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Expressing osteogenic growth peptide in the rabbit bone mesenchymal stem cells increased alkaline phosphatase activity and enhanced the collagen accumulation

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Abstract. – OBJECTIVES: The multipotent mesenchymal stem cells (MSCs) were distributed in the bone marrow stroma, and could generate all of the different skeletal cell lineages. The osteogenic differentiation of MSCs, which is indicated by the increased alkaline phosphatase (ALP) activity and the enhanced accumulation of collagen, could be induced by a tetradecapeptide termed osteogenic growth peptide (OGP). It has been hypothesized that the OGP induces the osteogenic differentiation of MSCs probably through regulating the fibroblast growth factor signaling pathways. Although the chemically synthesized OGP was widely applied to study the osteogenic differentiation of MSCs, transferring and expressing OGP gene in target cells is more desirable, especially for gene therapy, given the advantages and convenience on the stable expression of OGP.

MATERIALS AND METHODS: In this study, we attempt to test the effect of OGP gene transfection; we constructed a eukaryotic expression vector, pcDNA3.1-OGP, which contained the OGP-coding DNA fragment. Subsequently, the vector was transfected into the rabbit MSCs.

RESULTS: A significant increase of ALP activity was detected in the supernatant of pcDNA3.1-OGP transfected MSCs, and the enhanced collagen accumulation, which was inferred by the increased hydroxyproline content and RT-PCR.

CONCLUSIONS: These results implied that transfecting the OGP-expressing vectors into MSCs might induce the osteogenic differentiation of MSCs.

Key Words:

Osteogenic growth peptide, Mesenchymal stem cell, Gene transfection, Alkaline phosphatase activity, Collagen.

Introduction

The postnatal bone marrow is a complex tissue consisted of two distinct but interdependent compartments: the hematopoietic compartment that generates blood cells[1], and the stroma that provides the structural support for hematopoiesis[2]. The mesenchymal stem cells (MSCs) were distributed in the bone marrow stroma, and were progenitors of different cell lineages such as cartilage, bone, and adipocytes[3]. Because of the feasibility in cell isolation and cultivation[3-7], the multipotent MSCs have been widely investigated as a therapeutic agent in tissue engineering and gene therapy[8,9]. For example, it has been reported that after the induction by a combination of 4 factors, the MSCs could differentiate into skeletal muscle lineage cells and could be applied in muscle degeneration repair[10]. *In vitro* experiment has also shown that, under the stimulation of ascorbic acid, osteogenesis supplement, and beta-glycerophosphate, the cultivated MSCs were able to differentiate into osteoblast lineage cells[11], which was indicated by an increased alkaline phosphatase (ALP) activity and accumulated collagen[12].

The osteogenic growth peptide (OGP) is a tetradecapeptide that promotes the MSCs proliferation and differentiation into osteoblastic cells and, thus, enhances bone formation[13-15]. The natural OGP is highly abundant in the serum of human and other animals, and was initially isolated from the osteogenic phase of postablation regenerating bone marrow. It has been demonstrated that OGP was able to increase whole body bone mass through regulation and promotion of ROS, MC3T3, E1, and NH3T3 osteoblast cell lines[16,17]. Additionally, during the OGP therapy for bone fracture healing, Brager et al[18] observed increased TGF-β1 expression in the whole body and the local fracture. Moreover, the expression of 11A, 11B collagen and other factors also increased. Therefore, they concluded that OGP could regulate the TGF-β and the fibroblast growth factor, which were actively involved in signaling path-

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ways controlling the differentiation of MSCs to osteoblast lineage[12]. The peptide sequence of OGP is identical to the C-terminal amino acid sequences 89-102 of histone H4[19], because pre-OGP, the precursor of endogenous OGP, is encoded by a H4 mRNA and translated using an alternative initiation codon[20]. Experimental analyses showed that the 10-14 amino acid sequence was the bioactivity center of the OGP[21].

Although the OGP has been widely applied in the clinical treatment and scientific researches, most of the OGP used in the previous studies were derived from chemical synthesis[13-15]. The difficulties in controlling the concentration and amount of the chemically synthesized OGP largely restricted the clinical application of this peptide. However, the application of OGP as a measure of gene therapy for bone fracture or osteoporosis was also limited. In order to test the effect of OGP gene transfection and the feasibility in induction of the MSCs, the DNA fragment encoding OGP was recombined into the eukaryotic expression vector, $pcDNA_{3.1}$ plasmid, which was subsequently transfected into rabbit MSCs. The effect of the OGP transfection on the MSCs was evaluated by analyzing the ALP activity and collagen accumulation.

Materials and Methods

Isolation and Culture of Rabbit MSCs

The bone marrow cells were obtained from healthy New Zealand rabbits of 4-8 weeks-old, weighing about 2.0 kg. The rabbits were provided by the Laboratory Animal Center of Harbin Medical University. The rabbits were injected with sodium pentobarbital (30 mg/kg) for intravenous anesthesia. The No. 16 bone marrow puncture needle connected with 10 mL syringe which contained 2 mL heparin sodium (2500 U/mL) was used for puncture. Under aseptic condition, the puncture was conducted at the lateral tibial tubercle of the rabbit, and 2-3 mL of bone marrow fluid was obtained.

The bone marrow cell suspension was then injected into equal volume of Percoll solution (density: 1.082 g/mL) slowly. A clear interface was formed between the cell suspension and the Percoll solution. The MSCs were then isolated using the density gradient centrifugation technique. After centrifugation at 2500 r/m for 20 min, the white film formed by the mononuclear cells at the middle of the mixture was extracted and washed twice using PBS. Then the mononuclear cell suspension was centrifuged at 1500 r/m for 10 min. Supernatant was discarded and 10 mL Dulbecco's Modified Eagle Medium (DMEM) culture solution with 15% of fetal bovine serum (FBS) was added, and then the cell strainer filtration was applied to make the single-cell suspension. The MSCs in the suspension was subsequently incubated in 50 mL culture bottles at 37°C in humidified atmosphere of 5% CO_2. After 3 days of incubation, half volume of the culture medium was replaced and all the non-adherent cells were discarded. The replacement of the whole volume of the culture medium was conducted every 3 to 5 days thereafter. Cell passages were totally performed twice and the third passage was used for the subsequent research.

Construction of $pcDNA_{3.1}$-OGP Plasmid

The exogenous OGP gene was provided by the Biochemical Department of Harbin Medical University. The sequences of OGP gene were as follows:

Sense: 5'-AGCT ATG GCG CTT AAA CGC CAG GGC CGC ACA CTC TAC GGC TTC GGT GGT TAA-3';
Antisense: 5'-TCGA TTA ACC ACC GAA GCC GTA GAG TGT GCG GCC CTG GCG TTT AAG CGC CAT-3'.

Both the eukaryotic expression vector $pcDNA_{3.1}$ and the OGP gene fragment were digested with XhoI and HindIII. And then, the segments were ligated to construct the $pcDNA_{3.1}$-OGP recombinant plasmid.

The JM109 competent *Escherichia coli* cells were transformed by the constructed $pcDNA_{3.1}$-OGP plasmid and were, then, cultured at 37°C for 12 h on the Lysogeny Broth (LB) solid culture medium containing ampicillin (100 mg/L) and X-gal (20 g/L). After the cultivation, colonies were randomly selected for cultivation in liquid LB medium. Next, the plasmid was extracted from the transformed cells and then double-checked by BamHI digestion with electrophoresis on agarose gel and sequencing (Shang Hai Sangon Biotech, Shanghai, China).

Liposome Mediated $pcDNA_{3.1}$-OGP Transfection into Rabbit MSCs

When the third passage of MSCs reached 80% confluence, $pcDNA_{3.1}$-OGP plasmid, blank vector was respectively transfected into the MSCs

using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). G418 (300 g/mL) was added in the culture medium for the cells for screening at 24 hours after the transfection. The untransfected cells were cleaned out by the G418, and MSCs successfully transfected by plasmid survived in the G418 screening. Two weeks later, G418-resistant cell clones were collected and then cultivated with normal medium. The comparisons on ALP activity and collagen accumulation were then conducted among MSCs transfected with $pcDNA_{3.1}$-OGP plasmid, blank vector, and untransfected MSCs.

Detecting OGP Expression Using RT-PCR

Seventy-two hours after the transfection, 2×10^6 of rabbit MSCs were gathered from $pcDNA_{3.1}$-OGP transfected group, $pcDNA_{3.1}$ plasmid (blank vector) group, and untransfected control group, respectively. Total RNA of MSCs of the three groups was extracted using Trizol reagent according to the manufacturer's protocol (TianGen, Beijing, China). First-strand cDNA was synthesized with AMV Reverse Transcriptase (TaKaRa, Otsu, Shiga, Japan). The primers used in the RT-PCR were:

31OGPS9: ACTCTACGGCTTCGGTGGTT;
31OGPS113: CCACTGCTTACTGGCTTATCG;
31OGPA266: GTGAGGGTGACAGGAAAGGA.

31OGPS9 and 31OGPA266 amplified the cDNA of the primitive plasmid and the restructured plasmids, while the 31OGPS113 and 31OGPA266 specifically amplified the cDNA from $pcDNA_{3.1}$-OGP transfected MSCs. After 35 rounds of amplification the products were electrophoresed in 1% agarose gel, and stained with 10% ethidium bromide. The gel was illuminated with ultraviolet light.

Measuring ALP Activity and Hydroxyproline Content

The single cell suspension of G418-resistant MSCs transfected with $pcDNA_{3.1}$-OGP and blank vector, as well as the untransfected MSCs, were inoculated on a 24-well plate at a density of 2×10^4/mL in humidified atmosphere of 5% CO_2. Four parallel plates were set for each group. Supernatant was taken at 1, 2, 3, and 4, days to detect the ALP activity using ALP assay kit (Nanjing Jiancheng Bioengineering Institute, China).

Another 4 parallel plates of the same cell density were set for each MSCs group. After 24 h of incubation on the plates, the culture medium was replaced by serum-free medium with mineralization solution (β-sodium glycerophosphate 10 mmol/L and L-ascorbic acid 50 mg/L). After 2 weeks, the hydroxyproline, a specific ingredient of collagen which could indicate the synthesis and accumulation of collagen, was detected using the Chloramine-T method. The formula to calculate hydroxyproline content was:

$$\text{Hydroxyproline (mg/L)} = \text{Absorbance}_{\text{target fluid}} / \text{Absorbance}_{\text{standard fluid}} \times \text{concentration of standard solution} \times \text{dilution fold.}$$

RT-PCR of the Type I Collagen and β-actin mRNA

Two weeks after the adding of the mineralization solution in the medium, Total RNA of the different groups of MSCs was extracted as previously described. After the first strand synthesis, the expression of type I collagen and β-actin was detected using RT-PCR. The photodensity ratio of type I collagen/β-actin mRNA was used in the analysis, and 3 replicates were conducted.

Statistical Analysis

The statistical analyses were processed using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). ALP activity, the hydroxyproline content, and photodensity ratio of different groups were compared using *t* test. The level of significance was set at $p < 0.05$.

Results

Successful Construction of $pcDNA_{3.1}$-OGP Plasmid

After the ligation and recombination of OGP coding sequence with $pcDNA_{3.1}$ plasmid (Materials and Methods), the newly constructed plasmid was single digested with BamHI and electrophoresed on agarose gel. Primitive $pcDNA_{3.1}$ plasmid failing to incorporate OGP was cut into a chain of approximately 5.4 kb in length, while recombined $pcDNA_{3.1}$-OGP plasmid could not be digested and remained in circle shape (Figure 1). The sequencing of the reconstructed plasmid showed that the eukaryotic expression vector $pcDNA_{3.1}$-OGP was successfully constructed (Figure 2).

Cell Changed to Polygonal Shape After $pcDNA_{3.1}$-OGP Plasmid Transfection

Initially the rabbit MSCs were round-shaped. After inoculation of 48 hours, MSCs became

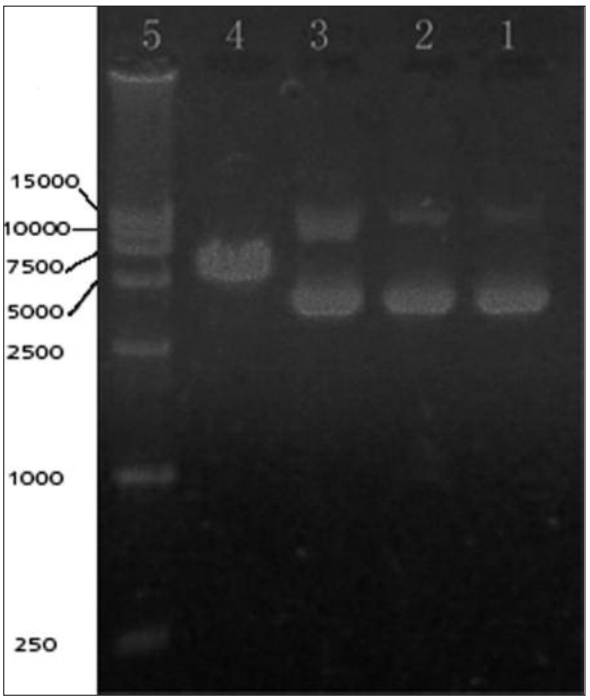

Figure 1. Electrophoresis of the reconstructed pcDNA$_{3.1}$-OGP plasmid. Lane 1: undigested pcDNA$_{3.1}$ plasmid; Lane 2: undigested pcDNA$_{3.1}$-OGP plasmid; Lane 3: BamHI digested pcDNA$_{3.1}$-OGP plasmid; Lane 4: BamHI digested pcDNA$_{3.1}$ plasmid; Lane 5: DNA marker. The length of the marker was also shown.

adherent, stretching to the triangle, polygon, and gradually turning into long spindle shape (Figure 3), which was the common shape before transfection. The G418 screening was performed after 24 h of transfection, and nearly 80% of the cultivated MSCs were killed in the next week. Two weeks after the G418 screening of the transfected MSCs, the pcDNA$_{3.1}$-OGP plasmid transfected MSCs formed colonies, and these transfected G418-resistant MSCs had polygonal shape, which was similar to the osteoblasts (Figure 4). In contrast, the MSCs transfected by blank vectors and the untransfected MSCs were still in long spindle shape (data not shown).

OGP Expressed in the pcDNA$_{3.1}$-OGP Transfected MSCs

In order to examine whether the OGP gene introduced by plasmid was expressed in the transfected MSCs, RT-PCR was conducted firstly using primers 31OGPS9 and 31OGPA266 (Materials and Methods). Fragments of 280 bp and 258 bp in length were detected after the amplification on cDNA of pcDNA$_{3.1}$-OGP transfected and pcDNA$_{3.1}$ transfected MSCs, respectively (data not shown). By contrast, using primers 31OGPS113 and 31OGPA266, which specifically amplified the cDNA of pcDNA$_{3.1}$-OGP group, a 154 bp product was observed for MSCs transfected by pcDNA$_{3.1}$-OGP, while there was no product for the MSCs transfected by blank vector (Figure 5). These results showed that, although transcription of both the blank vector and the reconstructed plasmid occurred in the transfected MSCs, exogenous OGP was specifically transcribed in rabbit MSCs transfected by pcDNA$_{3.1}$-OGP.

Increased ALP Activity in Rabbit MSCs Transfected by pcDNA$_{3.1}$-OGP

The ALP activity was measured at first, second, third, and fourth day after transfection. The ALP activity in the pcDNA3.1-transfected and untransfected control groups was basically unchanged (Figure 6). By contrast, the ALP activity in the pcDNA$_{3.1}$-OGP transfected MSCs kept increasing after the transfection (Figure 6). At each day, the ALP activity in the supernatant of cultivated MSCs transfected by pcDNA$_{3.1}$-OGP was significantly higher than that of the pcDNA$_{3.1}$-transfected MSCs and untransfected control ($p < 0.05$).

Increased C accumulation for pcDNA$_{3.1}$-OGP Transfected MSCs

In order to study the collagen accumulation of the cultured MSCs, mineralization solution was added to the culture medium and cultivated the MSCs for two weeks (Materials and Methods). Then the accumulation of collagen was examined by estimating the hydroxyproline content in the culture medium. The hydroxyproline content was

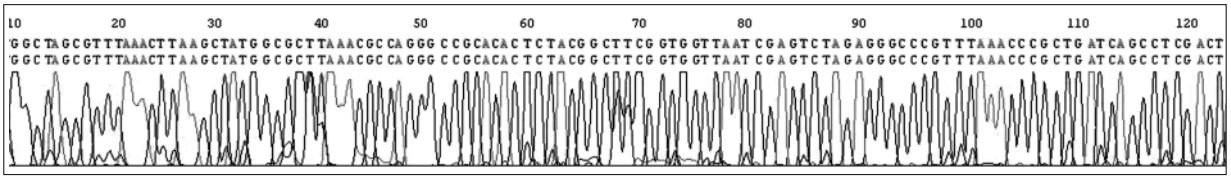

Figure 2. pcDNA$_{3.1}$-OGP plasmid sequencing result, the OGP gene fragment started from the base 28 in the figure.

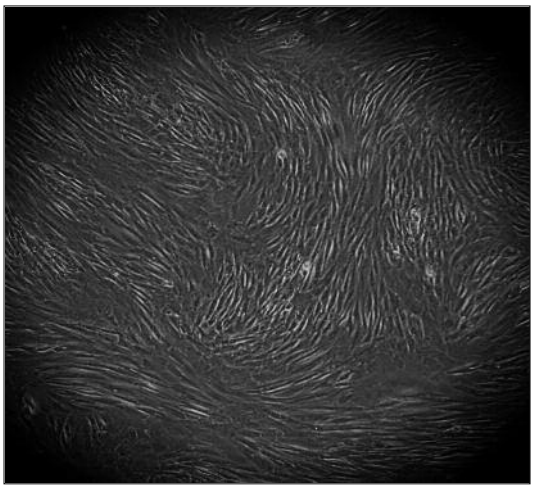

Figure 3. Long, spindle-shaped MSCs before transfection.

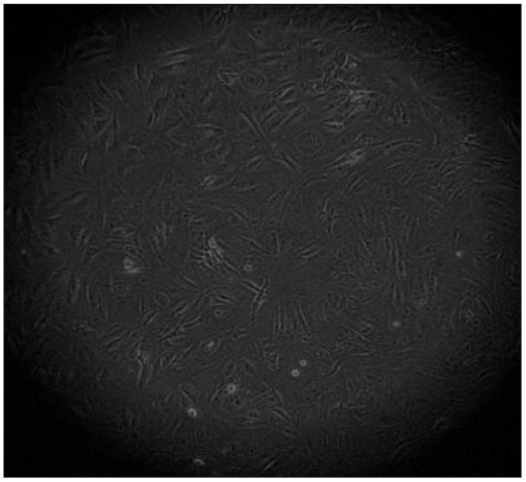

Figure 4. The polygonal-shaped pcDNA$_{3.1}$-OGP transfected MSCs.

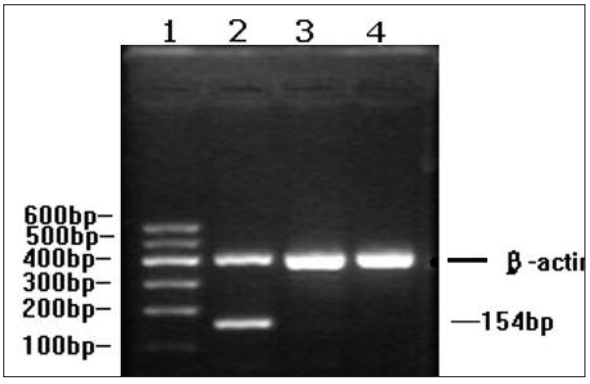

Figure 5. RT-PCR on cDNAs from different groups of MSCs. Lane 1: DNA marker; Lane 2: pcDNA$_{3.1}$-OGP transfected group; Lane 3: pcDNA$_{3.1}$ transfected group; Lane 4: untransfected control group. β-actin cDNA was used as positive control.

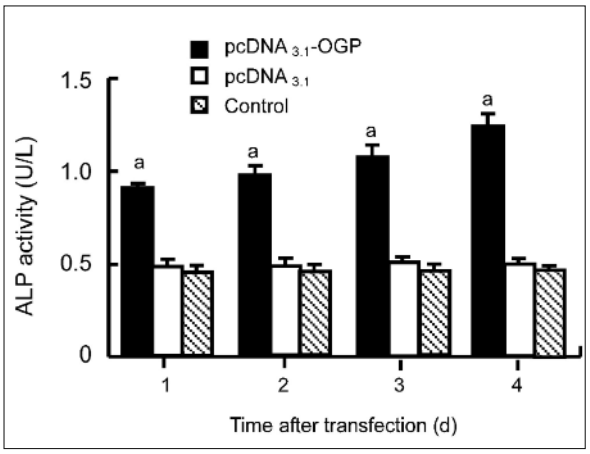

Figure 6. ALP activity in different groups of MSCs. a: Significantly higher ALP activity ($p < 0.05$) in the pcDNA$_{3.1}$-OGP transfected group compared with that of control groups.

significantly higher in the pcDNA$_{3.1}$-OGP transfected group than that in the pcDNA$_{3.1}$-transfected and the untransfected MSCs ($p < 0.01$, Figure 7). RT-PCR on the type I collagen mRNA also demonstrated that the expression of type I collagen was significantly higher in the pcDNA$_{3.1}$-OGP transfected MSCs than that in the pcDNA$_{3.1}$-transfected and the untransfected MSCs ($p < 0.05$, Table I).

Discussion

In this study, rabbit MSCs were transfected with pcDNA$_{3.1}$-OGP eukaryotic expression vector *in vitro*. Seventy-two hours after the transfection,

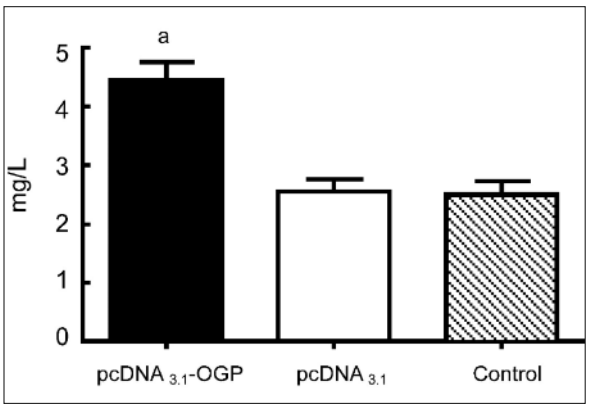

Figure 7. Hydroxyproline content in different groups of MSCs. a: Significantly higher hydroxyproline content ($p < 0.01$) in the pcDNA$_{3.1}$-OGP transfected group compared with that of control groups.

Table I. Photodensity ratio of the type I collagen/β-actin mRNA among different groups (n=3, mean ± SD).

MSCs group	Photodensity ratio
Untransfected controls	1.50 ± 0.15
$pcDNA_{3.1}$-transfected MSCs	1.33 ± 0.14
$pcDNA_{3.1}$-OGP transfected MSCs	2.88 ± 0.19

The type I collagen/β-actin photodensity ratio was significantly higher in the $pcDNA_{3.1}$-OGP transfected MSCs than that of $pcDNA_{3.1}$-transfected MSCs and untransfected controls ($p < 0.05$).

OGP gene transcription was detected using RT-PCR. Afterwards, an increase in ALP activity and the enhanced accumulation of collagen was detected. ALP and collagen were regarded as indicator of osteoblastic differentiation[22]. Therefore, the results of this study implied that the rabbit MSCs might started the differentiation into osteoblast lineage cells after $pcDNA_{3.1}$-OGP transfection. Much more efforts should be made to study whether expressing OGP in the MSCs triggered the osteoblastic differentiation. Although the chemically synthesized OGP was widely applied to scientific and clinical studies, it is the first time to transfer and express the exogenous OGP gene in MSCs. This study provided evidence that exogenous expression of OGP in MSCs was associated with the increased ALP activity and enhanced collagen accumulation *in vitro*.

The multipotent MSCs are appropriate seed cells in tissue engineering because they are easy to isolate and cultivate. Introducing differentiation-initiating factor genes into MSCs and express the gene *in vivo* is a prospective method for gene therapy. Although the MSCs have the potential to differentiate into various cell types, it is reported that their differentiation is not a spontaneous process, and is regulated by a number of cytokines *in vivo*. Previous studies have shown that the ossification process of MSCs was slow without stimulation of exogenous growth factors, and the original MSCs should be engineered before implantation[23,24]. Therefore, enhancing the differentiation of MSCs into osteoblasts stably and massively became a key issue in bone tissue engineering and related gene therapy.

Conclusions

The capability to promote MSCs differentiation into osteoblast lineage cells and the relatively low molecular weight made the OGP suitable for gene therapy of the bone fracture, osteoporosis, and other diseases of the bone. However, nobody has transferred OGP into MSCs for clinical gene therapy application so far. Further studies on the pathways regulated by OGP, transfecting OPG into MSCs *in vivo*, and controlling OGP expression was needed to facilitate the practical and clinical application of OGP as gene therapy treatment.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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