

RESEARCH ARTICLE

Vitamin C induces periodontal ligament progenitor cell differentiation via activation of ERK pathway mediated by PELP1

Yan Yan^{1,3*}, Wenfeng Zeng^{2*}, Shujun Song⁴, Fayun Zhang², Wenxi He¹, Wei Liang²✉, Zhongying Niu^{1,3}✉

¹ Department of Endodontics, School of Stomatology, Fourth Military Medical University, Xi'an, 710032, China

² Protein and Peptide Pharmaceutical Laboratory, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

³ Treatment Center of Oral Diseases, the 306th Hospital of People's Liberation Army, Beijing 100101, China

⁴ Department of Pathology and Experimental Medicine, the 306th Hospital of People's Liberation Army, Beijing 100101, China

✉ Correspondence: nzhongying@gmail.com (Z. Niu), weix@sun5.ibp.ac.cn (W. Liang)

Received April 17, 2013 Accepted June 4, 2013

ABSTRACT

The differentiation of periodontal ligament (PDL) progenitor cells is important for maintaining the homeostasis of PDL tissue and alveolar bone. Vitamin C (VC), a water-soluble nutrient that cannot be biosynthesized by humans, is vital for mesenchymal stem cells differentiation and plays an important role in bone remodeling. Therefore, the objective of this study was to determine the function and mechanism of VC in PDL progenitor cells osteogenic differentiation at the molecular level. We demonstrated that VC could induce the osteogenic differentiation and maturation of PDL progenitor cell without other osteogenic agents. During the process, VC preferentially activated ERK1/2 but did not affect JNK or p38. Co-treatment with ERK inhibitor effectively decreased the Vitamin C-induced expression of Runx2. ERK inhibitor also abrogated Vitamin C-induced the minimized nodules formation. PELP1, a nuclear receptor co-regulator, was up-regulated under VC treatment. PELP1 knockdown inhibited ERK phosphorylation. The overexpression of PELP1 had a positive relationship with Runx2 expression. Taken together, we could make a conclude that VC induces the osteogenic differentiation of PDL progenitor cells via PELP1-ERK axis. Our finding implies that VC may have a potential in the regeneration medicine and application to periodontitis treatment.

KEYWORDS periodontal ligament progenitor cells, Vitamin C, PELP1, ERK signaling pathway, osteogenic differentiation

INTRODUCTION

Periodontitis is a major infectious disease characterized by the irreversible destruction of the periodontal ligament (PDL), gingiva and alveolar bone. It is one of the main causes of tooth mobility and loss, which affects 5%–20% of the world population (Eklund and Burt, 1994; Desvarieux et al., 2003; Petersen et al., 2005). The conventional therapeutic strategy for periodontitis is to clear the infections and restore the structure and function of the PDL tissue and alveolar bone. With rapid development in stem cell research, stem cell-based hard and soft tissue regeneration has garnered a great deal of attention in the field of dentistry (Seo et al., 2004; Sonoyama et al., 2006; Liu et al., 2008; Ding et al., 2010). PDL progenitor cells, a type of mesenchymal stem cell (MSC), can differentiate into cementoblasts, osteoblasts, adipocytes, and collagen-forming cells under defined culture conditions, exhibit great therapeutic potential for alveolar bone repair (Seo et al., 2004; Mao et al., 2006). Therefore, the regulatory mechanism of PDL progenitor cells differentiation has become an important research topic.

VC, a major physiological antioxidant, is vital for the biosynthesis and function of collagen and extracellular matrix and shows great potential in regeneration medicine (Van Robertson et al., 1959; Mitoma and Smith, 1960; Peterkofsky, 1991). It is an indispensable supplement for promoting the proliferation and differentiation of a variety of mesenchymal stem cells by enhancing collagen biosynthesis, ALP activity, the expression of BSP and other extracellular matrix production (Franceschi et al., 1994; Shiga et al., 2003; Wang et al., 2006; Cao et al., 2012; Mekala et al., 2013). Evidences also indicate that VC can induce iPSCs (induced pluripotent stem cells) differentia-

*These authors contributed equally to the work.

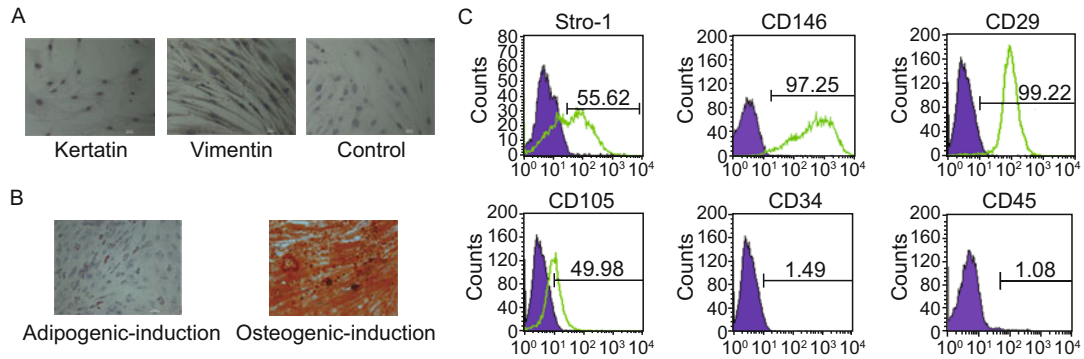


Figure 1. Characterization of periodontal ligament progenitor cells. The expression of keratin and vimentin in PDL progenitor cells were determined by immunocytochemistry (A). Oil Red O staining of PDL progenitor cells after 14 d of adipogenic differentiation. Alizarin Red S staining of PDL progenitor cells after 14 d of osteogenic differentiation (B). Flow cytometry analysis of mesenchymal surface markers on PDL progenitor cells. All microscopic photographs are shown at 200 \times magnification (C). All data are representative of 3 independent experiments.

tion and malignant cells apoptosis via ERK/MAPK pathway (Park et al., 2005; Cao et al., 2012). ERK/MAPK pathway lies in the central position in mesenchymal stem cells differentiation and skeletal development (Lai et al., 2001; Ge et al., 2007). ERK/MAPK pathway is also involved in the induction and activity of Runx2, a decisive transcription factor in mesenchymal stem cells osteogenic differentiation both *in vitro* and *in vivo* (Xiao et al., 2000; Franceschi et al., 2007; Ge et al., 2007). Moreover, other factors, such as mechanical stress, FGF-2 and IGF-1, also can mediate the differentiation of osteoblasts in a ERK/MAPK-dependent manner (Xiao et al., 2002; Kanno et al., 2007; Yu et al., 2012).

PELP1 (proline-, glutamic acid- and leucine-rich protein) is initially identified as a co-regulator of estrogen receptors (Vadlamudi et al., 2001) and can facilitate the non-genomic functions of ER in breast cancers (Vadlamudi et al., 2005). Working as the co-activator of RXR α and PPAR γ , PELP1 exhibits pro-differentiation effect (Vadlamudi et al., 2001; Haas et al., 2005; Singh et al., 2006). It has been reported that the cytoplasmic localization of PELP1 in MCF-7 can also augment ERK activation (Rayala et al., 2006). Thus we hypothesized that PELP1 may be involved in VC-induced ERK/MAPK activity during the differentiation of PDL progenitor cells. In the present study, we show that VC induces the osteogenic differentiation and maturation of PDL progenitor cells via ERK pathway. Further studies suggest that PELP1 is involved in this process. Our study may provide a possible application of VC in PDL tissue regeneration and treating periodontal diseases.

RESULTS

Characterization of PDL progenitor Cells

PDL progenitor cells were isolated from 5 individual patients according to the method of Seo et al. (2004). The immunocytochemistry results showed that the PDL progenitor cells were vimentin positive and keratin negative (Fig. 1A), suggesting a mesenchymal cell origin. PDL progenitor cells are a unique

mesenchymal stem cell population; therefore, we examined the stem cell markers on the PDL progenitor cells. The flow cytometry results demonstrated that the PDL progenitor cells were positive for Stro-1, CD146, CD29, and CD105 and negative for CD34 and CD45 (Fig. 1C), indicating the potential for differentiation into multiple cell types. The PDL progenitor cells differentiated into adipocytes and osteoblasts (Fig. 1B) as determined by Oil Red O and Alizarin Red S staining, respectively.

VC induced the osteogenic differentiation of PDL progenitor cells

It has been reported that VC can promote proliferation and differentiation of mesenchymal stem cells. Then, we firstly measured the cytotoxicity of VC on PDL progenitor cells with concentrations from 0 to 100 μ mol/L for 72 h and 96 h. As shown in Fig. 2A, all the concentrations of VC treatment exhibited proliferative effect rather than cytotoxicity. Next we investigated whether VC could induce osteogenic differentiation of PDL progenitor cells. After 14 d of osteo-induction by traditional osteo-induction medium or DMEM supplemented with different concentrations of VC, we observed mineralization nodules formation induced by VC in a dose dependent manner (Fig. 2C and 2D). Runx2 is the most important transcription factor for the osteogenic differentiation and controls the expression of bone forming genes in osteoblasts (Komori et al., 1997; Xiao et al., 2000; Gordon et al., 2007). So we detected whether VC could induce the expression of Runx2. Under 48 h of VC treatment, the expression of Runx2 showed an increase in a dose dependent manner too (Fig. 2B). These results suggest that VC is able to induce osteogenic differentiation of PDL progenitor cells by triggering the expression of Runx2.

The role of ERK pathway in VC-induced osteogenic differentiation of PDL progenitor cells

During the osteogenic differentiation of osteoblasts, Runx2 is

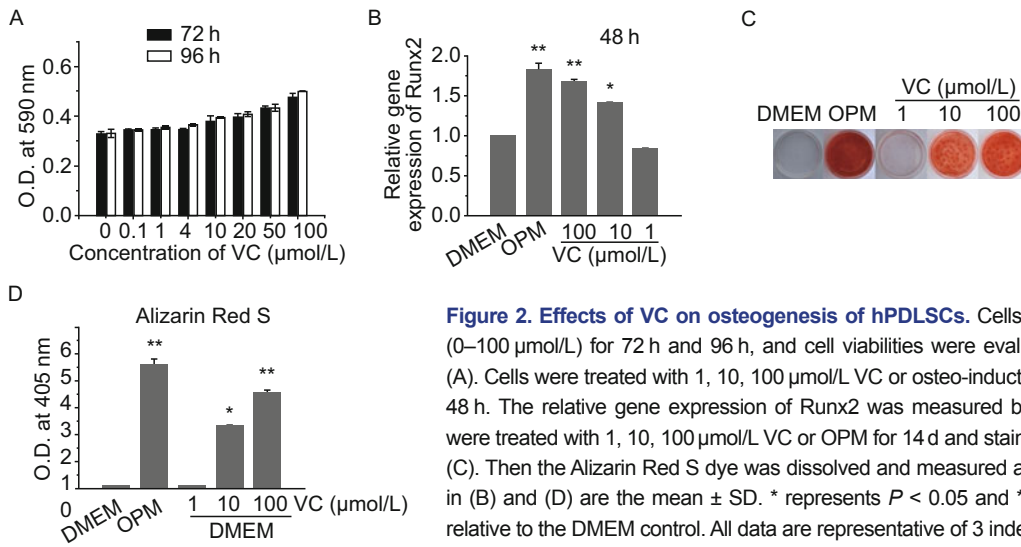


Figure 2. Effects of VC on osteogenesis of hPDLSCs. Cells were treated with VC (0–100 μmol/L) for 72 h and 96 h, and cell viabilities were evaluated by MTT assays (A). Cells were treated with 1, 10, 100 μmol/L VC or osteo-induction medium (OPM) for 48 h. The relative gene expression of Runx2 was measured by qRT-PCR (B). Cells were treated with 1, 10, 100 μmol/L VC or OPM for 14 d and stained with Alizarin Red S (C). Then the Alizarin Red S dye was dissolved and measured at 405 nm (D). The data in (B) and (D) are the mean ± SD. * represents $P < 0.05$ and ** represents $P < 0.01$ relative to the DMEM control. All data are representative of 3 independent experiments.

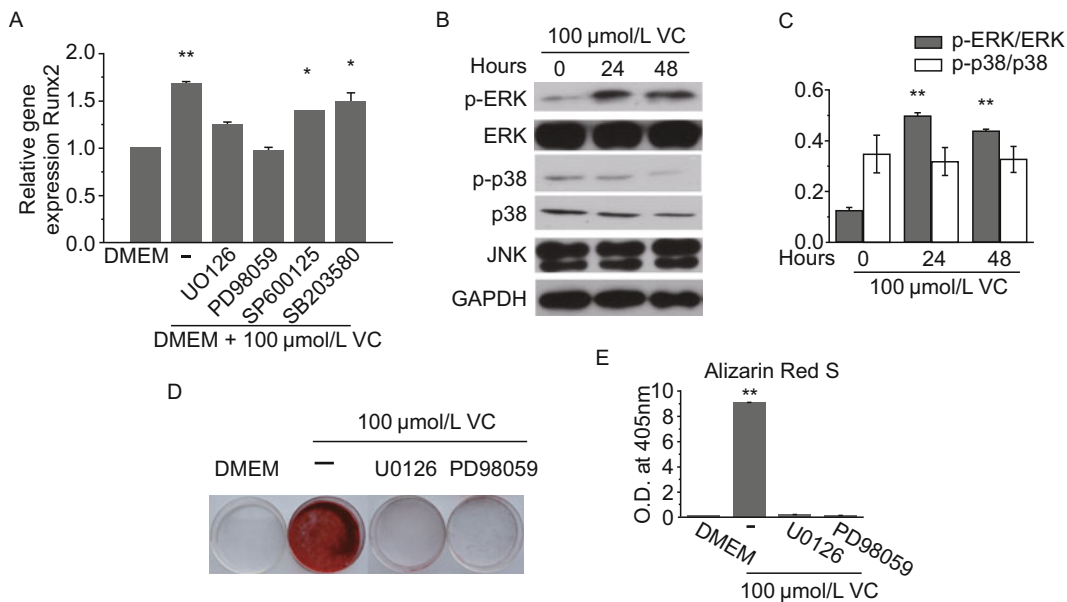


Figure 3. VC induces ERK phosphorylation and the activity of ERK is required for VC-induced osteogenesis of hPDLSCs. hPDLSCs were cultured with 100 μmol/L VC with or without U0126, PD98059, SP600125 and SB203580 for 48 h. The relative gene expression of Runx2 was detected by qRT-PCR (A). Cells were treated with 100 μmol/L VC for 24 h and 48 h. The phosphorylated ERK1/2 (p-ERK) and phosphorylated p38 (p-p38), ERK1/2, p38, JNK, GAPDH were detected by western blot (B) and relative expression of p-ERK1/2 and p-p38 was quantificated by Image J (C). Cells were treated with 100 μmol/L VC with or without U0126, PD98059 for 14 d. The mineralized nodules formation was detected by Alizarin Red S staining (D) and quantification was measured at 405 nm (E). The data in (A), (C), and (E) are the mean ± SD. * represents $P < 0.05$ and ** represents $P < 0.01$ relative to the DMEM control. All data are representative of 3 independent experiments.

regulated by various signaling pathways. Among these pathways, the MAPK pathway is well-studied (Xiao et al., 2000). Therefore, we explored whether MAPK signaling pathway was involved in VC-induced osteogenic differentiation. The expression of Runx2 was determined under the treatment of 100 μmol/L VC with or without inhibitors for MAPK signaling pathway for 48 h. As shown in Fig. 3A, VC alone could en-

hance the expression of Runx2 compared with DMEM control, and the ERK inhibitors (U0126 and PD98059) significantly inhibited VC-induced Runx2 expression whereas the p38 and JNK inhibitors (SB203580 and SP600125) showed little impacts. Then we tested the translational change of MAPK signaling pathway under 100 μmol/L VC treatment for 24 h and 48 h. We found that the phosphorylation of ERK increased at both

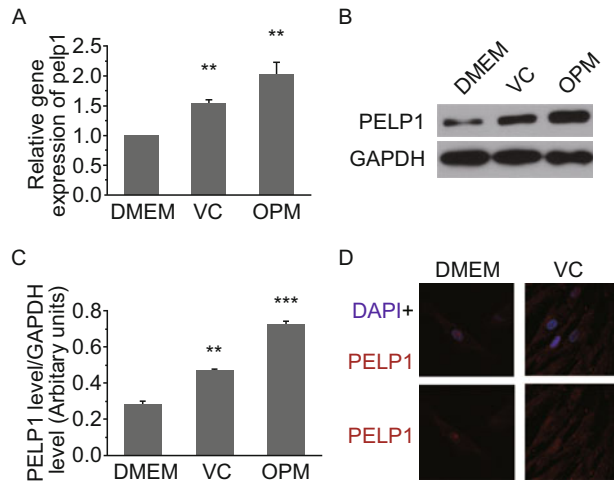


Figure 4. VC up-regulates the expression of PELP1 during VC-induced differentiation of hPDLSCs. hPDLSCs were treated with 100 $\mu\text{mol/L}$ VC for 48 h, and the relative gene expression of PELP1 was detected by qRT-PCR (A). The protein level of PELP1 was detected by western blot (B) and the relative expression of PELP1 was quantitated by Image J (C). The cytoplasmic and nucleic distributions of PELP1 were observed by confocal microscopy with or without 100 $\mu\text{mol/L}$ VC treatment for 48 h (D). The data in (A), and (C) are the mean \pm SD. ** represents $P < 0.01$ and *** represents $P < 0.005$ relative to the DMEM control. All data are representative of 3 independent experiments.

24 h and 48 h of VC treatment, yet phosphorylated p38 showed no change and phosphorylated JNK was not detected (Fig. 3B and 3C). Next we treated PDL progenitor cells under 100 $\mu\text{mol/L}$ VC with or without U0126 and PD98059 for 14 d and observed that ERK inhibition could significantly retard the VC-induced mineralization (Fig. 3D and 3E). These results indicate that ERK activation is necessary for VC-induced Runx2 expression and mineralization.

PELP1 was involved in VC-induced osteogenesis

Although evidences indicate that ERK activity is involved in VC-promoted cardiac progenitor cells proliferation and VC-induced acute myeloid leukemia cells apoptosis (Park et al., 2005; Cao et al., 2012), how VC activates ERK remains elusive. In the present study, we found that VC could up-regulate the expression of mRNA and protein levels of PELP1 (Fig. 4A–C). Other reports indicate that the cytoplasmic localization of PELP1 in MCF-7 breast tumor cells can augment ERK activation (Rayala et al., 2006). Thus we hypothesized that VC might employ the same mechanism to activate ERK during PDL progenitor cells differentiation. Then we investigated the localization of PELP1 under VC treatment and observed the expression of PELP1 increased predominantly in cytoplasm rather than in nucleus (Fig. 4D). Next we examined whether PELP1 was involved in VC-induced ERK activation. Our results showed that knockdown of PELP1 could decrease

VC-induced ERK activation and the expression of Runx2 (Fig. 5A, 5B and 5E). Overexpression of PELP1 could activate ERK and up-regulate the expression of Runx2 absent of VC (Fig. 5C–E). However, both treatments did not alter the phosphorylation of p38 nor induced the phosphorylation of JNK (Fig. 5E), which was consistent with our previous discovery (Fig. 3B). We can conclude that PELP1 is involved in VC-induced ERK activity.

DISCUSSION

Isolated from PDL tissue, PDL progenitor cells manifest great potential in regeneration medicine (Seo et al., 2004). The allogeneic PDL progenitor cells with low immunogenicity and marked immunosuppression and their autologous counterparts absent of those concerns can be both applied to regeneration therapy of inflammatory damages in associated tissues (Liu et al., 2008; Ding et al., 2010). However, so far there exist no efficient, convenient and safe methods to enhance the proliferation and differentiation of PDL progenitor cells. The present study demonstrates that VC has the potential to induce the osteogenic differentiation of PDLSCs via PELP1-ERK axis which has not been reported before.

VC, which can promote the speed and efficiency of iPSC generation, has become a hot topic in the regard of cell differentiation (Esteban et al., 2010). VC also can promote the differentiation of bone marrow-derived MSCs and MC3T3-E1 cells in a dose-dependent way (Shiga et al., 2003; Choi et al., 2008). Moreover, VC is essential for osteoblasts proliferation and differentiation via inducing ALP activity, up-regulating the expression of type X/I collagen, OCN, fibronectin and ALP, increasing the calcium deposition and the rate of procollagen secretion (Leboy et al., 1989; Franceschi et al., 1994; Shiga et al., 2003). VC modified functionalized biomaterials is able to control human MSCs proliferation, cellular aging and differentiation (Wang et al., 2006). In the present study, VC can induce the differentiation and maturation of PDL progenitor cells in a dose-dependent manner.

The PDL progenitor cells used in our study have been identified as mesenchymal stem cells. The process of mesenchymal stem cells differentiation is controlled by a serial of signal pathways. ERK/MAPK is one of the best-characterized pathways for the regulation of osteogenesis (Xiao et al., 2000; Yamaguchi et al., 2000). ERK activity is recognized as the decision maker during the process of embryonic stem cells differentiation (Kunath et al., 2007). And by activating ERK, VC enhances the cardiac differentiation of iPSCs and induces the apoptosis of acute myeloid leukemia cells (Park et al., 2005; Cao et al., 2012). So, whether ERK was involved in VC-induced osteogenic differentiation of PDL progenitor cells became our concern.

Runx2 is the most important transcription factor involved in the commitment of mesenchymal stem cells undergoing osteogenic differentiation, and Runx2 knockout mice lack osteoblasts and bone formation (Ducy et al., 1997; Komori et al.,

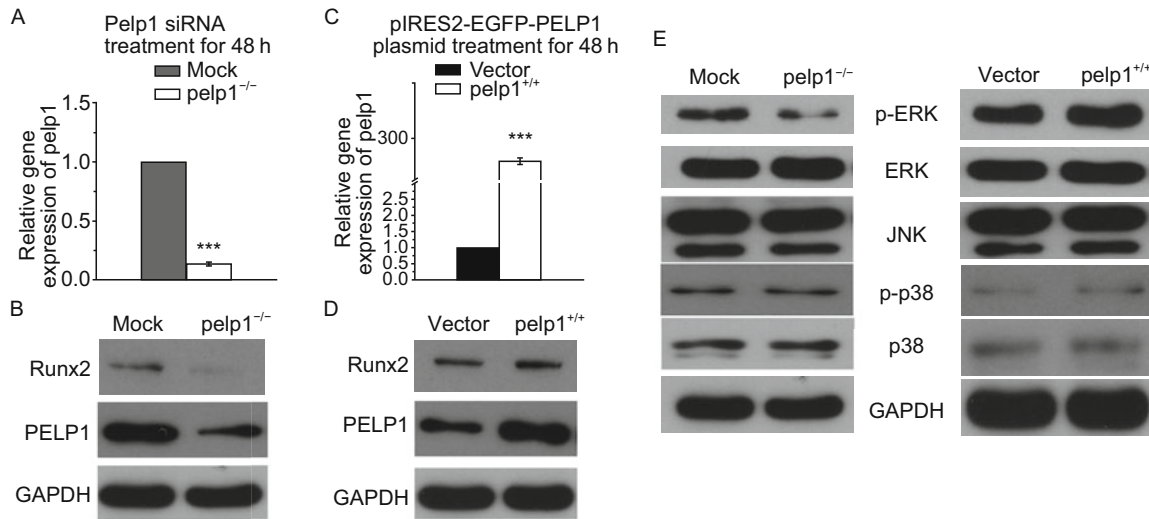


Figure 5. PELP1 is involved in VC-induced ERK activity. hPDLSCs were treated with 100 $\mu\text{mol/L}$ VC after PELP1 siRNA knockdown for 48 h. The expression of PELP1 at gene (A) and PELP1, Runx2 protein levels (B) were detected by qRT-PCR and western blot. hPDLSCs were treated with DMEM medium after PELP1 overexpression for 48 h. The expression of PELP1 at gene (C) and PELP1, Runx2 protein levels (D) were detected by qRT-PCR and western blot. (E) The phosphorylated ERK1/2 (p-ERK) and p38 (p-p38), ERK1/2, p38, JNK, GAPDH were detected by western blot and the cells were treated as in (A) and (C), respectively. The data in (A) and (C), are the mean \pm SD. *** represents $P < 0.005$ relative to the mock or vector control. All data are representative of 3 independent experiments.

1997; Yamaguchi et al., 2000; Xiao et al., 2005; Komori, 2008). The activity of ERK is essential for up-regulating the expression and transcriptional activity of Runx2 (Xiao et al., 2000; Lai et al., 2001). Our data are consistent with the observation that ERK was activated during VC-induced osteogenic differentiation and ERK blockade by specific inhibitor can inhibit VC-induced the expression of Runx2 and mineralized nodule formation. This finding suggests that ERK activation may be a possible molecular mechanism for the inductive effect of VC in the osteogenic differentiation of PDL progenitor cells.

Given ERK pathway is involved in the process that VC promotes PDL progenitor cells differentiation, how VC activates ERK pathway remains unclear. In our study, under VC treatment PELP1 was upregulated and the expression of PELP1 increased predominantly in cytoplasm. Initially identified as a co-regulator of estrogen receptors, PELP1 facilitates the non-genomic functions of ER in breast cancers through interacting with ERK/MAPK (Vadlamudi et al., 2001; Vadlamudi et al., 2005). And the cytoplasmic localization of PELP1 could augment ERK activation in MCF-7 breast tumor cells (Rayala et al., 2006). So these observations indicated that PELP1 might be involved in the process of VC-induced ERK activation. We examined the relationship between the expression of PELP1 and ERK activity using genetic methods. ERK activity and the expression of Runx2 decreased when PELP1 was knocked down under VC treatment and overexpression of PELP1 enhanced ERK activity and the expression of Runx2, indicating that PELP1 mediated ERK activation during VC-induced osteogenic differentiation of PDL progenitor cells.

In conclusion, VC promotes PDL progenitor cell differentia-

tion by PELP1-mediated ERK activation. This finding enriches our knowledge on the modulation mechanism of PDL progenitor cells differentiation and suggests that VC may possess the therapeutic potential in treating periodontitis.

MATERIALS AND METHODS

Isolation and culture of periodontal ligament progenitor cells

The Ethical Committee of the Stomatological Hospital of the Fourth Military Medical University approved the procedure for the isolation of human periodontal ligament progenitor cells in this study. Periodontal ligament (PDL) progenitor cells were obtained from extracted non-carries premolars or third molars of 5 patients aged 18–40 years old. The detailed procedure was based on the method of Seo et al. (2004). PDL progenitor cells from each donor were cultured and used at passage 3–4. The following experiments were repeated using the PDL progenitor cells for each donor, and the results shown were representative of the experiments.

Immunocytochemistry

PDL progenitor cells were subcultured onto 10×12 mm slides at 1×10^5 cells/well for 24 h. After the cells reached 60% confluence, they were fixed with ice-cold acetone for 30 min and incubated with 3% H_2O_2 . The cells were permeabilized with 1% Triton X-100, incubated with the monoclonal antibodies anti-vimentin (Santa Cruz, CA, USA), anti-keratin (Santa Cruz, CA, USA) or normal goat IgG as the negative control overnight at 4°C . The slides were then washed with PBS and incubated with secondary antibody for 1 h at room temperature. Next the slides were stained with 50 μL chromogen solution (DAB, Boster). Then slides were counterstained with hematoxylin for 30 s and observed under a light microscope (Leica).

Table 1. Primers used in qRT-PCR experiments

	Forward primer (5'→3')	Reverse primer (5'→3')
PELP1	TCAGTAATGCACGTCTCAGTTC	CCGAAGCCAAGACACACAG
Runx2	TGGTTACTGTCATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA
18S	CAGCCACCCGAGATTGAGCA	TAGTAGCGACGGGCGGTGTG

Characterization of periodontal ligament progenitor cells

PDL progenitor cells (60×10^4) were plated in 10-cm culture dishes and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) until they reached 70% confluence. The cells were then washed with PBS, digested with 0.25% trypsin and harvested by centrifugation at 450 g for 5 min. For cell surface staining, the PDL progenitor cells were stained with fluorescently labeled PE-conjugated antibodies in staining buffer (2% FBS and 0.09% sodium azide in PBS) for 30 min at 4°C. The antibodies used for staining were PE-conjugated anti-Stro-1, PE-conjugated anti-CD146, PE-conjugated anti-CD29, PE-conjugated anti-CD105, PE-conjugated anti-CD34 and PE-conjugated anti-CD45. All antibodies were purchased from eBioscience except anti-CD146, which was a kind gift from Prof. Yan Xiyun (Institute of Biophysics, CAS). The cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA).

Adipogenic, osteogenic differentiation and staining

PDL progenitor cells were seeded at a density of 3×10^4 cells/well in a 6-cm culture dish with DMEM supplemented with 10% FBS. Adipogenic differentiation was induced with 0.5 mmol/L isobutylmethylxanthine, 0.5 μmol/L dexamethasone, 1 μg/mL insulin and 60 μmol/L indomethacin. Osteogenic differentiation of the PDL progenitor cells were induced with osteogenic induction medium (DMEM containing 10% FBS, 10 mmol/L β-glycerophosphate, 10 nmol/L dexamethasone, and 50 μmol/L VC). The medium was changed every 3 d. On day 14, the cells were fixed with 4% paraformaldehyde for 30 min and then stained with Oil Red O or 2% Alizarin Red S for 5 min at room temperature. The cells were then counterstained with hematoxylin for 30 s and observed under a light microscope (Leica).

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

PDLSCs were seeded on 96-well plates at a density of 8×10^3 cells/well and treated with VC at concentrations of 0, 0.1, 1, 4, 10, 20, 50 and 100 μmol/L, respectively. At 72 h or 96 h after VC treatment, the proliferation/survival of the cells was evaluated by MTT assay.

Cell culture and treatments

For osteogenic differentiation, 5×10^4 cells were seeded in 6-cm culture dishes and treated with either osteo-induction medium or 100 μmol/L VC for 14 d. The mediums were changed every 3 d.

For RNA and protein extraction, cells were planted in 6-well culture plates in proper density and treated with 100 μmol/L VC with or without inhibitors for indicated times.

PELP1 transfection and siRNA knockdown of PELP1

Full length PELP1 gene was cloned from MCF-7 cells by RT-PCR,

and then was inserted into the purified pIRES2-EGFP plasmid. The plasmids and siRNAs were transfected into the PDL progenitor cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The mediums were replaced with basic medium with or without 100 μmol/L VC 6 h after transfection the siRNAs or plasmids. After an additional 48 h, RNA from the treated cells was collected and used for qRT-PCR analysis. The siRNAs targeting PELP1 and the control siRNA were purchased from Invitrogen.

RNA extraction and qRT-PCR assay

Cells were seeded in 6-well tissue culture plates and harvested with TRIzol reagent (Invitrogen). The total RNA was immediately extracted, and reverse transcription PCR was performed with the Transgen TransScript One-Step cDNA Removal and cDNA Synthesis SuperMix according to the manufacturer's instructions. Real-time quantitative PCR was performed using the Transgen TransSmart™ Green qPCR SuperMix UDG. The PCR primers used for the amplification were as follows (Table 1). The relative mRNA expression levels for PELP1 and Runx2 were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The 18S gene was used as the internal control.

Western blot

PDL progenitor cells were lysed in RIPA lysis buffer containing protease inhibitors (Sigma) and phosphatase inhibitors (Sigma). The cellular debris was removed by centrifugation at 12,000 r/min for 15 min at 4°C. The proteins (20 μg) were heated with 4× SDS sample and separated by 12% SDS-PAGE gel electrophoresis and then transferred to 0.45 μm PVDF membranes (Millipore, Bedford, CA). After blocking, the membranes were incubated with the following primary antibodies: PELP1, 1:2500 (Bethyl Laboratories Inc.); Runx2, 1:200 (Abcam); ERK1/2, 1:1000 (CST); p-ERK1/2, 1:1500 (CST); JNK, 1:1000 (CST); p-JNK, 1:1000 (CST); P38, 1:1000 (CST); p-P38, 1:1000 (CST); and GAPDH, 1:1000 (CST) at 4°C overnight. On the second day, the membranes were washed with TBST and incubated with secondary antibodies (1:1500, CST) before visualization.

Confocal microscopy

Cells were treated with DMEM with or without 100 μmol/L VC for 48 h. Then culture medium was removed and cells were washed with ice-cold PBS. Then cells were fixed with 4% paraformaldehyde for 15 min and then blocked with normal goat serum for 30 min at room temperature. The cells were incubated with monoclonal anti-PELP1 antibody at a 1:100 dilution at 4°C overnight. Then the primary antibody was removed and the cells were incubated with the Alexafluor 568-labeled goat-anti-rabbit antibody for 1 h at room temperature in the dark. Sections were washed three times with ice-cold PBS, and counterstained the nucleus with DAPI for 10 min. Then samples were analyzed and

photographed in PBS FV1000 confocal microscopy (Olympus).

Statistical analysis

All experiments were performed with triplicate samples and repeated at least three times. The results are represented as the mean \pm SD. A two-tailed Student's *t*-test was used to compare the values between groups. A *P*-value of less than 0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

We thank Prof. Xiyun Yan (Protein & Peptide Pharmaceutical Laboratory, Institute of Biophysics) for kindly providing the CD146 antibody. We also thank Junfeng Hao and Zhenwei Yang from The Core Facilities-Experimental Animal Pathological Laboratory and the Lab of Cell Biology, Institute of Biophysics, respectively, for help with the ICC staining and qRT-PCR assays.

This work is supported by grants: the National Basic Research Program (973 Program) (No. 2011CB707705), National Natural Science Foundation of China (Grant No. 11202229).

ABBREVIATIONS

ALP, alkaline phosphatase; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERK, extracellular regulated protein kinases; iPSC, induced pluripotent stem cell; JNK, c-jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MSC, mesenchymal stem cell; NR, nuclear receptor; OCN, osteocalcin; PDLC, periodontal ligament cell; PELP1, proline-, glutamic- and leucine-rich protein; PPAR γ , peroxisome proliferator-activated receptor γ ; Runx2, runt-related transcription factor 2; RXR α , retinoid X receptor α ; VC, Vitamin C

COMPLIANCE WITH ETHICS GUIDELINES

Yan Yan, Wenfeng Zeng, Shujun Song, Fayun Zhang, Wenxi He, Wei Liang and Zhongying Niu declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000(5). Informed consent was obtained from all patients for being included in the study.

REFERENCES

- Cao, N., Liu, Z., Chen, Z., Wang, J., Chen, T., Zhao, X., Ma, Y., Qin, L., Kang, J., Wei, B., et al. (2012). Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells through promoting the proliferation of cardiac progenitor cells. *Cell Res* 22, 219–236.
- Choi, K.M., Seo, Y.K., Yoon, H.H., Song, K.Y., Kwon, S.Y., Lee, H.S., and Park, J.K. (2008). Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation. *J Biosci Bioeng* 105, 586–594.
- Desvarieux, M., Demmer, R.T., Rundek, T., Boden-Albala, B., Jacobs, D.R., Jr., Papapanou, P.N., and Sacco, R.L. (2003). Relationship between periodontal disease, tooth loss, and carotid artery plaque: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Stroke* 34, 2120–2125.
- Ding, G., Liu, Y., Wang, W., Wei, F., Liu, D., Fan, Z., An, Y., Zhang, C., and Wang, S. (2010). Allogeneic periodontal ligament stem cell therapy for periodontitis in swine. *Stem Cells* 28, 1829–1838.
- Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L., and Karsenty, G. (1997). *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell* 89, 747–754.
- Eklund, S.A., and Burt, B.A. (1994). Risk factors for total tooth loss in the United States; longitudinal analysis of national data. *J Public Health Dent* 54, 5–14.
- Esteban, M.A., Wang, T., Qin, B., Yang, J., Qin, D., Cai, J., Li, W., Weng, Z., Chen, J., Ni, S., et al. (2010). Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell Stem Cell* 6, 71–79.
- Franceschi, R.T., Ge, C., Xiao, G., Roca, H., and Jiang, D. (2007). Transcriptional regulation of osteoblasts. *Ann N Y Acad Sci* 1116, 196–207.
- Franceschi, R.T., Iyer, B.S., and Cui, Y. (1994). Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res* 9, 843–854.
- Ge, C., Xiao, G., Jiang, D., and Franceschi, R.T. (2007). Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol* 176, 709–718.
- Gordon, J.A., Tye, C.E., Sampaio, A.V., Underhill, T.M., Hunter, G.K., and Goldberg, H.A. (2007). Bone sialoprotein expression enhances osteoblast differentiation and matrix mineralization in vitro. *Bone* 41, 462–473.
- Haas, D., White, S.N., Lutz, L.B., Rasar, M., and Hammes, S.R. (2005). The modulator of nongenomic actions of the estrogen receptor (MNAR) regulates transcription-independent androgen receptor-mediated signaling: evidence that MNAR participates in G protein-regulated meiosis in *Xenopus laevis* oocytes. *Mol Endocrinol* 19, 2035–2046.
- Kanno, T., Takahashi, T., Tsujisawa, T., Ariyoshi, W., and Nishihara, T. (2007). Mechanical stress-mediated Runx2 activation is dependent on Ras/ERK1/2 MAPK signaling in osteoblasts. *J Cell Biochem* 101, 1266–1277.
- Komori, T. (2008). Regulation of bone development and maintenance by Runx2. *Front Biosci* 13, 898–903.
- Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., et al. (1997). Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89, 755–764.
- Kunath, T., Saba-El-Leil, M.K., Almousailleakh, M., Wray, J., Meloche, S., and Smith, A. (2007). FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* 134, 2895–2902.
- Lai, C.F., Chaudhary, L., Fausto, A., Halstead, L.R., Ory, D.S., Avioli, L.V., and Cheng, S.L. (2001). Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. *J Biol Chem* 276, 14443–14450.
- Leboy, P.S., Vaia, L., Uschmann, B., Golub, E., Adams, S.L., and Pacifici, M. (1989). Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. *J Biol Chem* 264, 17281–17286.
- Liu, Y., Zheng, Y., Ding, G., Fang, D., Zhang, C., Bartold, P.M., Gronthos, S., Shi, S., and Wang, S. (2008). Periodontal ligament stem

- cell-mediated treatment for periodontitis in miniature swine. *Stem Cells* 26, 1065–1073.
- Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408.
- Mao, J.J., Giannobile, W.V., Helms, J.A., Hollister, S.J., Krebsbach, P.H., Longaker, M.T., and Shi, S. (2006). Craniofacial tissue engineering by stem cells. *J Dent Res* 85, 966–979.
- Mekala, N.K., Baadhe, R.R., Rao Parcha, S., and Prameela Devi, Y. (2013). Enhanced proliferation and osteogenic differentiation of human umbilical cord blood stem cells by L-ascorbic Acid, in vitro. *Curr Stem Cell Res Ther* 8, 156–162.
- Mitoma, C., and Smith, T.E. (1960). Studies on the role of ascorbic acid in collagen synthesis. *J Biol Chem* 235, 426–428.
- Park, S., Park, C.H., Hahm, E.R., Kim, K., Kimler, B.F., Lee, S.J., Park, H.K., Lee, S.H., Kim, W.S., Jung, C.W., et al. (2005). Activation of Raf1 and the ERK pathway in response to l-ascorbic acid in acute myeloid leukemia cells. *Cell Signal* 17, 111–119.
- Peterkofsky, B. (1991). Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *Am J Clin Nutr* 54, 1135S–1140S.
- Petersen, P.E., Bourgeois, D., Ogawa, H., Estupinan-Day, S., and Ndiaye, C. (2005). The global burden of oral diseases and risks to oral health. *Bull World Health Organ* 83, 661–669.
- Rayala, S.K., Hollander, P., Balasenthil, S., Moll, P.R., Bean, A.J., Vadlamudi, R.K., Wang, R.A., and Kumar, R. (2006). Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) interacts with PELP1 and activates MAPK. *J Biol Chem* 281, 4395–4403.
- Seo, B.M., Miura, M., Gronthos, S., Bartold, P.M., Batouli, S., Brahimi, J., Young, M., Robey, P.G., Wang, C.Y., and Shi, S. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 364, 149–155.
- Shiga, M., Kapila, Y.L., Zhang, Q., Hayami, T., and Kapila, S. (2003). Ascorbic acid induces collagenase-1 in human periodontal ligament cells but not in MC3T3-E1 osteoblast-like cells: potential association between collagenase expression and changes in alkaline phosphatase phenotype. *J Bone Miner Res* 18, 67–77.
- Singh, R.R., Gururaj, A.E., Vadlamudi, R.K., and Kumar, R. (2006). 9-cis-retinoic acid up-regulates expression of transcriptional coregulator PELP1, a novel coactivator of the retinoid X receptor alpha pathway. *J Biol Chem* 281, 15394–15404.
- Sonoyama, W., Liu, Y., Fang, D., Yamaza, T., Seo, B.M., Zhang, C., Liu, H., Gronthos, S., Wang, C.Y., Wang, S., et al. (2006). Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS One* 1, e79.
- Vadlamudi, R.K., Manavathi, B., Balasenthil, S., Nair, S.S., Yang, Z., Sahin, A.A., and Kumar, R. (2005). Functional implications of altered subcellular localization of PELP1 in breast cancer cells. *Cancer Res* 65, 7724–7732.
- Vadlamudi, R.K., Wang, R.A., Mazumdar, A., Kim, Y., Shin, J., Sahin, A., and Kumar, R. (2001). Molecular cloning and characterization of PELP1, a novel human coregulator of estrogen receptor alpha. *J Biol Chem* 276, 38272–38279.
- Van Robertson, W.B., Hiwett, J., and Herman, C. (1959). The relation of ascorbic acid to the conversion of proline to hydroxyproline in the synthesis of collagen in the carrageenan granuloma. *J Biol Chem* 234, 105–108.
- Wang, Y., Singh, A., Xu, P., Pindrus, M.A., Blasioli, D.J., and Kaplan, D.L. (2006). Expansion and osteogenic differentiation of bone marrow-derived mesenchymal stem cells on a vitamin C functionalized polymer. *Biomaterials* 27, 3265–3273.
- Xiao, G., Jiang, D., Ge, C., Zhao, Z., Lai, Y., Boules, H., Phimpilai, M., Yang, X., Karsenty, G., and Franceschi, R.T. (2005). Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. *J Biol Chem* 280, 30689–30696.
- Xiao, G., Jiang, D., Gopalakrishnan, R., and Franceschi, R.T. (2002). Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. *J Biol Chem* 277, 36181–36187.
- Xiao, G., Jiang, D., Thomas, P., Benson, M.D., Guan, K., Karsenty, G., and Franceschi, R.T. (2000). MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem* 275, 4453–4459.
- Yamaguchi, A., Komori, T., and Suda, T. (2000). Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr Rev* 21, 393–411.
- Yu, Y., Mu, J., Fan, Z., Lei, G., Yan, M., Wang, S., Tang, C., Wang, Z., Yu, J., and Zhang, G. (2012). Insulin-like growth factor 1 enhances the proliferation and osteogenic differentiation of human periodontal ligament stem cells via ERK and JNK MAPK pathways. *Histochem Cell Biol* 137, 513–525.