Cell Proliferation

Ectopic study of tissue-engineered bone complex with enamel matrix proteins, bone marrow stromal cells in porous calcium phosphate cement scaffolds, in nude mice

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Abstract

Objective: This study aimed to investigate the potential of enamel matrix proteins (EMPs) on promoting osteogenic differentiation of porcine bone marrow stromal cells (pBMSCs), as well as new bone formation capabilities, in a tissue-engineered bone complex scaffold of EMPs, pBMSCs and porous calcium phosphate cement (CPC).

Materials and methods: Effects of EMPs on pBMSCs *in vitro* was first determined by alkaline phosphatase (ALP) activity, von Kossa staining assay and mRNA expression of *ALP*, *bone sialoprotein* (*BSP*) and *osteocalcin* (*OCN*) genes. Next, an ectopic new bone formation test was performed in a nude mouse model with four groups: CPC scaffold alone; CPC scaffold + EMPs; CPC scaffold + pBMSCs; and CPC scaffold + EMPs + pBMSCs, for 2 or 4 weeks.

Results: ALP activity, von Kossa assay and mRNA expressions of *ALP*, *BSP* and *OCN* genes were all significantly higher with 150 μg/ml EMP treatment *in vitro*. In nude mice, new bone formation was detected only in the CPC scaffold + EMPs + pBMSCs group at 2 weeks. At 4 weeks, in the tissue-engineered construct there was significantly higher bone formation ability than other groups.

Conclusions: EMPs promoted osteogenic differentiation of pBMSCs, and the tissue-engineered complex of EMPs, pBMSCs and CPC scaffold may be a valuable alternative to be used in periodontal bone tissue engineering and regeneration.

Introduction

Periodontitis is a chronic inflammatory disease, which undermines the tooth supporting apparatus – including periodontal ligament, cementum and alveolar bone, and leads to odontoseisis and tooth loss (1). The ultimate goal of periodontal therapy is to regenerate supporting tissues destroyed during the disease process. As the breakdown of alveolar bone has a direct correlation with odontoseisis and tooth loss, alveolar bone regeneration is critical for periodontal restoration. Alveolar bone restoration has been deemed the beginning of analyses of problems involved in periodontal tissue engineering (2).

Several clinical techniques have been developed to promote periodontal regeneration, including guided tissue regeneration, bone grafting and use of bioactive agents (2). Among the above mentioned methods, enamel matrix proteins (EMPs) belong to one of the widely studied bioactive agents commercially available. They are derived from the enamel matrix of pre-erupted porcine tooth germs and are composed of amelogenins, which comprise more than 90% of EMPs, as well as other non-amelogenin matrix proteins or enzyme components (3,4). EMPs are largely hydrophobic proteins and share high degrees of homology across different species (5–7). Due to their important roles in development of the periodontium, EMPs have been used in

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pre-clinical and clinical studies to restore periodontal ligament, cementum and alveolar bone. To obtain a better therapeutic efficacy, EMPs have also been used in combination with other materials such as barrier membranes or bone grafts, in recent studies (8–10), although it is controversial in terms of whether there are additional benefits by combining the proteins with certain materials (11).

Recent advances in tissue engineering and regenerative medicine have provided opportunities for regeneration of various kinds of tissue. This has suggested a new paradigm based on appropriate cells, effective bioactive agents and scaffold materials for periodontal regeneration. For example, ideal cells should be non-immunogenic, highly proliferative and easy to harvest, and have the ability to differentiate into various cell types with specialized functions (12). Bone marrow stromal cells (BMSCs) which can be easily isolated and expanded *in vitro*, may be a feasible choice. BMSCs are pluripotent with the ability to differentiate into a variety of phenotypes, which depends on the environment in which they are cultured, and they have been identified as a major source of osteogenic cells (13,14). It has also been reported that BMSCs could be used successfully to form cementum, periodontal ligament and alveolar bone *in vivo* after transplantation into periodontal defects in beagles (15). These results suggest that BMSCs could be an important cell source for periodontal regeneration. More importantly, there have been reports that EMPs could promote cell adhesion, proliferation and differentiation, including increase in alkaline phosphatase activity and matrix mineralization of BMSCs (16,17). These ideas have aroused our interest into whether EMPs could be used together with BMSCs to promote new bone formation *in vivo*.

In this study, we have explored effects of EMPs on porcine BMSCs (pBMSCs) *in vitro,* and evaluated the ability of new bone formation by use of a tissue-engineered bone complex of EMPs, pBMSCs and porous calcium phosphate cement (CPC), in a dorsal subcutaneous model in nude mice. To our knowledge, there seems to be lack of research appearing in the scientific literature of using EMPs, pBMSCs and CPC for tissue-engineering applications.

Materials and methods

EMP preparation

Porcine EMPs were extracted, according to protocols reported by Moe *et al.* (18) and Shu Rong *et al.* (19), from secretory-stage enamel matrix obtained from 6-month-old pig non-erupted, developing permanent germs. Briefly, unmineralized enamel matrix was scraped from germ surfaces then treated with 0.5 mol/l acetic acid and 20% (w/v) trichloroacetic acid, and finally, lyophilized under −56 °C vacuum to obtain EMP powder. After being analysed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis, the lyophilized powder was dissolved in 5 mmol/l acetic acid at concentration of 4 mg/ml, sterilized by filtration and stored at −80 °C (20).

Animal model

Three 3 month old male healthy crossbred pigs, weight 20–25 kg, and eight 5-week-old male nude Balb/c mice weight 20 ± 2 g were used in the study. All procedures concerning animals were approved by the Animal Research Committee of the Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine (JYLL-10016).

Cell culture

Under general anaesthesia with ketamine (10 mg/kg) and xylazine (3 mg/kg), in the region of 4 ml bone marrow was aspirated from ilium of a single pig, with a 16-gauge bone marrow aspiration needle, and then was transferred into a pre-heparinized centrifuge tube. Bone marrow specimens were flushed out with Dulbecco's modified Eagle's medium (DMEM, high glucose; Gibco, Grand Island, NY, USA) and pBMSCs were cultured in DMEM with 10% foetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C, in an atmosphere with 95% humidity and 5% CO_2. Media were changed after 24 h to remove non-adherent cells and was then renewed every 3 days. When confluence of 80–90% was reached pBMSCs were released from the culture substratum using trypsin/EDTA (0.25% w/v trypsin, 0.02% EDTA), and were seeded in fresh petri dishes (10 cm in diameter) at density of 1.0×10^5 cells/ml. Cells at passage 2–3 were used for the following study.

EMP treatment

pBMSCs were detached with trypsin/EDTA, and then seeded in six-well plates at 1.0×10^5 cells/ml density. They were then incubated in culture media containing 50 or 150 μg/ml EMP, as experimental groups, and in media without EMPs as control group. All culture media were changed every 3 days.

Alkaline phosphatase activity and von Kossa assay

All groups of pBMSCs were cultured for a further 14 days, and alkaline phosphatase (ALP) activity was

evaluated by ALP staining kit according to manufacturer's instructions. Briefly, cells were fixed for 10 min at 4 °C and incubated in a mixture of naphthol AS-MX phosphate and fast blue BB salt (ALP kit; Hongqiao, Shanghai, China) (21). Areas stained purple were designated as ALP positive. After being treated with EMP for 21 days, mineralized nodules of all groups were assayed by von Kossa staining. Cells were fixed in 70% ethanol and stained with 5% silver nitrate and put under ultraviolet light for 10 min, then treated with 5% NaS_2O_3 for 2 min and washed in distilled water (22).

RNA extraction and real-time PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen) plus RNA purification kit (Invitrogen) according to manufacturer's instructions at 3 and 6 days after EMP treatment. Quality and quantity of RNA extracted were analysed using an Rneasy Mini kit (Qiagen, Hilden, Germany). Following RNA extraction, reverse transcription was carried out with PrimeScript[TM] RT reagent kit (TaKa-Ra Bio, Otsu, Japan). A quantity of 20 µl reverse transcription reaction volume contained 1 µg total RNA according to the manufacturer's recommendations. Sequences of primers for alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OCN) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as the calibrator gene for normalization, were synthesized commercially (Shengong Co. Ltd., Shanghai, China) from known porcine sequences (Table 1). Real-time PCR analysis of osteogenic marker genes was performed using MyiQ[TM] Single Color Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Briefly, 10 µl SYBR Premix Ex Taq[TM], 0.4 µl forward, 0.4 µl reverse primer and 2.0 µl cDNA template were added to a final reaction volume of 20 µl. All samples were assayed in triplicate ($n = 3$) and three independent experiments were performed. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression levels relative to GAPDH and control group.

Preparation of EMPs/pBMSCs/CPC construct

CPC scaffolds were manufactured and provided by Re-bone Biomaterial Co. Ltd. (Shanghai, China). Average diameter of scaffold pores was 400 µm and average porosity was 70% (Fig. 1). In this study, CPC granules of dimensions of Ø 4 × 2 mm were used. CPC scaffolds were dropped into 150 µg/ml EMP solution for 3 h at 37 °C, then flash frozen and lyophilized (20,23). pBMSCs were seeded on to CPC scaffolds according to the method reported by Maniatopoulos *et al.* (24). Briefly, pBMSCs were released from culture substratum using trysin/EDTA (0.25% w/v trypsin, 0.02% EDTA), centrifuged to remove supernatant, then resuspended in serum-free medium at a density of 2×10^7 cells/ml. Cell suspensions were added to the CPC scaffolds to final saturation. Surgical procedures were performed immediately after seeding saturation was reached.
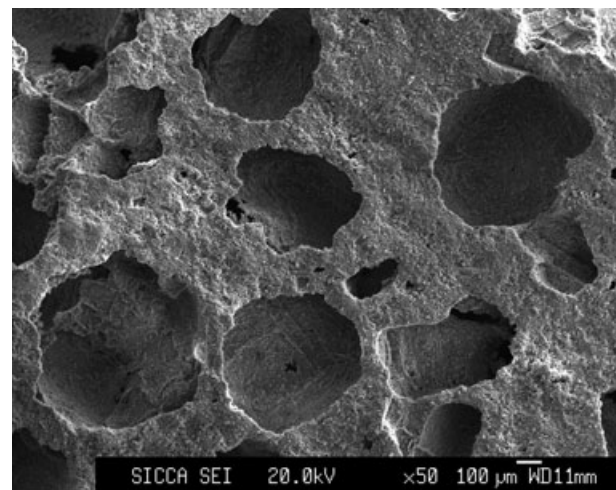

Figure 1. SEM observation of microstructure of the three-dimensional porous structure of CPC scaffold (×50).

Table 1. The sequences of primers for GAPDH, ALP, BSP and OCN

Genes	Primer sequence (forward/reverse)	Product size (bp)	Annealing temperature (°C)	Accession number
GAPDH	5′-GGTCGGAGTGAACGGATTTGGC-3′ 5′-AGCCTTGACTGTGCCGTGGAAT-3′	172	57	AF017079.1
ALP	5′-CAAAGGCTTCTTCTTGCTGGTG-3′ 5′-CAAAGGTAAAGACGTGGGAGTGG-3′	185	58	AY145131.1
BSP	5′-ACCAGCACC AACAGCACAGAGG-3′ 5′-GTTCAAGCCCACCATTCGGAGA-3′	169	59	L10363.1
OCN	5′-GGCGCTTCTATGGCATAGCCT-3′ 5′-GGGATGATGGGGACCTTACACTT-3′	139	60	AY150038.1

Surgical procedures

Eight 5-week-old male nude Balb/c mice were enrolled in this study. Each mouse received 4 constructs from the following different groups: ① CPC scaffold alone; ② CPC scaffold + 150 µg/ml EMPs; ③ CPC scaffold + pBMSCs and ④ CPC scaffold + 150 µg/ml EMPs + pBMSCs, and all constructs were harvested at 2 or 4 weeks with four samples for each group at both time points. All constructs were implanted under dorsal subcutaneous skin with a distance of more than 5 mm between each implant as adopted by Tsuda *et al.* (25). Briefly, animals were anaesthetized by intraperitoneal injection of pentobarbital (Nembutal 3.5 mg/100 g) after light ether inhalation. Mid-longitudinal skin incisions were made in the back of each mouse and subsequently four separate subcutaneous pockets were created with two on each flank, by blunt dissection through the incision. Four constructs were placed into the subcutaneous pockets then skin incisions were sutured. At the time of surgery, location information of all the implants was recorded accurately.

Implant harvest and histological analysis

Mice were sacrificed at either 2 or 4 weeks post-surgery by intraperitoneal overdose injection of pentobarbital, and implants were harvested for histological analysis. Briefly, after fixation in 10% neutral buffered formalin, implants were decalcified in 10% EDTA for 2 weeks, then embedded in paraffin wax. Five micrometre serial sections were cut parallel to cross sections, for haematoxylin and eosin (H&E) staining. Percentage of new bone formation in each implant was calculated by mean value of three sections selected from each of the three equally divided parts, cut parallel to the cross section, by complete random sampling method, which was further used to calculate mean value for each group. For each section, H&E staining result was observed and recorded using an OLYMPUS BX51 microscope, then computer-assisted histomorphometric measurement of new bone formation was performed using a PC-based image analysis system (Image-Pro Plus System; Media Cybernetics, Silver Spring, MD, USA). New bone formation in each section was expressed as percentage of new bone area in observed areas per whole implant.

Statistical analysis

Statistically significant differences ($P < 0.05$) between various groups were determined using ANOVA and SNK *post hoc*, for real-time PCR analysis and Independent Samples *t*-test for new bone area assay. All statistical analyses were carried out using SPSS 16.0 statistical software package (Chicago, IL, USA). All data are presented as mean ± standard deviation.

Results

Cell culture and EMP treatment

Cell morphology in each group is shown in Fig. 2. pBMSCs grew well after being treated with 50 µg/ml EMP (Fig. 2b,e) or 150 µg/ml EMP (Fig. 2c,f), without obvious observed cell death. More importantly, pBMSCs at 3 days after being treated with 50 µg/ml EMP and 150 µg/ml EMP, displayed spindle and polygonal morphology as shown in Fig. 2b,c. When treatment time was extended to 6 days, pBMSCs almost reached confluence and gradually showed morphology of polygonal to square shape (Fig. 2e,f). This trend of change to cell morphology was more pronounced in pBMSCs treated with 150 µg/ml EMPs, whereas pBMSCs untreated with EMPs always maintained short fibroblasts-like morphology.

Alkaline phosphatase activity and von Kossa assay

Fourteen days after EMP treatment, alkaline phosphatase staining was stronger and more intense in 150 µg/ml EMP-treated pBMSCs (Fig. 3c) than in those treated with 50 µg/ml EMPs (Fig. 3b) or untreated pBMSCs (Fig. 3a). In addition, pBMSCs 21 days after EMP treatment revealed more pronounced von Kossa staining of mineralized nodules in 150 µg/ml EMP treated pBMSCs (Fig. 3f), compared to 50 µg/ml EMP treated (Fig. 3e) or untreated ones (Fig. 3d).

Real-time PCR analysis of osteogenic markers

Real-time PCR analysis of osteogenic differentiation markers was performed to compare differential gene expression at 3 and 6 days, after pBMSCs were treated with 50 µg/ml EMPs, 150 µg/ml EMPs or left untreated. Compared to expression of *ALP* gene in three groups, expression level in 150 µg/ml EMP treated pBMSCs showed dramatic up-regulation than 50 µg/ml EMP treated or untreated pBMSCs at both 3 and 6 days; however, no significant difference was found between 50 µg/ml EMP treated and untreated pBMSCs (Fig. 4a). Also, it was revealed that gene expression of *BSP* was significantly enhanced in 150 µg/ml EMP treated pBMSCs compared to untreated pBMSCs both at 3 and 6 days, although there was less notable difference found in 50 µg/ml EMP treated pBMSCs than untreated ones at day 3 (Fig. 4b). Finally, gene expression level of *OCN* in pBMSCs treated with 150 µg/ml EMPs showed significant up-regulation compared to 50 µg/ml EMP treated or
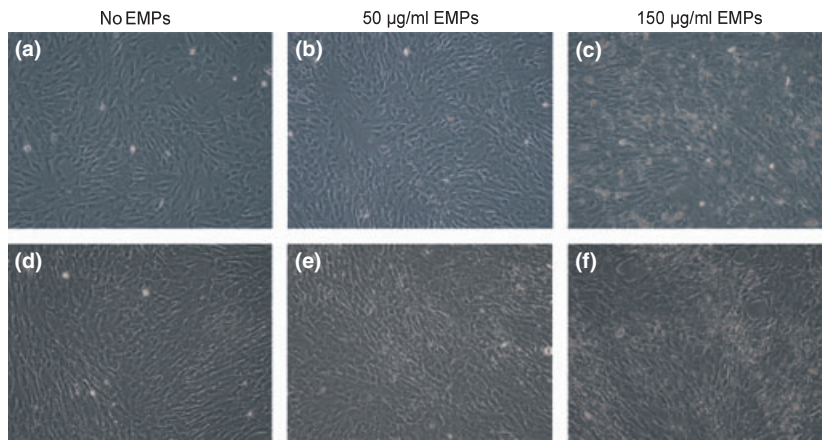
No EMPs   50 µg/ml EMPs   150 µg/ml EMPs

Figure 2. Cell culture and EMP treatment. Cell morphology of pBMSCs treated with 0, 50 and 150 µg∕ml EMPs at 3 days (a, b, c) and 6 days (d, e, f) respectively. pBMSCs without being treated with EMPs always maintained short fibroblasts-like morphology, while pBMSCs treated with EMPs displayed spindle and polygonal morphology at day 3, and showed a morphology of polygonal or squarish by day 6 (reverse phase contrast microscope, ×100).
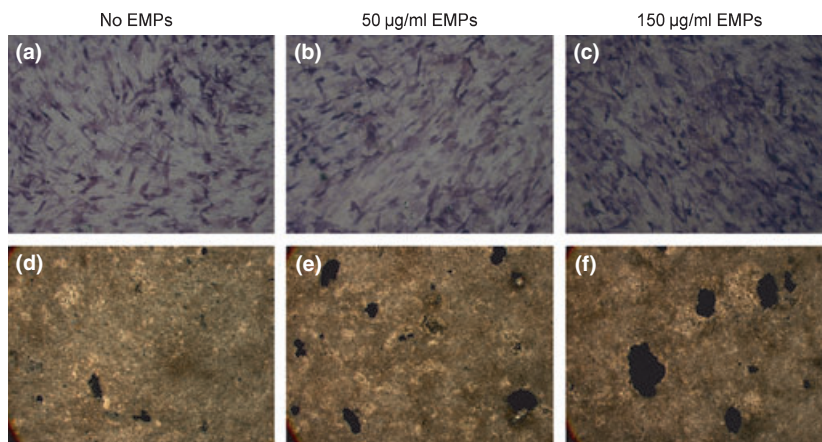
No EMPs   50 µg/ml EMPs   150 µg/ml EMPs

Figure 3. *In vitro* analysis of osteogenic differentiation of pBMSCs with or without EMP treatment. (a, b, c) Alkaline phosphatase expression of pBMSCs treated with 0, 50 and 150 µg∕ml EMPs for 14 days (reverse phase contrast microscope, ×100). (d, e, f) von Kossa staining for mineralized nodules of pBMSCs treated with 0, 50 and 150 µg∕ml EMPs for 21 days (reverse phase contrast microscope, ×16).

untreated cells at both 3 and 6 days. Gene expression in pBMSCs treated with 50 µg∕ml EMP was also significantly enhanced at 6 days compared to untreated pBMSCs (Fig. 4c). Taken together, these data support the notion that EMPs enhanced osteogenic differentiation of pBMSCs and the effect was dose-dependent and related to time in culture.

Histological analysis of new bone formation

Two weeks after surgery, half the implants were harvested and no inflammation was found in any group. No new bone formation was found in CPC scaffold alone, CPC scaffold + 150 µg∕ml EMPs, and CPC scaffold + pBMSCs groups. However, a hint of new bone formation was noted in CPC scaffold + 150 µg∕ml EMPs + pBMSCs group (Fig. 5). Four weeks post-operation, all remaining implants were harvested and again no inflammation was found in all groups. New bone formation was observed in CPC scaffold + pBMSCs group, and CPC scaffold + 150 µg∕ml EMPs + pBMSCs group; there was no obvious new bone formation observed in

CPC scaffold alone or CPC scaffold + 150 µg∕ml EMPs groups. Percentage of new bone areas was $18.52 \pm 1.03\%$ in CPC scaffold + 150 µg∕ml EMPs + pBMSCs group compared to $11.63 \pm 0.95\%$ in CPC scaffold + pBMSCs group – this was statistically different ($P < 0.05$) (Fig. 6). These results indicate that EMPs had enhanced bone formation ability using this tissue-engineering method, *in vivo*.

Discussion

Bone tissue-engineering methods are generally centred on delivery of osteoinductive growth factors, implantation of osteogenic cells and combination with osteoconductive scaffolds, to promote bone regeneration.

EMPs, secreted and synthesized by cells of the Hertwig's epithelial root sheath, are actively involved in embryogenesis of cementum, periodontal ligament and periodontal supporting bone (26) EMPs can be extracted from developing porcine tooth germs, the major component of which is amelogenin and its cleavage products, a family of hydrophobic proteins that make up over 90% of
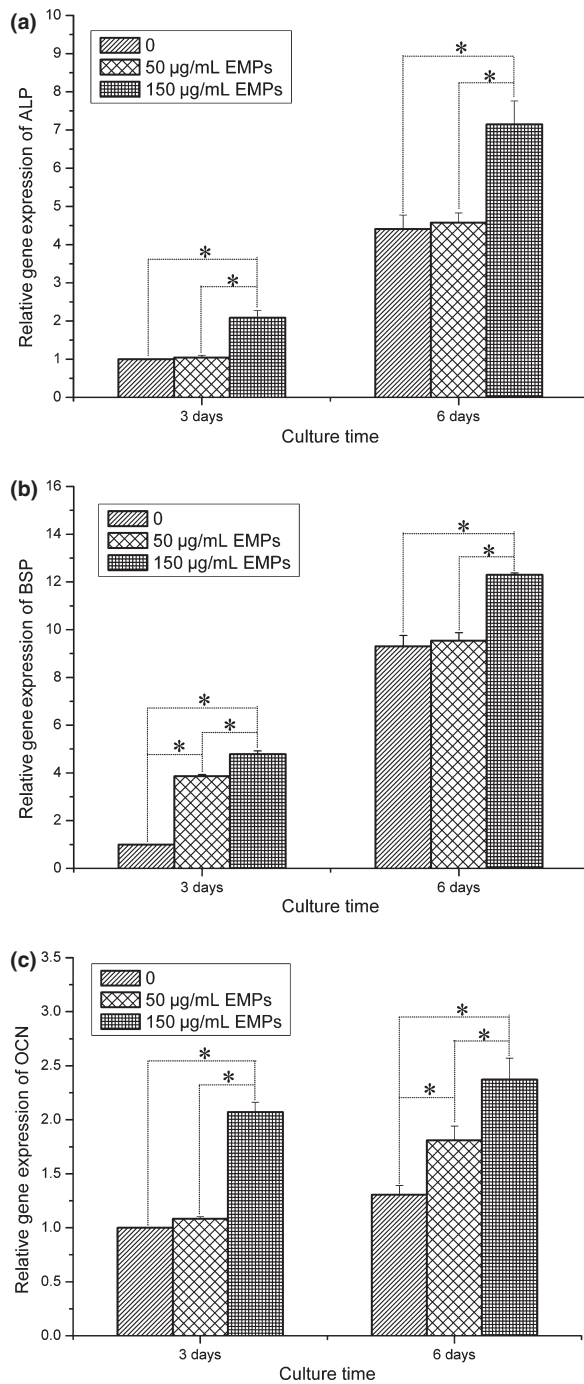
Figure 4. Real-time PCR analysis of osteogenic differentiation gene expression in pBMSCs treated with 0, 50 and 150 μg∕ml EMPs at 3 and 6 days. (a) alkaline phosphatase (ALP), (b) bone sialoprotein (BSP), (c) osteocalcin (OCN). All values normalized to GAPDH (*$P < 0.05$).

the total protein content (27). The remaining components of EMPs include ameloblastin (also called amelin or sheathlin), amelotin, tuftelin and enamelin. Amelogenins can self-aggregate into supramolecular aggregates,

so-called nanospheres, and play a crucial role in regulating initiation and growth of hydroxyapatite crystals during formation of enamel (28,29). Besides, in the context of periodontal tissue regeneration, it is clear that EMPs have functions other than enamel biomineralization. For example, EMPs have been available as a therapeutic agent (brand name Emdogain), since 1997. Emdogain consists of an enamel matrix derivative, water, and a carrier, propylene glycol alginate. Clinically, Emdogain is used for periodontal regeneration of teeth affected by periodontitis, root coverage procedures and tooth replantation, although mechanism of its function is still regarded to be obscure (30).

For the success of bone tissue engineering, porous scaffolds which act as a temporary carriers for anchorage-dependent cells are also an important factor. Ideal scaffolds require three-dimensional interconnected porous structures to provide sufficient space for cell migration, adhesion, proliferation and for new bone tissue ingrowth (31). Suitable pore size and porosity is beneficial for cell infiltration, bone ingrowth and internal mineralized bone formation (32,33). CPC adopted in this study has composition and structure close to natural bone minerals, excellent bone biocompatibility, osteoconductivity and bioresorbability, and is easy to shape (34–37). Average pore size of 400 μm and average porosity of 70% are suitable for cell infiltration and bone ingrowth. As *in vitro* morphological features of cells cultured on scaffolds partially reflects biocompatibility of the scaffolds, pBMSCs need to be able to attach, spread and proliferate well on the CPC scaffold, indicating whether the scaffold would well support good cell outcome. Thus, CPC was considered to be an ideal material for BMSCs to construct tissue-engineered bone in the current study.

According to the study of Song *et al.* (17) as well as our previous experimental results, EMPs with concentration of 50 and 150 μg∕ml were used here to explore the effect of EMPs on pBMSCs in osteogenic differentiation *in vitro*. ALP activity and mineralized nodule formation, makers of osteogenic differentiation and commitment of pBMSCs towards the osteoblastic phenotype, were observed at 14 and 21 days after EMP treatment, respectively. 150 μg∕ml EMP-treated pBMSCs showed strong expression of ALP and a significant increase in mineralized nodules when compared to 50 μg∕ml EMP treated, or untreated cells. However, less apparent differences were clearly observed between 50 μg∕ml EMP treated and untreated pBMSCs. These findings indicated that pBMSCs treated with 150 μg∕ml EMP had been directed towards, and specifically enhanced, osteogenic differentiation, and that the effect of EMPs was dose-dependent, which results were further confirmed by real-time PCR analysis of osteogenic makers.
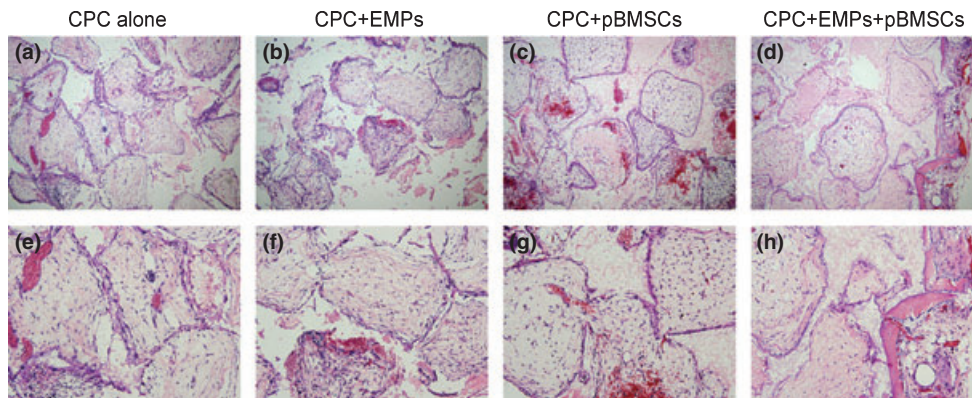
Figure 5. Representative histological images of implants at 2 weeks post-surgery. (a) and (e) CPC alone group, (b) and (f) CPC + 150 μg/ml EMPs group, (c) and (g) CPC + pBMSCs group, (d) and (h) CPC + 150 μg/ml EMPs + pBMSCs group (a, b, c, d ×100; e, f, g, h ×200). A slight amount of new bone formation was found in the CPC + 150 μg/ml EMPs + pBMSCs group, whereas no new bone was found in other groups.
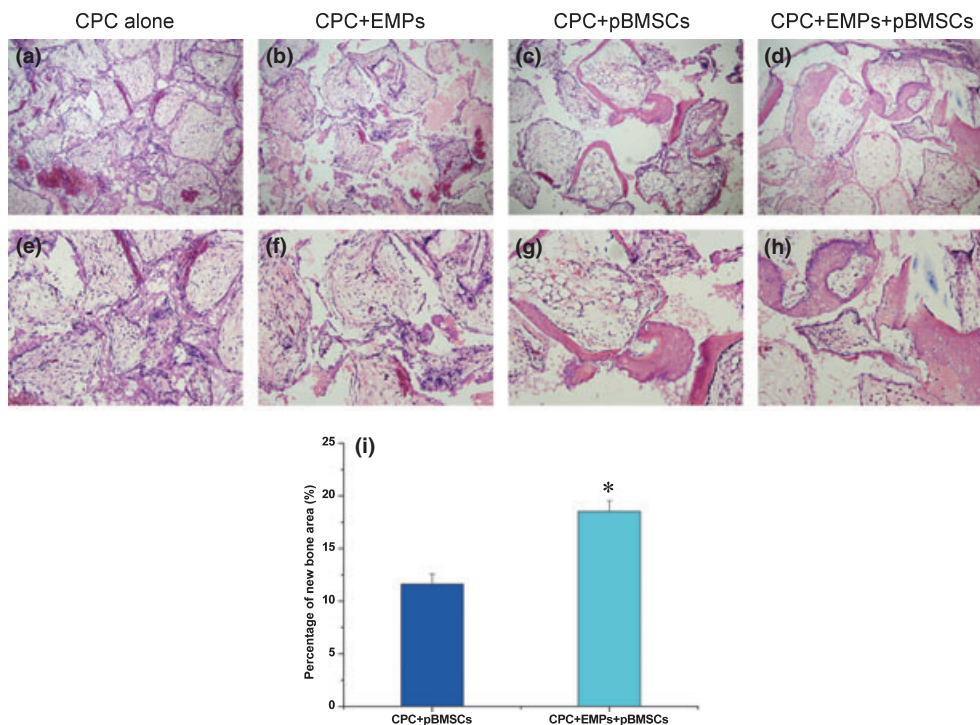

Figure 6. Representative histological image and new bone area assessment of implants at 4 weeks post-surgery. (a) and (e) CPC alone group, (b) and (f) CPC + 150 μg/ml EMPs group, (c) and (g) CPC + pBMSCs group, (d) and (h) CPC + 150 μg/ml EMPs + pBMSCs group (a, b, c, d ×100, e, f, g, h ×200). Apparent new bone formation was observed in CPC + pBMSCs group and CPC + 150 μg/ml EMPs + pBMSCs group, but still no new bone was found in CPC alone and CPC + 150 μg/ml EMPs groups. (i) Percentage of new bone area in CPC + 150 μg/ml EMPs + pBMSCs group was significantly higher than that in CPC + pBMSCs group by histomorphometric analysis ($n = 4$, *$P < 0.05$).

EMP treated pBMSCs presented a defined sequence of gene expression during osteogenic differentiation and maturation. ALP, an early maker of osteogenic differentiation, was significantly up-regulated in 150 μg/ml EMP treated pBMSCs compared to untreated cells at both 3 and 6 days, while no obvious up-regulation was observed in the 50 μg/ml EMP treated group. Results were consistent with those of ALP staining. Besides, BSP was considered to be intermediate osteogenic differentiation maker (38), expression of which, similar to that of ALP, was also significantly increased in the 150 μg/ml EMP treated group at 3 and 6 days, although a less significant increase was

found in the 50 μg/ml EMP treated group only at 3 days. Finally, OCN, which was reported to be a later marker of osteogenic differentiation corresponding with matrix deposition and mineralization (21), also showed clear increases in the 150 μg/ml EMP treated group at 3 and 6 days, in comparison with the control group. In contrast, expression of *OCN* gene showed less significant up-regulation at 6 days in the 50 μg/ml EMP treated pBMSCs. These results further indicated that osteogenic differentiation effects of EMPs on pBMSCs was both dose-dependent and time-dependent. This implies that EMPs might have played an important role in commitment of mesenchymal progenitor cells to the osteogenic lineage. Similarly, there have been reports that EMPs could significantly enhance ALP activity and *in vitro* mineralized nodule formation in rat BMSCs (16). Likewise, Iwata *et al.* fractionated enamel matrix extracts from developing porcine teeth and found that an osteoinductive fraction (OFE) containing mainly 20, 23 and 25 kDa proteins could enhance ALP activity and *in vitro* mineralized nodule formation, as well as lead to up-regulated ALP, BSP and OCN mRNA expression in ST2 cells, a mouse bone marrow stromal cell line (39). However, other studies have reported an inhibitory effect of EMPs on expression of OCN (40,41). Guida *et al.* have shown that EMPs can down-regulate type I collagen synthesis and ALP activity of human BMSCs, whereas decrease in OCN synthesis was not statistically significant (42). In a heterogeneous cell population from rat bone marrow, EMPs had no significant effect on cell proliferation, ALP activity and mRNA expression of type I collagen, OCN and ALP (43). Discrepancies between different reports may be due to differences in cell types, culture conditions, methods used, concentration and timing of EMP addition to cultures (44). Nevertheless, according to the current *in vitro* experimental results, gene expression of *ALP, BSP* and *OCN* in 150 μg/ml EMP treated group were all significantly increased at both time points, thus 150 μg/ml was considered to be the effective protein concentration in inducing osteoblastic differentiation and thus was adopted for our further *in vivo* investigation.

In our study, we used an ectopic osteogenesis model in nude mouse to investigate bone formation by implantation of porous CPC scaffolds combined with 150 μg/ml EMPs and seeded with pBMSCs, without extra osteogenic medium induction. Two weeks post-surgery, new bone formation was observed only in the CPC scaffold + EMPs + pBMSCs group. Four weeks post-surgery, a small amount of bone formation was also detected in the CPC scaffold + pBMSCs group, while there was no indication of new bone formation in the remaining CPC scaffold alone or the CPC scaffold + EMPs group. Formation of small amounts of new bone in the CPC scaffold + pBMSCs group may be attributed to self-osteogenic differentiation tendency of BMSCs, while significantly more newly formed bone in the group of CPC scaffolds + 150 μg/ml EMPs + pBMSCs was found compared to the group with CPC scaffold + pBMSCs. Results revealed that the tissue-engineering technique by implantation of the bone complex of EMPs, pBMCs and CPC scaffold can accelerate new bone formation and augment the amount of new bone. While CPC alone or simply combining CPC with EMPs was not sufficient enough to induce new bone formation under the current formulation here.

It can thus be concluded from our study that EMPs promote osteogenic differentiation of pBMSCs *in vitro* and benefit new bone formation *in vivo* when combined with pBMSCs and a CPC scaffold. The results suggested that a tissue-engineered complex of EMPs, pBMSCs and CPC scaffold will be a valuable alternative to be used in periodontal bone tissue engineering and regeneration.

Acknowledgements

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