Periodontal ligament-associated protein-1 delays rat periodontal bone defect repair by regulating osteogenic differentiation of bone marrow stromal cells and osteoclast activation

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Received May 13, 2016; Accepted November 28, 2017

DOI: 10.3892/ijmm.2017.3312

Abstract. The aim of the present study was to assess the roles of periodontal ligament-associated protein-1 (PLAP-1) in the osteogenic differentiation of rat bone marrow stromal cells (rBMSCs) and in osteoclast activation during the repair of rat periodontal bone defects. Male, 6-week-old, Wistar rats treated with periodontal bone defects were randomly assigned to 3 groups: The PLAP-1-transfected rBMSC group (PLAP-1 group), the empty vector-transfected rBMSC group (vector group) and the normal rBMSC group (control group). Specimens were obtained at 2, 4 and 6 weeks post-surgery. Histological observation and micro-computed tomography were applied to evaluate the repair effect. The bone defect areas of the mandible were dissected for western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Osteogenesis-associated proteins, including alkaline phosphatase (ALP), bone sialoprotein (BSP), runt-related transcription factor 2 (Runx2), Osterix (Osx) and osteocalcin (OC), as indicators of rBMSC-induced osteogenesis, were examined by RT-qPCR and western blotting. Osteoclasts were identified and quantified using tartrate-resistant acid phosphatase staining. Meanwhile, the receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) ratio was quantified to assess osteoclast activation by western blotting. The repair effect of the PLAP-1 group was significantly worse than that of the vector and control groups. In the PLAP-1 group, newly formed and mineralized bones were significantly less in quantity than that in the other two groups ($P<0.05$), and the expression of osteogenic proteins (ALP, BSP, Runx2, Osx and OC) was also reduced ($P<0.01$). However, there was no significant difference between the vector and control groups. The RANKL/OPG ratio was upregulated in the PLAP-1 group due to decreased OPG protein expression and a simultaneous increase in RANKL protein expression ($P<0.01$), and more osteoclasts were activated in the PLAP-1 group ($P<0.01$). In conclusion, the present study found that PLAP-1 delays rat periodontal bone defect repair by inhibiting osteogenic differentiation and promoting osteoclast activation, mainly dependent on the upregulation of the RANKL/OPG ratio.

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Key words: periodontal ligament-associated protein-1, bone marrow stromal cells, periodontal bone defect, micro-computed tomography, osteoclast activation

Introduction

As a member of the small leucine-rich repeat proteoglycan (SLRP) family (1,2), periodontal ligament-associated protein-1 (PLAP-1) plays an important role in maintaining the homeostasis of the periodontium (3,4), and protects the periodontal ligament from excessive osteogenesis by the negative regulation of the osteoblastic differentiation of periodontal fibroblasts (5) and periodontal ligament stem cells (PDLSCs) into mineralized tissue-forming cells (3,6).

Dental follicle stem cells (DFSCs) and PDLSCs are potentially able to differentiate into the periodontal lineage (7), and are therefore of value in dental tissue engineering (8). Bone marrow stromal cells (BMSCs) also have multilineage differentiation potential (9). Combined with biomaterials, BMSCs have been incorporated into repair different bone defects, including periodontal bone defects, in a number of studies (10-12). The majority of studies of tissue engineering have focused on osteogenesis-promoting factors, including osterix (Osx), bone morphogenetic protein-2 (BMP-2), alkaline phosphatase (ALP), osteocalcin (OC) and bone sialoprotein (BSP) (13-15), but our knowledge on osteogenesis-inhibiting factors, such as PLAP-1, and their molecular mechanisms is insufficient (16-18).

PLAP-1 is an important marker of the periodontal ligament. PDL cells have been demonstrated to be multipotent, with the microenvironment-dependent ability for differentiation into osteoblasts or cementoblasts. As a negative regulator, PLAP-1 inhibits periodontal ligament mineralization. To investigate the roles of PLAP-1 in the cytodifferentiation and mineralization of BMSCs, rat BMSCs overexpressing PLAP-1 were established in our previous study. The vector expressing the PLAP-1 gene

was transfected into BMSCs and stable transfectants that were overexpressing PLAP-1 were established (19). The study showed that the overexpression of the PLAP-1 gene inhibits the differentiation of rat BMSCs (rBMSCs) into osteoblast-like cells *in vitro* (19) and delays rat critical-size skull defect repair *in vivo* (18). However, the molecular mechanisms of PLAP-1 in the osteogenic differentiation of BMSCs and in osteoclast activation in periodontal bone defect repair remain unclear.

Osteoblasts are vital for bone formation and for the maintenance of a dynamic equilibrium within bone tissues. RANKL/RANK signaling regulates osteoclast differentiation and activation in bone modeling and remodeling (20-22). OPG confers a protective effect over bone, preventing excessive resorption by binding to RANKL and impeding it from binding to RANK. Therefore, the OPG/RANKL ratio is a fundamental determinant in bone (23).

In the present study, osteogenesis-associated proteins, including ALP, BSP, runt-related transcription factor 2 (Runx2), Osx and OC, as indicators of rBMSC-induced osteogenesis (22), were examined to assess the role of PLAP-1 in the osteogenic differentiation of BMSCs. Osteoclast number and the RANKL/OPG ratio were quantified to analyze osteoclast activation. PLAP-1 is an important marker of the periodontal ligament (24) and studying its mechanism of action in the osteogenic differentiation of BMSCs and in osteoclast activation may assist in furthering dental tissue engineering (25).

Materials and methods

Animals. Male, 6-week-old, Wistar rats (n= 24; weight, 260-300 g; Laboratory Animal Center, Shandong University, Shandong, China), which were acclimated for 1 week prior to the experiments, were maintained on a normal hard food diet, with water *ad libitum*. The animals were housed in cage racks, with a 12-h light/12-h dark cycle (light on from 8:00 AM to 8:00 PM) at ambient temperature (22-24˚C) and 45% relative humidity. Experiments used in this study were conducted according to the guidelines for Animal Experimentation of Shandong University. The study was approved by the Ethics Committee of the School of Stomatology, Shandong University. Rats that received a periodontal bone defect of 5x2x1 mm according to a previously described procedure (26) were randomly allocated to 3 groups according to differentially transfected-rBMSCs: PLAP-1 group (collagen membranes with PLAP-1 lentivirus-transfected rBMSC were transferred to the periodontal bone defects), vector group (collagen membranes with empty vector lentivirus-transfected rBMSC were transferred to the periodontal bone defects) and control group (collagen membranes with normal rBMSC were transferred to the periodontal bone defects). For statistical analysis, 8 animals were present in each group.

Cell culture. Primary rBMSCs were harvested from 4-week-old Wistar rats as previously described (9). Briefly, the proximal end of the femora and the distal end of the tibiae were excised. α-minimal essential medium (α-MEM; Gibco, Grand Island, NY, USA), supplemented with 20% fetal bovine serum (FBS; Gibco), 200 IU/ml penicillin and 200 mg/ml streptomycin (Solarbio, Beijing, China) was used to flush the marrow gently from the shafts with a 25-gauge needle. A single-cell suspension was obtained by gently aspirating the cells sequentially through 20- and 23-gauge needles. The bone marrow cells were then seeded into culture flasks (Takara Bio, Inc., Otsu, Japan) at a cell density of $4.0x10^5$ cells/cm^2 and cultured using α-MEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin. The culture medium was changed every 3 days, and the cells were subcultured 1:3 at subconfluence. The adherent cells after one subculture were termed rBMSCs. Cells (3-5 passages) were subsequently used for experiments.

Overexpression of the PLAP-1 gene in rBMSCs. The protocol of overexpression of the PLAP-1 gene in rBMSCs was the same as previously described (18,27). rBMSCs were then plated in 6-well plates ($1x10^5$ cells/well) and transfected with viral stocks of pPBABE-hygro-PLAP-1, pPBABE-hygro or empty vector in the presence of polybrene (6 μg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 8 h. At 24 h post-transfection, the rBMSCs were subjected to hygromycin B selection (50 μg/ml) for 2 weeks. The stably transduced rBMSCs were used for the following experiments.

Cell seeding. Type I collagen membranes (Sigma-Aldrich; Merck KGaA) were trimmed into 5x2x1-mm pieces and sterilized for usage as previously described (21). The differentially transfected-rBMSCs ($1x10^4$) were suspended in 5-μl α-MEM, seeded on each surface of the collagen scaffolds and cultured for 3 h in the incubator in 5% CO_2 at 37˚C. After anesthetization by intraperitoneal injection of 10% chloral hydrate (0.4 g/kg body weight), Bilateral bone defects were created at the buccal aspect of the mandibular molar. The signs of peritonitis were not observed following the administration of 10% chloral hydrate. A defect of 5x2x1 mm was made in the mandibular body using a dental drill driven at a low speed with irrigation of 0.9% sodium chloride. Type I collagen membranes and differentially transfected-rBMSCs were applied to fill in the defect. The wounds were closed with nylon 4-0 sutures.

Tissue preparation. The rats were euthanized in order to minimize pain and distress. The rats were sacrificed at 2, 4 and 6 weeks post-surgery. Anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 g/kg body weight), the rats were fixed with 4% paraformaldehyde (PFA) via systemic circulation fixation for 30 min, and then the detached mandible was further fixed immediately in 4% PFA for another 12 h at 4˚C. The specimens were then demineralized in 10% EDTA for 3 months. The demineralized tissues were dehydrated by gradient ethanol, cleared with xylene and embedded in paraffin. Serial sections of 5 μm in thickness were sliced in the buccolingual direction. The specimens of mandibular bone for the western blotting and RT-qPCR analyses were not fixed. The defect areas were cut from the mandibular bone with 1-mm margins using bone cutting forceps and then rapidly frozen in liquid nitrogen and stored at -80˚C.

Micro-computed tomography (micro-CT) imaging and analysis. For analysis of the alveolar bone loss, fixed mandible samples were scanned using a PerkinElmer micro-CT (PerkinElmer, Inc., Waltham, MA, USA) at 90 kV and 88 μA. All scans were reoriented prior to analysis to uniformly align the scan axes and anatomical positions. The specimens were scanned at a resolution of 10 μm, ensuring that the defect areas were encompassed. The three-dimensional (3D) volume viewer and analyzer software (Analyze 12.0 and SimpleViewer version 5.1.2;

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Genes	Upstream primer (5'-3')	Downstream primer (3'-5')
PLAP-1	CCTGGTAGGAGGGCTGGATT	AGGGGTTCACTGGCTCTTTG
ALP	GGAGATGGATGAGGCCATCG	CGTCCACCACCTTGTAACCA
BSP	GCCACATCTCAGGGGTAAC	TGCATCTCCAGCCTTCTTGG
Runx2	CAGACACAATCCTCCCCACC	GCCAGAGGCAGAAGTCAGAG
OSX	GGATGGCGTCCTCTCTGCTTGAG	AGGGAGCTGGGTAGGCGTCC
OC	CAGGTGCAAAGCCCAGCGACT	AGGGGATCTGGGTAGGGGGCT
GAPDH	TGATGGGTGTGAACCACGAG	CCCTTCCACGATGCCAAAGT

PLAP-1, periodontal ligament-associated protein-1; ALP, alkaline phosphatase; BSP, bone sialoprotein; Runx2, runt-related transcription factor 2; OSX, osterix; OC, osteocalcin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PerkinElmer, Inc.) were used for the visualization and quantification of two-dimensional (2D) and 3D data on a personal computer output and a standardized gray scale value was used to visualize only mineralized tissues.

RT-PCR. Total RNA was extracted from the cells and bone defect tissues with RNAiso Plus (Takara Bio, Inc.) according to the manufacturer's instructions. A total of 1.0 mg RNA (in a 20-ml reaction volume) was reverse transcribed using the PrimeScript RT Reagent kit with gDNA Eraser (Takara Bio, Inc.). RT-PCR amplifications labeled with SYBR Premix Ex Taq (Takara Bio, Inc.) were performed in a Roche LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) at 95˚C for 30 sec, then at 95˚C for 5 sec and 60˚C for 30 sec for a total of 40 cycles. The primer sequences for PLAP-1, ALP, BSP, Runx2, Osx and OC (19) were designed with Primer-BLAST software from the National Center for Biotechnology Information (Bethesda, MD, USA) nucleotide sequence database (Table I). Relative expression was normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ method (28).

Western blotting. The frozen samples were homogenized with hypotonic lysis buffer (Solarbio). Protein concentrations were determined by bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China), and the curves in the BSA protein standard curves were used. Equal amounts of total proteins (20 μg per lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The membranes was washed three times and blocked with 5% skimmed milk (BD, Mr Ng Nanjing Biological, Nanjing, China) at room temperature for 1 h. Next, the membranes were incubated with antibodies against PLAP-1 (diluted 1:1,000; A3883-45C-AP; US Biological, Salem, MA, USA), ALP (diluted 1:1,000; ab95462), BSP (diluted 1:1,000; ab52128), Runx2 (diluted 1:1,000; ab23981), Osx (diluted 1:1,000; ab209484), OC (diluted 1:1,000; ab13420) (all Abcam, Cambridge, MA, USA), OPG (diluted 1:500; bs-0431R) or RANKL (diluted 1:500; bs-0747R) (both Bioss, Beijing, China) overnight at 4˚C. Secondary antibodies, horseradish peroxidase-linked goat anti-rabbit IgG (diluted 1:5,000; CW0156S; CW Biotech, Beijing, China), were then applied. The blots were visualized using enhanced chemiluminescence reagents (EMD Millipore, Billerica, MA, USA), and quantified by densitometric analysis [ImageJ (x64); 1.48u; National Institutes of Health, Bethesda, MD, USA]. Equal protein loading was shown by stripping and incubation with an anti-GAPDH antibody (diluted 1:5,000; CW0100S; CW Biotech).

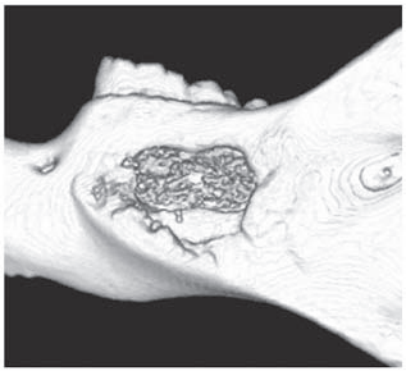

Figure 1. Three-dimensional periodontal bone defect in the mandible as observed by micro-computed tomography.

Tartrate-resistant acid phosphatase (TRAP) staining. Sections were deparaffinized using xylene, hydrated in gradient ethanols and gently washed twice with prewarmed, filtered water (37˚C). The sections were then fixed with stationary liquid for 20 sec and stained with TRAP (Sigma-Aldrich; Merck KGaA) for 60 min at 37˚C. The TRAP-stained cells were then counterstained with hematoxylin (Solarbio) at room temperature for 5 min, and examined under a light microscope. $TRAP^+$ multinucleated cells containing three or more nuclei were counted as osteoclasts. Osteoclasts were quantified by imaging five fields of view under 100-fold magnification and directly counting the number of $TRAP^+$ cells.

Statistical analysis. Statistically significant differences ($P<0.05$) between the various groups were measured using one-way analysis of variance and Student-Newman-Keuls test. All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± standard deviation.

Results

Micro-CT images. Newly formed mineralized bone could be found in the defects at 4 weeks post-surgery (Fig. 1).

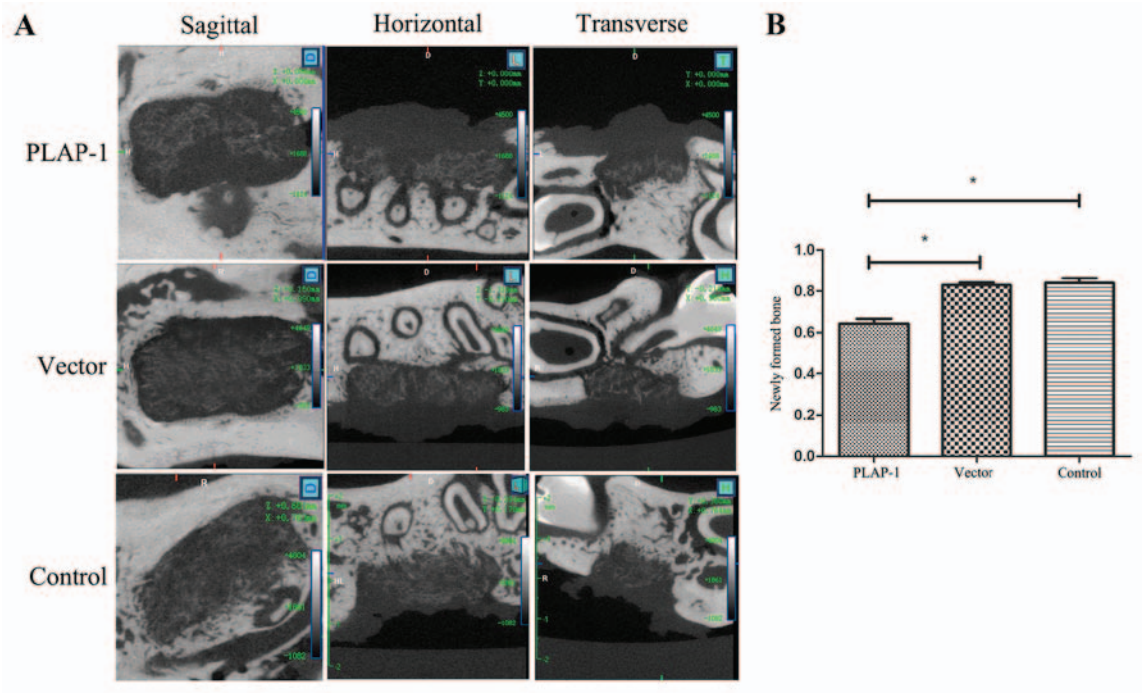

Figure 2. Micro-computed tomography images of periodontal bone defects at 4 weeks post-surgery. In the PLAP-1 group, newly formed mineralized bone could be observed in the defect. The newly formed mineralized bone showed a trabecular structure. (A) The mineralized bone in the PLAP-1 group was markedly less in quantity compared with that in the control and vector groups. The new mineralized bone area in the PLAP-1 group was markedly smaller than that in the vector and control groups (*P<0.05). (B) There were no significant differences in the amount of newly formed bone between the vector and control groups. PLAP-1, periodontal ligament-associated protein-1.

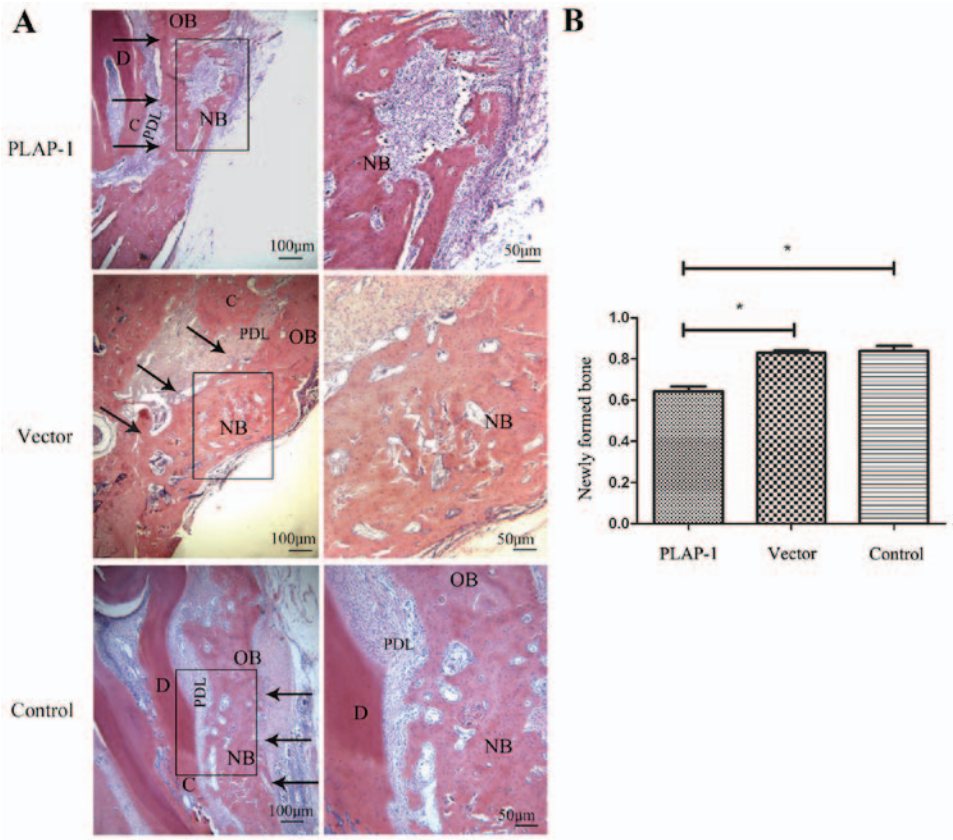

Figure 3. Histological observation of the periodontal bone defects at 6 weeks post-surgery. In the PLAP-1 group, new bone had filled the majority of the periodontal defects; the newly formed bone showed a trabecular structure and mature lacunae could be observed. (A) In the vector and control groups, newly formed bone had almost filled the defects, but a number of bone lacunae remained at the new bone area. The newly formed bone area was analyzed using Image Pro-Plus 6.0. The new bone area in the PLAP-1 group was markedly smaller than that in the vector and control groups (*P<0.05). (B) There were no significant differences in the amount of newly formed bone between the vector and control groups. PLAP-1, periodontal ligament-associated protein-1; C, cementum; D, dentin; NB, new bone; OB, old bone; PDL, periodontal ligament.

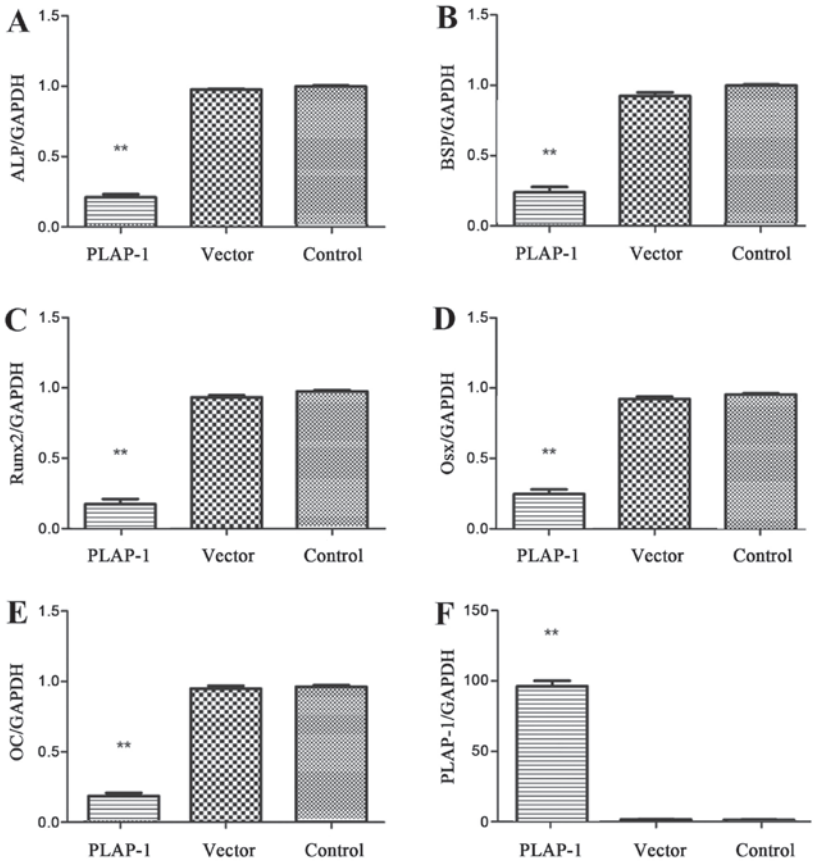

Figure 4. Expression of PLAP-1-overexpressing rat bone marrow stromal cells and oteogenesis-associated proteins in rat periodontal bone defects at 4 weeks post-surgery usingreverse transcription-quantitative polymerase chain reaction. (A) ALP, (B) BSP, (C) Runx2, (D) OSX and (E) OC mRNA expression was deceased in the PLAP-1 group compared with that in the vector and control groups (**P<0.01). (F) PLAP-1 mRNA expression was elevated significantly after gene transfection (P<0.01). PLAP-1, periodontal ligament-associated protein-1; ALP, alkaline phosphatase; BSP, bone sialoprotein; Runx2, runt-related transcription factor 2; Osx, osterix; OC, osteocalcin

The newly formed mineralized bone showed a trabecular structure. The mineralization density of the new bone was lower than that of normal bone (Fig. 2A). The new mineralized bone in the control and vector groups was significantly greater in quantity than that in the PLAP-1 group (P<0.05). There was no significant difference in the amount of newly formed bone between the vector and control groups (Fig. 2B).

Histological observation. Histological observation showed that newly formed bone had filled the majority of the defects of the PLAP-1 group at 6 weeks post-surgery. Newly formed bone trabeculae and lacunae were visible in the new bone. Osteoblasts, multinucleated osteoclasts and bone resorption pits could be viewed, which indicated that osteogenesis and bone resorption occurred simultaneously in the PLAP-1 group.

In the vector and control groups, newly formed bones had almost filled the defects, but there were a lot of bone lacunae at the new bone area. Osteoblasts could be observed at the edge of the new bone. Few osteoclasts could be found in the two groups (Fig. 3A). The newly formed bone proportion was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). The amount of new bone in the PLAP-1 group was significantly less than that in the vector and control groups (P<0.05). There was no significant difference

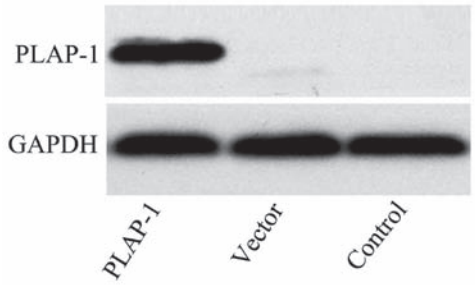

Figure 5. Expression of PLAP-1-overexpressing rat bone marrow stromal cells. PLAP-1 expression was elevated significantly following gene transfection (P<0.01). PLAP-1, periodontal ligament-associated protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

in the amount of newly formed bone between the vector and control groups (Fig. 3B).

RT-qPCR. The mRNA expression of osteogenesis-associated proteins in rat periodontal bone defects at 4 weeks post-surgery was detected using RT-PCR. ALP, BSP, Runx2, Osx and OC mRNA expression was deceased in the PLAP-1 group compared with that in the vector and control groups (Fig. 4A-E) (P<0.01).

PLAP-1 expression in rBMSCs was elevated significantly following transfection with pPBABE-hygro-PLAP-1

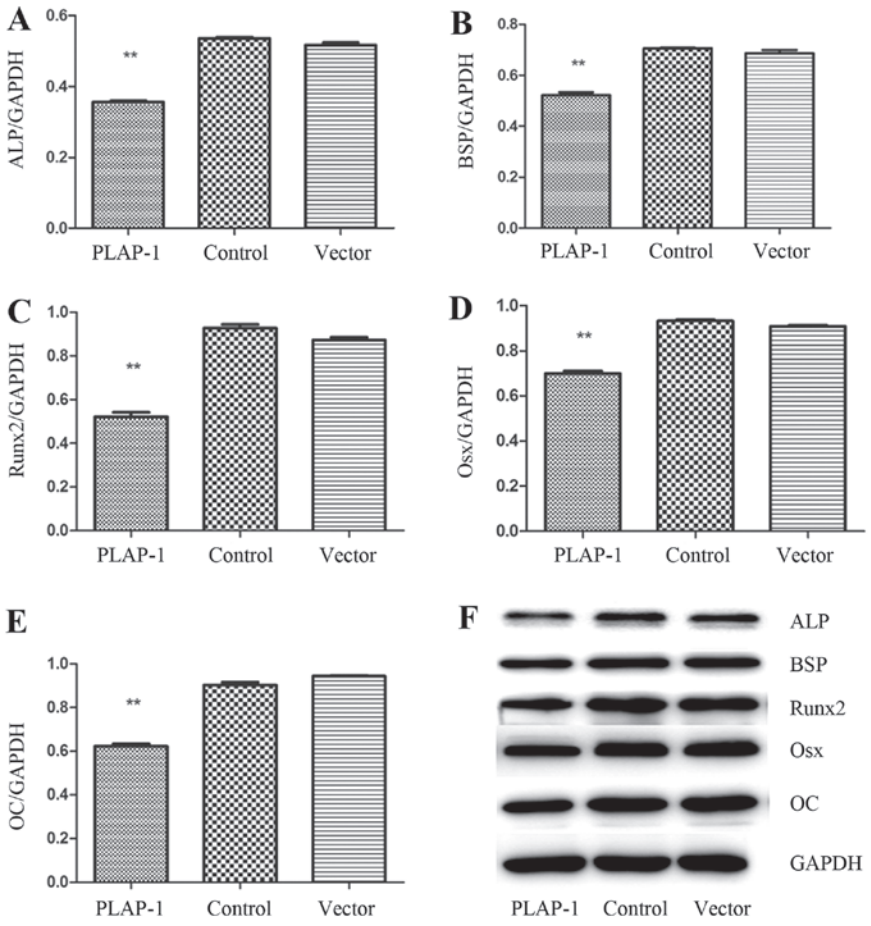

Figure 6. Expression of osteogenesis-associated proteins in rat periodontal bone defects at 4 weeks post-surgery as determined by western blotting. (A) ALP, (B) BSP, (C) Runx2, (D) Osx) and (E) OC expression was deceased in the PLAP-1 group compared with that in the vector and control groups (P<0.01). (F) Overall western blotting results. PLAP-1, periodontal ligament-associated protein-1; ALP, alkaline phosphatase; BSP, bone sialoprotein; Runx2, runt-related transcription factor 2; Osx, osterix; OC, osteocalcin

compared with that in rBMSCs transfected with empty vector and normal rBMSCs (Figs. 4F and 5).

Western blotting. The expression of ALP, BSP, Runx2, Osx and OC in rat periodontal bone defects at 4 weeks post-surgery was also detected using western blotting. ALP, BSP, Runx2, Osx and OC expression was deceased in the PLAP-1 group compared with that in the vector and control groups (P<0.01) (Fig. 6A-F), which was similar to the mRNA expression results.

Expression of RANKL and OPG protein in rat periodontal bone defects at 2, 4 and 6 weeks was detected by western blotting. RANKL protein expression was upregulated during rat periodontal bone defect repair, and higher expression was observed in the PLAP-1 group compared with the control group (Fig. 7A). This trend was reversed for OPG; OPG was downregulated compared with the higher expression of the control group from 2 to 6 weeks (Fig. 7B). The RANKL/OPG ratio was upregulated in the PLAP-1 group compared with that in the control group (P<0.01) (Fig. 7C and D).

TRAP staining. TRAP staining of the periodontal defects was applied at 6 weeks. TRAP is highly expressed by osteoclasts, which reflects osteoclast activity. A number of $TRAP^+$ multinucleated cells were found in the PLAP-1 group. By contrast, $TRAP^+$ cells were hardly detectable at 6 weeks in the control group (Fig. 8A). The number of $TRAP^+$ cells in the periodontal defects of the PLAP-1 group was significantly higher than that in the control group (P<0.01) (Fig. 8B).

Discussion

It is widely known that clinical periodontal tissue regeneration in patients with serious periodontitis is difficult to achieve (29). Besides the conventional approach of anti-inflammatory therapy, dental tissue engineering has been used to obtain periodontal tissue regeneration. Seeding cells, including DFSCs, PDLSCs and BMSCs, have been incorporated into the repair of periodontal bone defects (30). BMSCs have a multilineage differentiation potential, highly proliferative capacity and the ability to differentiate into several cell lineages, including muscle, bone, cartilage, epithelium, fat and neural progenitors (31,32). BMSCs have been applied in studies associated with osteoblast differentiation and bone regeneration. In the present study, rBMSCs were applied as a cellular model to investigate the functions of PLAP-1 in osteoblast differentiation and osteoclast activation.

According to histological observations and micro-CT examinations, PLAP-1 inhibited rat periodontal defect repair. The formation and mineralization of new bone was less prominent in the PLAP-1 group. The suppression of PLAP-1

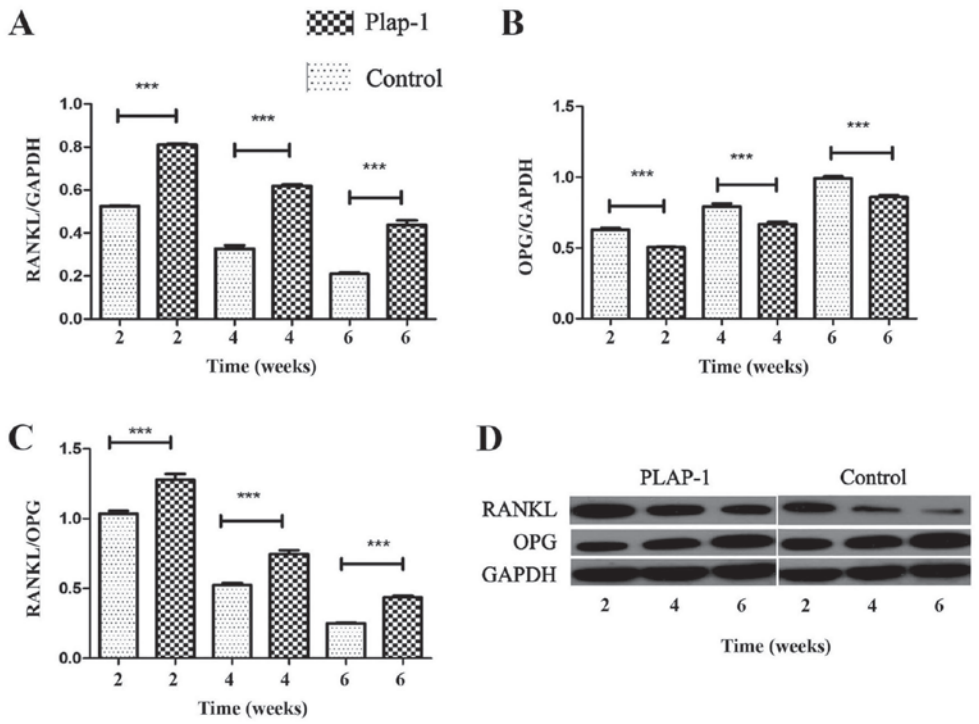

Figure 7. Expression of RANKL and OPG protein in rat periodontal bone defects at 2, 4 and 6 weeks as determined by western blotting. (A) RANKL protein expression was upregulated during rat periodontal bone defect repair, and higher expression was observed in the PLAP-1 group. This trend was reversed for OPG. (B) OPG was downregulated from 2 to 6 weeks, and higher expression was observed in the control group. (C) The PLAP-1 gene in bone marrow stromal cells led to an increase in the RANKL/OPG ratio. (D) Expression of RANKL and OPG protein in the PLAP-1 and control groups. PLAP-1, periodontal ligament-associated protein-1; RANKL, receptor activator of nuclear factor κB ligand; OPG, osteoprotegerin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (***P<0.01).

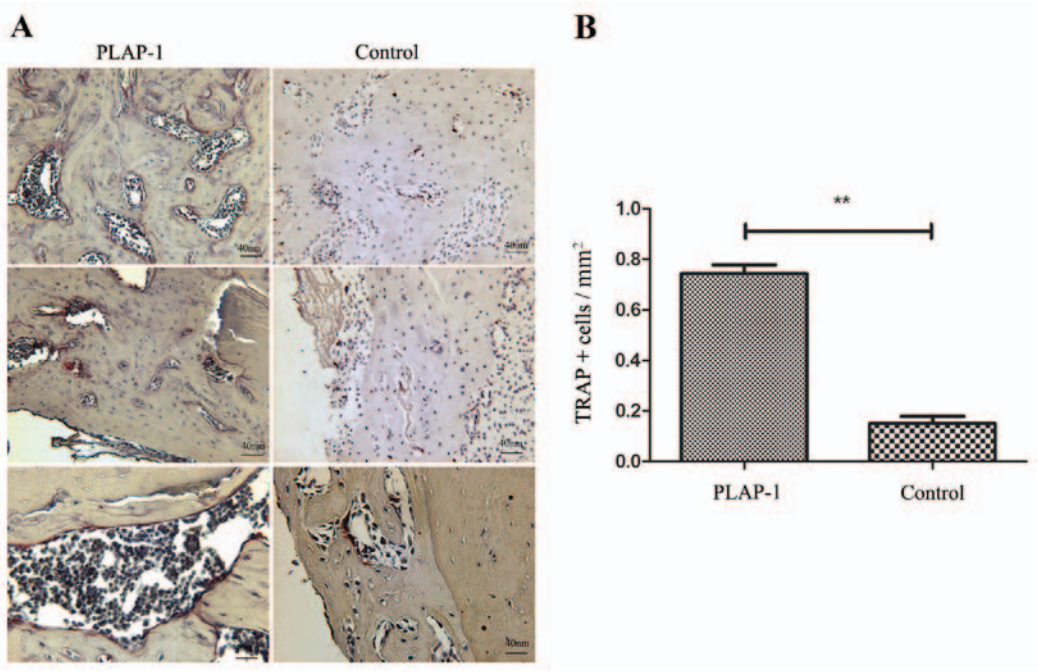

Figure 8. TRAP staining of the periodontal defects in the PLAP-1 and control groups at 6 weeks. (A) Numerous TRAP+ osteoclasts were observed adjacent to bone lacunae in the PLAP-1 group. However, few TRAP+ cells were detectable in the control group. (B) A higher number of TRAP+ cells were observed in the periodontal defects of the PLAP-1 compared with that in the control group (**P<0.01 vs. control). Scale bar, 40 nm. PLAP-1, periodontal ligament-associated protein-1; TRAP, tartrate-resistant acid phosphatase.

would therefore be useful for periodontal bone formation and regeneration. Hence, a better understanding of the cellular and molecular mechanisms behind the function of PLAP-1 is vital.

Osteoblast markers are well documented to primarily include ALP, BSP, Runx2, Osx and OC. By observing the levels of osteoblast markers, osteoblast differentiation from

rBMSCs can be speculated upon. The present RT-PCR and western blotting results showed that PLAP-1 reduced the expression levels of these markers at 4 weeks post-surgery compared with that in the vector and normal groups; the osteogenic differentiation of rBMSCs was restrained *in vivo*. The same effects in osteoblast differentiation have been reported (19). PLAP-1 regulates periodontal ligament cell cytodifferentiation and mineralization through BMP-2 activity. PLAP-1 inhibits the effect of BMP by binding to BMP receptor, which indicates that PLAP-1 forms part of the negative feedback mechanism of BMP-2 (33).

Osteoclast formation, activation and survival is regulated in normal bone modeling and remodeling by RANKL/RANK signaling. Osteoclast number and activity can increase if there is a change in the RANKL/OPG ratio (34). During the progression of rat periodontal bone defect repair, osteoclast precursors are attracted from the invading blood vessels close to newly formed bone trabeculae. Multinucleated osteoclasts are formed by the fusion of these precursors with each other, and the osteoclasts then resorb the majority of the newly formed bone, leaving only a limited number of trabeculae. The osteoblasts lay down new bone on certain surface regions of the surviving trabeculae where there had previously been osteoclastic resorption and a great deal of this new bone is then resorbed by osteoclasts in a remodeling process (35,36).

As aforementioned, there was no significant difference between the vector and control groups in the present study. So only the PLAP-1 and control groups were included in the following experiments. RANKL and OPG expression in bone defect tissues was detected at 2, 4 and 6 weeks during rat periodontal bone defect repair. Higher RANKL/OPG ratio expression was observed in the PLAP-1 group, that is, the PLAP-1 gene in the BMSCs led to an increase in the RANKL/OPG ratio, which was further confirmed by TRAP staining. A greater number of $TRAP^+$ cells was observed in the PLAP-1 group than in the control group even at the late stage of defect repair. An extensive bone remodeling process was observed in the PLAP-1 group. Overexpression of PLAP-1 promoted osteoclast activation dependent on the upregulated RANKL/OPG ratio.

Taken together, the present results showed that PLAP-1 suppressed the differentiation of rBMSCs into osteoblasts and promoted osteoclast activation in the rat periodontal bone defects model. PLAP-1 exhibited positive effects on bone remodeling by promoting osteoclastogenesis and reducing osteoblast differentiation, leading to an inhibited repair effect.

The molecular mechanism of PLAP-1 in osteoblast differentiation, osteoclastogenesis and bone remolding require further investigation to promote bone functional regeneration. It is of great importance to reveal the negative feedback regulation between PLAP-1 and positive growth factors in mineralized tissues under physiological and pathological conditions.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 81271138), the Open Foundation of Shandong Provincial Key Laboratory of Oral Biomedicine (grant no. SDKQ201403) and the Shandong Province Natural Science Foundation (grant no. ZR2015PH017).

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