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Effects of parathyroid hormone-related protein on osteogenic and adipogenic differentiation of human mesenchymal stem cells

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Abstract. – OBJECTIVE: This work aims to investigate the effects of parathyroid hormone-related peptide (PTHrP) (1-86) on osteogenic and adipogenic differentiation of human mesenchymal stem cells (hMSCs) and the related mechanisms.

MATERIALS AND METHODS: hMSCs were isolated and cultured *in vitro*. They were divided into control group, osteogenesis group, adipogenesis group, osteogenesis+PTHrP group and adipogenesis+PTHrP group. The cell proliferation and differentiation, and expression levels of osteopontin (OPN) and lipoprotein lipase (LPL) mRNA were observed.

RESULTS: The proliferation rates of hMSCs in osteogenesis+PTHrP and adipogenesis+PTHrP group were significantly higher than that in control group, respectively ($p < 0.01$). The alkaline phosphatase (ALP)-positive osteoblasts firstly appeared in osteogenesis+PTHrP group, and Sudan IV -positive adipocytes firstly appeared in adipogenesis group. The expression level of OPN mRNA in osteogenesis+PTHrP group was significantly higher than that in osteogenesis group ($p < 0.05$), and that in adipogenesis+PTHrP group was also higher than adipogenesis group ($p < 0.05$). The expression level of LPL mRNA in osteogenesis+PTHrP group was significantly lower than that in osteogenesis group, and that in adipogenesis+PTHrP group was also lower than adipogenesis group ($p < 0.05$).

CONCLUSIONS: The osteogenesis and adipogenesis are related to each other during the induced differentiation of hMSCs. PTHrP (1-86) can promote the osteogenic differentiation and inhibits the adipogenic differentiation for hMSCs.

Key Words:
Parathyroid hormone-related protein (1-86), Human mesenchymal stem cells, Osteogenesis, Adipogenesis, Differentiation.

Introduction

Parathyroid hormone-related protein (PTHrP) is a polypeptide which is found in research of malignant tumor induced hypercalcemia. There are three original translation products for PTHrP, including PTHrP (1-139), PTHrP (1-141) and PTHrP (1-173). It is expressed in skin, bone marrow, brain, angiocarpy, thyroid, parathyroid and bone, and exerts the function through the autocrine-paracrine pathway[1-4]. They are transformed into series of polypeptides with different biological activities by pos-translational processing with a variety of hormone precursor invertases. The structure and function of the N-terminal amino acid is very similar to that of parathyroid hormone (PTH)[5-8]. PTHrP and PTH act on the same type I receptor (PTHIR), and have similar effects on regulation of calcium and phosphorus metabolism[9,10].

PTH is a single-chain polypeptide with 84 amino acids, which is synthesized and secreted by parathyroid chief cells. It has biological activities of regulating calcium and phosphorus metabolism and maintaining calcium homeostasis in extracellular fluid, playing an important role in balancing and regulating bone metabolism. As confirmed by previous studies, intermittent administration of low-dose PTH can promote the osteogenesis, increase bone density and decrease bone fracture. In addition, PTH has synergistic or accumulating effect for promotion of osteogenesis, when used together with bone resorption inhibitors[11-13]. Therefore, PTH has been a classical drug for osteogenesis for a long time. hPTH (1-34) is the earliest artificially synthesized amino acid fragment with activity of total PTH molecular. It is commonly used for prevention and treatment of osteoporosis. However, PTH has double effect of promoting bone formation and bone absorption. Continuous use of high-dose PTH can inhibit the osteoblastic function and promote bone absorption, leading to loss of bone mass[14-16]. The mouse experiment also finds that high dose of PTH can induce the formation of osteosarcoma[17]. Unlike PTH, PTHrP is a pure ana-

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bolic agent, and only selectively promotes the osteogenesis, without activating the bone absorption[18]. Therefore, PTHrP is superior to PTH in treatment of osteoporosis, with a more extensive application prospect.

At present, the study on promotion function of PTHrP on osteogenesis is limited to 1-36 N-terminal amino acids, relatively less for other fragments[19,20]. In recent years, the research on PTHrP (1-86) has caused more and more attention. PTHrP (1-86) with biological activity has been successfully expressed in *Escherichia coli* using recombinant DNA technology[1]. This provides a favorable support for the further research.

Mesenchymal stem cells (MSCs) derive from the mesoderm, and exist widely in systemic connective tissue and mesenchyme, especially in bone marrow tissue. MSCs have potential of high proliferation, self-renewal and multipotent differentiation[21,22]. More previous literature focus on the hematopoietic cells and osteoblasts in bone marrow stroma, but little attention is paid to the most abundant bone marrow adipocytes.

In this paper, PTHrP (1-86) was used for intervention on hMSCs *in vitro*. The osteogenic and adipogenic differentiation of hMSCs was observed.

Materials and Methods

Specimen Collection

Five bone marrow specimens were collected from patients with traumatic femoral-head replacement surgery (3 males and 2 females, aged 37-45 years). All patients had been diagnosed without metabolic bone disease, acute or chronic infection, or hematologic disease. This study had obtained consent from all patients. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Tianjin Hospital. Written informed consent was obtained from all participants.

Separation, Culture and Grouping of hMSCs

5-10 mL of bone marrow was drawn from patients, and added into a 50 mL centrifuge tube containing 1 mL of heparin and 15 mL of Hank's solution, followed by thorough mixing to obtain bone marrow suspension. After centrifugation at 1000 rpm for 10 min, the fat layer was removed, and then Hank's solution with amount equal to bone marrow was added, followed by thorough mixing. Equal amount of lymphocyte separation medium (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) was slowly added along the centrifuge tube wall. After centrifugation at 2500 rpm for 30 min, the intermediate mononuclear cell layer was carefully sucked out. After washing with Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Sigma, St. Louis, MO, USA) for 2 times, the sediment was mixed with 10% fetal bovine serum (FBS)-DMEM/F12. Then the mixture was transferred to a cell culture flask and placed in incubator for culture (37°C, 5% CO_2 and 95% saturated humidity).

After 3 days of culture, half culture fluid was replaced by new culture fluid. The total culture fluid was replaced after another 2-3 days, considering the growth of cells. When the fusion rate of hBMSCs reached 80%, 0.25% trypsin was used for cell digestion and passage. The third generation cells were selected for experiments. The cells were divided into control group (cultured with 10% FBS-DMEM/F12); osteogenesis group (cultured with 10% FBS-DMEM/F12 containing 1×10^{-8} mol/L dexamethasone, 10×10^{-3} mol/L sodium β-glycerophosphate, and 50 mg/L Vitamin C (above three reagents were provided by Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China[23]); osteogenesis+PTHrP group (cultured with 10% FBS-DMEM/F12 containing 1×10^{-8} mol/L dexamethasone, 10×10^{-3} mol/L sodium β-glycerophosphate, 50 mg/L Vitamin C, and 40 nmol/L PTHrP (PeproTech Inc., Rocky Hill, CT, USA); adipogenesis group (cultured with 10%FBS-DMEM/F12 containing 1×10^{-8} mol/L dexamethasone and 10 mg/L insulin[23]), adipogenesis+PTHrP group (cultured with 10% FBS-DMEM/F12 containing 1×10^{-8} mol/L dexamethasone, 10 mg/L insulin, and 40 nmol/L PTHrP).

Determination of hBMSCs Proliferation

The third generation cells (concentration, 5×10^4 /mL) were inoculated in 96-well plate in each group for culture with different mediums. The culture liquid was replaced by new culture fluid per 48 h. MTT assay was used to determine the cell proliferation rate on the 2nd, 4th, 6th, and 8th day, respectively.

Staining of Osteoblasts and Adipocytes

The third generation cells (concentration, 1×10^5 /mL) were inoculated in 6-well plate in each group for culture with different mediums. The culture liquid was replaced by new culture

Table I. Primer sequences for each gene.

Gene	Upstream sequence	Downstream sequence
OPN	5'-CTAGGCATCACCTGTGCCATACC-3'	5'-CAGTGACCAGTTCATCAGATTCATC-3'
LPL	5'-ATGGAGAGCAAAGCCCTGCTC-3'	5'-TACAGGGCGGCCACAAGTTTT-3'
β-actin	5'-CGTTGACATCCGTAAAGAC-3'	5'-TGGAAGGTGGACAGTGAC-3'

fluid per 48 h. The alkaline phosphatase (ALP) staining of osteoblasts and Sudan IV staining of adipocytes were conducted on the 10th, 14th, and 21st day, respectively. The operation for ALP staining was as follows: The coverslip was taken out, and fixed with cold 95% ethanol for 20 min. Then it was stained using α-naphthol phosphate (Koch-Light Laboratories Ltd., Bucks, England) as substrate and fast red as coupling agent. Finally, the microscopic examination was conducted.

The operation for Sudan IV staining was as follows: The coverslip was taken out, washed with phosphate buffer for 2 times and fixed with formaldehyde calcium for 15 min. Then, it was washed with distilled water for 2 times, followed by staining with 1% Sultan IV solution (1 g of Sudan IV was dissolved in 70% ethanol water solution) for 20 min. After washing with phosphate buffer, the microscopic examination was conducted.

Expressions of Osteopontin and Lipoprotein Lipase

The third generation cells (concentration, 1×10^5/mL) were inoculated in 6-well plate in each group for culture with different mediums. The culture liquid was replaced by new culture fluid per 48 h. The total RNA was extracted using TRIzol and the concentration was determined on the 3rd, 5th, 10th, and 21st day. The cDNA was synthesized by reverse transcription. The related genes were detected using SYBR Green PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) in real-time fluorescent quantitative PCR analyzer (ABI 7300, Foster City, CA, USA) in condition as follows: 95°C, 30 s, 95°C, 5 s, 60°C, 31 s; 40 cycles. The relative expression levels of osteopontin (OPN) and lipoprotein lipase (LPL) mRNA were calculated from Ct values using 2^{-DDt} method. The primer sequences for each gene were shown in Table I.

Statistical Analysis

Statistical analysis was performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. One-way ANOVA was conducted for comparison of measurement data among different groups. $p < 0.05$ was considered as statistically significant.

Results

Cell Proliferation

As shown in Table II, the proliferation rates of hMSCs in osteogenesis+PTHrP and adipogenesis+PTHrP group were significantly higher than that in control group, respectively ($p < 0.01$). There was no significant difference between osteogenesis group and osteogenesis+PTHrP group for each detection time ($p > 0.05$). The difference between adipogenesis group and adipogenesis+PTHrP group was not significant in early stage, but significant since the 4th day ($p < 0.05$).

ALP staining and Sudan IV Staining

Table II. Proliferation rate of hMSCs in each group (n = 6).

Group	Proliferation rate of hMSCs (%)			
	The 2nd day	The 4th day	The 6th day	The 8th day
Control group	0.284 ± 0.011	0.355 ± 0.020	0.393 ± 0.022	0.504 ± 0.052
Osteogenesis group	0.566 ± 0.030*	0.694 ± 0.053*	1.109 ± 0.040*	1.337 ± 0.034*
Osteogenesis+PTHrP group	0.555 ± 0.025*	0.686 ± 0.034*	1.077 ± 0.061*	1.226 ± 0.044*
Adipogenesis group	0.395 ± 0.021*	0.497 ± 0.012*	0.616 ± 0.035*	0.753 ± 0.031*
Adipogenesis+PTHrP group	0.373 ± 0.016*	0.431 ± 0.022*,§	0.527 ± 0.023*,§	0.635 ± 0.023*,§§

Note: *$p < 0.01$, compared with control group; §$p < 0.05$ and §§$p < 0.01$, compared with adipogenesis group.

Results of ALP staining of osteoblasts were shown in Figure 1. On the 10th day, the ALP-positive osteoblasts in osteogenesis+PTHrP group firstly appeared, and the staining in control group and osteogenesis group was negative, respectively. On the 14th day, the positive cells in osteogenesis group appeared. On the 21st day, the positive rate and positive strength in each group increased, and the positive strength in osteogenesis+PTHrP group was higher than the other two groups.

Results of Sudan IV staining of adipocytes were shown in Figure 2. In adipogenesis group, small amount of red tiny lipid droplets appeared on the 10th day. On the 14th day, the number of lipid droplets increased but the arrangement was disordered. On the 21st day, a large number of lipid droplets could be observed. In adipogenesis+PTHrP group, the lipid droplets appeared on 14th day, and the number was lower than adipogenesis group. There was almost no positive cell in control group for each detection time.

Expressions of OPN and LPL mRNA

As shown in Figure 3, the expression levels of OPN mRNA in osteogenesis group and osteogenesis+PTHrP group were significantly higher than that of control group, respectively ($p < 0.05$), and that in early stage of osteogenesis+PTHrP group was significantly higher than osteogenesis group ($p < 0.05$). The expression levels of OPN mRNA in adipogenesis group and adipogenesis+PTHrP group were lower than that in control group, respectively, but that in adipogenesis+PTHrP group was higher than adipogenesis group ($p < 0.05$).

Figure 4 showed that, the expression levels of LPL mRNA in osteogenesis group and osteogenesis+PTHrP group were higher than that in control group, respectively, and that in osteogenesis+PTHrP group was lower than osteogenesis group ($p < 0.05$). The expression levels of LPL mRNA in adipogenesis group and adipogenesis+PTHrP group were significantly higher than that in control group, and that in adipogenesis+PTHrP group was lower than adipogenesis group ($p < 0.05$).

Discussion

In this study, the effects of PTHrP (1-86) on osteogenic and adipogenic differentiation of hMSCs are analyzed. The cell proliferation, ALP staining, Sudan IV staining and expression of OPN mRNA in hMSCs with and without PTHrP induction were compared. Results find that, PTHrP (1-86) can promote the osteogenic differentiation of hMSCs and inhibits the adipogenic differentiation.

Recent researches find that, PTHrP has strong effect of promoting osteogenesis in fetuses and adults[24,25]. In Casado-Diaz et al's study[26], PTHrP (1-36) is added to the osteogenic induction medium to intervene the hMSCs. At the 6th day, the ALP activity and mRNA level are significantly increased, indicating that PTHrP mainly affects the early markers of osteogenic differentiation. In this study, the proliferation rates of hMSCs in PTHrP

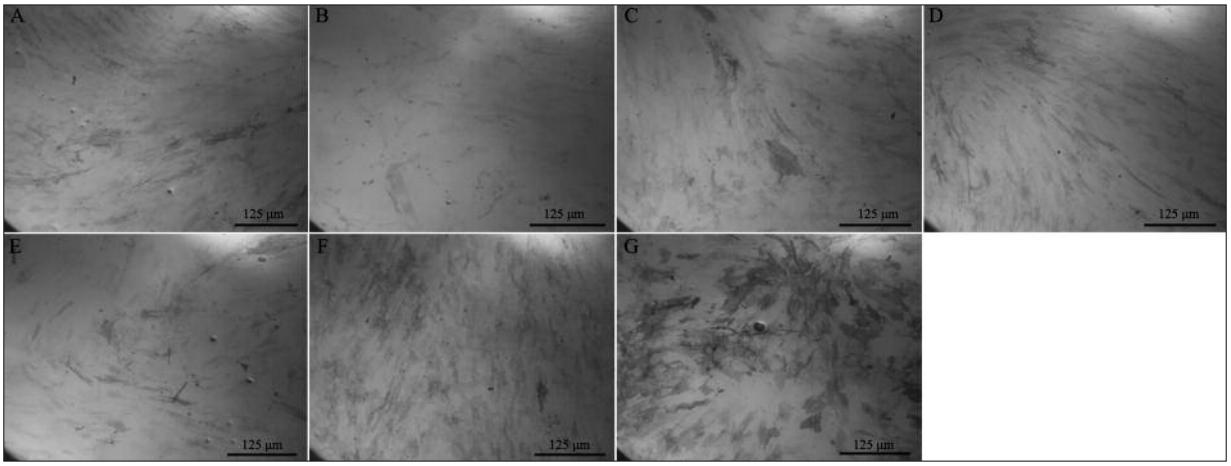

Figure 1. ALP staining of osteoblasts. ***A,*** Osteogenesis+PTHrP group (the 10th day). ***B,*** Control group (the 14th day). ***C,*** Osteogenesis group (the 14th day). ***D,*** Osteogenesis+PTHrP group (the 14th day). ***E,*** Control group (the 21st day). ***F,*** Osteogenesis group (the 21st day). ***G,*** Osteogenesis+PTHrP group (the 21st day).

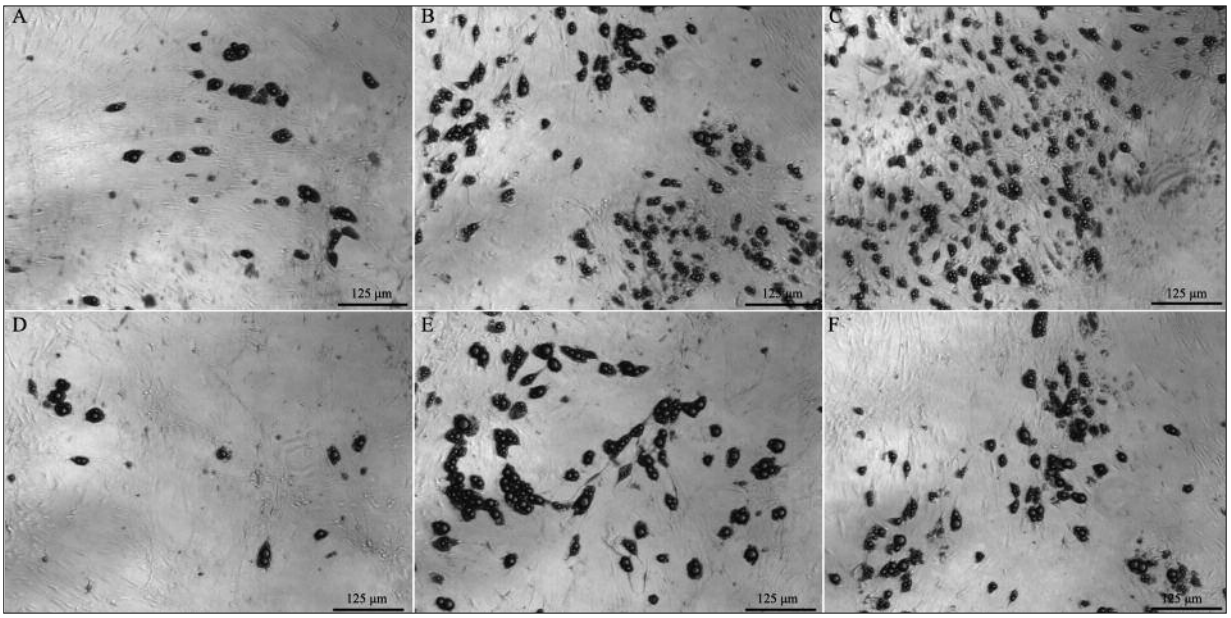

Figure 2. Sudan IV staining of adipocytes. ***A,*** Adipogenesis group (the 10th day). ***B,*** Adipogenesis group (the 14th day). ***C,*** Adipogenesis group (the 21st day). ***D,*** Control group (the 21st day). ***E,*** Adipogenesis+PTHrP group (the 14th day). ***F,*** Adipogenesis+PTHrP group (the 21st day).

intervention groups are significantly higher than that in control group. There was no significant difference of cell proliferation between osteogenesis group and osteogenesis+PTHrP group for each detection time. The difference between adipogenesis group and adipogenesis+PTHrP group was not significant in early days, but significant since the 4th day (Table I). This indicates that, PTHrP (1-86) does not directly promote the proliferation. It may maintain the advantage of osteoblasts by inhibiting proliferation of adipocytes in later stage. The ALP-positive cells in osteogenesis+PTHrP group firstly appear on the 10th day. With the extension of culture time, the positive cells appear in osteogenesis group, and the positive strength in osteogenesis+PTHrP group is higher than other two groups (Figure 1). As shown in this study, the expression level of OPN mRNA in osteogenesis+PTHrP group is significantly higher than that of osteogenesis group. It reaches the peak on the 10th day (Figure 3A). This has further proved the potential synergistic effect of PTHrP (1-86) on osteogenesis.

The positive lipid droplets in adipogenesis+

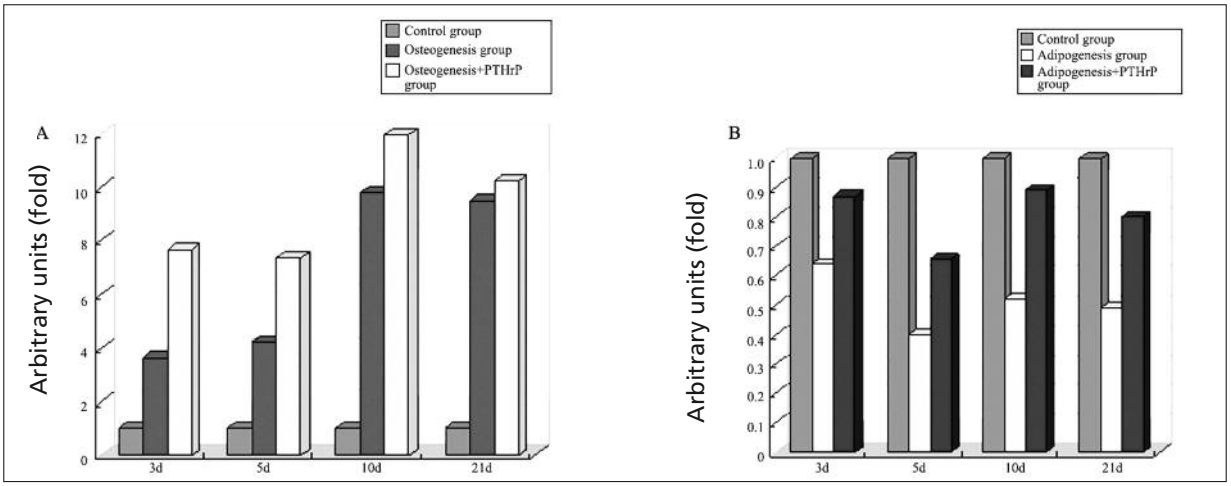

Figure 3. The expression of OPN mRNA in osteogenesis groups ***(A)*** and adipogenesis groups ***(B)***.

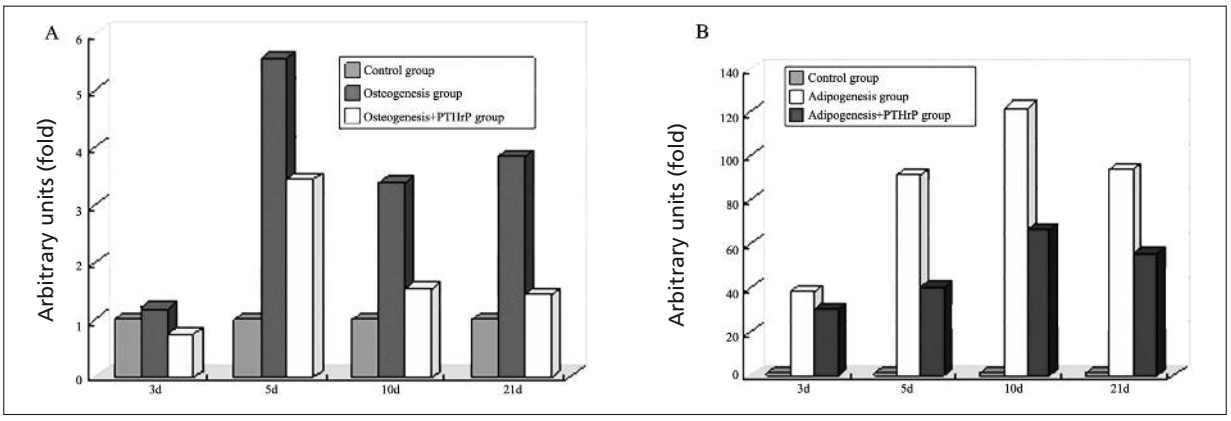

Figure 4. The expression of LPL mRNA in osteogenesis groups ***(A)*** and adipogenesis groups ***(B)***.

PTHrP group appear later than adipogenesis group, and the number of lipid droplets is relative less than adipogenesis group (Figure 2). The expression level of LPL mRNA in adipogenesis+PTHrP group was lower than that in adipogenesis group (Figure 3B), indicating that PTHrP (1-86) has inhibitory effect on adipogenesis for MSCs. Similar results of previous studies show that, addition of excessive PTHrP or its N-fragment can inhibit the adipogenic differentiation of 3T3-L1 and C3H10T1/2 cell line[26-28], and daily administration of PTH (1-34) can decrease the expression level of adipogenic gene in rabbit[29].

This study also shows that, there are expression of OPN and LPL mRNA both in osteogenesis groups and adipogenesis groups. The expression levels of OPN and LPL mRNA in osteogenesis group are higher than that in control group, respectively. The reasons may be that, dexamethasone can induce the differentiation both of osteoblasts and adipocytes. Some scholars[30,31] have observed the induction effect of dexamethasone on rabbit marrow mesenchymal stem cells and find that, dexamethasone can not only promote the adipogenic differentiation, but also promote the osteogenic differentiation. This indicates that, a certain amount of dexamethasone plays an important role in induced osteogenic and adipogenic differentiation, and can be used as an initiation factor in marrow mesenchymal stem cell differentiation[30,31].

In adipogenesis groups, the expression level of OPN mRNA is significantly lower than control group, while the LPL mRNA expression level is significantly higher. This suggests that, both osteogenesis and adipogenesis exist during induced-differentiation of MSCs, and there is correlation between them. Results of this study are in accordance with the view of Owen[32] that, there are common precursor cells for in adult marrow osteoblasts and other cell lines such as adipocytes. As confirmed by previous investigation, MSCs have potential of intrinsic osteogenic and adipogenic differentiation, and there is a transdifferentiation between osteoblasts and adipocytes. The adipogenesis ability decreases during induced osteogenesis, and the osteogenesis ability decreases during adipogenic induction[33-35]. After intervention by PTHrP, the expression level of OPN mRNA increases, while the LPL expression level decreases. This further indicates that, PTHrP (1-86) fragments have effects of promoting osteogenic differentiation and inhibiting adipogenic differentiation.

This study is focused on the effects of N-PTHrP on osteogenic and adipogenic differentiation of hMSCs. However, many physiological characteristics of C terminal and whole peptide and their effects on MSCs are not very clear. In addition, the direct activation effect of PTHrP on osteoclasts is still controversial. The signal pathway in proliferation promotion and its clinical application still need to be further investigated.

Conclusions

The osteogenesis and adipogenesis are related to each other during the induced differentiation of hMSCs. PTHrP (1-86) can promote osteogenic differentiation and inhibit adipogenic differentiation for hMSCs. This study can further elaborate the action mechanism of PTHrP on promotion of osteogenesis, from perspective of

stem cell differentiation, and provide a theoretical and experimental basis for developing new type of anti-osteoporosis drug. In addition, it can widen the train of thought for studying *in vitro* directed differentiation of hMSCs, and provide a foundation for developing new therapeutic means for osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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