T3-E1 cells.

Although clinically introduced as an injectable drug for osteoporosis treatment, PTH still presents several drawbacks and possible complexity.[27] Therefore, PTH was modified into various biological small active peptides, such as PTH (1–31), PTH (1–34), and PTH (1–38).[12] PTH is the major regulator of calcium and phosphorus metabolism and skeletal metabolism,[28] the action of which is mediated through PTH receptor. PTH is a G-protein-coupled plasma membrane receptor expressed in osteoblasts but not in osteoclasts in bone tissue.[29] PTH appears to exert the same anabolic effect on bone because of the identical (1–31) amino acids from the N-terminus of PTH that perform key functions on the osteogenic effect.[30] Human PTH (1–34), teriparatide, consists of the first 34 amino acids of PTH. Teriparatide has been proven to be an effective drug of osteoporosis[31] and exhibits an evident osteogenic capacity. However, teriparatide is only administered by injection and shows short duration. In addition, this drug could not thoroughly expose the active sites.[17]

On this basis, our group has optimized the structure of PTH according to our experience on the BMP-2-related peptide P24, and Ser was phosphorylated in the N-terminal of PTH (1–34). Meanwhile, 3 repetitive Asps were increased in the C-terminal and denoted by PTH-related peptide PTHrP-1. Three possible explanations describe bone formation about PTHrP-1 in vitro in this study. First, PTHrP-1 was derived from the knuckle epitope of the amino acid sequence of PTH that can induce bone formation.[32] Second, phosphorylated Ser and Asp are abundant in the phosphoprotein, which may promote nucleation and self-assembly mineralization of apatite.[18,33,34] Third, the anionic group (phosphorylated Ser) can make the peptide more adhesive to calcium ion and phosphate ion, as well as accelerate nucleation and mineralization. Moreover, PTHrP-1 may thoroughly expose active sites by repetitive Asp binding to HA-binding sites firmly[35] or combining with the specific cell surface receptor efficiently through mechanisms of intercellular communication and signal transduction system.[18] In the present study, with increasing concentration of PTHrP-1 in the culture medium (0, 50, 100, and 200 μg/mL), the time of changing cells into bone-like structure occurred in advance, which showed that PTHrP-1 could effectively promote the osteogenic differentiation of MC3T3-E1 cells, thus confirming cellular compatibility and bone-inducing activity.

Related works reported that PTH (1–34) could exert anabolic or catabolic effects according to concentration and mode of administration. In general, high and sustained doses of PTH (1–34) lead to bone resorption, but intermittent doses with higher amounts or infusion of low doses result in bone formation.[13,36,37] In the present work, depending on intermittent doses, the 200 and 100 ng/mL PTHrP-1 concentration groups showed significantly increased ALP activity, OCN concentration, as well as Col-I, OPN, and OCN mRNA and protein expression levels in comparison with the other 2 groups. However, no significant difference was found between the former 2 groups. Our results show that concentrations of 200, 100, and 50 ng/mL of PTHrP-1 are osteogenic doses that lead to bone formation, and the recommended osteogenic dose is 100 ng/mL. To continuously increase the amount and/or duration of PTHrP-1, we need further study whether the bone induction results will be affected.

As we know, the MC3T3-E1 cells are currently recognized as seed cells in tissue engineering and have been extensively used in osteogenesis experiments in vitro.[38,39] In our study, we synthesized a novel biologically PTH-related peptide, PTHrP-1, and examined its dose-dependence on the osteogenic induction of MC3T3-E1 cells in vitro, which could improve the proliferation and differentiation of MC3T3-E1 cells and maintained their morphology. However, Zhang et al[40] reported that 3 different cell models were utilized for differentiation studies in vitro in order to determine whether there was variability among cell lines or there were cell type-specific effects. Perhaps PTHrP-1 also could improve the proliferation and differentiation of bone marrow stromal cells (BMSc) or other cell systems, which would be reported in the subsequent experiments.

## 5. Conclusions

This study has shown that the PTH-related peptide, PTHrP-1, is a novel biologically active peptide that shows good biocompatibility with MC3T3-E1 cells and could improve the osteogenic differentiation of MC3T3-E1 cells. A PTHrP-1 preferable concentration of 100 ng/mL could effectively promote MC3T3-E1 cells into osteoblasts.

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