

Effect of FDC-SP on the phenotype expression of cultured periodontal ligament cells

Na Wei^{1,2}, Haiyang Yu¹, Shulin Yang³, Xingmei Yang¹, Quan Yuan¹, Yi Man², Ping Gong²

¹State Key Laboratory of Oral Disease, Sichuan University, Chengdu, China

²Department of Dental Implants, West China College of Stomatology, Sichuan University, Chengdu, China

³The Seventh People's Hospital, Chengdu, China

Submitted: 15 May 2010

Accepted: 31 August 2010

Arch Med Sci 2011; 7, 2: 235-241

DOI: 10.5114/aoms.2011.22073

Copyright © 2011 Termedia & Banach

Corresponding author:

Prof. Ping Gong

No. 14, Third Section Renmin
Nan Road Chengdu, Sichuan
610041, China

Phone: +86 28 85 503 579

Fax: +86 28 85 582 167

E-mail:

dentistgong@hotmail.com

Abstract

Introduction: Recently, a novel protein, follicular dendritic cell secreted protein (FDC-SP), has been identified in human periodontal ligament (PDL) tissue and a biomolecular study suggested that the expression of FDC-SP might be associated with the expression of the PDL phenotype. The purpose of this study was to test the effect of FDC-SP on the proliferation and phenotype of PDL cells.

Material and methods: Periodontal ligament cells obtained following the 3rd passage were exposed to various concentrations of FDC-SP. The cell proliferation was monitored by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Then, as a measure of osteogenic activity, the alkaline phosphatase (ALP) activity was recorded after 4, 7, and 14 days using *p*-nitrophenylphosphate as a substrate. Finally, total RNA was extracted and RT-PCR was performed for gene analysis.

Results: The results indicated that PDL cells exposed to 50 ng/ml FDC-SP could proliferate more rapidly. RT-PCR results showed that the mRNA expression of epidermal growth factor receptor (EGFR) was obviously upregulated and the mRNA expression of osteocalcin (OCN) and bone sialoprotein (BSP) were downregulated in PDL cells exposed to FDC-SP. Moreover, two groups of PDL cells exposed to FDC-SP showed a significant decrease of ALP activity during all the culture days.

Conclusions: In sum, the findings observed in this study suggest that FDC-SP in PDL cells could positively affect the proliferation and act as a fibroblastic phenotype stabilizer by inhibiting their differentiation into mineralized tissue-forming cells.

Key words: follicular dendritic cell secreted protein, periodontal ligament cells, phenotype, proliferation.

Introduction

Millions of people are afflicted with periodontal diseases that cause destruction of supporting structures of the teeth. The process can lead to the loss of attachment and even contribute to atheroma formation and the ensuing clinical complications [1]. The treatment of periodontal diseases has long been a challenge in clinical periodontics. Despite considerable research efforts in this area, regeneration of the periodontal ligament (PDL) has still proved to be an elusive goal of periodontal therapy and remains a subject of intense interest to dentists and dental scientists, with the molecular and cellular bases of PDL tissue still poorly understood [2, 3].

Therefore, an understanding of the intrinsic functions of PDL tissue is required.

Periodontal ligament is an unmineralized connective tissue that connects cementum and alveolar bone and plays quite important roles such as a shock absorber against the mastication impact and receptor of biting forces [4, 5]. Periodontal ligament cells are a heterogeneous population of cells that can differentiate into fibroblasts, cementoblasts, and osteoblasts [6, 7]. Moreover, some studies have shown that about 3.9% of the PDL cells termed PDLSCs could be induced to differentiate into a Schwann cell phenotype [8]. Interestingly, despite the mechanical stress of mastication or orthodontic forces during orthodontic movement, under physiological conditions, the PDL always maintains its width unmineralized. It has thus been speculated that PDL cells possess regulatory mechanisms to stabilize the fibroblastic phenotype and inhibit mineralization [9].

In 2005, a novel protein, follicular dendritic cell secreted protein (FDC-SP), was identified in human PDL tissue, but not detected in the cultured PDL cells [10]. Follicular dendritic cell secreted protein is a small secretory protein having molecular properties similar to statherin [11]. Statherin has been suggested to play an important role in the inhibition of spontaneous precipitation of calcium salts in saliva. Therefore, it is tempting to speculate that FDC-SP also prevents calcium precipitation in PDL that is surrounded by mineralized tissues, alveolar bone and cementum. Moreover, a bio-molecular study has suggested that the expression of FDC-SP may be associated with the expression of the PDL phenotype [10], and it is assumed that FDC-SP may promote differentiation of periodontal ligament cells towards a more fibroblastic phenotype. However, the mechanism of FDC-SP in expression of the PDL phenotype is still unclear.

Many investigations have been carried out to demonstrate that PDL cells have the ability to differentiate into osteoblast-like cells [12-14]. However, the precise functions of the PDL cells to stabilize the fibroblastic phenotype and maintain the PDL structural integrity remain largely unknown. It is necessary to obtain a better understanding of the phenotypic characteristics of PDL cells in order to develop purposeful strategies to influence their reparative capacities.

The aim of this study was to elucidate the role of FDC-SP in the proliferation and phenotype change of PDL cells. First, PDL cells were derived from human periodontal ligament by enzymatic digestion and were exposed to various concentrations of FDC-SP during the cell culture. After that, the cell proliferation, alkaline phosphatase (ALP)

activity and mRNA expression of EGFR, OCN and BSP genes were examined.

Material and methods

Cell culture

Human periodontal ligament cells were scraped from the middle third of the roots of premolars extracted from healthy human donors for orthodontic reasons (donors' age: 12 and 14 years old). Informed consent had been obtained from patients and parents, and an approved protocol of the ethics committee of the Sichuan University had been followed before tooth extraction. Briefly, extracted teeth were washed twice with phosphate-buffered saline (PBS) (5× penicillin and streptomycin, and 1× fungizone), then PDL tissue attached to the mid-third of the root was removed with a surgical scalpel and was teased into 1-2 mm³ fragments in tissue culture plates. PDL cells were released from the scraped samples by a 30-min enzymatic digestion (0.05% trypsin and 0.15% collagenase; Sigma, St Louis, MO, USA). After centrifugation, single cell suspensions were prepared and cultured in α -MEM containing 10% fetal bovine serum and 0.5% antibiotics, at 37°C in an atmosphere of 100% humidity and 5% CO₂ until the cells were 90% confluent. Then the cultures were washed twice with PBS, and the cells were dissociated from the flasks with 0.25% trypsin in 1 ml EDTA (GIBCO) for 5 min at 37°C. After centrifugation, the cells were replated at 1×10^4 cells/cm² and were further cultured as first-passage cells, replenishing the culture medium as above. Periodontal ligament cells at passage 3 were used for the following experiment.

Preparation and application of follicular dendritic cell secreted protein

A sample of 0.5 mg of the FDC-SP (ANASPEC, San Jose, CA) was dissolved in 500 μ l double-distilled water. Then the FDC-SP solution was diluted to 50 μ g/ml with PBS and bovine serum albumin (BSA). pH of resulting solution was adjusted to 7.1.

Cell proliferation assay

Periodontal ligament cells obtained following the 3rd passage were seeded in a 96-well plate at a density of 5×10^3 cells/well and cultured in unsupplemented medium containing 10% FBS/ α -MEM (group A) or medium supplemented with 10 ng/ml (group B), 50 ng/ml (group C), 100 ng/ml (group D), 500 ng/ml (group E) FDC-SP respectively. The cell proliferation was monitored after 1, 3, 5 and 7 d by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl

tetrazolium bromide (MTT; Sigma-Aldrich) assay. Three parallel replicates were read for each sample.

Alkaline phosphatase activity

Periodontal ligament cells were seeded in a 24-well plate at a density of 5×10^4 cells/well and exposed to varying concentrations of FDC-SP (0 ng/ml, 50 ng/ml, 100 ng/ml). The medium was changed every 48 h. As a measure of osteogenic activity, the ALP activity was recorded after 4, 7, and 14 days. Briefly, PDL cells were rinsed twice with PBS after removal of the culture media, then 1 ml of 10 mM Tris-HCl buffer, pH 8.5, was added. The cell layer was scraped off the plates and homogenized by sonication three times on ice (10 s each time). ALP activity was assayed using *p*-nitrophenylphosphate as a substrate. The amount of *p*-nitrophenol produced was measured spectrophotometrically at a wavelength of 410 nm and quantified against a standard curve. The rate of increase in absorbance at this wavelength is directly proportional to ALP activity in the sample. Alkaline phosphatase levels were normalized to the total protein content of cells at the end of the experiment.

Total RNA extraction and RT-PCR analysis

Periodontal ligament cells were seeded in a 24-well plate at a density of 5×10^4 cells/well and exposed to FDC-SP (50 ng/ml). And PDL cells cultured in unsupplemented medium containing 10% FBS/ α -MEM served as a control. The medium was changed every 48 h. The total cellular RNA from each incubation was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The RNA was then treated with DNase and cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan). The primer sets used for PCR were as follows: EGFR, 5'-GAGTAACAAGCTCACGCAGTTG-3'/5'-GAGGACATAACCAGCCACCTC-3' (product size: 169 bp); osteocalcin, 5'-CTCACACTCCTCGCCCTATTG-3'/5'-GCCTGGGTCTTCACTACCT-3' (product size: 142 bp); BSP, 5'-AGGAGGAGGAAGAAGAGGAGACT-3'/5'-CATAGCCCCAGTGTGTAGCAGA-3' (product size: 114 bp) and β -actin, 5'-GAATTCATGTTTGAGACCTTCAA-3'/5'-CCGGATCCATCTCTTGCTCGAAGTCCA-3' (product size: 326 bp). Real-time polymerase chain reaction (PCR) was performed using the software ABI PRISM 7300 SYSTEM SDS SOFTWARE. PCR amplification reactions were carried out in a total volume of 20 μ l in PCR master mix containing 10 μ l of SYBR Premix Ex Taq (Takara, Shiga, Japan), 0.4 μ l of Rox Reference Dye (Takara, Shiga, Japan), 0.8 μ l of PCR Forward Primer, 0.8 μ l of PCR Reverse Primer and 2 μ l of the reverse transcription product filled up to 20 μ l with RNase-free H₂O. Reactions for each gene

were performed in the following cycling conditions: 95°C for 10 s, and then 40 cycles of 95°C for 5 s followed by 60°C for 31 s. Melting analysis and agarose gel electrophoresis were performed to confirm the specificity of the PCR products obtained using each primer pair. The relative expression levels of genes were analysed using the $2^{-\Delta\Delta C_t}$ method [15] by normalizing with β -actin house-keeping gene expression, and presented as fold increase relative to the control group.

Western blot analysis

Treated cells were subjected to lysis buffer (Keygen total protein extraction kit, Keygen Biotech., Nanjing, Jiangsu, China). The cytosolic fraction was collected as the supernatant after centrifugation at 14,000 g at 4°C for 15 min and assayed quantitatively with the BCA method. After boiling for 5 min, 20-25 μ l of the lysate (50 μ g of protein) was applied to SDS-12% PAGE at 120 V for 5 h, and the proteins in the gel were transferred to a PVDF membrane (Millipore Corp., Bedford, MA, USA). After blocking, the membranes were probed with 1 : 1,000 dilutions of the anti-EGFR, anti-OCN, and anti-BSP (Cell Signaling Technology), followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1 : 6,000) at 37°C for 1 h. Reactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Corp., Arlington Heights, IL, USA). The density of the bands was computer analysed by a densitometer (Quantity One, Bio-Rad, USA).

Statistical analysis

All the experiments were performed in triplicate. Each value was expressed as mean \pm standard deviation (SD). A one-way ANOVA was used to compare the means of different data sets, and *p* values less than 0.05 were considered to be significant.

Results

Cell proliferation assay

To examine the effects of concentration of FDC-SP on PDL cell proliferation, the cell numbers in each culture were estimated by an MTT assay. The results of the cell proliferation assay are presented in Figure 1. At day 1, there was no significant difference between the five groups. At days 3, 5 and 7, there was a significantly greater number of cells in group C containing 50 ng/ml FDC-SP than other groups (*p* < 0.05). There was no significant difference between group A and group B (*p* > 0.05), and a conclusion was drawn that 10 ng/ml FDC-SP may not be enough to promote the PDL cell proliferation. Periodontal ligament cells

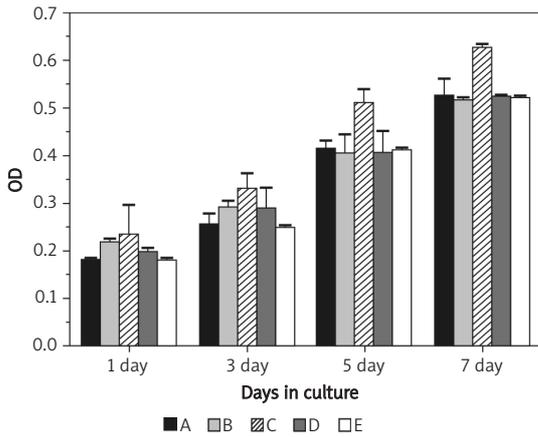


Figure 1. Results of an MTT assay showing relative proliferation rates between different concentrations of FDC-SP (group A: 0 ng/ml, group B: 10 ng/ml, group C: 50 ng/ml, group D: 100 ng/ml, group E: 500 ng/ml). Results represent the mean ± SD. The intra-experiment coefficient of variation (CV) ranged from 0.63% to 14.46%. The inter-experiment coefficient of variation (CV) was 12.00%, 11.52%, 10.45% and 8.54% respectively

cultured in 50 ng/ml FDC-SP showed the highest cell proliferation activity, while addition of 100 ng/ml or 500 ng/ml FDC-SP produced a decreased rate of cell proliferation in comparison with 50 ng/ml FDC-SP. It is believed that a higher concentration of FDC-SP than 50 ng/ml might have induced cellular damage during culture.

Alkaline phosphatase activity

To determine the effect of FDC-SP on the osteogenic differentiation of PDL cells, ALP activity, an early marker of odontoblast differentiation [16, 17], was measured. Figure 2 depicts the values of ALP

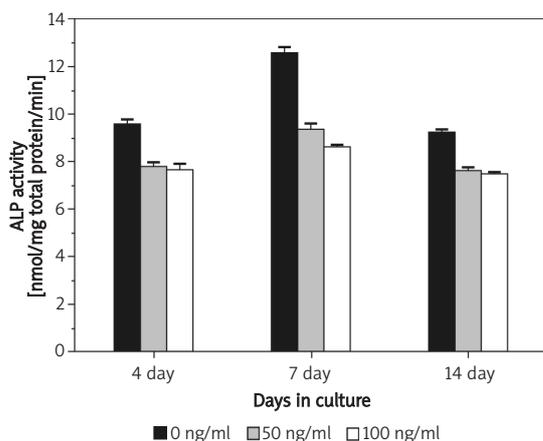


Figure 2. Alkaline phosphatase activity in PDL cells exposed to varying concentrations of FDC-SP (0 ng/ml, 50 ng/ml, 100 ng/ml) after 4, 7 and 14 days of culture. Results represent the mean ± SD. The intra-experiment CV ranged from 1.03% to 3.12%. The inter-experiment CV was 12.77%, 20.66% and 12.00% respectively

activity over protein concentration. The results showed that addition of 50 ng/ml and 100 ng/ml of FDC-SP significantly decreased the ALP activity in PDL cells ($p < 0.05$). Higher levels of ALP activity were always observed for cells harvested at day 7 compared to those harvested on days 4 and 14 ($p < 0.05$). There was no significant difference of ALP activity between two FDC-SP addition groups on days 4 and 14. However, evident enhanced ALP activity was observed in PDL cells cultured in 50 ng/ml FDC-SP medium compared to those cultured in 100 ng/ml FDC-SP medium on day 7 ($p < 0.05$).

Effects of FDC-SP on EGFR, OCN and BSP levels in PDL cells

To determine the role of FDC-SP for PDL cell phenotype, we examined the expression of mRNA for EGFR, OCN, and BSP by RT-PCR. We noted that expression of EGFR was upregulated in PDL cells exposed to FDC-SP (50 ng/ml) in comparison with control cells. Epidermal growth factor receptor mRNA was improved over 2-fold on day 4 and 4-fold on day 8 (Figure 3A). Moreover, lower expression of osteogenic genes was observed in PDL cells exposed to FDC-SP. For example, OCN, a marker of terminal osteogenic differentiation, demonstrated obvious decreased mRNA expression; less than 0.4-fold on day 4, and less than 0.6-fold on day 8 downregulation of this gene was noted (Figure 3B). And the BSP gene was also downregulated during the entire observation period (0.4- and 0.7-fold on day 4 and 8 respectively) (Figure 3C).

Western blotting was performed to examine the effect of FDC-SP on PDL cell phenotype. When cells were treated with 50 ng/ml FDC-SP for 8 days, EGFR protein was upregulated, and OCN and BSP protein were downregulated (Figure 4). This is in agreement with RT-PCR results on day 8.

Discussion

Once tissue destruction has occurred, one of the major goals of periodontal therapy is to regenerate the affected tissues to their original architecture and function. To date, attempts to regenerate periodontal tissues have focused almost exclusively on regenerating lost alveolar bone [18-20]. However, reconstruction of the periodontium is not just a simple matter of regenerating one tissue but involves at least three quite diverse and unique tissues. The management of the PDL has always been a challenge in clinical periodontics. There is a growing requirement to regenerate PDL for the treatment of periodontitis and implant dentistry [21]. In response to this, cell-based therapies are receiving increasing attention [22, 23].

Periodontal ligament cells consist of heterogeneous cell populations that can differentiate into

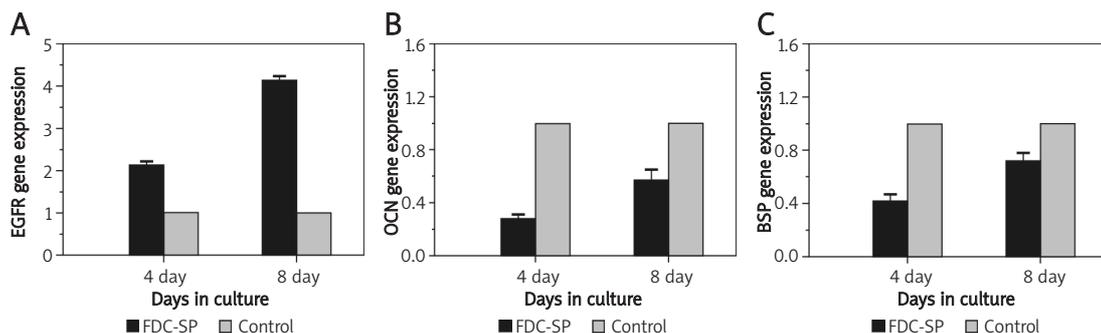


Figure 3. Expression of phenotype associated markers by PDL cells exposed to FDC-SP (50 ng/ml) and by control. **A** – The level of EGFR mRNA expression. The intra-experiment CV was 3.44% on day 4 and 2.7% on day 8. The inter-experiment CV was 51.46% on day 4 and 86.36% on day 8. **B** – The level of OCN mRNA expression. The intra-experiment CV was 12.58% on day 4 and 14.21% on day 8. The inter-experiment CV was 80.33% on day 4 and 38.34% on day 8. **C** – The level of BSP mRNA expression. The intra-experiment CV was 13.02% on day 4 and 8.54% on day 8. The inter-experiment CV was 57.79% on day 4 and 23.04% on day 8. Results represent the mean \pm SD

fibroblasts, cementoblasts, and osteoblasts [6, 7]. Evidence is accumulating that the phenotype of PDL cells can be regulated by some cytokines [24-26]. In a clinical setting, it is essential to control the PDL cells to be directed along fibroblastic or osteogenic pathways for periodontal regeneration. One of the most important findings of the present study was that FDC-SP could act as a fibroblastic phenotype stabilizer by inhibiting PDL cell differentiation into mineralized tissue-forming cells through an EGFR pathway and maintain the PDL structural integrity. The findings suggested that FDC-SP plays an important role to induce the fibroblastic differentiation of PDL cells and, therefore, might be useful for controlling PDL cell differentiation and mineralization for PDL regeneration.

It was demonstrated that PDL cells exposed to 50 ng/ml FDC-SP could proliferate more rapidly, and there was no significant difference among other groups. From these results, it would be a reasonable assumption that FDC-SP may play a crucial role in the proliferation of PDL cells, and the proliferation may be regulated by the concentration of FDC-SP.

Some studies have implicated the EGF/EGFR system in the process of phenotype regulation in different cell populations [27-29] and both pre-osteoblasts and prechondrocytes have numerous EGFR *in vivo*. However, the number diminished dramatically as their differentiation progressed. Fully differentiated osteoblasts and chondrocytes did not have any significant number of EGFR [30]. Moreover, EGFR on PDL cells had an inhibitory effect on ALP activity and functions as a negative regulator of their differentiation into mineralized tissue forming cells. Thus, the upregulation of EGFR on PDL fibroblasts was considered to play an important role in maintaining their phenotype, balancing the population of fibroblasts in the PDL by inhibiting their differentiation into mineralized tissue-forming cells [31-34]. In this study, the mRNA expression of

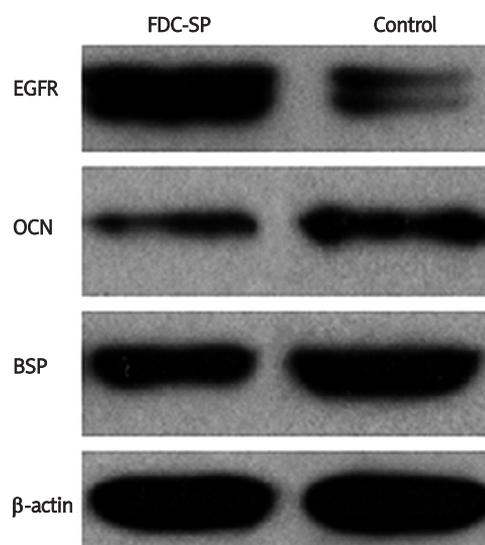


Figure 4. Expression of EGFR, OCN and BSP protein after cells were treated with 50 ng/ml FDC-SP for 8 days. Epidermal growth factor receptor protein was upregulated, OCN and BSP protein were downregulated. Similar data were obtained from 3 independent experiments

EGFR was obviously upregulated in PDL cells exposed to FDC-SP (over 2-fold on day 4 and 4-fold on day 8). These findings may indicate that FDC-SP enhances the PDL fibroblastic phenotype through an EGFR pathway.

Alkaline phosphatase is a glycoprotein thought to be involved in the formation of minerals in calcifying tissues [35-37]. From the results, two groups of PDL cells exposed to FDC-SP showed a significant decrease of ALP activity during all the culture days. These findings were consistent with the previous speculation that FDC-SP may prevent calcium precipitation in PDL according to amino acid sequence analysis [11].

Osteocalcin is a major phosphorylated extracellular matrix protein in mineralised tissues,

whereas BSP, an early marker of bone formation, is believed to be involved in the proliferation and migration of osteogenic cell populations. RT-PCR results showed that the mRNA expression of OCN and BSP were downregulated in PDL cells exposed to FDC-SP, which was in accordance with the results of ALP observations. More importantly, the decrease of ALP, OCN and BSP activation was accompanied by an increase of EGFR content. These observations suggested the novel role of FDC-SP to be a negative regulator of PDL cell differentiation into osteoblastic cells and maintain the PDL fibroblastic phenotype, which may be through an EGFR pathway.

In conclusion, the findings observed in this study suggest that FDC-SP in PDL cells could positively affect their proliferation and act as a fibroblastic phenotype stabilizer by inhibiting their differentiation into mineralized tissue-forming cells through an EGFR pathway. These findings, together with the results reported previously [10], provide information to support the hypothesis that FDC-SP may be a potential factor associated with the expression of the PDL phenotype, and maintaining the PDL structural integrity that is surrounded by mineralized tissues, alveolar bone and cementum. Further studies are needed to establish signal transduction mechanisms and second messenger pathways operating in PDL cells as a result of FDC-SP interaction.

Acknowledgments

This study was carried out and completed in the State Key Laboratory of Oral Diseases (Sichuan University). The work was sponsored by grants from the State Key Laboratory of Oral Diseases and the Ministry of Education of the People's Republic of China (20090181120025).

References

- Shanker J, Kakkar VV. Role of periodontal infection in cardiovascular disease: a current perspective. *Arch Med Sci* 2009; 5: 125-34.
- Tu QS, Zhang J, James L, et al. Cbfa1/Runx2-deficiency delays bone wound healing and locally delivered Cbfa1/Runx2 promotes bone repair in animal models. *Wound Rep Reg* 2007; 15: 404-12.
- Bartold PM, Narayanan AS. Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol* 2006; 40: 29-49.
- Linde J, Karring T. The anatomy of the periodontium. In: Linde J (ed.). *Textbook of clinical periodontology*. 2nd ed. Munksgaard, Copenhagen 1989; 19-69.
- Ralph WJ. Tensile behaviour of the periodontal ligament. *J Periodont Res* 1982; 17: 423-6.
- Nohutcu RM, McCauley LK, Koh AJ, Somerman MJ. Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization in vitro. *J Periodontol* 1997; 68: 320-7.
- McCulloch CA, Bordin S. Role of fibroblast subpopulations in periodontal physiology and pathology. *J Periodontol Res* 1991; 26: 144-54.
- Li XJ, Liao DP, Gong P, Tang H. Neural differentiation of periodontal ligament stem cells induced by neurotrophic Schwann cells factors. *Arch Med Sci* 2009; 5: 313-20.
- Kato C, Kojima T, Komaki M, et al. S100A4 inhibition by RNAi up-regulates osteoblast related genes in periodontal ligament cells. *Biochem Biophys Res Commun* 2005; 326: 147-53.
- Nakamura S, Terashim T, Yoshid T, et al. Identification of genes preferentially expressed in periodontal ligament: specific expression of a novel secreted protein, FDC-SP. *Biochem Biophys Res Commun* 2005; 338: 1197-203.
- Schlesinger DH, Hay DI. Complete covalent structure of statherin, a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva. *J Biol Chem* 1997; 272: 1689-95.
- Hayami T, Zhang Q, Kapila Y, Kapila S. Dexamethasone's enhancement of osteoblastic markers in human periodontal ligament cells is associated with inhibition of collagenase expression. *Bone* 2007; 40: 93-104.
- Lecic P, McCulloch CA. Periodontal ligament cell populations: the central role of fibroblasts in creating a unique tissue. *Anat Rec* 1996; 245: 327-41.
- Murakami Y, Kojima T, Nagasawa T, Kobaysashi H, Ishikawa I. Novel isolation of alkaline phosphatase-positive subpopulation from periodontal ligament fibroblasts. *J Periodontol* 2003; 74: 780-6.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC(T)} method. *Methods* 2001; 25: 402-8.
- Yokose S, Kadokura H, Tajima Y, et al. Establishment and characterization of a culture system for enzymatically released rat dental pulp cells. *Calcif Tissue Int* 2000; 66: 139-44.
- Lee DH, Lim BS, Lee YK, Yang HC. Effects of hydrogen peroxide (H₂O₂) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biol Toxicol* 2006; 22: 39-46.
- Hosoya A, Ninomiya T, Hiraga T, et al. Alveolar bone regeneration of subcutaneously transplanted rat molar. *Bone* 2008; 42: 350-7.
- Inoue T, Osada H, Shiigai T, Fujiseki M, Shimono M. An experimental study of osteogenesis by autografted dental pulp, periodontal ligament, and bone marrow in vivo. *Bull Tokyo Dent Coll* 1993; 34: 183-90.
- Akizuki T, Oda S, Komaki M, et al. Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. *J Periodont Res* 2005; 40: 245-51.
- Yuan Q, Gong P, Li XY, et al. Enhanced proliferation and differentiation of osteoblasts induced by co-culturing with Schwann cells. *Arch Med Sci* 2008; 3: 242-8.
- Picinich SC, Mishra PJ, Mishra PJ, Glod J, Banerjee D. The therapeutic potential of mesenchymal stem cells. Cell- and tissue-based therapy. *Expert Opin Biol Ther* 2007; 7: 965-73.
- Mimeault M, Hauke R, Batra SK. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. *Clin Pharmacol Ther* 2007; 82: 252-64.
- Matsuda N, Yokoyama K, Takeshita S, Watanabe M. Role of epidermal growth factor and its receptor in mechanical

- stress-induced differentiation of human periodontal ligament cells in vitro. *Arch Oral Biol* 1998; 43: 987-97.
25. Li L, Yu JF, Yan W, Guang W, Yin D. Effect of estrogen receptor beta on the osteoblastic differentiation function of human periodontal ligament cells. *Arch Oral Biol* 2008; 53: 553-7.
 26. Kitagawa M, Kudo Y, Iizuka S, et al. Effect of F-spondin on cementoblastic differentiation of human periodontal ligament cells. *Biochem Biophys Res Commun* 2006; 349: 1050-6.
 27. Serrero G. EGF inhibits the differentiation of adipocyte precursors in primary cultures. *Biochem Biophys Res Commun* 1987; 146: 194-202.
 28. Serrero G, Mills D. Physiological role of epidermal growth factor on adipose tissue development in vivo. *Proc Nat Acad Sci USA* 1991; 88: 3912-6.
 29. Taketani Y, Ota J. Epidermal growth factor stimulates cell proliferation and inhibits functional differentiation of mouse mammary epithelial cells in culture. *Endocrinology* 1983; 113: 871-7.
 30. Cho MI, Garant PR, Lee YL. Radiographic evidence for binding of epidermal growth factor by hard tissue stem cells and periodontal ligament fibroblasts. In: *The biological mechanisms of tooth eruption and root resorption*. Davidovitch Z (ed.) EBSCO Media, Birmingham 1988; 133-44.
 31. Cho MI, Garant PR. Expression and role of epidermal growth factor receptors during differentiation of cementoblasts, osteoblasts, and periodontal ligament fibroblasts in the rat. *Anatomical Record* 1996; 1245: 342-60.
 32. Matsuda N, Kumar NM, Ramakrishnan PR, Lin WL, Genco RJ, Cho MI. Evidence for up-regulation of epidermal growth factor receptors on rat periodontal ligament fibroblastic cells associated with stabilization of phenotype in vitro. *Arch Oral Biol* 1993; 38: 559-69.
 33. McCulloch CA, Melcher AH. Cell density and cell generation in the periodontal ligament of mice. *Am J Anat* 1983; 167: 43-58.
 34. Canalis E. Effects of hormones and growth factors on alkaline phosphatase activity and collagen synthesis in cultured rat calvaria. *Metab Clin Exp* 1983; 32: 14-20.
 35. Wolgens JH, Bervoets TJ, Bronckers AL, Lyaruu DM. Organ culture of tooth germs: relationship between alkaline phosphatase and mineralization in vitro. *J Biol Buccale* 1982; 10: 191-8.
 36. Beertsen W, Van den Bos T. Calcification of dentinal collagen by cultured rabbit periosteum: the role of alkaline phosphatase. *Matrix* 1989; 9: 159-71.
 37. Beertsen W, Van den Bos T. Alkaline phosphatase induces the deposition of calcified layers in relation to dentine: an in vitro study to mimic the formation of a fibrillar acellular cementum. *J Dent Res* 1991; 70: 176-81.