Mangiferin promotes the osteogenic differentiation of human periodontal ligament stem cells via TGF-β/SMAD2 signaling

YINGZHI GU, LI ZHANG and YUXING BAI

Department of Orthodontics, Beijing Stomatological Hospital, Capital Medical University, Beijing 100050, P.R. China

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Abstract. Mangiferin (MAG) is a polyphenolic compound present in mangoes. This compound suppresses inflammation and decreases bone destruction. This study aimed to determine whether MAG directly promotes proliferation and osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs). Cell proliferation and osteogenic differentiation experiments were performed in hPDLSCs, and MAG was used as a stimulator during osteogenic induction. Alkaline phosphatase (ALP) activity and Alizarin red staining were analyzed, and the expression of osteogenesis-associated genes was investigated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis to determine the effect of MAG on the osteogenic differentiation of hPDLSCs. Galunisertib was used to selectively inhibit TGF-β/SMAD2 signaling. Western blotting was performed to study the underlying mechanism. Cell Counting Kit-8 assay showed that MAG did not promote the proliferation of hPDLSCs. MAG (200 μM) significantly promoted ALP activity, mRNA levels of alkaline phosphatase biomineralization associated, collagen type 1, and runt-related transcription factor-2, protein levels of SMAD5, alkaline phosphatase and bone morphogenetic protein 2 protein expression and mineralized nodule formation in hPDLSCs. Furthermore, MAG significantly promoted the phosphorylation of SMAD2. Galunisertib inhibited the activation of SMAD2 and partially reversed the MAG-mediated promotion of hPDLSC osteogenic differentiation. These data indicated that MAG promoted osteogenic differentiation of hPDLSCs potentially through TGF-β/SMAD2 signaling. Therefore, MAG may help improve periodontal bone loss.

Correspondence to: Professor Yuxing Bai, Department of Orthodontics, Beijing Stomatological Hospital, Capital Medical University, 4 Tiantanxili, Dongcheng, Beijing 100050, P.R. China
E-mail: byuxing@ccmu.edu.cn

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Introduction

Periodontal disease is a chronic inflammatory disease that is the leading cause of tooth loss in adults (1). This disease is characterized by an inflammatory response around the teeth, which is primarily caused by oral microbial biofilms and maintained by an uncoordinated immune inflammatory response, ultimately leading to progressive destruction of the tissues supporting the teeth (2). This condition is related to many systemic diseases, such as vascular disease, diabetes and heart disease (3). To the best of our knowledge, no suitable method for periodontal tissue regeneration has yet been developed.

The periodontal ligament contains pluripotent periodontal stem cells that express mesenchymal stem cell surface markers and show self-renewal and pluripotency (4). Periodontal ligament stem cells (PDLSCs) form cementum/periodontal ligament-like structures after transplantation *in vivo*, which indicates that PDLSCs play an important role in the reconstruction and regeneration of periodontal tissue, providing a new prospect for periodontal tissue regeneration (5). In addition, animal experiments have shown that PDLSCs from different sources (including human, canine, and porcine) can initiate the homing effect and promote the regeneration of periodontal tissue after implantation (6,7). Transplantation of PDLSCs effectively regenerates alveolar bone in alveolar defects in minipigs, showing encouraging results in preclinical trials (8,9). Due to their proliferative and pluripotent differentiation abilities, as well as their ability to form Sharpey fibers and cementum-like structures, PDLSCs are considered optimal seeding cells for periodontal engineering (10).

Researchers have increasingly examined new plant and fruit bioactive compounds that can be used to combat chronic disease and certain types of cancer (11,12). Mangiferin (MAG), a natural polyphenolic compound commonly found in mango and papaya, exhibits beneficial biological activities, including antioxidant, antitumor, antiviral, antidiabetic and immunomodulatory activities (13-18). MAG has also been reported to have anti-osteoclast activity for the treatment and prevention of bone disease (19). To the best of our knowledge, however, the effect of MAG on the osteogenic differentiation of PDLSCs has not been reported.

The present study examined the effect of MAG on the proliferation and osteogenic differentiation of human PDLSCs (hPDLSCs) *in vitro* as well as the molecular pathways

that are involved. It was hypothesized that MAG can promote the differentiation of hPDLSCs into osteoblasts and may be a potential drug for periodontal tissue regeneration.

Materials and methods

Primary culture of hPDLSCs. The first or second premolars that were removed from healthy individuals due to orthodontic needs were collected at the Beijing Stomatological Hospital after obtaining verbal patient consent, with approval of the Ethics Committee of Capital Medical University in China. A total of 10 patients (4 male, 6 females) aged 18-23 years were recruited from January 2021 to July 2021. Periodontal tissue was scraped from the middle third of the root of a healthy premolar extracted by orthodontic treatment. Tissues were minced and digested in equal volumes with collagenase type I (3 mg/ml) and neutral protease (4 mg/ml) for 1 h at 37˚C. The isolated cells were then cultured in α-MEM with 10% fetal bovine serum and 1% penicillin/streptomycin (all Gibco) and placed in a 37˚C and 5% CO_2 cell incubator. Cells at third and fourth passage were used for the subsequent experiments.

Cell viability assay. The effect of MAG on viability of hPDLSCs was determined by Cell Counting Kit-8 (CCK-8) assays. Briefly, hPDLSCs were cultured in 96-well plates ($1x10^4$ cells/well) and cultured with 0, 50, 100, 200 or 500 μmol/l MAG (labeled MAG0, MAG50, MAG100, MAG200 or MAG500) for 7 days. Then, 10 μl CCK-8 reagent (Dojindo Laboratories, Inc.) was added dropwise to each well for 2 h. The optical density values in each well were measured at a wavelength of 490 nm under a microplate reader (Omega Bio-Tek, Inc.).

Osteogenic induction. The osteogenic medium consisted of 15% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 U/ml streptomycin, 1% glutamine (Gibco; Thermo Fisher Scientific, Inc.), 10 nmol/l dexamethasone (MilliporeSigma), 0.2 mmol/l ascorbic acid (MilliporeSigma) and 10 mmol/l sodium β-glycerophosphate (MilliporeSigma) in α-MEM (Gibco). MAG at 0, 200 and 500 μmol/l (labeled as MAG0, MAG200, MAG500) were selected for hPDLSC treatment in subsequent cell experiments.

Alkaline phosphatase (ALP) activity assay. ALP activity in hPDLSCs was assessed using an ALP activity assay kit (MilliporeSigma) and absorbance was measured at a wavelength of 520 nm on a microplate reader. ALP staining was performed using an ALP staining kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

Alizarin red staining. Alizarin red staining was used to observe mineral deposition in each group. Cultured cells were fixed in 95% ethanol for 30 min and then stained with 0.1% Alizarin red staining solution (pH 4.2) for 20 min at room temperature. After cells were washed with PBS, 100 mmol/l cetylpyridinium chloride (MilliporeSigma) was added to each well and incubated at room temperature for 30 min. The absorbance was measured at a wavelength of 562 nm on a microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) detection of osteogenesis-related genes. Total cellular RNA was extracted from hPDLSCs using TRIzol (Invitrogen, Thermo Fisher Scientific, Inc.) and a total of 2 μg RNA/sample was reverse transcribed to complementary DNA (cDNA) using a cDNA RT kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The expression of the osteogenesis-related genes ALP, biomineralization associated (ALPL), collagen type 1 (COL1) and runt-related transcription factor 2 (RUNX2) was determined by RT-qPCR using TaqMan Gene Expression Assay (Invitrogen, Thermo Fisher Scientific, Inc.), then qPCR was performed with denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 15 sec. The expression of the housekeeping gene GAPDH was used as an internal reference. Data were analyzed using the $2^{-\Delta\Delta Cq}$ relative expression method (20). The primer sequences are listed in Table I.

Western blot analysis. Cells were harvested, washed and lysed in immunoprecipitation assay buffer (Beyotime Institute of Biotechnology). The protein content was determined using the BCA method. Proteins were separated by 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20 μg protein per lane) and blotted onto polyvinylidene fluoride membranes. After blocking with Quick Blot Buffer (Applygen Technologies Inc.) at room temperature for 30 min, the proteins were detected by overnight incubation with rabbit polyclonal primary antibodies against RUNX2 (1:1,000, ab23981; Abcam), ALP (1:1,000, ab83295; Abcam), COL1 (1:1,000, ab233080, Abcam), HSP90 (1:1,000, ab13495; Abcam), TGF-β1 (1:1,000, ab92486; Abcam), p-SMAD2 (1:1,000, 3108T; Cell Signaling Technology), SMAD2 (1:1,000, 5339T; Cell Signaling Technology), SMAD3 (1:1,000, 9523T; Cell Signaling Technology) and β-actin (1:1,000, ab8227; Abcam) at 4˚C. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG secondary antibody (1:2,000, cat. no. ab6721; Abcam) for 1 h at room temperature. Specific complexes were visualized using SuperEnhanced chemiluminescence detection kit (Applygen Technologies, Inc.). Band intensities were quantified using ImageJ 1.53 software (National Institutes of Health). The background was subtracted, and the signal of each target band was normalized to that of HSP90 or β-actin.

Galunisertib treatment of hPDLSCs. hPDLSCs were cultured for 5 days at 37˚C in 4 groups as follows: untreated control, 200 μM MAG, 100 nM galunisertib or 100 nM galunisertib + 200 μM MAG. After multiple washes with PBS, hPDLSCs were harvested and subjected to western blot analysis and alizarin red staining analysis.

Statistical analysis. Statistical analysis was performed using SPSS 10.2 (SPSS, Inc.). One-way ANOVA and Tukey's post hoc test was used to assess differences between groups and P-values were calculated using unpaired Student's t test. All data are presented as the mean ± standard deviation (SD). A 95% confidence level ($P<0.05$) was considered to indicate a statistically significant difference. The number of replicates in each experiment is indicated in the figure legends.

Table I. Reverse transcription-quantitative polymerase chain reaction primers used.

Gene name	Sequence, 5'→3'
GAPDH	
Forward	CGGACCAATACGACCAAATCCG
Reverse	AGCCACATCGCTCAGACACC
ALPL	
Forward	GACCTCCTCGGAAGACACTC
Reverse	TGAAGGGCTTCTTGTCTGTG
COL1	
Forward	GCTGATGATGCCAATGTGGTT
Reverse	CCAGTCAGAGTGGCACATCTTG
RUNX2	
Forward	GGAATGCCTCTGCTGTTATGAA
Reverse	GCTTCTGTCTGTGCCTTCTG

ALPL, alkaline phosphate, biomineralization associated; COL1, collagen type 1; RUNX2, runt-related transcription factor 2.

Results

MAG has no effect on hPDLSC viability. Five concentrations of MAG (0, 50, 100, 200 and 500 μM) were selected for the experiments. The effect of different amounts of MAG on the viability of hPDLSCs was assessed using CCK-8 assays. The cell viability of hPDLSCs gradually increased from day 1 to 7, and different concentrations of MAG had no effect on the cell viability of hPDLSCs on days 1, 3, 5 and 7 compared with that of the MAG0 group (Fig. 1A). However, MAG500 significantly reduced cell viability compared with MAG50 and MAG10 at 7 days. Therefore, 0, 200 and 500 μM MAG were chosen for subsequent experiments.

MAG promotes osteogenic differentiation of hPDLSCs. ALP activity is important for bone mineralization and is a useful biochemical marker of bone formation (21). Both MAG200 and MAG500 significantly increased the ALP activity of hPDLSCs on day 4 of osteogenic induction (Fig. 1B). The ALP activity of the MAG200 group was the highest and was ~1.5 times that of the MAG0 group. The ALP activity of the MAG500 group was ~1.2 times that of the MAG0 group.

Next, the expression of osteogenic genes, including ALPL, COL1, and RUNX2, at 4 and 7 days was analyzed. MAG200 significantly increased the expression of ALPL on the 4th day (Fig. 1C). On both the 4 and 7th days, the expression of RUNX2 was significantly different from that of the MAG0 group. On day 7, neither MAG200 nor MAG500 had a significant effect on the expression of ALPL and COL1.

The protein levels of the osteogenesis-related genes COL1, RUNX2, SMAD5, ALP and bone morphogenetic protein 2 (BMP2) were semiquantified using western blotting (Fig. 2A and B). The SMAD5, ALP, and BMP2 protein levels in hPDLSCs in the MAG200 group were significantly upregulated after 7 days compared with the MAG0 group.

In addition to proteins associated with bone formation, mineralization is another marker for assessing osteogenesis (22). Calcium deposition was assessed to study the mineralization of cultured hPDLSCs in different groups. MAG treatment increased calcium nodules compared with the MAG0 group (Fig. 2C and D). However, the formation of mineralized nodules was not proportional to the concentration of MAG, with MAG200 resulting in the most mineralized nodules. As a confirmation of osteogenic induction, ALP staining performed after 4 days of culture showed positive staining for all of the MAG-containing groups (Fig. 2E). In particular, ALP activity was significantly upregulated in the MAG200 group compared with that in the MAG0 group. This was in accordance with the aforementioned ALP activity results. These results suggest that MAG served an important role in promoting mineralization and has a strong ability to induce osteogenic differentiation of hPDLSCs.

MAG promotes osteogenesis via the TGF-β/SMAD2 signaling pathway. To analyze the mechanism by which MAG promotes osteogenic differentiation, four key proteins of the TGF-β/SMAD2 signaling pathway, TGF-β, p-SMAD2, SMAD2 and SMAD3 were analyzed by western blotting. Galunisertib is a small molecule inhibitor of TGF-β receptor I kinase (23). The expression of TGF-β1 in the MAG200 group in the presence of galunisertib was significantly lower compared with the MAG200 group, indicating that galunisertib successfully inhibited expression of TGF-β1 (Fig. 3A and B). Western blotting showed that the expression levels of p-SMAD2, SMAD2 and SMAD3 were significantly upregulated in the MAG200 group after hPDLSCs were cultured for 7 days. Addition of the pathway inhibitor galunisertib partially reversed the upregulation of protein expression caused by MAG (Fig. 3A and B). The ratio of p-SMAD2/SMAD2 in the MAG200 + galunisertib group was significantly lower than the MAG200 group, indicating that galunisertib decreases SMAD2 phosphorylation (Fig. 3C). Alizarin red staining and calcium deposits quantification also demonstrated that the addition of galunisertib could partially inhibit the effect of MAG in promoting the osteogenic differentiation of hPDLSCs (Fig. 3D and E). Collectively, these data indicated that MAG activates the TGF-β/SMAD2 signaling pathway during osteogenic differentiation.

Discussion

Periodontal tissue is a complex group of tissues consisting of gingiva, periodontal ligament, cementum, and alveolar bone (24,25). Given this complexity, regeneration of lost or damage periodontal tissue due to periodontitis remains a challenge for current treatments (26-28). PDLSCs can generate structures similar to the cementum/periodontal ligament *in vivo*, suggesting the important role of PDLSCs in periodontal regeneration (29). PDLSCs exhibit pluripotent differentiation and are good candidates for tissue engineering due to their ability to promote regeneration of dental and nondental tissues (30).

A number of previous studies have shown that MAG inhibits the inflammatory response (31-33). Studies have shown that MAG has a notable inhibitory effect on intracellular adhesion

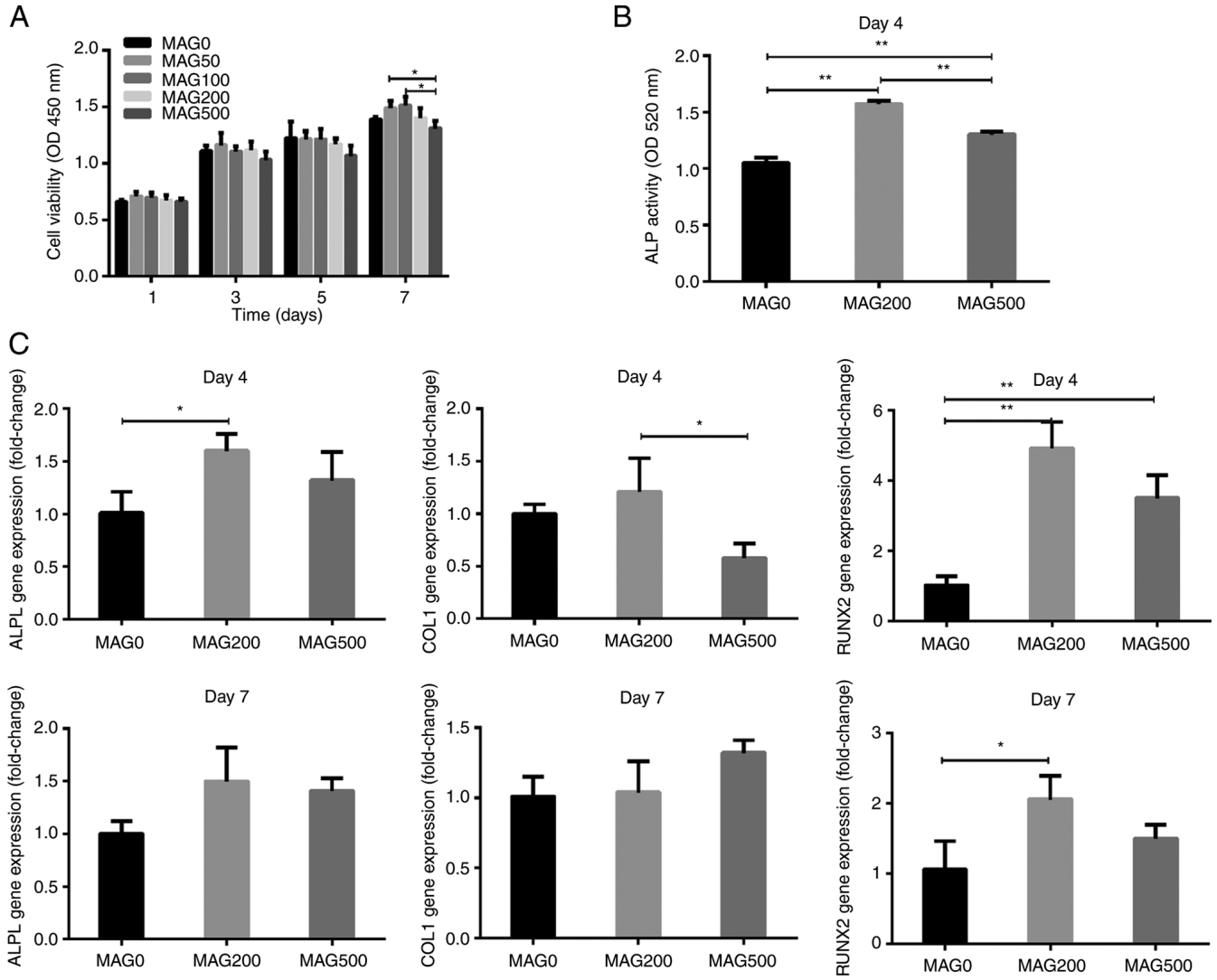

Figure 1. Proliferation and osteogenic differentiation of hPDLSCs following MAG treatment. (A) Proliferation of hPDLSCs following MAG stimulation at different concentrations (0, 50, 100, 200, 500 μmol/ml) was evaluated by a Cell Counting Kit-8 assay on days 1, 3, 5 and 7. (B) ALP activity of hPDLSCs treated with 0, 200, and 500 μmol/ml MAG on day 4. (C) Effects of MAG on mRNA expression of osteogenic differentiation markers. hPDLSCs were treated with MAG at concentrations of 0, 200 and 500 μmol/ml, and the mRNA levels of COL1, ALPL and RUNX2 were determined via reverse transcription-quantitative PCR on days 4 and 7 post-MAG treatment. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01. ALPL, alkaline phosphate, biomineralization associated; COL1, collagen type 1; RUNX2, runt-related transcription factor 2; hPDLSC, human periodontal ligament stem cells; OD, optical density; MAG, mangiferin.

molecule and endothelial leukocyte adhesion molecule expression, which are required for the transportation of leukocytes during inflammation (34-36). Orally administered MAG (50 mg/kg body weight, once daily) was used to treat periodontitis in mice for 8 weeks; MAG was found to markedly inhibit alveolar bone loss, TNF-α production and NF-κB in gingival epithelial cells and phosphorylation of the JAK1-STAT1/3 pathway (37). Therefore, MAG has good therapeutic potential for prevention and treatment of periodontitis.

Researchers have found that MAG promotes bone tissue regeneration (38-42). Sekiguchi *et al* (39) showed that MAG can promote osteoblastic bone formation by promoting cell proliferation and inducing cell differentiation through RUNX2 in pre-osteoblast MC3T3-E1 cells. Demeyer *et al* (40) prepared MAG-loaded chitosan-silica hybrid nanocomposite scaffolds using sol gel synthesis and freeze-drying processes; investigation of biomineralization and cell viability showed that the addition of bioactive MAG further promoted the effects of hybrid nanocomposite scaffolds in guided bone regeneration applications. Li *et al* (41) used a freeze-drying technique to prepare MAG-loaded poly(D, L-lactide-co-glycolide) scaffolds. The scaffolds were then implanted into the alveolar bone defect of diabetic rats and bone repair was examined using hematoxylin and eosin staining. Under diabetic conditions *in vitro*, the MAG-loaded scaffolds increased the histological score of bone regeneration and improved delayed alveolar bone defect healing in diabetic rats. Huh *et al* (42) isolated mesenchymal stem cells (MSCs) from the subchondral bone of rabbits and treated them with MAG and/or IL-1β. MAG induced chondrogenic differentiation of MSCs by upregulating the expression of several key chondrogenic markers, including TGF-β, BMP-2, and BMP-4. The experimental results of the present study are similar to those of the aforementioned experiments. In the present study, hPDLSCs were used as the

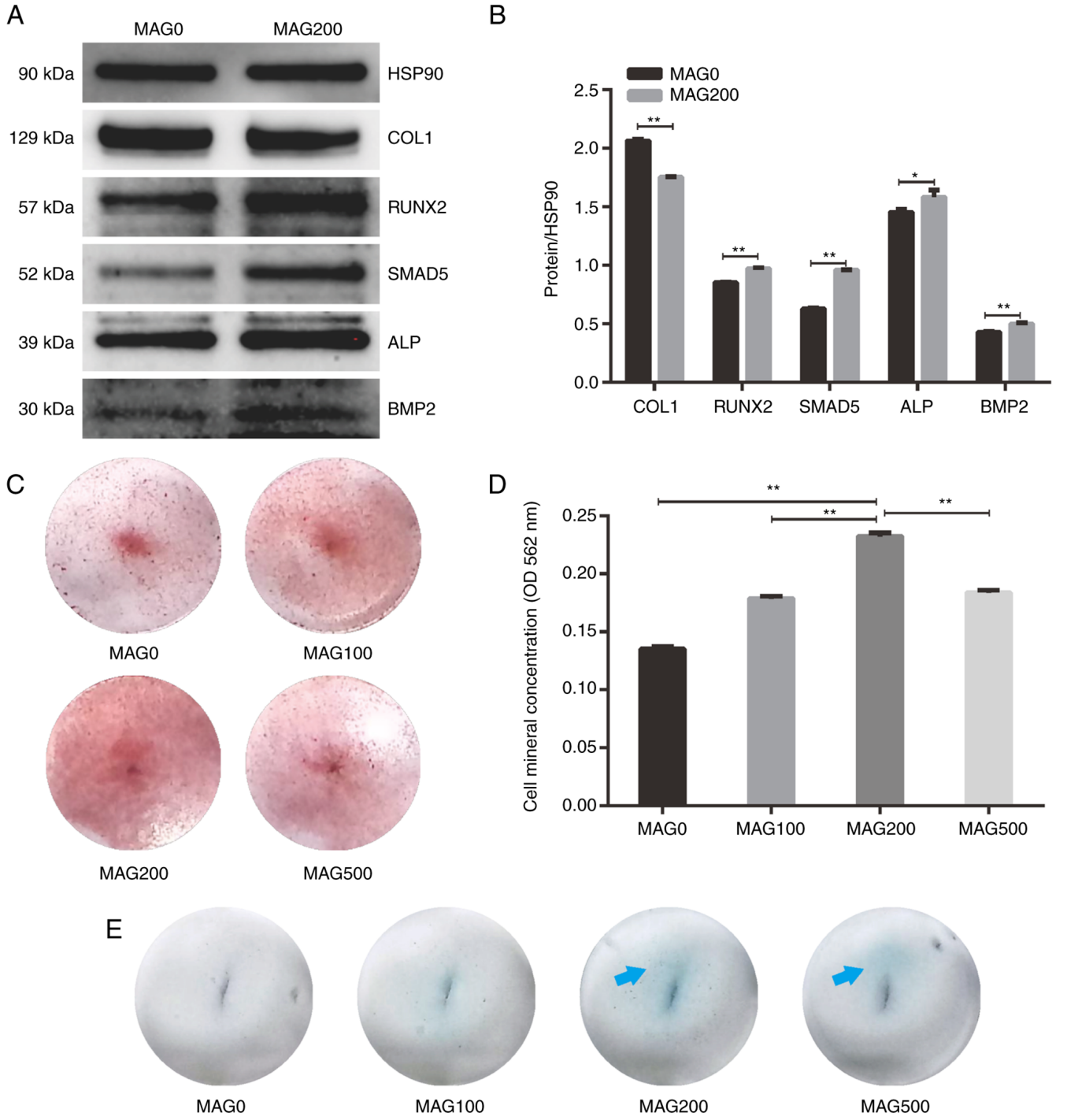

Figure 2. Western blot analysis and Alizarin red staining of hPDLSCs following MAG treatment. (A) Western blot analysis of hPDLSCs treated with 0 and 200 μM MAG on day 7. (B) Semi-quantitative analysis of protein expression. (C) Representative Alizarin red staining images following MAG stimulation at different concentrations (0, 100, 200, 500 μmol/ml) in hPDLSCs at 1x magnification. (D) Concentration of Alizarin-stained mineral deposits. (E) Representative alkaline phosphatase staining images following MAG stimulation at different concentrations (0, 100, 200, 500 μmol/ml) in hPDLSCs at 1xmagnification. Blue arrows indicate positive staining for ALP. Data are presented as the mean ± SD (n=4). *P<0.05, **P<0.01. hPDLSCs, human periodontal ligament stem cells; OD, optical density; MAG, mangiferin; ALP, alkaline phosphate; COL1, collagen type 1; RUNX2, runt-related transcription factor 2; BMP2, bone morphogenetic protein 2; HSP90, heat shock protein 90.

research object, and MAG was found to promote the osteogenic differentiation of hPDLSCs. This result indicated that MAG may promote the regeneration of periodontal tissue by promoting the osteogenic differentiation of stem cells and may be a potential drug for periodontal treatment.

Osteoblast differentiation is critical for bone formation and involves the TGF-β/SMAD signaling pathway in bone morphogenesis (43). SMAD proteins are intermediate molecules that transmit the signal generated by the binding of TGF-β to its receptor from the cytoplasm to the nucleus, thereby playing an important role in signal transmission and regulation of transcription of downstream target genes (44). During signal transduction in the TGF-β/SMAD pathway, TGF-β first binds to its type II receptor and activates its type I receptor. Activated type I

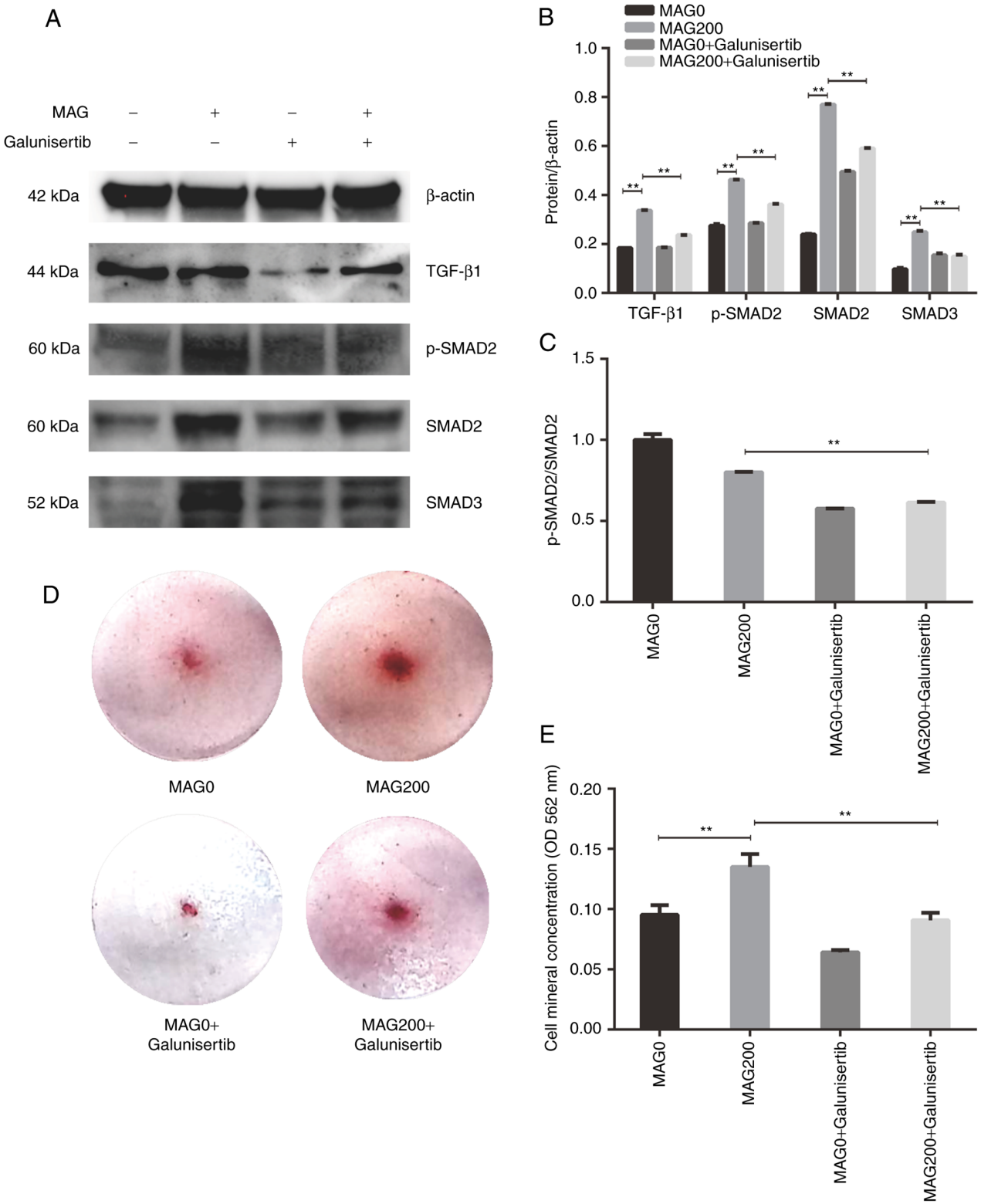

Figure 3. Effect of MAG on the activation of TGF-β/SMAD2 signaling during osteogenic differentiation of hPDLSCs. hPDLSCs were divided into four treatment groups as follows: Control, MAG, galunisertib, and MAG + galunisertib. (A) Western blotting was performed to determine protein expression of TGF-β1, p-SMAD2, SMAD2 and SMAD3 after 7 days of MAG treatment at 0 and 200 μmol/ml. β-actin served as the loading control. (B) Quantitative analysis of protein expression. (C) Quantitative analysis of the ratio of p-SMAD2/SMAD2 protein expression. (D) Images of Alizarin red-stained hPDLSCs in the four groups at 1x magnification. (E) Concentration of Alizarin red-stained mineral deposits. Data are presented as the mean ± SD (n=4). **P<0.01. p, phosphorylated; hPDLSCs, human periodontal ligament stem cells; OD, optical density; MAG, mangiferin.

receptors promote the phosphorylation of SMAD2 or SMAD3 at the C-terminus, and these molecules bind with SMAD4 and translocate into the nucleus, thereby affecting osteoblast proliferation and differentiation (45-47). Therefore, compounds or drugs that activate the SMAD signaling pathway through the TGF-β or BMP pathways can modulate osteoporosis (48).

Previous studies have shown that SMAD2/3 serves an important role in the regulation of bone formation (49) and the expression of SMAD2/3 and phosphorylated (p)-SMAD2/3 was found to be elevated during cementoblast differentiation and mineralization (50). Typically, activation of the TGF-β/SMAD signaling pathway mediates the phosphorylation of SMAD2/3, which dimerizes with SMAD4 and translocates to the nucleus, leading to transcription of downstream genes to direct cell differentiation (51). In the present study, western blot analysis showed that the addition of MAG could upregulate the expression of p-SMAD2, SMAD2, and SMAD3 in hPDLSCs, which revealed that MAG could activate the TGF-β/SMAD2 signaling pathway.

In the TGF-β/SMAD2 signaling pathway, serine in SMAD2 is directly phosphorylated by the TGF-β1 receptor, resulting in SMAD2 activation. Galunisertib (LY2157299 monohydrate) is a small molecule inhibitor of TGF-β receptor I kinase that specifically decreases SMAD2 phosphorylation and eliminates activation of the classic pathway (52). In the present study, addition of galunisertib partially reversed the MAG-mediated upregulation of the protein expression of p-SMAD2 and SMAD2. Alizarin red staining also indicated that galunisertib treatment could partially inhibit the effect of MAG in promoting the osteogenic differentiation of hPDLSCs. Collectively, these data indicated that MAG promotes the osteogenic differentiation of hPLDSCs by activating the TGF-β/SMAD2 signaling pathway.

In conclusion, MAG can promote osteogenic differentiation of hPDLSCs, and the TGF-β/SMAD2 signaling pathway was involved in this process. In addition, given the potential of MAG in antibacterial treatment and treatment of inflammation, studying the regulatory effect of MAG on bone regeneration has implications for the clinical treatment of periodontal disease. MAG may be an effective drug for preventing periodontitis and promoting periodontal bone regeneration.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YG and YB conceived the original idea and performed experiments. YG and LZ confirm the authenticity of all the raw data. LZ analyzed the data. YG, LZ and YB wrote the manuscript. YB supervised the project and give final approval of the version to be published. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The first or second premolars that were removed from healthy individuals due to orthodontic needs were collected at the Beijing Stomatological Hospital (Beijing, China) after obtaining patient verbal consent with approval of the Ethics Committee of Capital Medical University (approval no. KJ-2021-016-C-01-CS).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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