IF 1.158

Archives of Oral Biology (2005) 50, 923–928

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Archives of Oral Biology

www.intl.elsevierhealth.com/journals/arob

MEPE is downregulated as dental pulp stem cells differentiate

He Liu [a,b], Wu Li [a], Songtao Shi [c], Stefan Habelitz [a], Cen Gao [a], Pamela DenBesten [a,*]

[a] University of California, San Francisco, Growth and Development, 521 Parnassus Avenue, Rm C734, Box 0640, San Francisco, CA 94143-0640, USA
[b] Peking University School of Stomatology, Beijing, China
[c] NIDCR, Bethesda, MD, USA

Accepted 1 March 2005

KEYWORDS
MEPE;
DSP;
Proliferation;
Differentiation;
Dental pulp stem cells;
DPSC;
Osteogenesis Superarray;
TGF-β

Summary Previous studies on dental pulp cell culture have described heterogenous mixtures of cells that differentiate into odontoblasts and form mineralized dentin.
Objective: The aim of this study was to characterize the matrix extracellular phosphoglycoprotein (MEPE) expression by dental pulp stem cells (DPSC), related to cell differentiation.
Design: DPSC differentiation to form mineralized nodules was characterized by Alizarin red staining and micro-Raman spectroscopy. Osteogenesis SuperArray analysis was used to broadly screen for osteogenesis-related genes altered by DPSC differentiation. Relative levels of expression of MEPE and DSP were determined by semiquantitative RT-PCR and Western blot.
Results: Mineral analysis showed that as DPSC differentiated, they formed a carbonated hydroxyapatite mineral. Differentiation was initially marked by upregulation by Runx2, TGFβ-related genes, EGFR and genes involved in collagen metabolism. ALP activity first increased, as DPSCs reached confluence but later decreased when cells further differentiated three weeks after confluence. MEPE was the only marker that was downregulated as DPSCs differentiated.
Conclusion: DPSC differentiation can be characterized by downregulation of MEPE as other markers of DPSC differentiation, such as DSP, are upregulated. Expression of MEPE related to DSP and can be used to monitor DPSC as they are used for studies of odontoblast differentiation, tissue engineering or vital pulp therapy. The downregulation of MEPE as DPSC differentiate, suggests that MEPE is an inhibitor of mineralization.

Introduction

Under conditions, such as caries and trauma, dental pulp cells have the ability to proliferate and

* Corresponding author. Tel.: +1 415 502 7828; fax: +1 415 476 1499.
E-mail address: pkdb@itsa.ucsf.edu (P. DenBesten).

0003-9969/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2005.03.003

differentiate into odontoblasts to repair the damage.[17,18] These odontoblasts are believed to come from a subgroup of precursor cells residing in the dental pulp. *In vitro* studies have shown that cells from the dental pulp can attach to culture dishes, and these cells have been used to study pulp cell differentiation into odontoblast-like cells.[1,4,23,33] Recently, the isolation of clonogenic post-natal dental pulp stem cells (DPSC) has been described.[10,11,19] DPSCs are single-colony-derived cells that are capable of self-renewal and have multi-lineage differentiation capacity, including differentiation into adipocytes and neural-like cells, as well as odontoblast-like cells. DPSCs were shown to regenerate a dentin-pulp-like complex when transplanted into nude mice,[10,11] and show promise in future applications for tissue engineering of dental tissues.

A number of markers for cell differentiation have been identified in mixed pulp cell cultures that differentiate and mineralize *in vitro*.[4,23,33] When these cells are grown in the presence of β-glycerolphosphate, alkaline phosphatase synthesis increases prior to mineralization.[12,20,22,33,36] Other markers of differentiation include the increased fibronectin, which is secreted in the initial stage of odontoblast differentiation and remains throughout odontogenesis.[26,34,35] Type I collagen is upregulated as the cells differentiate,[1,3,6] and osteonectin is expressed in young odontoblasts but not in pulp cells.[8,24] Dentin sialophosphoprotein (DSPP), as well as DMP1, are expressed by differentiating odontoblasts.[2,3,5] Dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are cleaved products of DSPP,[16] and DSP has been used as a late marker for odontoblast formation.[19,20]

While all of these molecules enhance differentiation and mineralization, the role of the recently identified matrix extracellular phosphoglycoprotein (MEPE), in dentin is not known. MEPE is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family.[7,9,15] Ablation of the MEPE gene in a mouse model resulted in increased numbers of osteoblasts and enhanced mineralization.[9] Similarly, recent studies of an active fragment of MEPE, named Dentonin or AC100, enhanced DPSC proliferation.[14] However Siggelkow et al.[28] recently showed that when primary human osteoblasts were cultured in the presence of 5 mM β-glycerophosphate, ascorbate and dexamethasone, they showed a progressive inhibition of MEPE gene expression.

The purpose of the present study was to characterize gene expression and mineralization associated with DPSC differentiation. The possibility of using MEPE as differentiation marker was explored.

Materials and methods

DPSCs were isolated as described by Gronthos et al.[11] The cells were cultured and collected at 80% confluence, complete confluence, 1–3 weeks after confluence, respectively, ALP activity was measured using alkaline phosphatase activity kit (Sigma Diagnostics 104). The total amount of protein in the supernatant was measured by BioRad Commassie Blue protein assay (Bio-Rad Laboratories, Hercules, CA), and the activity of ALP was calculated as pmol *p*-nitrophenol/h/μg protein.

To induce mineral formation, the cells were plated at a density of 1.5×10^4/ml on 100 mm dishes (Primaria, Falcon) and were cultured at 37 °C, in 5% CO_2 in MEM with 10% fetal bovine serum (FBS), 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone (Sigma) 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. When nodule formation was detected, the samples were fixed with 4% paraformaldehyde overnight and stained for calcium by Alizarin red staining. The mineral phase formed in nodules was analysed using micro-Raman spectroscopy (HR 800, Jobin Ivon, Horiba Group, Japan) with a 20 mW He–Ne laser at a wavelength of 632.8 nm. Spectra were obtained in the range from 400 to 3400 cm^{-1} using a 50× optical lens sampling an area of approximately 5 μm × 5 μm.

Osteogenesis gene SuperArray

DPSCs were cultured and collected at 80% confluence and after complete confluence. Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). GEArray Q series human osteogenesis gene array kit was obtained from SuperArray Inc. (Bethesda, MD), and total RNA was used as a template to generate biotin-16-dUTP labelled cDNA probes according to the manufacturer's instructions. The cDNA probes were denatured and hybridized at 60 °C with the SuperArray membrane, which was washed and exposed using a chemiluminescent substrate and analysed according to the manufacturer's instructions.

Semiquantitative RT-PCR

DPSCs were cultured and collected at 80% confluence, complete confluence, 1–3 weeks after confluence, respectively. Total mRNA was isolated using RNeasy Mini Kit (Qiagen Inc.). The same amount of total mRNA was used for reverse-transcription by using an oligo-dT primer followed by PCR with a 50 μl reaction system including, 30 μl H_2O, 5 μl 10 × PCR buffer, 1.5 μl 50 mM $MgCl_2$ 1 μl 15 mM

dNTP, 0.5 μTaq, 2 μl template, 5 μl upper primer, 5 μl lower primer. Primers included GAPDH (sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′), ALP (sense, 5′-GACCCGTCACTCTCCGAGATG-3′; antisense, 5′-CTGCGCCTGGTAGTTGTTGTG-3′), MEPE (sense, 5′-GGTTATACAGATCTTCAAGAGAGAG-3′; antisense, 5′-GTTGGTACTTTCAGCTGCATCACT-3′). The reaction was incubated in a PCR cycler at 94 °C for 3 min for 1 cycle and then 94 °C for 30 s, 55 °C for 30 s for GAPDH and ALP and 60 °C for 30 s for MEPE, followed by 72 °C 1 min for 35 cycles, and a final 10-min extension at 72 °C. After amplification, the reaction was analysed by 1% agarose gel electrophoresis.

Western blot

DPSCs were cultured and collected in SDS lysis buffer at 80% confluence, complete confluence, and at the initiation of mineralization. The samples were sonicated briefly, boiled for 1.5 min and centrifuged at 10,000 rpm for 5 min. The protein in the supernatant was measured using a BioRad Commassie Blue protein assay (Bio-Rad Laboratories, Hercules, CA). The protein was concentrated and adjusted to same concentration for each sample. Equal amounts of protein were loaded onto a 15% polyacrylamide gel and separated by electrophoresis. Proteins were then transferred to a nitrocellulose membrane using a 180 mA current for 1.5 h. The membrane was blocked in 5% milk overnight at room temperature and was incubated with anti-MEPE (a gift from Acologix Inc., Emmeryville, CA), anti-DSP (gift from Dr. Shi, NIDCR, Bethesda, MD) or anti-actin (Santacruz Biotechnology). After washing in PBS, the membrane was incubated with the appropriate secondary antibody for 0.5 h, and positive bands were detected using an ECL chemiluminecence detection system (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's instructions. The relative intensity of DSP- and MEPE-specific bands was digitalized and compared using NIH image (public domain image processing and analysis software developed at the National Institutes of Health).

Results

When DPSCs were cultured with ascorbic acid, β-glycerophosphate and dexamethasone, nodule-like structures could be seen scattered within the cultured cells after 6 weeks. These nodules stained positive with Alizarin red (Fig. 1). The extracted mineral was characterized by micro-Raman spectroscopy. Fig. 2 shows a spectrum obtained in the range of 400–1500 cm^{-1} exhibiting an intensive peak at 960 cm^{-1} and a small band at 1070 cm^{-1}. The intensive peak at 960 cm^{-1} is characteristic of the ν_1-stretching mode of PO_4 groups in apatite.[21] The small band at 1070 cm^{-1} might indicate the presence of carbonate groups incorporated into the apatite lattice.[21] No other calcium phosphate phase was observed. A series of weak and broad bands were observed around 1300 and 1450 cm^{-1} as well as around 2800 cm^{-1}, as shown in the insert of Fig. 3. These bands are attributed to amide and C–H vibration modes of remnants of organic phases, e.g. collagen type.[27]

ALP activity increased when the cells grew more confluent and differentiated, but decreased 3 weeks after confluence (Fig. 3). The osteogenesis-related cDNA array (Fig. 4) showed that as the cells reached the confluence, most of the osteogenesis-related genes were upregulated. Fibronectin and osteonectin were expressed strongly in DPSCs at both time points. The genes most strongly upregulated (>10 times) were

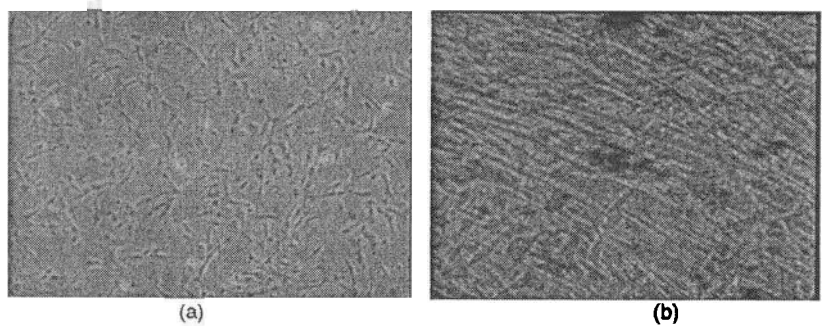

Figure 1 Alizarin red staining. (A) DPSCs did not spontaneously form the mineralized nodules. (B) With dexamethasone, β-glycerophosphate and ascorbic acid, DPSCs induced nodule formation after 6 weeks of culture, as indicated by Alizarin red.

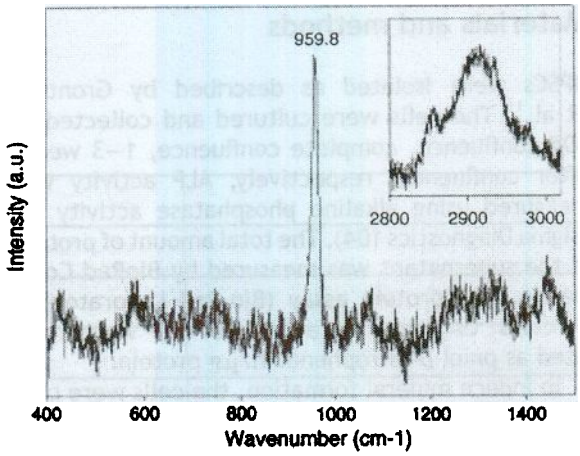

Figure 2 Raman spectrum of mineral extracted from odontoblast nodules at 6 weeks showed a strong peak at 960 cm^{-1}, indicating that the mineralizing nodule contained hydroxyapatite as the only calcium phosphate phase. The insert shows the spectrum at higher wavenumbers with peaks around 2900 cm^{-1}, characteristic for C–H stretching modes of protein remnants.

TGFβ3, SMAD3, SMAD8, Cathepsin K, TNFα and VEGF-C. In addition, genes involved in collagen metabolism (COL16α1, COL2α1, CD36L1), as well as TGFβ-related genes (TGFβ2, TGFβ receptor I) Runx2, EGFR were also upregulated (>3 times). These results were duplicated in two separate experiments.

Semi-quantitative RT-PCR showed that as ALP was upregulated, MEPE expression decreased (Fig. 5). Comparisons of relative levels of MEPE and DSP protein secretion by differentiating DPSC by Western blot analysis, confirmed that MEPE protein secretion decreased as cells differentiated, while DSP protein secretion increased relative to actin (Fig. 6).

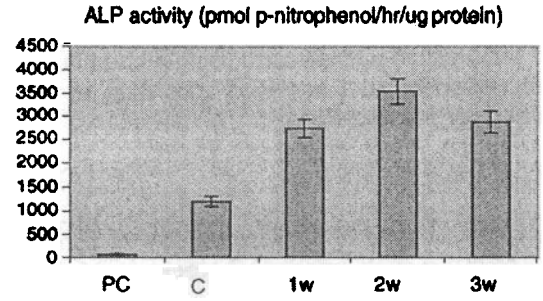

Figure 3 ALP activity measurement. ALP activity increased after the cells reached confluence but decreased at 3 weeks after confluence. PC: preconfluent; C: confluent; 1w: 1 week after confluence; 2w: 2 week after confluence; 3w: 3 week after confluence.

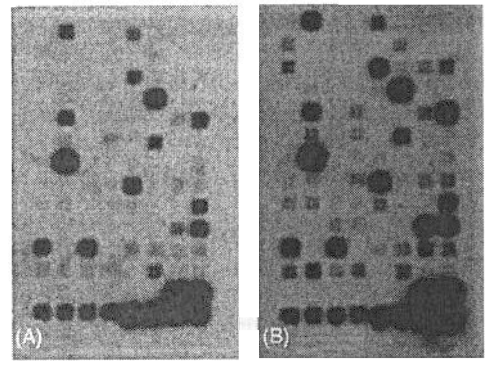

Figure 4 Osteogenesis SuperArray. (A) Genes present at 80% confluence. (B) Genes present at complete confluence. Upregulation of osteogenesis-related genes is evident when comparing the two sample membranes.

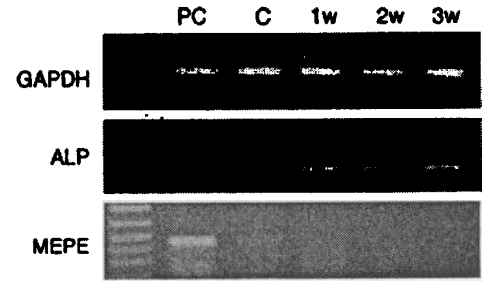

Figure 5 Semiquantitative RT-PCR. ALP expression increased while MEPE expression decreased as cells differentiated relative to GAPDH. PC: preconfluent; C: confluent; 1w: 1 week after confluence; 2w: 2 week after confluence; 3w: 3 week after confluence.

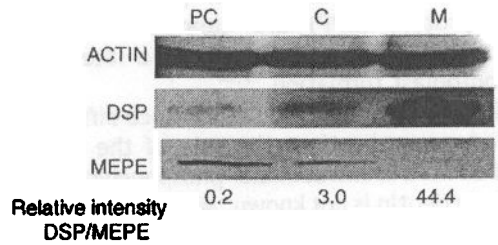

Figure 6 Western blot. Actin was used as a control. DSP expression was upregulated while MEPE expression was downregulated as the cells differentiated. The relative intensity of the different bands was measured by NIH image and ration of DSP/MEPE proteins is shown below the images. PC: preconfluent; C: confluent; M: initiation of mineralization.

Discussion

Mineralization of the dentin matrix is a hallmark of functional, and fully differentiated odontoblasts.[20] Dental pulp cells have been shown to form mineralized nodules in culture.[1,31,32] In our study, DPSCs in

culture were induced to mineralize with addition of ascorbic acid, β-glycerophosphate and dexamethasone, resulting in the formation of a hydroxyapatite with small quantities of carbonate characteristic for biological apatites.[14]

As DPSC differentiated, genes associated with osteoblast differentiation were upregulated. In particular, TGFβ-related genes (TGFβ3, TGFβ2, TGFβ receptor I, SMAD3, SMAD8), genes related to collagen metabolism (COL16α1, COL2α1, TNFα, CD36L1, Cathepsin K), Runx2, VEGF-C and EGFR were significantly upregulated when cells reached the confluence. Both at 80% confluence and complete confluence, fibronectin and osteonectin were expressed strongly, while ALP and osteocalcin were below detectable levels by this array analysis. These resutts indicate that even when the cells reach confluence, they are still at an early stage of differentiation.[24,26]

The identification of TGFβ3, TGFβ2, TGFβ receptor I, SMAD3 and SMAD8 upregulation at the initial stages of cell differentiation, emphasizes the importance of this family of growth factors in odontoblast development. Sloan et al. have shown that TGFβ stimulates the matrix secretion and initiate the odontoblast cytodifferentiation.[29] However, overexpression of TGFβ in a transgenic mouse model resulted in reduced dentin mineralization and decreased DSPP expression,[30] implying that TGFβ may maintain initial stages of odontoblast differentiation, and inhibit odontoblast terminal differentiation.

Increased ALP activity as dental pulp cells differentiate, corresponds to previous studies by Yokose et al. in rat dental pulp cell culture.[33] DSP expression increased during differentiation of DPSCs and increased dramatically after the appearance of mineral in agreement with previous studies on dental pulp cell culture.[4,5]

Of all the proteins and growth factors analysed, only MEPE expression decreased as DPSCs differentiated. The decrease in MEPE expression by differentiating DPSCs, identified by RT-PCR and confirmed by Western blot, differs from osteoblast culture where MEPE expression is reported to increase as the cells differentiate.[13,25] However, a recent study by Siggelkow et al. of MEPE in human osteoblasts in culture showed that MEPE was downregulated, as the cells differentiate and mineralize.[28] In that study, the authors also noted the discrepancy mouse studies, when MEPE was maximally expressed during mineralization, and suggested that in the human system, MEPE is most active in the proliferative and early maturation stage of formation.

The downregulation of MEPE in DPSCs as they reached confluence and initiated mineralization suggests that similar to human osteoblast, MEPE may inhibit differentiation and mineralization of DPSC. This result also suggests that MEPE along with DSP can be used as a marker for odontoblast differentiation.

Acknowledgements

This study was supported by the Lee Hysan Foundation (HL) and NIDCR P01-DE09859.

References

1. About I, Bottero MJ, de Denato P, Camps J, Franquin JC, Mitsiadis TA. Human dentin production *in vitro*. *Exp Cell Res* 2000;**258**(1):33–41.
2. Begue-Kirn C, Ruch JV, Ridall AL, Butler WT. Comparative analysis of mouse DSP and DPP expression in odontoblasts, preameloblasts, and experimentally induced odontoblast-like cells. *Eur J Oral Sci* 1998;**106**(Suppl. 1):254–9.
3. Bleicher F, Couble ML, Farges JC, Couble P, Magloire H. Sequential expression of matrix protein genes in developing rat teeth. *Matrix Biol* 1999;**18**(2):133–43.
4. Couble ML, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M, Magloire H. Odontoblast differentiation of human dental pulp cells in explant cultures. *Calcif Tissue Int* 2000;**66**(2):129–38.
5. D'Souza RN, Cavender A, Sunavala G, Alvarez J, Ohshima T, Kulkarni AB, et al. Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions *in vivo*. *J Bone Miner Res* 1997;**12**(12):2040–9.
6. Domon S, Shimokawa H, Yamaguchi S, Soma K. Temporal and spatial mRNA expression of bone sialoprotein and type I collagen during rodent tooth movement. *Eur J Orthod* 2001;**23**(4):339–48.
7. Fisher LW, Fedarko NS. Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. *Connect Tissue Res* 2003;**44**(Suppl. 1):33–40.
8. Garcia JM, Martins MD, Jaeger RG, Marques MM. Immunolocalization of bone extracellular matrix proteins (type I collagen, osteonectin and bone sialoprotein) in human dental pulp and cultured pulp cells. *Int Endod J* 2003;**36**(6):404–10.
9. Gowen LC, Petersen DN, Mansolf AL, Qi H, Stock JL, Tkalcevic GT, et al. Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. *J Biol Chem* 2003;**278**(3):1998–2007.
10. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;**81**(8):531–5.
11. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Post-natal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 2000;**97**(25):13625–30.
12. Hotton D, Mauro N, Lezot F, Forest N, Berdal A. Differential expression and activity of tissue-nonspecific alkaline phosphatase (TNAP) in rat odontogenic cells *in vivo*. *J Histochem Cytochem* 1999;**47**(12):1541–52.
13. Igarashi M, Kamiya N, Ito K, Takagi M. In situ localization and *in vitro* expression of osteoblast/osteocyte factor 45 mRNA during bone cell differentiation. *Histochem J* 2002;**34**(5):255–63.

14. Liu H, Li W, Gao C, Kumagai Y, Blacher RW, DenBesten PK. Dentonin, a fragment of MEPE, enhanced dental pulp stem cell proliferation. *J Dent Res* 2004;83(6):496–9.
15. MacDougall M, Simmons D, Gu TT, Dong J. MEPE/OF45, a new dentin/bone matrix protein and candidate gene for dentin diseases mapping to chromosome 4q21. *Connect Tissue Res* 2002;43(2–3):320–30.
16. MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT. Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4. Dentin phosphoprotein DNA sequence determination. *J Biol Chem* 1997;272(2):835–42.
17. Murray PE, About I, Lumley PJ, Smith G, Franquin JC, Smith AJ. Postoperative pulpal and repair responses. *J Am Dent Assoc* 2000;131(3):321–9.
18. Murray PE, Kitasako Y, Tagami J, Windsor LJ, Smith AJ. Hierarchy of variables correlated to odontoblast-like cell numbers following pulp capping. *J Dent* 2002;30(7–8):297–304.
19. Nakashima M, Mizunuma K, Murakami T, Akamine A. Induction of dental pulp stem cell differentiation into odontoblasts by electroporation-mediated gene delivery of growth/differentiation factor 11 (Gdf11). *Gene Ther* 2002;9(12):814–8.
20. Narayanan K, Srinivas R, Ramachandran A, Hao J, Quinn B, George A. Differentiation of embryonic mesenchymal cells to odontoblast-like cells by overexpression of dentin matrix protein 1. *Proc Natl Acad Sci USA* 2001;98(8):4516–21.
21. Nishino M, Yamashita S, Aoba T, Okazaki M, Moriwaki Y. The laser-Raman spectroscopic studies on human enamel and precipitated carbonate-containing apatites. *J Dent Res* 1981;60(3):751–5.
22. Nuki K, Bonting SL. Quantitative histochemistry of the developing hamster tooth: alkaline phosphatase and lactic dehydrogenase. *J Histochem Cytochem* 1961;9:117–25.
23. Onishi T, Kinoshita S, Shintani S, Sobue S, Ooshima T. Stimulation of proliferation and differentiation of dog dental pulp cells in serum-free culture medium by insulin-like growth factor. *Arch Oral Biol* 1999;44(4):361–71.
24. Papagerakis P, Berdal A, Mesbah M, Peuchmaur M, Malaval L, Nydegger J, et al. Investigation of osteocalcin, osteonectin, and dentin sialophosphoprotein in developing human teeth. *Bone* 2002;30(2):377–85.
25. Petersen DN, Tkalcevic GT, Mansolf AL, Rivera-Gonzalez R, Brown TA. Identification of osteoblast/osteocyte factor 45 (OF45), a bone-specific cDNA encoding an RGD-containing protein that is highly expressed in osteoblasts and osteocytes. *J Biol Chem* 2000;275(46):36172–80.
26. Sawada T, Nanci A. Spatial distribution of enamel proteins and fibronectin at early stages of rat incisor tooth formation. *Arch Oral Biol* 1995;40(11):1029–38.
27. Schulze KA, Balooch M, Balooch G, Marshall GW, Marshall SJ. Micro-Raman spectroscopic investigation of dental calcified tissues. *J Biomed Mater Res A* 2004;69(2):286–93.
28. Siggelkow H, Schmidt E, Hennies B, Hufner M. Evidence of downregulation of matrix extracellular phosphoglycoprotein during terminal differentiation in human osteoblasts. *Bone* 2004;35(2):570–6.
29. Sloan AJ, Smith AJ. Stimulation of the dentine-pulp complex of rat incisor teeth by transforming growth factor-beta isoforms 1–3 *in vitro*. *Arch Oral Biol* 1999;44(2):149–56.
30. Thyagarajan T, Sreenath T, Cho A, Wright JT, Kulkarni AB. Reduced expression of dentin sialophosphoprotein is associated with dysplastic dentin in mice overexpressing transforming growth factor-beta 1 in teeth. *J Biol Chem* 2001;276(14):11016–20.
31. Tsukamoto Y, Fukutani S, Shin-Ike T, Kubota T, Sato S, Suzuki Y, et al. Mineralized nodule formation by cultures of human dental pulp-derived fibroblasts. *Arch Oral Biol* 1992;37(12):1045–55.
32. Ueno A, Kitase Y, Moriyama K, Inoue H. MC3T3-E1-conditioned medium-induced mineralization by clonal rat dental pulp cells. *Matrix Biol* 2001;20(5–6):347–55.
33. Yokose S, Kadokura H, Tajima Y, Fujieda K, Katayama I, Matsuoka T, et al. Establishment and characterization of a culture system for enzymatically released rat dental pulp cells. *Calcif Tissue Int* 2000;66(2):139–44.
34. Yoshiba K, Yoshiba N, Nakamura H, Iwaku M, Ozawa H. Immunolocalization of fibronectin during reparative dentinogenesis in human teeth after pulp capping with calcium hydroxide. *J Dent Res* 1996;75(8):1590–7.
35. Yoshiba N, Yoshiba K, Nakamura H, Iwaku M, Ozawa H. Immunoelectron-microscopic study of the localization of fibronectin in the odontoblast layer of human teeth. *Arch Oral Biol* 1995;40(2):83–9.
36. Yoshiki S, Kurahashi Y. A light and electron microscopic study of alkaline phosphatase activity in the early stage of dentinogenesis in the young rat. *Arch Oral Biol* 1971;16(10):1143–54.