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## RESEARCH PAPER

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# Enamel matrix derivative enhances the proliferation and osteogenic differentiation of human periodontal ligament stem cells on the titanium implant surface

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**ABSTRACT.** Periodontal ligament stem cells (PDLSCs) have mesenchymal-stem-cells-like qualities, and are considered as one of the candidates of future clinical application in periodontal regeneration therapy. Enamel matrix derivative (EMD) is widely used in promoting periodontal regeneration. However, the effects of EMD on the proliferation and osteogenic differentiation of human PDLSCs grown on the Ti implant surface are still no clear. Therefore, this study examined the effects of EMD on human PDLSCs *in vitro*. Human PDLSCs were isolated from healthy participants, and seeded on the surface of Ti implant disks and stimulated with various concentrations of EMD. Cell proliferation was determined with Cell Counting Kit-8 (CCK-8). The osteogenic differentiation of PDLSCs was evaluated by the measurement of alkaline phosphatase (ALP) activity, Alizarin red staining, and real-time polymerase chain reaction (qRT-PCR) and Western blotting, respectively. The results indicated that EMD at concentrations (5–60  $\mu\text{g/ml}$ ) increased the viability and proliferation of PDLSCs. The treatment with 30 and 60  $\mu\text{g/ml}$  of EMD significantly elevated ALP activity, augmented mineralized nodule formation and calcium deposition, and upregulated the mRNA and

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protein levels of Runx-2 and osteocalcin (OCN) in the PDLSCs grown on the Ti surface. Further investigation found that EMD treatment did not change the protein levels of phosphatidylinositol-3-kinase (PI3K), p-PI3K, Akt and mTOR, but significantly upregulated the phosphorylated levels of Akt and mTOR. Collectively, these results suggest that EMD stimulation can promote the proliferation and osteogenic differentiation of PDLSCs grown on Ti surface, which is possibly associated with the activation of Akt/mTOR signaling pathway.

**KEYWORDS.** Akt, Enamel matrix derivative, mTOR, osteogenic differentiation, osteoblasts, periodontal ligament stem cells

## INTRODUCTION

Dental implant therapy is one of the most important and effective prosthodontic therapy options for partially and completely edentulous patients.<sup>1</sup> Currently, the use of dental implants has widely become an accepted treatment in many clinical situations.<sup>2</sup> Titanium (Ti) devices have been widely used as major biomaterials in dental implant therapy for many years and their rough surface can stimulate the differentiation of bone-forming osteoblasts.<sup>3,4</sup> Although many evidence indicates that implant therapy is an efficient and safe choice and the brilliant success of dental implants has been made, low bone quality and unpredictable osseointegration still result in the failure of dental implant therapy.<sup>5,6</sup>

Periodontal disease is a chronic inflammation that leads to the destruction of periodontal ligament, cementum and bone.<sup>7</sup> PDLSCs discovery provides a new prospect for periodontal tissue regeneration because they were found to form a cementum/PDL-like structure after *in vivo* transplantation.<sup>8</sup> Additionally, many studies have indicated that PDLSCs have multipotent differentiation ability toward a chondrogenic, osteoblastic, and adipogenic phenotype under defined conditions.<sup>9-11</sup> Chemical induction of osteoblastic phenotype in PDLSCs through the application of various growth factors and biomaterials, such as platelet-derived growth factor (PDGF) and enamel matrix derivative (EMD), has been studied extensively.<sup>12,13</sup>

Enamel matrix derivative is an extracellular matrix derivative obtained from porcine tooth buds,<sup>14</sup> and has been reported to be very effective in regeneration of cementum, periodontal ligament and bone.<sup>15</sup> Although it was found that

EMD can stimulate cellular proliferation and mineralization of preosteoblasts and periodontal ligament cells, and plays an important role in the cell differentiation necessary for periodontal regeneration,<sup>16,17</sup> other studies have pointed out that EMD also reduces the differentiation of osteoblasts.<sup>18,19</sup> In addition, some studies indicated that the application of EMD contributing to osteoblasts differentiation and bone formation around Ti implants remains controversial.<sup>20,21</sup> Therefore, in this study, we investigated the effect of EMD on the proliferation and osteogenic differentiation of PDLSCs grown on the surface of Ti disks.

## RESULTS

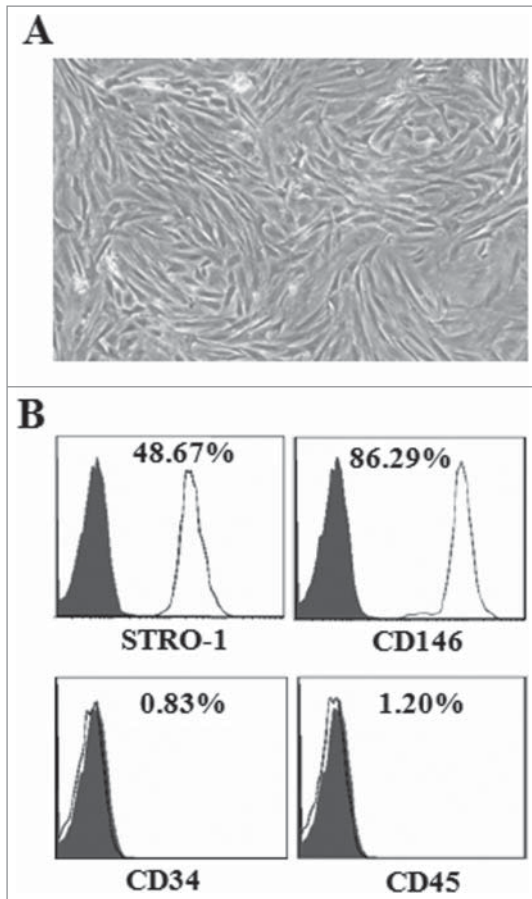
### *Isolation and identification of human PDLSCs*

Light microscopy demonstrated that the primary human PDLSCs were spindle-shape (Fig. 1A). The results of flow cytometry analysis revealed that these isolated cells strongly expressed STRO-1 and CD146 (48.67% and 86.29%, respectively) cell surface antigens, and were negative for CD34 (0.83%) and CD45 (1.20%), as shown in Fig. 1B. These data suggested that these cells isolated from human periodontal ligament tissues belonged to PDLSCs.

### *Effect of EMD on the proliferation of PDLSCs*

Cell proliferation was evaluated using CCK-8 assay to detect the viability of PDLSCs grown on Ti implant surfaces. The results showed that

FIGURE 1. Morphologic and phenotypic analysis of PDLSCs. (A) PDLSCs showed a fibroblast-like spindle shape under the microscope view. Original magnification:  $\times 400$ . (B) Flow cytometric analysis of surface markers in the isolated PDLSCs.

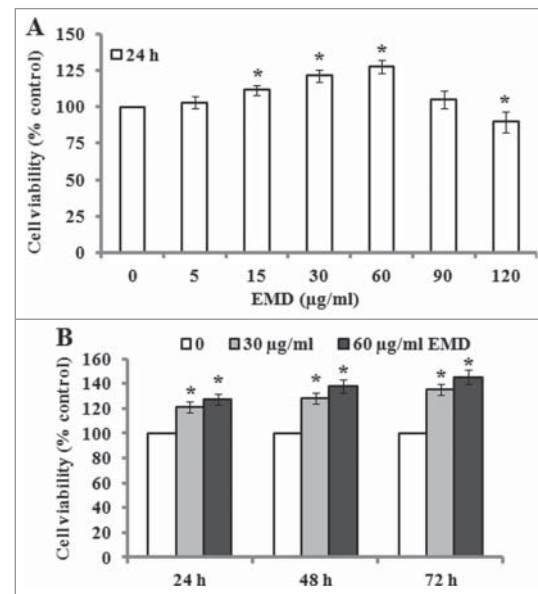


5–60  $\mu\text{g/ml}$  of EMD treatment of 24 h resulted in a dose-dependent increase in the viability of PDLSCs, but more higher doses (such as 90–120  $\mu\text{g/ml}$ ) of EMD led to an inhibitory effect on cell viability (Fig. 2A). Additionally, a time-dependent increase of cell viability was observed when the PDLSCs were incubated with 30 and 60  $\mu\text{g/ml}$  of EMD for 24, 48 and 72 h (Fig. 2B).

### ***EMD promotes the differentiation of PDLSCs into osteoblasts***

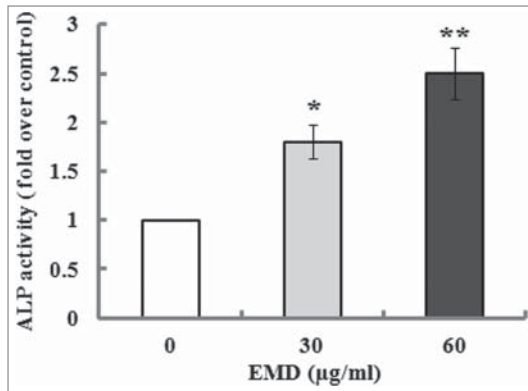
ALP is an early marker for dental mesenchymal cell differentiation toward osteoblastic

FIGURE 2. Effect of EMD on the viability of PDLSCs. (A-B) The viability of PDLSCs was determined by CCK-8 assay after the cells were treated with various concentrations (0, 5, 15, 30, 60, 90 and 120  $\mu\text{g/ml}$ ) of EMD for 24 h or with 30 and 60  $\mu\text{g/ml}$  of EMD for 24, 48 and 72 h. Values of changes caused by EMD treatment in the viability of PDLSCs were normalized to the control, and the representative results from 3 independent experiments are shown. \* $P < 0.05$  vs. the control.



phenotype. Therefore, ALP activity was determined after 7 d of induction culture containing 30 and 60  $\mu\text{g/ml}$  of EMD. The results indicated that EMD significantly enhanced ALP activity in the PDLSCs when compared with the untreated cells (Fig. 3). In addition, Alizarin red staining and a quantitative biochemical colorimetric assay displayed that the EMD-enhanced calcium deposition and mineralized nodule formation were observed after 21 d of culture (Fig. 4). On the other hand, the gene expression levels of osteogenic markers such as Runx-2 and OCN in the PDLSCs were detected after 14 d culture. The results of real-time PCR indicated that the mRNA levels of Runx-2 and OCN were significantly elevated in comparison to the control (Fig. 5A). The results of western blot analysis confirmed that the protein levels o

FIGURE 3. Effect of EMD on the ALP activity of PDLSCs. After 7 d of incubation in the induction medium added with 30 and 60  $\mu\text{g/ml}$  of EMD, the ALP activity of PDLSCs was determined and normalized to the control (untreated cells), which was arbitrarily set to 1. \* $P < 0.05$  and \*\* $P < 0.01$  vs. the control.



Runx-2 and OCN were significantly increased by 30 and 60  $\mu\text{g/ml}$  of EMD treatment (Fig. 5B and C).

### *EMD activates Akt signaling in PDLSCs*

Previous studies have indicated phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) play an important role in the regulation of cell survival, proliferation, and cell differentiation.<sup>22,23</sup> Accordingly, we examined the protein levels of PI3K/Akt and their downstream mTOR and their phosphorylation states in the PDLSCs after 48 h incubation with 30 and 60  $\mu\text{g/ml}$  of EMD. The results demonstrated that EMD treatment did not change the expression levels of PI3K, p-PI3K, Akt and mTOR, but significantly upregulated the phosphorylated levels of Akt and mTOR (Fig. 6), which suggested that the

FIGURE 4. Effect of EMD on osteogenic differentiation of PDLSCs. (A) After 21 d of incubation in the induction medium added with 30 and 60  $\mu\text{g/ml}$  of EMD, mineralized deposits was evaluated by Alizarin red S staining. (B) The deposition of Alizarin staining was extracted, and the absorbance value of released dye was determined at 562 nm using a microplate reader. \* $P < 0.05$  and \*\* $P < 0.01$  vs. the control.

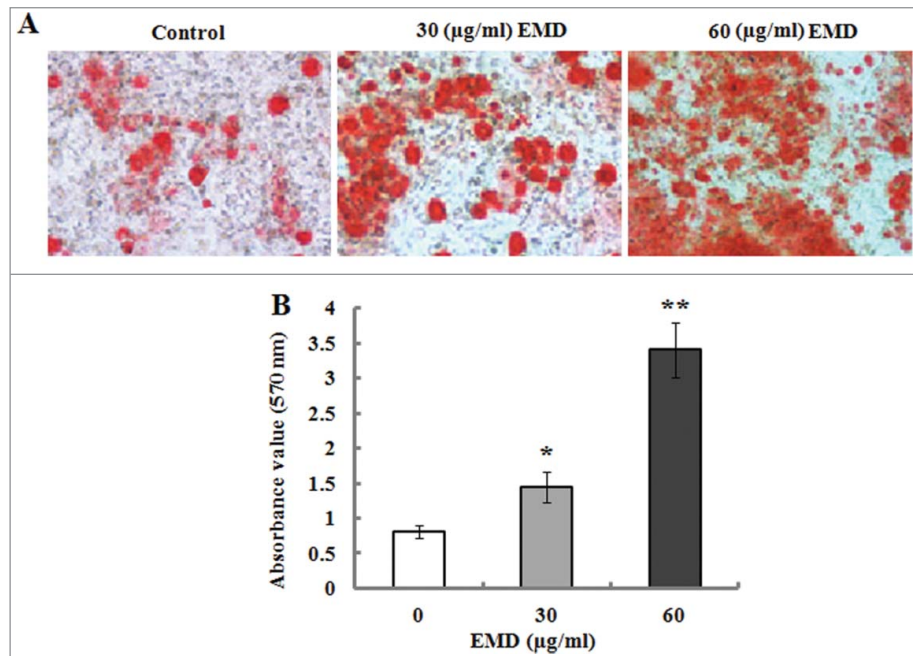
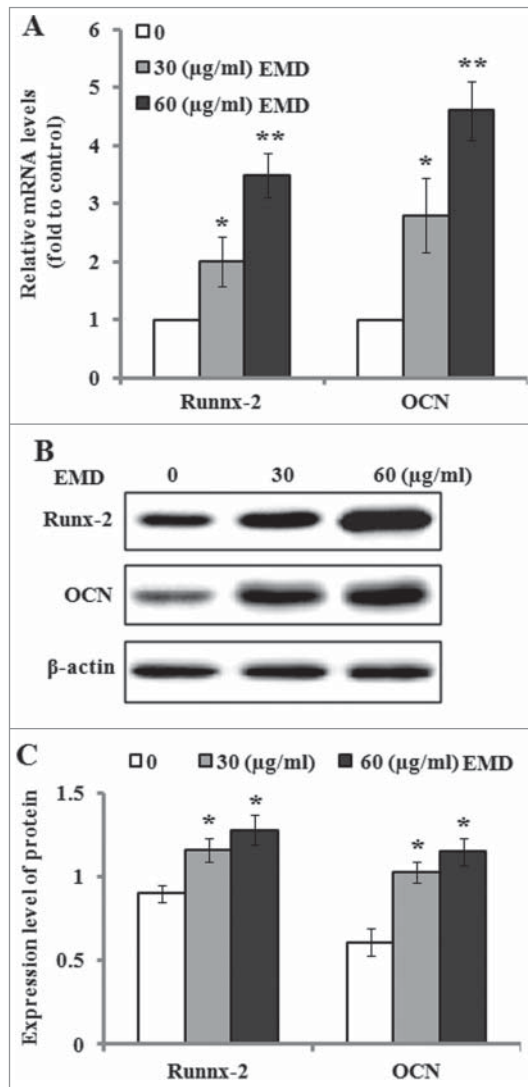


FIGURE 5. Effects of EMD on the expression levels of Runx-2 and OCN. (A) The relative mRNA level of Runx-2 and OCN in the PDLSCs was detected by real time PCR after 14 d of induction culture containing 30 and 60  $\mu\text{g/ml}$  of EMD. (B) Representative western blot images for the expression of Runx-2 and OCN in the PDLSCs after 14 d of osteogenic induction containing 30 and 60  $\mu\text{g/ml}$  of EMD. (C) Relative protein level of Runx-2 and OCN were quantified by the densitometry of each band and normalized to  $\beta$ -actin signal. \* $P < 0.05$  vs. the control.



activation of Akt/mTOR signaling was correlated with EMD-induced osteogenic differentiation of PDLSCs.

## DISCUSSION

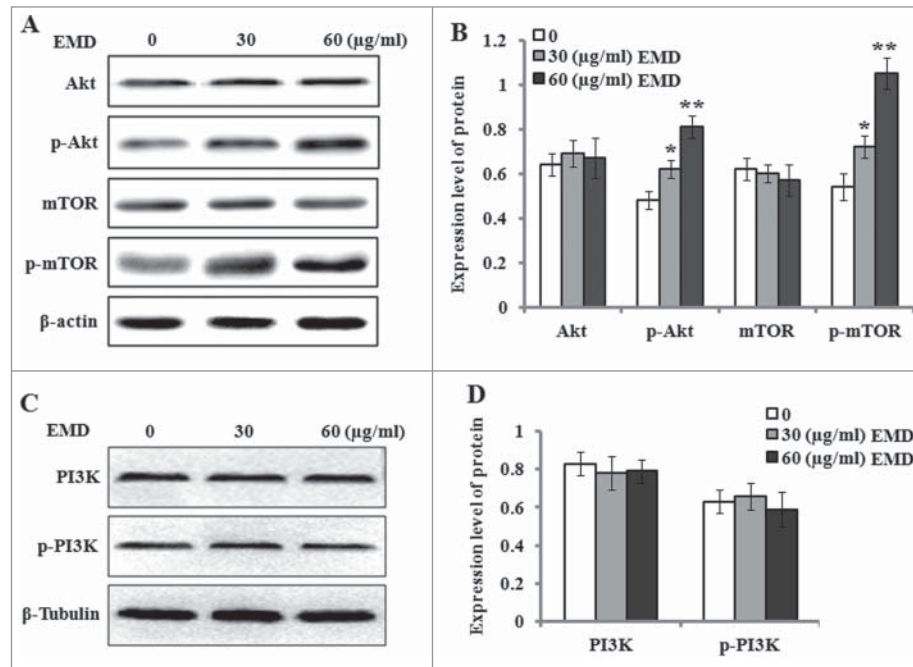
To determine the applicability of EMD for enhancing PDLSCs-based bone regeneration modalities, we evaluated the effects of EMD on proliferation and osteogenic differentiation of human PDLSCs grown on the surface of Ti implant disks. EMD at various concentration was added in the medium in which PDLSCs were cultured. The results showed that the proliferation of PDLSCs was significantly increased by 15, 30 and 60  $\mu\text{g/ml}$  of EMD after 24 h of incubation. However, more than 90  $\mu\text{g/ml}$  of EMD treatment resulted in an inhibitory role on PDLSCs viability. Therefore, 30 and 60  $\mu\text{g/ml}$  of EMD were chosen for evaluation on osteogenic differentiation of PDLSCs based on these findings. The results indicated that EMD promoted osteogenic differentiation of PDLSCs, upregulated the expression of Runx-2 and OCN, and activated Akt/mTOR signaling pathway.

PDLSCs are known to possess multi-differentiation potential and exhibit similar properties to those described for bone marrow-derived mesenchymal stem cells (MSCs).<sup>24</sup> In this study, the PDLSCs were isolated from healthy participants, and these cells positively expressed the mesenchymal stem-cell markers STRO and CD146, did not express haematopoietic stem-cell markers CD34 and CD45. These results confirmed that these isolated PDLSCs possess similar characteristics as bone marrow-derived MSCs, and are also consistent with previous reports.<sup>25,26</sup>

EMD is a purified extract of porcine enamel matrix, and has been documented as a promising biomaterial for *in vivo* periodontal regeneration.<sup>27</sup> However, the influences of EMD on different cell types and osteogenic differentiation of MSCs remain still controversial.<sup>12,19,28</sup> Additionally, the combined application of Ti implants and EMD seems to be a promising approach for a further improvement in the clinical implant outcome.<sup>29</sup> However, a lack of agreement exists regarding the effect of EMD on bone formation around Ti implants.<sup>20,30</sup> In the present study, the influence of EMD on osteogenic differentiation of PDLSCs seeded on the surface of Ti implants was evaluated.



FIGURE 6. EMD activates the Akt signal pathway in the osteogenic differentiation of PDLSCs. PDLSCs were incubated in differentiation medium without or with 30 and 60  $\mu\text{g/ml}$  EMD, and cell lysates were then prepared and subjected to SDS-PAGE analysis. (A) Representative western blot images for the expression of Akt, p-Akt, mTOR and p-mTOR. (B) Relative protein levels of Akt, p-Akt, mTOR and p-mTOR were quantified by the densitometry of each band and normalized to  $\beta$ -actin signal. (C-D) Representative western blot images for the expression of PI3K and p-PI3K, and their relative protein levels were quantified by the densitometry of each band and normalized to  $\beta$ -Tubulin signal. \* $P < 0.05$  and \*\* $P < 0.01$  vs. the control.



The results of Alizarin red staining showed that the formation of mineralized and calcium deposition was apparently increased after 21 d of incubation in the osteogenic induction medium supplemented with EMD. ALP is an indicator of new bone formation, and its high-level expression is induced during early osteogenic differentiation,<sup>31</sup> and so it has been commonly studied as an early maker of osteogenic differentiation. Our findings demonstrated that ALP activity was significantly elevated in the PDLSCs after 7 d of incubation in the presence of EMD in comparison with the control cells. In addition, the expression levels of osteogenic differentiation related genes such as Runx-2 and OCN were upregulated as determined using real time PCR and Western blot analysis after osteogenic induction with EMD. Runx-2 is the bone specific transcription factor, and has a

major impact on osteogenesis by stimulating osteoblast differentiation gene transcription.<sup>32</sup> Additionally, OCN is also an osteoblast-specific marker, and it is present during the late stages of osteoblast differentiation.<sup>33</sup> Furthermore, previous studies have confirmed that OCN is the target gene of Runx-2 and is modulated by Runx-2.<sup>34,35</sup> Therefore, the upregulation of OCN expression tended to correlated positively with the expression of Runx-2.<sup>36</sup> In this study, the changes in Runx-2 and OCN expression suggested that EMD could promote the osteogenic differentiation of PDLSCs grown on Ti implant surface.

Many studies have indicated that PI3K/Akt/mTOR signaling pathway, which can be activated by various extracellular stimuli, plays an important role in the regulation of cell motility, proliferation, cell cycle progression, and cell

differentiation.<sup>37,38</sup> Recent studies showed that Mechano-growth factor promoted the growth rate and osteogenic differentiation of mesenchymal stem cells via PI3K/Akt pathway.<sup>23</sup> In the present study, our results demonstrated that EMD treatment did not change the protein levels of PI3K, p-PI3K, Akt and mTOR, but up-regulated the phosphorylated forms of p-Akt and p-mTOR. These results indicated that the activation of Akt/mTOR signaling pathway occurred in the EMD-mediated osteogenic differentiation of PDLSCs grown on the Ti surface. Interestingly, some studies demonstrate that mTOR activation contributes to osteoblast differentiation in mouse or rat bone marrow stromal cells,<sup>39,40</sup> as opposed to suppression in human embryonic stem cells.<sup>41</sup> These apparent discrepancies are probably associated with cell-type or various methodological differences.

In conclusion, our results indicate that EMD can increase the viability and proliferation of PDLSCs at a proper concentration range, and enhance the osteogenic differentiation of human PDLSCs grown on Ti implant surface. Additionally, EMD treatment elevates ALP activity and up-regulates Runx-2 and OCN expression and the phosphorylated levels of Akt and mTOR. These data seem to suggest that the activation of Akt/mTOR signal pathway involves in the EMD-mediated osteogenic differentiation of PDLSCs. Therefore, EMD could be a useful tool for further promoting implant osseointegration and enhancing clinical outcome.

## MATERIALS AND METHODS

### Materials

EMD powder was obtained from Institut Straumann (Basel, Switzerland), and dissolved in 0.1% acetic acid to prepare a concentration of 10 mg/ml of EMD as the stock solution that was diluted to the working concentration with culture medium. The final dose of acetic acid was less than 0.001% throughout the following studies, and did not display any effect on the parameters investigated in the present study. Pure Ti disks with a cylindrical shape with

1 mm thickness and 15 mm diameter were commercially available from Institut Straumann. The Ti disks with coarse-grit-blasted and acid-etched surface were prepared as described previously.<sup>42,43</sup>

### PDLSCs isolation and cultivation

PDLSCs isolation and culture were performed as described previously,<sup>44</sup> with some modifications. Briefly, periodontal ligament tissues from healthy teeth extracted for orthodontic purpose were immediately washed and minced into small pieces, and enzymatically digested with type I collagenase (0.75 mg/ml) for 30 min. Single-cell suspensions were then obtained and incubated with Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The informed consents were obtained from the subjects who donated the periodontal ligament tissues, and reviewed and approved by the Ethics Committee of Chinese PLA General Hospital (Beijing, China).

To identify the phenotype of PDLSCs, the cells (Passage 3) were incubated with FITC or PE-conjugated monoclonal antibodies against human CD34, CD45, CD146 and STRO-1 (Cell Signal Technology, USA) for 30 min in the dark, and then subjected to flow cytometric analysis using CellQestPro acquisition software.

The isolated PDLSCs were then seeded on the Ti disks in 24-well cell culture plates at a density of  $1 \times 10^4$  cells in 500  $\mu$ l culture medium added without or with 5, 15, 30, 60, 90 and 120  $\mu$ g/ml of EMD, and cultured for indicated time to evaluate cell proliferation/viability and osteogenic differentiation.

### Cell viability

Cell Counting Kit 8 (CCK-8; Genomeditech, Shanghai, China) was used to evaluate the effect of EMD on cell viability. Briefly, PDLSCs were incubated with various

concentrations (0, 5, 15, 30, 60, 90 and 120  $\mu\text{g/ml}$ ) of EMD for 24 h or with 30 and 60  $\mu\text{g/ml}$  of EMD for 24, 48 and 72 h, respectively. 50  $\mu\text{l}$  of CCK-9 reagents were then added into each well and incubated for another 4 h at 37°C. Subsequently, 100  $\mu\text{l}$  of the resulting suspension containing water-soluble formazan produced by WST-8 tetrazolium salt upon reduction by cellular mitochondria respiratory chain, were transferred into 96-well plates. Absorbance value was then determined at 570 nm using automatic reader for microtiter plates (BMG Labtech PHERA star FS, Germany). The experiments were performed 3 times.

### ***Osteogenic differentiation of PDLSCs***

The cells at passage 3 were seeded in 24-well plates at a density of  $1 \times 10^4$  cells/ml in 500  $\mu\text{l}$  of growth medium, and incubated overnight. Growth medium was replaced with induction medium consisting of DMEM, 10% FBS, 10 nM dexamethasone (Sigma-Aldrich, USA), 10 mM  $\beta$ -glycerophosphate (Sigma-Aldrich), 200  $\mu\text{M}$  ascorbate-2-phosphate (Sigma-Aldrich), 100 U/ml penicillin/streptomycin. The medium was replaced every 2 d. EMD (0, 30 and 60  $\mu\text{g/ml}$ ) was added and kept in the cell culture. For the analysis of alkaline phosphatase (ALP) activity, the cells were differentiated for 7 d. The real-time polymerase chain reaction (PCR) analysis and the western blot analysis were performed at day 14, and mineralized nodules by Alizarin red S staining were evaluated at 21.

### ***Measurement of alkaline phosphatase (ALP) activity***

The cells were incubated in the induction medium for 7 days, and were then lysed by 0.1% Triton X-100. The activity of ALP was determined using a commercially available assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Enzyme activity was measured by absorbance at 520 nm using a

microplate reader (BMG Labtech PHERA star FS, Germany).

### ***Alizarin red S staining-based calcium deposition analysis***

After induction culture for 3 weeks, the cells were washed with PBS and fixed with 4% paraformaldehyde, and then stained using 2% Alizarin red for 20 min at room temperature to evaluate calcium deposition. Alizarin red quantification was performed as described previously.<sup>45</sup> Briefly, 1 ml of 10% cetylpyridinium chloride (Sigma-Aldrich, USA) was added into each well to extract the deposition, followed by collection of the solution, and the light absorbance at 562 nm was determined with a microplate reader (BMG Labtech PHERA star FS, Germany).

### ***Real-time polymerase chain reaction***

Total RNA was extracted with Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and cDNA was then reversely synthesized using OligodT primer and PrimeScript<sup>®</sup> RTase (Takara, Dalian, China) following to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR Green Premix Ex Taq<sup>™</sup> kit (Takara) using a specific thermal cycler (Applied Biosystems, NY, USA). The specific primers used for Runx-2, OCN, and GAPDH in this study were as follows: Runx-2 forward 5'-GCAGTCCCAAGCATTTTCAT-3', and reverse 5'-CACTCTGGCTTTGGGAAGAG-3'; OCN forward 5'-GTGCAGAGTCCAGCAAAGGT-3', and reverse 5'-AGACTGCGCC TGGTAGTTGT-3'; GAPDH forward 5'-CATCAAGAAGGTGGTGAAGCAGG-3', and reverse 5'-AAAGGTGGAGGAGT

GGGTGTCG-3'. The samples were subjected to 40 cycles of amplification at 95°C for 30 sec, 55°C for 45 sec, and 70°C for 30 sec. Critical threshold (CT) values obtained from qPCR were analyzed using the  $2^{-\Delta\Delta\text{CT}}$  method for relative gene expression level. The results were presented relative to the control value, which was arbitrarily set to 1.



### Western blot analysis

After culture in the indicated conditions for 2 weeks, total proteins were extracted from the PDLSCs with lysis buffer, and the protein concentration of each sample was determined with BCA assay kit (Bio-Rad Laboratories, USA). Subsequently, lysate proteins were resolved by electrophoresis, followed by transfer to polyvinylidene difluoride (PVDF) membranes, and blotted with antibodies against Runx-2, OCN, PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR and  $\beta$ -actin (dilution, 1: 500). The integrated optical density values of each blot were analyzed using an ImageQuant LAS 4000 (Fuji-film, Tokyo, Japan), and expressed as a relative expression level against  $\beta$ -actin protein.

### Statistical analysis

All of the data were presented as the mean  $\pm$  SD. All statistical analyses were performed using a SPSS software (version 11.0). The comparisons among different groups were performed by Student's *t*-test. *P* value < 0.05 was considered statistically significant.

### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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