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Prunella vulgaris L protects glucocorticoids-induced osteogenesis inhibition in bone marrow mesenchymal stem cells through activating the Smad pathway

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Abstract. – OBJECTIVE: To elucidate the role of *Prunella vulgaris L* (PVL) in protecting glucocorticoids (GC)-induced osteogenesis inhibition, thereafter, protecting the deterioration of osteoporosis (OP).

MATERIALS AND METHODS: Cell Counting Kit-8 (CCK-8) assay was conducted to assess the influence of PVL treatment on MSCs viability. Osteogenesis in MSCs was induced by Dexamethasone (DEX) stimulation. Regulatory effects of PVL on osteogenesis-related gene expressions, ALP activity, and mineralization ability in DEX-induced MSCs were determined. At last, protein levels of p-Smad1/5/9 and total-Smad1/5/9 influenced by DEX and PVL were measured by Western blot.

RESULTS: PVL treatment did not pose a time- or dose-dependent influence on MSCs viability. DEX induction in MSCs downregulated ALP, RUNX2, Bglap, and Osterix. ALP activity and mineralization in DEX-induced MSCs were suppressed. Downregulated osteogenesis-related genes decreased ALP activity and mineralization in MSCs undergoing DEX stimulation were partially reversed by PVL treatment. Moreover, the downregulated p-Smad1/5/9 level in DEX-induced MSCs was elevated by PVL treatment, while total-Smad1/5/9 was not affected.

CONCLUSIONS: PVL alleviated GC-induced suppression in MSCs osteogenesis by activating the Smad pathway, thereafter, protecting the deterioration of OP.

Key Words:
Prunella vulgaris L, Smad, Glucocorticoids, Osteogenesis.

Introduction

Glucocorticoids (GCs) are the preferred drugs for the treatment of many chronic diseases and autoimmune diseases, such as rheumatoid arthritis, connective tissue disease, inflammatory bowel disease, chronic obstructive pneumonia, asthma, etc.[1]. It is reported that approximately 1-2% population in different ages worldwide are treated with long-term GCs[2]. Nevertheless, long-term administration of GCs would result in many adverse events. Glucocorticoid-induced osteoporosis (GIO) is the main cause of secondary OP. Notably, low stress fractures resulted by GIO pose severe pain and burden on affected people[3].

Prunella vulgaris L (PVL) is a plant belonging to the Lamiaceae family, which is traditionally applied for anti-inflammatory, anti-allergic, anti-oxidant, and anti-viral treatment[4]. Some studies[5-8] have shown that the triterpenoids and their saponins, which are enriched in PVL, have a pronounced anti-tumor activity.

Smad protein is a direct substrate for Transforming Growth factor-β (TGF-β). Eight members of Smad have been found in mammals. They can be divided into three subfamilies based on their structures and functions: R-smad, Co-Smad, and I-Smad. They are able to indirectly mediate the signal transduction of TGF-β by binding to other transcription factors in the nucleus or directly regulate DNA transcription[9,10]. It is reported that Smad is capable of promoting osteoblast formation through activating the TGF-β pathway[11]. In this paper, we analyzed the protective role of PVL in GC-induced inhibition of MSCs osteogenesis and the potential mechanism.

Materials and Methods

Cell Culture

MSCs were prepared for suspension with (5-8)×10^4/mL. Cells were cultured in α-Modified

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Eagle's Medium (α-MEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 UI/mL penicillin and 100 μg/mL streptomycin. Fourth-generation MSCs were used for functional experiments.

DEX Treatment

Cells in good growth were digested and prepared for suspension with (5-8)×10^4/mL. 100 μL of suspension was applied in each well of a 96-well plate. After overnight cell culture, medium containing 1 μM dexamethasone (DEX) was replaced.

Osteogenesis Induction

Mesenchymal stem cells (MSCs) were inoculated in a 6-well plate. After cells were grown to 60-70% confluence, osteogenic medium (α-MEM containing 10% fetal bovine serum, 0.1 μmol/L DEX, penicillin 100 UI/mL and streptomycin 100 μg/mL, 10 mmol/L β-glycerophosphate and 50 mg/L Vitamin C) was applied.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 4 replicates per well. Viabilities in MSCs treated with 0, 5, 10 or 20 μM PVL were determined at day 1, 3, 7 or 14, respectively. Before viability determination, 20 μL of CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) was applied per well. After 3-h incubation, values at 450 nm wavelength were measured.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. Through reverse transcription of RNA, the extracted complementary deoxyribose nucleic acid (cDNA) was used for PCR detection by SYBR Green method (TaKaRa, Otsu, Shiga, Japan). The primer sequences were listed as follows: ALP, forward: 5′-ACCACCACGAGAGTGAACCA-3′ and reverse: 5′-CGTTGTCTGAGTACCAGTCCC-3′; RUNX2, forward: 5′-GGGTAAGACTGGT-CATAGGACC-3′ and reverse: 5′-CCCAGT-ATGAGAGTAGGTGTCC-3′; Bglap, forward: 5′-AAAGCC TGGTGATGCAGAGT-3′ and reverse: 5′-CTAGACTGGGCCGTAGAAGC-3′; Osterix, forward: 5′-AGGAGGCACAAAGAAGCCATAC-3′ and reverse: 5′-AGGGAAGGGTGGGTAGTCATT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5′-TTCTTTTGCGTCGCCAGCCGA-3′ and reverse: 5′-GTCACCACCCGCCCAATACGA-3′.

Western Blot

Cellular protein was isolated using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and electrophoresed. Protein sample was loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were immersed in phosphate-buffered saline (PBS) containing 5% skim milk for 2 h, reacted with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Band exposure was achieved by enhanced chemiluminescence (ECL) and processed by Image Software.

ALP Activity Determination

MSCs were washed with pre-cold PBS for three times and lysed in pre-cold 1% Triton X-100 on ice for 30 min. Cell lysate was prepared by centrifugation at 4°C, 12,000 g/min for 5 min. Suspension was applied in a 96-well plate (30 μL/well). Value at 405 nm was measured and normalized to that of total protein concentration.

ALP Staining

MSCs were washed with PBS twice, reacted in 70% ethanol for 10 min and ALP buffer (0.15 M NaCl, 0.15 M Tris-HCl, 1 mM $MgCl_2$, pH9.5) for 15 min. Subsequently, cells were cultured in NBT-BCIP solution at 37°C, in the dark for 30 min. Images were captured under a microscope.

Alizarin Red Staining (ARS)

After osteogenesis induction in MSCs, cells were washed with PBS, reacted in 2 ml of 4% methanol for 30 min and washed again with PBS. Subsequently, MSCs were dyed in 1 ml of alizarin red (ARS)-Tris-HCL solution (pH4.3) for 3-5 min. Visible mineralized nodules were captured under an inverted microscope

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean ± SD (standard deviation). The *t*-test was used for analyzing differences between two groups. $p<0.05$ indicated the significant difference.

Results

Influences of PVL On MSCs Viability

MSCs were treated with 0, 5, 10 or 20 μM PVL for 1, 3, 7 or 14 days, respectively. In the same time point, no significant difference in MSCs viability was observed after treatment of different concentrations of PVL. In addition, time duration of PVL treatment did not significantly affect MSCs viability as well (Figure 1A). It is demonstrated that PVL treatment had little impact on MSCs viability.

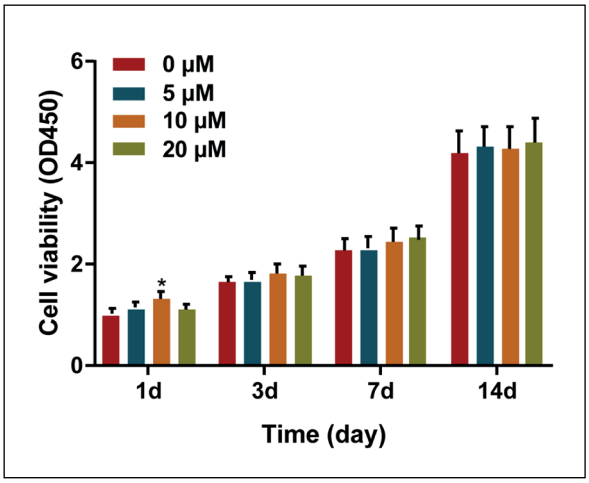

Figure 1. Influences of PVL on MSCs viability. MSCs were treated with 0, 5, 10 or 20 μM PVL for 1, 3, 7 or 14 days. Their viabilities were detected by CCK-8 assay.

PVL Protected Downregulation of Osteogenesis-Related Genes In GC-Induced MSCs

MSCs were subjected to osteogenesis by 1 μM DEX treatment. DEX treatment markedly downregulated ALP (Figure 2A), RUNX2 (Figure 2B), Bglap (Figure 2C), and Osterix (Figure 2D). Interestingly, 10 μM PVL treatment markedly reversed their downregulated levels. The results suggested that PVL protected downregulation of osteogenesis-related genes in GC-induced MSCs.

PVL Protected GC-Induced Osteogenesis Suppression In MSCs

Similarly, protein level of RUNX2 was downregulated in MSCs undergoing DEX treatment, which was further enhanced by PVL administration (Figure 3A). Both ALP activity and ALP-positive staining were reduced in DEX-induced MSCs than those of controls, and PVL treatment reversed the decreased trends (Figure 3B, 3C). ARS suggested that the inhibited mineralization ability in DEX-induced MSCs was partially reversed by PVL treatment (Figure 3C). These results revealed that PVL protected GC-induced osteogenesis suppression in MSCs.

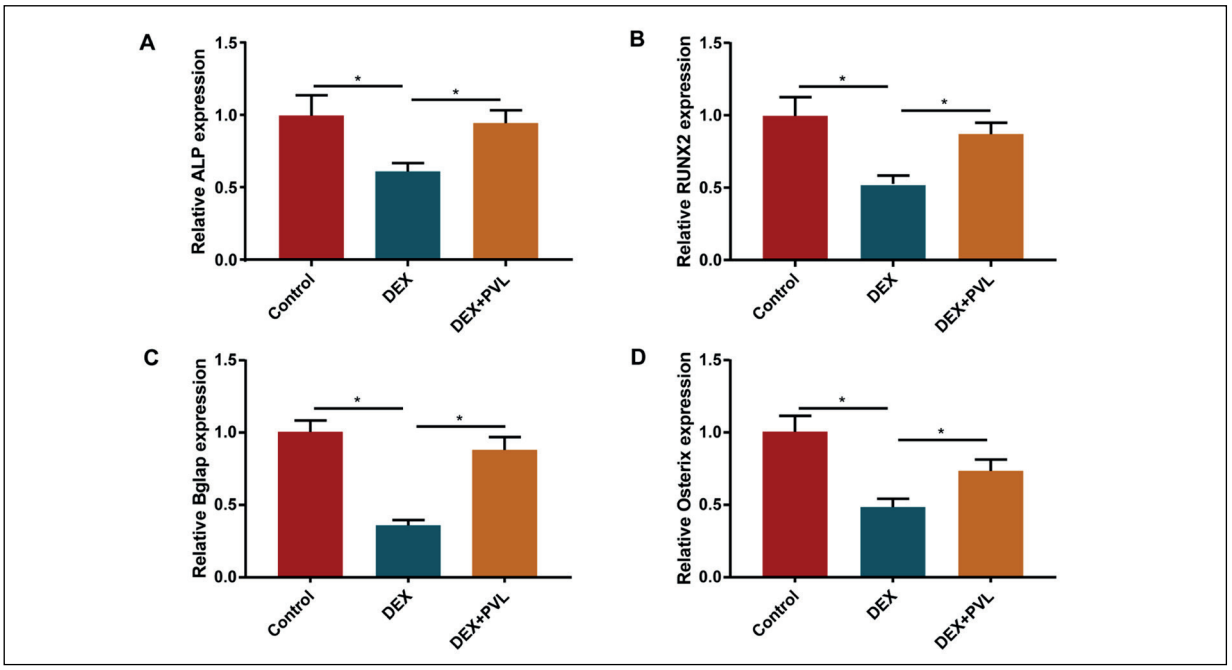

Figure 2. PVL protected downregulation of ALP (**A**), RUNX2 (**B**), Bglap (**C**), and Osterix (**D**) in GC-induced MSCs.

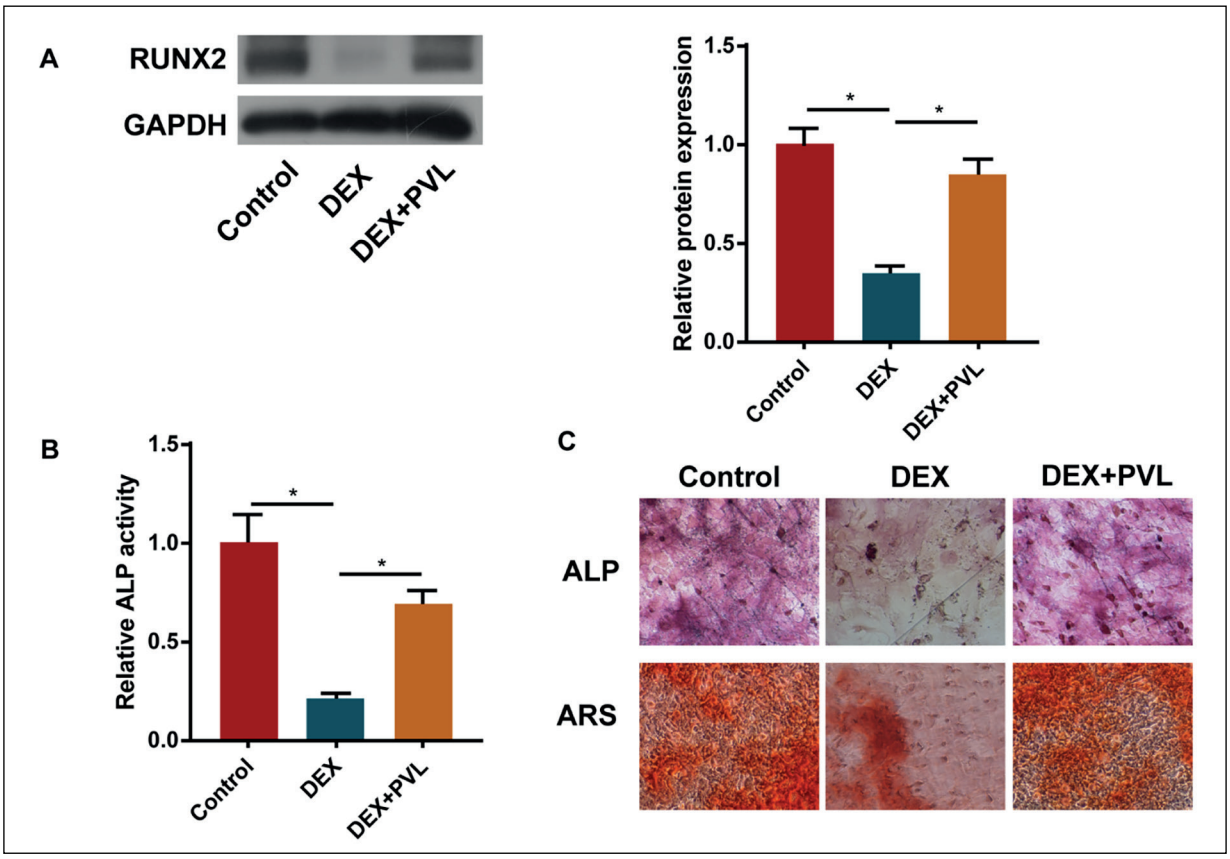

Figure 3. PVL protected GC-induced osteogenesis suppression in MSCs. MSCs were treated with blank control, DEX, or DEX+PVL. **A,** Protein level of RUNX2; **B,** ALP activity; **C,** ALP staining and ARS (magnification: 400×).

PVL Regulated Osteogenesis By Activating the Smad Pathway

Western blot analyses uncovered that protein level of p-Smad1/5/9 was downregulated in MSCs undergoing DEX treatment, and PVL protected such a downregulated trend. However, either DEX or PVL treatment affected the protein level of total-Smad1/5/9 (Figure 4). These results suggested that PVL regulated osteogenesis by activating the Smad pathway.

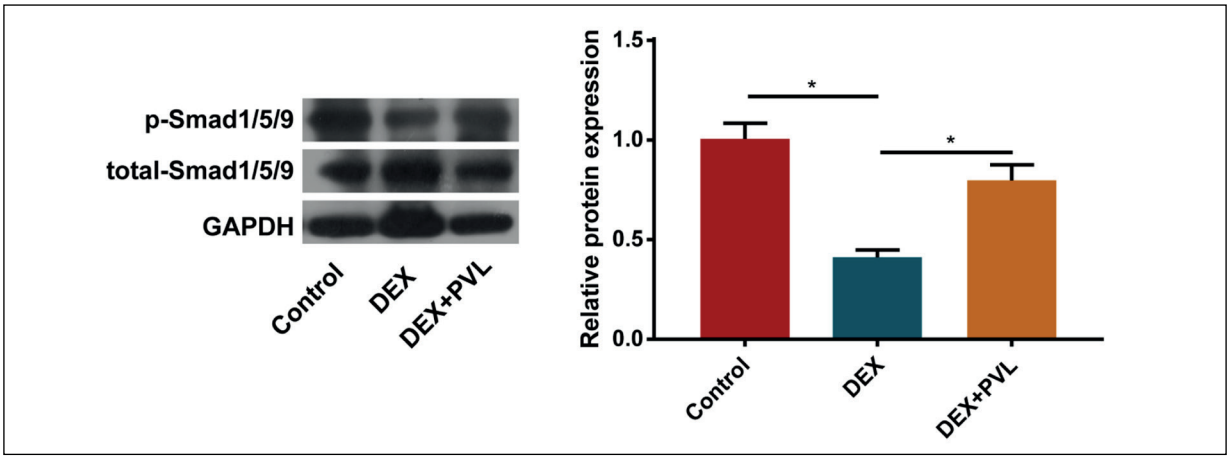

Figure 4. PVL regulated osteogenesis by activating the Smad pathway. Protein levels of p-Smad1/5/9 and total-Smad1/5/9 in MSCs treated with blank control, DEX, or DEX+PVL.

Discussion

GIO is a skeletal complication resulted from long-term or high-dose administration of GCs[12,13]. Shen et al[14] have confirmed that bone metabolism disorder is the major pathogenesis of OP.

In vivo osteogenesis in MSCs is a complex process regulated by a variety of mechanisms. Under normal physiological conditions, osteogenesis and bone resorption are in equilibrium. Inhibited bone formation and stimulated bone resorption eventually lead to imbalanced bone metabolism[15]. During the process of osteogenesis, osteoprogenitor cells, as stem cells of bone tissues, are differentiated into osteoblasts and thus maintain bone metabolism homeostasis[16]. In addition to supplement to osteoblast number, MSCs osteogenesis also triggers paracrine secretion of endogenous growth factors, which further stimulates bone formation[17]. ALP increases significantly during the early stage of osteogenic differentiation, and the level of ALP activity can reflect the degree of osteogenic differentiation[18,19]. Runx2, an important transgenic gene factor, is related to osteogensis[20]. Osterix is regulated by Runx2 during osteogenic differentiation[21]. Moreover, Bglap is involved in embryonic bone formation and are activated during bone remodeling[22,23]. In our present study, we found that DEX induction downregulated osteogenesis-associated genes, reduced ALP activity, and mineralization ability in MSCs, which were all reversed by PVL treatment.

Multiple pathways and cytokines are involved in osteogenesis[24]. Santibanez et al[25] demonstrated the critical role of the TGF-β/Smads pathway in bone remodeling. TGF-β is a cytokine secreted by osteoblasts. The subtype TGF-β1 has the highest proportion among TGF-β members in bone tissues. It is the vital coupling factor coordinating bone formation and resorption[26]. The Smads family is known to be the only signal transduction molecule of TGF-β1 in the cytoplasm[27]. TGF-β1 activates the downstream Smad2/3 protein by phosphorylation. Phosphorylated Smad2/3 (p-Smad2/3) binds to Smad4 to form a heterodimer and translocates into the nucleus, thereafter regulating transcription of target genes. Smad7 is an inhibitor in the TGF-β/Smads pathway. It blocks Smad2/3 phosphorylation and thus inactivates the TGF-β1 pathway[28,29]. In our research, the downregulated p-Smad1/5/9 level in DEX-induced MSCs was elevated by PVL treatment.

Collectively, GC induction inhibited MSCs osteogenesis, which was alleviated by PVL treatment by activating the Smad pathway. Our findings provided novel directions for prevention and treatment of OP.

Conclusions

Taken together, these results revealed that PVL alleviated GC-induced suppression in MSCs osteogenesis by activating the Smad pathway, thereafter protecting the deterioration of OP.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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