J Periodont Res 2009; 44: 199–210
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JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01106.x

Apical tooth germ cell-conditioned medium enhances the differentiation of periodontal ligament stem cells into cementum/periodontal ligament-like tissues

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Yang Z-H, Zhang X-J, Dang N-N, Ma Z-F, Xu L, Wu J-J, Sun Y-J, Duan Y-Z, Lin Z, Jin Y. Apical tooth germ cell-conditioned medium enhances the differentiation of periodontal ligament stem cells into cementum/periodontal ligament-like tissues. J Periodont Res 2009; 44: 199–210. © 2009 The Authors. Journal compilation © 2009 Blackwell Munksgaard

Background and Objective: Limitations of current periodontal regeneration modalities in both predictability and extent of healing response, especially on new cementum and attachment formation, underscore the importance of restoring or providing a microenvironment that is capable of promoting the differentiatiation of periodontal ligament stem cells (PDLSCs) towards cementoblast-like cells and the formation of cementum/periodontal ligament-like tissues. The aim of this study was to investigate the biological effect of conditioned medium from developing apical tooth germ cells (APTG-CM) on the differentiation and cementogenesis of PDLSCs both *in vitro* and *in vivo*.

Material and Methods: Using the limiting dilution technique, single-colony-derived human PDLSCs were isolated and expanded to obtain homogeneous populations of PDLSCs. Morphological appearance, cell cycle analysis, bromodeoxyuridine incorporation, alkaline phosphatase (ALP) activity, mineralization behavior, gene expression of cementoblast phenotype and *in vivo* differentiation capacities of PDLSCs co-cultured with APTG-CM were evaluated.

Results: The induced PDLSCs exhibited several characteristics of cementoblast lineages, as indicated by the morphological changes, increased proliferation, high ALP activity, and the expression of cementum-related genes and calcified nodule formation *in vitro*. When transplanted into immunocompromised mice, the induced PDLSCs showed tissue-regenerative capacity to produce cementum/periodontal ligament-like structures, characterized by a layer of cementum-like mineralized tissues and associated periodontal ligament-like collagen fibers connecting with the newly formed cementum-like deposits, whereas control, untreated PDLSCs transplants mainly formed connective tissues.

Conclusion: Our findings suggest that APTG-CM is able to provide a cementogenic microenvironment and induce differentiation of PDLSCs along the cementoblastic lineage. This has important implications for periodontal engineering.

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Key words: periodontal ligament stem cell; cell differentiation; cementogenesis; periodontal regeneration

Accepted for publication March 23 2008

Predicable regeneration of the attachment apparatus following periodontal disease has long been the major goal of periodontal therapy. In pursuit of treatments that can reverse the bony defect, including regeneration of new bone, new cementum and supportive periodontal ligament (PDL), various reconstructive procedures, such as bone autografts, allografts, root surface conditioning, guided tissue regeneration and various growth factors, have been explored over the years (1,2). Unfortunately, these treatment modalities are limited in both predictability and extent of healing response, especially on new cementum and attachment formation (3–5). Periodontal regeneration requires new connective tissue attachment to the root surface, a process that involves the formation of new cementum on previously exposed root surfaces, the synthesis of Sharpey's fibers and their insertion into newly formed cementum. From a biological perspective, the occurrence of this predictable periodontal regeneration depends upon two crucial factors: the availability of appropriate cell types and a favorable local environment, that is, the presence of the cues and signals necessary to recruit and stimulate these cells (6). The two factors need to be precisely co-ordinated both temporally and spatially.

The recent identification of human postnatal PDL stem cells (PDLSCs) within the periodontal ligament represents a significant development in the progress towards predictable periodontal regeneration (7). It has also been recognized that if triggered appropriately, adult periodontal ligament stem cells are capable of differentiating towards cementoblast-like cells and of secreting cementum/PDL-like tissues *in vitro* and *in vivo* (8,9). In terms of emerging evidence showing that local environment is a major regulator of how cells respond to environmental cues and signals (10,11), much emphasis has been placed on the underlying biological mechanisms that regulate PDLSCs differentiation (6). It is generally believed that tooth development relies on reciprocal and reiterated molecular signals between epithelium and mesenchyme to control morphogenesis and development. The reciprocal and sequential nature of these inductions has been found to be the basis for advancing differentiation of dental tissues (12,13). During the past few years, one major discovery from research targeting crown development has been the recognition of niche areas, specifically the enamel knot, and cervical loop/apical bud regions, which appear at key times to deliver the crucial cues to advance crown development (14). Do 'niche' regions for root/periodontal tissue development exist, similar to the enamel knot region? During tooth development, the periodontium is formed by the apical mesenchyme of the tooth germ, which includes the dental follicle surrounding the enamel organ and dental papilla situated within the developing pulp, while the inner and outer enamel epithelia fuse below the level of the crown cervical margin to produce a bilayered epithelial sheath termed Hertwig's epithelial root sheath. Epithelial stimuli derived from this root sheath seem to be responsible for the differentiation of follicle cells into cementoblasts and papilla cells into odontoblasts (15–18). Therefore, the apical portion of the developing tooth germ, which consists of the dental follicle, dental papilla and Hertwig's epithelial root sheath, is the development primordium and 'stem cell niche' of the tooth root.

In fact, previous studies have shown that periodontal regeneration during wound healing is likely to share many common biological processes with those of periodontal development (2). To create the most odontogenic microenvironment, Yu *et al.* used tooth germ cell-conditioned medium (TGC-CM) to induce dental pulp stem cells and generated a regular-shaped dentin–pulp complex containing distinct dentinal tubules and predentin *in vivo* (19). Accordingly, from a development perspective, the present study was designed to mimic the microenvironment of root/periodontal tissue development and to investigate the biological effect of apical tooth germ cell-conditioned medium (APTG-CM) on the proliferation and differentiation of PDLSCs. The characteristics of induced PDLSCs were assessed by *in vitro* morphological appearance, cell cycle analysis, bromodeoxyuridine (BrdU) incorporation, alkaline phosphatase (ALP) activity, mineralization behavior, gene expression of cementoblast phenotype and the tissue-regenerative capacity *in vivo*.

Material and methods

Apical tooth germ cell-conditioned medium

All experiments involving the use of animals were reviewed and approved by the Animal Care Committee of Fourth Military Medical University. The tooth germs were isolated as previously described (20), with some modifications. Eight-day postnatal Sprauge–Dawley rats were killed by cervical dislocation and their mandibles were aseptically removed and dissected free of adherent tissues. Twenty developing mandibular first molar germs were surgically isolated under a stereomicroscope (Leica MZ9.5; Leica Microsystèmes SA, Rueil-Malmaison, France) and placed in α-minimum essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 0.292 mg/mL glutamine (Invitrogen, Carlsbad, CA, USA), 100 μ/mL pencillin and 100 μg/mL streptomycin (Gibco BRL) and 100 μM/L ascorbic acid (Sigma, St Louis, MO, USA). A transection was made along the apical hard tissue margin of the enamel crown, and almost the entire Hertwig's epithelial root sheath with associated apical mesenchyme (dental follicle and dental papilla) was removed (Fig. 1). The apical portions of tooth germ were minced into < 1 mm^3 pieces and enzymatically treated in a mixture of Dispase I (0.3 U/mL; Gibco BRL) and type I collagenase (60 U/mL; Gibco BRL) for 1 h at 37°C. Cells were gently dissociated by trituration, then washed in α-MEM, pelleted and counted. Single-cell suspensions were washed again with α-MEM supplemented with 10% FBS, then placed into 75 cm^2 culture flasks (Costar, Cambridge, MA, USA) at 1×10^5 cells/mL and grown in a humidified

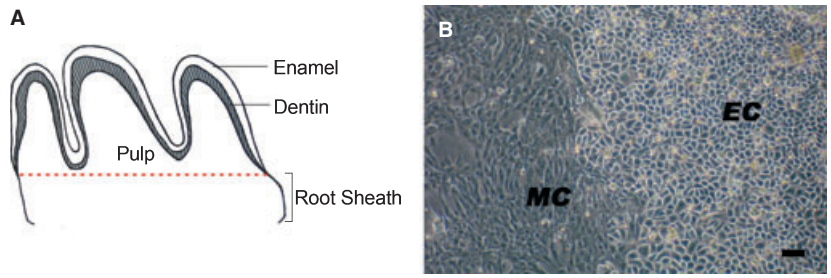

Fig. 1. Isolation and culture of APTGs. (A) Schematic diagram of the isolation of APTGs. The APTGs were dissociated along the apical margin of hard tissue (red dotted line). (B) Primary APTGs were heterogeneous, containing both cobblestone-like epithelial and spindle-shaped mesenchymal cell types. Scale bar respresents 100 μm. EC, epithelial cell; MC, mesenchymal cell.

atmosphere of 5% CO_2 and 95% air at 37°C.

The culture medium of primary apical tooth germ cells (APTGs) containing both epithelial and mesenchymal cells was changed every 48 h until full confluence. Three days after the last medium change, the media were collected and centrifuged at 2000 g for 15 min. Then the supernatants, which were mixed with an equal volume of fresh α-MEM supplemented with 10% FBS, were used as APTG-CM and stored at −20°C for PDLSCs culture. The experiment for APTGs culture was repeated 40 times for collecting the medium.

Isolation of PDLSCs

Periodontal ligament cultures were aseptically isolated and enriched as previously described (7,21,22). Briefly, normal human third molars ($n = 4$) extracted for orthodontic treatment purposes from three individuals (15–18 years of age) were collected after obtaining written informed consent. Periodontal ligament tissue was gently scraped from the surface of the middle part of the root, minced into 1 mm^3 cubes and placed into six-well culture dishes (Costar, Cambridge, MA, USA). The explants were grown in α-MEM supplemented with 10% FBS, 0.292 mg/mL glutamine, 100 μ/mL pencillin and 100 μg/mL streptomycin and 100 μM/L ascorbic acid. The cultures were maintained at 37°C in a humidified atomosphere of 5% CO_2, and 95% air, and cultures obtained from each donor were processed separately.

To isolate putative stem cells, subconfluent primary cultures of periodontal ligament cells were cloned using the limiting dilution technique as previously reported (7,23). Single-colony-derived strains were isolated and expanded to obtain homogeneous populations of PDLSCs. From the second passage, PDLSCs were grown in the presence or in the absence of APTG-CM, which was regularly changed every 48 h. The PDLSCs were observed and photographed under phase-contrast inverted microscopy (CK40-F200, Olympus Optical Co. Ltd, Tokyo, Japan) to evaluate their morphological appearance.

To assess colony-forming efficiency, subconfluent cultures (second passage) of stem cells were fixed with 70% ethanol and then stained with 0.1% Crystal Violet (Jiayuan Co., Hangzhou, China). Aggregates of over 50 cells were counted as a colony under microscopic observation. The stem cell nature of isolated cells was confirmed by immunostaining for monoclonal mouse anti-human STRO-1 antibody (MAB1038, R&D Systems, Inc., Minneapolis, MN, USA) and fluorescence-activated cell sorting (FACS) analysis for STRO-1 and CD146 (R&D Systems, Inc.) according to the manufacturer's protocol. Induction of calcification and adipogenesis were as previously reported (24,25). Mineral nodule formation was detected by staining the cultures with 2% Alizarin Red (Sigma). The adipogenic cultures were fixed in 70% ethanol for 15 min and stained with fresh Oil Red O solution (Sigma) for 2 h.

Cell cycle analysis

Following 14 days co-culture with APTG-CM, single-cell suspensions of PDLSCs were harvested by trypsin–ethylenediaminetetraacetic acid (EDTA) digestion and washed twice with phosphate-buffered saline (PBS). The cells were then fixed in cold 70% dehydrated alcohol at −4°C for 24 h. After washing again, cell suspensions were stained with propidium iodide (100 mg/mL; Sigma) at 4°C for 30 min, and subjected to Elite ESP flow cytometry (Beckman Coulter Inc., Fullerton, CA, USA) for cell cycle analysis. One million cells were counted per sample.

Detection of bromodeoxyuridine (BrdU) incorporation

Following 14 days co-culture with APTG-CM, single-cell suspensions of PDLSCs were seeded into 24-well plates at a density of 1×10^4 cells per well. After serum starvation-induced cell cycle synchrony, 10 μM BrdU (Sigma-Aldrich, St Louis, MO, USA) was added 4 h prior to fixation with 4% polyoxymethylene. Then BrdU incorporation in proliferating cells was revealed using immunostaining against anti-BrdU antibody (1:100 dilution; Boster Biotechnology, Wuhan, China). Cells containing densely brown-stained nuclei with clear morphology were considered BrdU positive, and they were counted in five fields per well (center and at 3, 6, 9 and 12 o'clock). Results are expressed as the percentage of BrdU-positive cells out of total cells counted.

Alkaline phosphatase activity assay

For quantitative analysis of ALP activity, single-cell suspensions of PDLSCs were seeded at a density of 1×10^3 cells per well into 96-well plates. After 4, 8, 12 and 16 days of co-culture with APTG-CM, the ALP activity of PDLSCs was detected with a commercially available assay kit (Zhongsheng Co., Beijing, China). Briefly, cells were washed three times in PBS and incubated in Triton X-100 (3 ml/L in PBS) overnight at 4°C. One

hundred microlitres of *p*-nitrophenol phosphate substrate solution was added to each well, and the cells were incubated for 40 min at 37°C; then the reaction was stopped with 0.1 M NaOH. The optical density value was measured at 405 nm in a spectrophotometer using a microplate reader.

In vitro mineralization assay

The PDLSCs (1×10^5 per well) were initially cultured to confluence in α-MEM, containing 10% fetal bovine serum, in six-well culture dishes. Then, the samples were grown in differentiation conditions (α-MEM supplemented with 10% FBS, 100 μ/mL penicillin and 100 μg/mL streptomycin, 50 μg/mL of ascorbic acid and 2 mM sodium β-glycerophosphate), with or without APTG-CM. At day 14, day 21 or day 28, the samples were fixed with 4% polyoxymethylene for 0.5 h. Alizarin Red staining was performed to determine mineralization as previously reported (24). The mineralized nodules were imaged and analyzed with Leica Q-Win (Leica, Cambridge, UK) image analysis system.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The PDLSCs were cultured in APTG-CM for 21 days, and the total RNA was extracted from each sample using TRIzol reagents (Invitrogen Life Technologies) and then quantified. Two micrograms of total RNA was reverse transcribed with reverse transcriptase (Promega, WI, USA) according to the manufacturer's instructions. All PCR experiments were performed with *Taq* polymerase (Promega). The primers used were as follows: 5′-GAA CCACTTCCCCACCTTTT-3′ and 5′-TCTGACCATCATAGCCATCG-3′ for bone sialoprotein (BSP, GenBank accession no.: NM_004967); 5′-ATGAGAGCCCTCAGACTCCTC-3′ and 5′-CGGGCCGTAGAAGCGCC-GATA-3′ for osteocalcin (OCN, GenBank accession no.: NM_199173); 5′-ATGGGCACATCAAGCACTGA-3′ and 5′-CCCCATTAGTGTCATCC-TGC-3′ for cementum-derived protein (CP-23, GenBank accession no.: NM_001048212); 5′-AAGTACTGGC-GAGACCAAGC-3′ and 5′-AGAG-GGCCACGAAGGGGAACT-3′ for Alkaline phosphatase (ALP, GenBank accession no.: NM_000478); 5′-CTGACCTTCCTGCGCCTGATG-TCC-3′ and 5′-GTCTGGGGCACCA-ACGTCCAAGGG-3′ for collagen type I (COLI, GenBank accession no.: NM_000088); and 5′-CAGGCTGTG-CTATCCCTGTA-3′ and 5′-CATA-CCCCTCGTAGATGGGC-3′ for the internal quantitative control, β-actin (Actin, GenBank accession no.: NM_001101). The cycling conditions consisted of the initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 56°C for 30 s, and extension at 72°C for 1 min. The PCR products were separated and visualized on 1.5% agarose gel containing 5 g/L ethidium bromide.

In vivo differentiation assay

Five single-colony-derived PDLSC clones treated and untreated with APTG-CM were assessed for their potential to form mineralized matrix upon transplantation into five 6-week-old mice with severe combined immunodeficiency. For a single transplant, PDLSCs co-cultured with APTG-CM for 21 days were trypsinized, and approximately 2.0×10^6 cells were mixed with 40 mg of ceramic bovine bone (CBB; Research and Development Center for Tissue Engineering, Fourth Military Medical University, Xi'an, China) powders and then implanted into subcutaneous pockets on the backs of immunodeficient mice as described previously (26,27). As a control, the untreated PDLSCs from the same single-colony-derived clones were implanted into the other side of the same host. All procedures were performed under institutionally approved guidelines for the use of animals in research. The implants were recovered at 6 weeks post-transplantation, fixed with 4% paraformaldehyde, decalcified with buffered 10% EDTA (pH 8.0), and then processed for routine histological examination (hematoxylin and eosin staining).

Statistical analysis

Statistical significance was assessed by chi-squared test and independent samples *t*-test (student's unpaired *t*-test). The difference was considered to be statistically significant at $p < 0.05$. The SPSS 12.0 software package (SPSS Inc., Chicago, IL, USA) was used for the statistical tests.

Results

Isolation and characterization of PDLSCs

Previous study has demonstrated that human PDL-derived mesenchymal stem cells have the ability to form adherent clonogenic cell clusters of fibroblast-like cells, termed periodontal ligament stem cells (PDLSCs), similar to those observed for different mesenchymal stem cell populations (7). In the present study, using the limiting dilution technique, we isolated and purified putative clonogenic PDLSCs as previously reported (Fig. 2). The isolated PDLSCs were capable of forming adherent colonies, characteristic of other stromal stem cell populations, and majority of the cells retained their fibroblastic spindle shape (Fig. 3A–C). Crystal Violet staining revealed that 50–80% of PDLSCs possessed replicative potential. In most of the cases, cellular growth was maintained for four or more passages. With extended passaging, however, some of the culture dishes for the single strains exhibited the morphology characteristic of senescent cells and was discarded (Fig. 2F). *Ex vivo* expanded PDLSCs demonstrated positive immunostaining for early mesenchymal stem cell surface molecule STRO-1 (Fig. 3D), previously also found to be present in bone marrow stromal cells (BMSCs) and dental pulp stem cells (DPSCs) (28,29). Approximately 14.1% of the PDLSCs stained positive for STRO-1 and 88.4% for CD146 as assessed by flow cytometric analysis (Fig. 3G,H).

To investigate the differentiation potential of PDLSCs, multiple colony-derived PDLSCs at passage six were grown in the presence of 50 μg/mL L-ascorbate-2-phosphate, 10 nM dexa-

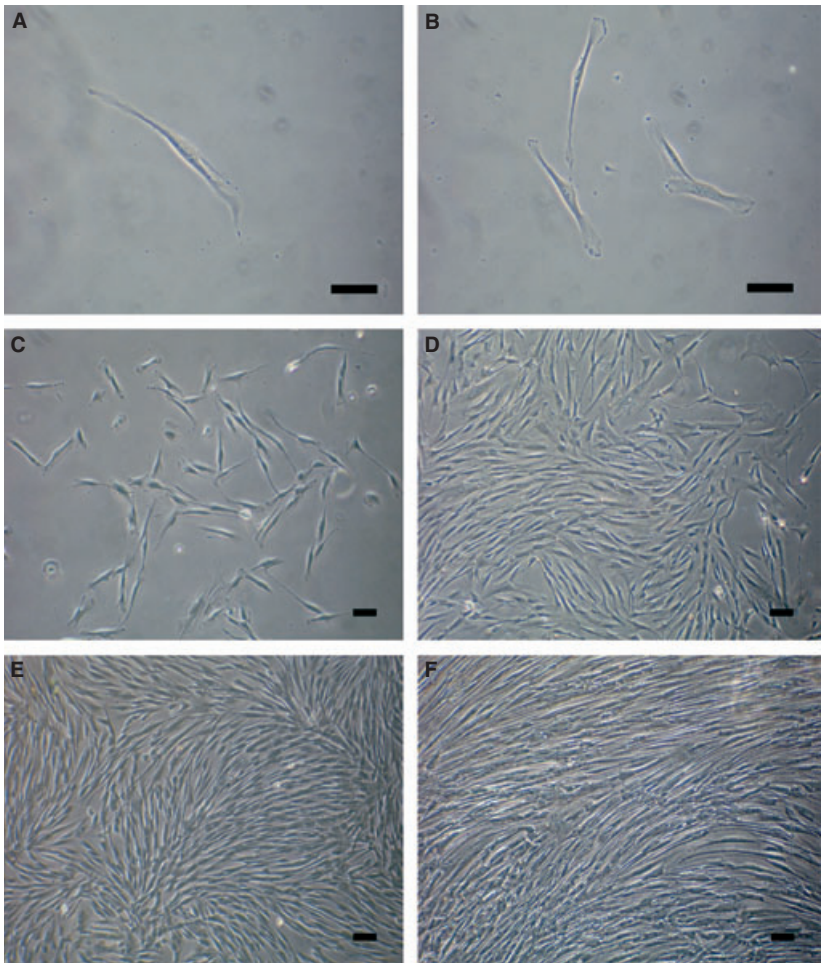

Fig. 2. Isolation and expansion of PDLSCs using the limiting dilution technique. (A) A single fibroblast 1 day after explantation. (B) At day 3, the colony consisted of four cells. (C) Part of the same colony at day 7; the colony consisted of about 50 cells. (D) Peripheral part of the same colony at day 14; the colony consisted of more than 1000 cells. (E) Some culture dishes were passaged and expanded to obtain homogeneous populations of PDLSCs. (F) With prolonged passaging, part of single strains of PDLSCs exhibited morphology characteristic of senescent cells and were discarded. Scale bar represents 100 μm.

methasone and 10 nM inorganic phosphate to induce mineralization *in vitro* as described previously. After 4 weeks of induction, cultured PDLSCs formed extensive amounts of Alizarin Red-positive mineral deposits throughout the adherent layers (Fig. 3E). Moreover, PDLSCs were capable of undergoing an adipogenic differentiation with an adipogenic-inductive cocktail (Fig. 3F).

Morphological appearance and proliferation activity of PDLSCs co-cultured with APTG-CM

After co-culturing with APTG-CM for 3 weeks, changes in cell morphology of confluent monolayer PDLSCs cultures were evaluated using an inverted light microscope (Olympus Optical Co. Ltd, Japan). Figure 4 shows representative phase-contrast micrographs of human PDLSCs in standard and inducing monolayer culture conditions. It is evident that cells in standard medium did not change their characteristic spindle-shaped morphology (Fig. 4-A,B). However, in the co-culture group, some cells lost their initially fibroblastic spindle morphology and exhibited shorter spindle or round shapes, resembling cemento/osteoblasts, after 3 weeks of co-culture (Fig. 4C). Moreover, the proportion of these round cells continued to grow with culture time. In some co-culture groups, round cells even constituted the majority of the whole population after five weeks. Throughout the entire culture period, neither of the standard cultures showed transition from spindle-shaped fibroblastic cells to round cells.

To determine the effect of APTG-CM on the proliferation activity of PDLSCs, cell cycle analysis and BrdU incorporation were performed to investigate whether APTG-CM could affect cell proliferation *in vitro*. As shown in Fig. 5, PDLSCs co-cultured with APTG-CM presented a significantly higher percentage of cells in S (24.2%) and G2M phases (7.5%) and a lower percentage of cells in G0G1 phase (68.3%) compared with untreated cells ($\chi^2 = 18.02$, $P < 0.01$), suggesting that APTG-CM may exert a stimulating effect on the proliferation of PDLSCs (Fig. 5A,B). The result of BrdU incorporation, which is representative of a significant proportion of cells in S-phase, showed that there were more BrdU-positive cells in the APTG-CM-treated group than in the untreated group (Fig. 5C), further confirming that APTG-CM could enhance the proliferation of PDLSCs. In general, during the entire co-culture period, PDLSCs cultured with APTG-CM underwent a dramatic change in cellular morphology and proliferation activity at the same time.

Alkaline phosphatase activity and mineralization behavior of PDLSCs co-cultured with APTG-CM

It is known that ALP represents an early marker for dental mesenchymal cell differentiation towards the cemento/osteoblastic phenotype (30). In the present study, the ALP activity of PDLSCs treated with or without APTG-CM was measured and resulted in considerably higher levels in the co-culture group. This effect of APTG-CM on PDLSCs increased with culture time during the 16 day culture period. As shown in Fig. 6, ALP activity in induced cultures was steadily elevated by day 4, and continued to climb until peaking at day 12, indicating that

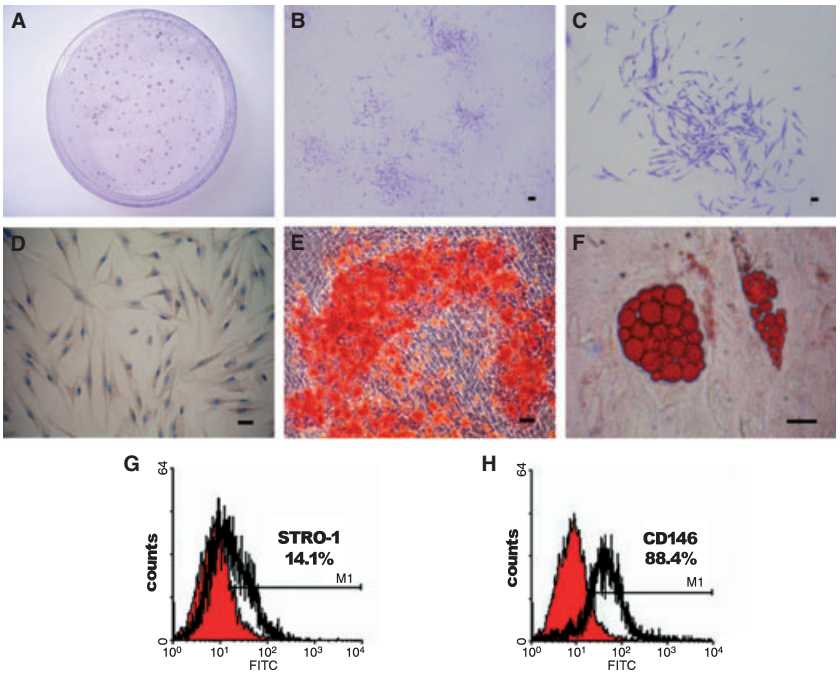

Fig. 3. Characteristics of adult human PDLSCs. (A–C) Stained with Crystal Violet, the number of fibroblastic colony forming units was determined after 10 days. Cell morphology was typical of fibroblast-like cells. (D) Immunocytochemical staining showed that cultured PDLSCs expressed STRO-1. (E) When human PDLSCs were cultured in osteogenic inductive conditions containing L-ascorbate-2-phosphate, dexamethasone and inorganic phosphate for 4 weeks, mineralized nodules were shown by Alizarin Red staining. (F) Cultured human PDLSCs formed lipid clusters that stained positive for Oil Red O following 4 weeks of adipogenic induction in the presence of 0.5 mM isobutylmethylxanthine, 0.5 mM hydrocortisone, 60 mM indomethacin and 10 mg/mL insulin. (G,H) Flow cytometric analysis of *ex vivo* expanded PDLSCs revealed expression of STRO-1 (14.1%) and CD146 (88.4%). Scale bar represents 100 μm, except in (B), where it represents 300 μm.

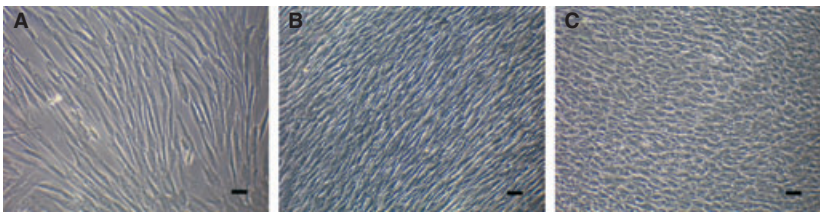

Fig. 4. Morphological changes of PDLSCs co-cultured with APTG-CM. (A) Untreated PDLSCs at day 3 exhibited characteristic spindle-shaped morphology. (B) After 3 weeks of standard culture, untreated PDLSCs showed no obvious morphological changes. (C) After 3 weeks of co-culture, some cells lost their initially fibroblastic spindle morphology and exhibited a shorter spindle or round shape, resembling cementoblast-like cells. Scale bar represents 100 μm.

co-cultured cells continually differentiate into hard tissue-forming cells. After that, ALP activity declined gradually between days 12 and 16, which might be correlated with increasing mineral deposition (Fig. 7).

The PDLSCs were also studied for their ability differentiate into cementoblast-like cells and to mineralize the extracellular matrix *in vitro* when they were cultured in the presence of APTG-CM. Cells grown in control medium with ascorbic acid and sodium β-glycerophosphate showed minimal mineralized nodules throughout the culture period as measured by Alizarin Red staining. By contrast, differentiation conditions with APTG-CM, ascorbic acid and β-glycerophosphate caused a statistically significant increase in mineralized nodule formation. Figure 7 graphically illustrates that less calcium was detected at any time in control cultures; however, cultures treated with APTG-CM showed a heavily deposited mineralized matrix between days 21 and 28. Moreover, this mineralization pattern was distributed in a uniform manner throughout the culture dish (data not show). Taken together, our results indicate that APTG-CM can enhance ALP activity of PDLSCs; meanwhile, the absence of mineral in cultures grown in control medium further supports the possibility that APTG-CM can promote the differentiation of PDLSCs into mineralized tissue-forming cells, such as cementoblast-like cells.

Gene expression of PDLSCs co-cultured with APTG-CM

To investigate the effect of APTG-CM on the expression of genes responsible for differentiation of PDLSCs, RT-PCR analysis was performed. The PDLSCs in control medium showed high expression of COLI and ALP mRNA, but no BSP and OCN mRNA, which are known usually to be expressed in cementoblasts (31,32). After 21 days co-culture with APTG-CM, high expression of BSP and OCN mRNA could be detected in induced cells (Fig. 8). This indicates that APTG-CM can stimulate the differentiation of PDLSCs by promoting the expression of BSP and OCN transcripts. As a local regulator of cementoblast differentiation, CP-23 was found in mature cementum, cementoblasts and periodontal ligament cells but not osteoblasts (33). In this study, the expression levels of mRNA for CP-23 were the same in treated and untreated groups (Fig. 8). The different mRNA expression patterns of bone-related proteins suggest that PDLSCs co-cultured with APTG-

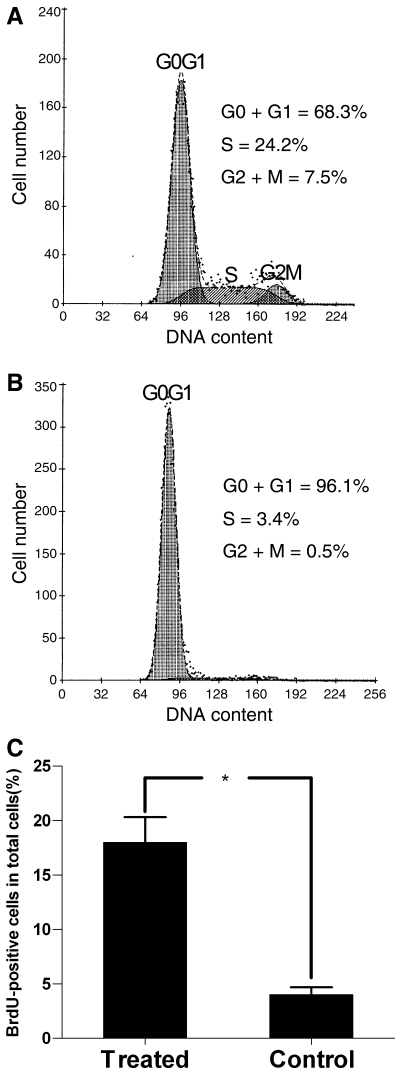

Fig. 5. Cell proliferation of PDLSCs co-cultured with APTG-CM. (A) Representative cell cycle distributions of treated PDLSCs. More induced PDLSCs appeared to escape G0G1 arrest by shifting to S (24.2%) and G2M phases (7.5%). (B) Representative cell cycle distributions of untreated PDLSCs; 96.1% of the cells were arrested in G0G1 phase. (C) BrdU incorporation assay showed that the percentage of BrdU-positive cells in the experimental group was higher than that in the control group; $*p < 0.05$ vs. untreated group.

CM present some molecular properties of cementoblast-like cells.

Differentiation of PDLSCs *in vivo* co-cultured with APTG-CM

It has been demonstrated that PDLSCs were able to form cementum/periodontal ligament-like tissues upon *in vivo* transplantation (7–9). To investigate the capacity of differentiation of PDLSCs co-cultured with APTG-CM, an *in vivo* differentiation assay was performed using immunodeficient mice. This model is well established and it has been repeatedly demonstrated in this system that the mineralized matrix formed by transplanted cells is of donor origin (9,34–36). Thus, this *in vivo* assay offers an excellent screening tool for testing the commitment of transplanted cells towards a particular phenotype. Six weeks after transplantation, the induced PDLSC implants generated cementum-like mineralized tissues lining the CBB surfaces and PDL-like fibrous tissues interfaced with the newly formed cementum-like deposits (Fig. 9A); the mineralized structure resembled cellular cementum with cementocyte-like cells embedded in the matrix (Fig. 9B). Significantly, large collagen bundles were inserted perpendicularly into cementum-like tissues (Fig. 9C, arrows), mimicking the physiological attachment of natural Sharpey's fibers in the periodontal ligament. In contrast, untreated PDLSC transplants seldom formed cementum/PDL-like tissues (Fig. 9D,E). Of five selected single-colony strains of PDLSCs, all treated colonies showed a capacity to generate cementum/PDL-like structures, while out of the corresponding untreated colonies, only one colony formed small amounts of cementum-like tissues within the transplants (Fig. 9D) and the remaining four did not form cementum/PDL-like structures *in vivo* (Fig. 9E). No mineralized or PDL-like tissues were observed within the CBB alone (Fig. 9F).

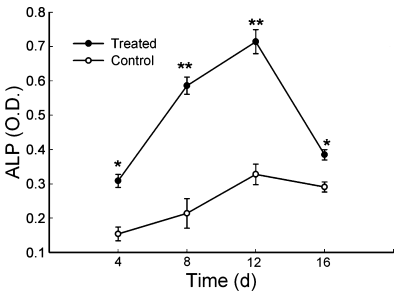

Fig. 6. Alkaline phosphatase (ALP) activity of PDLSCs co-cultured with APTG-CM. Note that the ALP activity in the treated group was threefold higher than in the control group at day 8. Data are expressed as means ± SD ($n = 6$); $*p < 0.05$, $**p < 0.01$ vs. untreated group.

Discussion

Adult stem cells require a special environment and are therefore usually found in the so-called stem cell niche (37). It is known that in normal homeostasis, differentiation of the stem cells is most likely to be triggered by instructive and stimulatory signals provided by the local environment (38,39). The unpredictability of current available regeneration techniques, especially on new cementum and attachment formation, is attributed to the lack of an optimal microenvironment in some fashion (6). Although in the last few years, PDLSCs have been isolated, cultured and characterized *in vitro*, the stem cell niches, which regulate differentiation of PDLSCs, are only beginning to be understood (40). At present, the origin of PDLSCs is still not clear. Ten Cate has postulated that dental follicle precursors remain in adult PDL. During periodontal development, the dental follicle compartment is progressively replaced by PDL (41). Recent studies show that dental follicle compartments exhibit properties similar to the mesenchymal progenitor cells present in adult PDL, suggesting that PDLSCs may be derived from dental follicles (42). During tooth development, the periodontium is formed by the apical mesenchyme (dental follicle and dental papilla) after the completion of crown formation, while the inner and outer enamel epithelia extend apically to form the bilayered Hertwig's epithelial root sheath. It has been claimed that epithelial–mesenchymal interactions which take place between Hertwig's epithelial root sheath and underlying root mesenchyme seem to play an important role in root/periodontal tissue development (15). Accordingly, the similarity between periodontal regeneration during wound healing

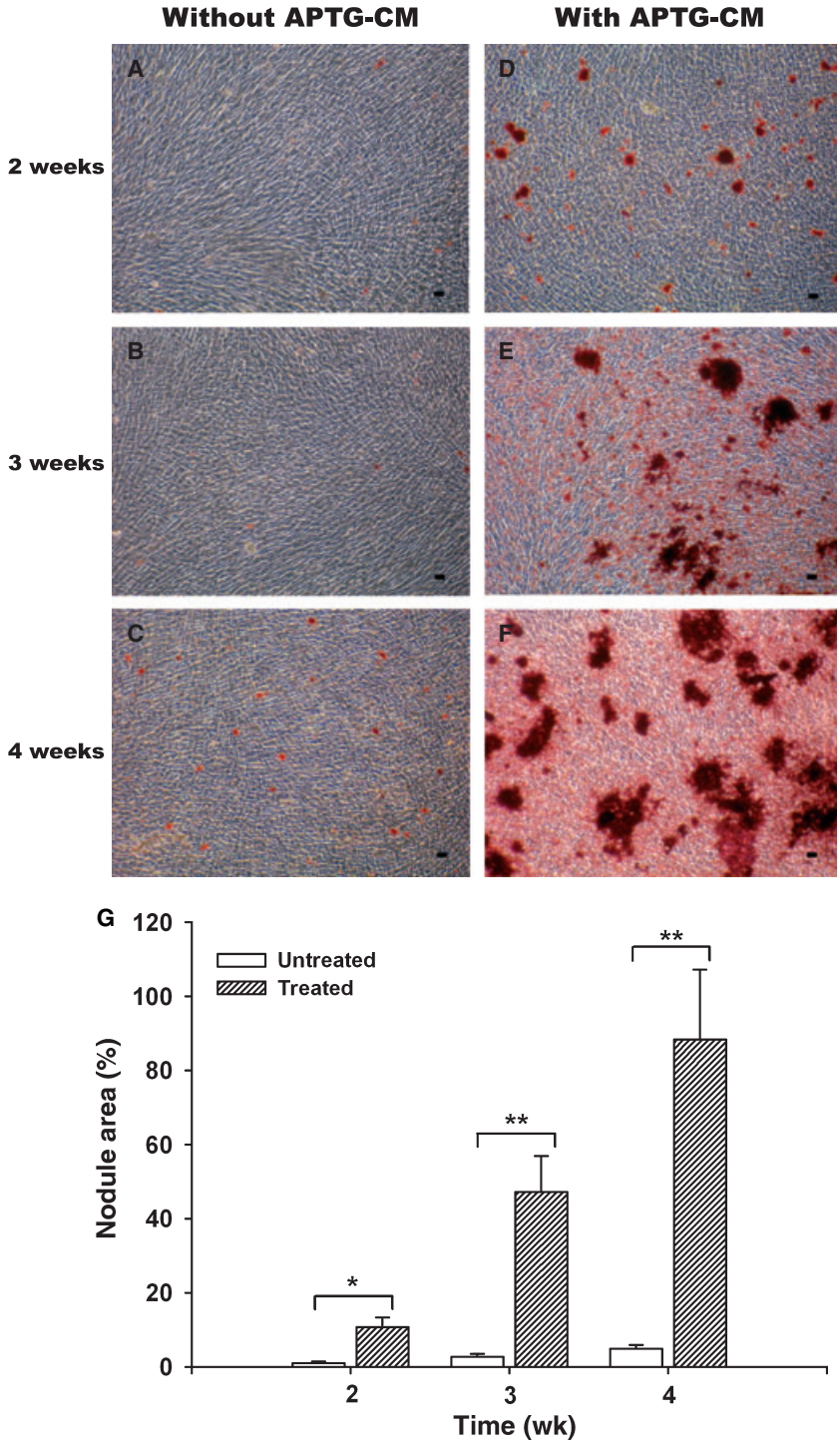

Fig. 7. Mineralization potential of PDLSCs co-cultured with APTG-CM. (A–C) The PDLSCs grown in differentiation conditions (α-MEM supplemented with 10% FBS, 100 μ/mL penicillin and 100 μg/ml streptomycin, 50 μg/mL of ascorbic acid and 2 mM sodium β-glycerophosphate) without APTG-CM hardly deposited any mineralized matrix throughout the culture period as measured by Alizarin Red staining. (D–F) Cultures treated with APTG-CM showed acceleration in the formation of mineral at different time points of culture. (G) Mean ± SD nodule area was significantly higher in cultures exposed to APTG-CM; $*p < 0.05$, $**p < 0.01$ vs. untreated group. Scale bars represent 100 μm.

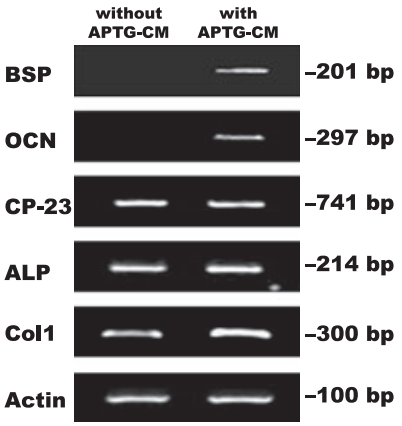

Fig. 8. Gene expression of PDLSCs co-cultured with APTG-CM. Osteocalcin (OCN), bone sialoprotein (BSP) and cementum-derived protein (CP-23) gene expression levels served as a marker for cementoblast differentiation. After 21 days co-culture with APTG-CM, there was high expression of OCN and BSP mRNA in induced PDLSCs, while untreated PDLSCs were lacking OCN and BSP expression.

and periodontal development may require that events during early cementogenesis be recapitulated (2). Thus, based on the principles of cell and developmental biology, it can be assumed that APTGs as the root/periodontal tissue development primordium may provide the 'periodontal niche' for PDLSCs.

To date, the exact mechanisms that regulate the differentiation of mesenchymal precursors into cementoblasts during periodontal development have not been elucidated. Although enamel matrix derivative (EMD), bone marrow stromal cells (BMP) and other growth factors have been proposed to mimic the inductive role of Hertwig's epithelial root sheath products (43,44), no defined molecular factor specifically inducing the cementoblastic phenotype is known. It is very difficult to mimic the cementogenic microenvironment using only a few growth factors, which are not likely to provide the complete repertoire of the molecules needed. Further, the combined effect of these molecules may be additive, synergistic or antagonistic, and the timing of their use may be critical (45). In fact, during the course of wound healing and periodontal development, the spectrum

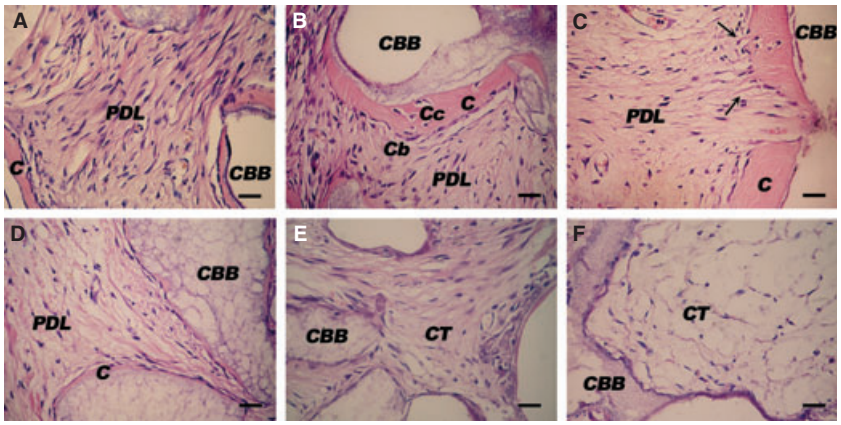

Fig. 9. Differentiation potential of PDLSCs *in vivo* co-cultured with APTG-CM. (A) The PDLSCs co-cultured with APTG-CM showed the tissue-regenerative capacity to produce cementum-like mineralized deposits (*C*) on the surface of the ceramic bovine bone (*CBB*) powders and PDL-like collagen fibers (*PDL*) connecting with the newly formed cementum-like tissues. (B) It is clear that there are cementoblast-like cells (*Cb*) at the cementum–PDL-like interface and cementocyte-like cells (*Cc*) embedded within the mineralized matrix. (C) Collagen bundles (arrows) were inserted into cementum-like tissues, similar to the physiological attachment of natural Sharpey's fibers in normal periodontal ligament. (D,E) Untreated PDLSCs transplants seldom formed cementum/PDL-like structures; only one colony formed small amounts of cementum-like tissues lining the CBB surfaces. (F) No mineralized or PDL-like tissues were observed within the CBB alone. Scale bars represent 100 μm. *CT*, connective tissue.

and concentration of molecules change continuously. A great number of substances, which include growth factors, other soluble mediators and extracellular matrix components, are present in the local microenviroment (43). This may to some extent explain why regeneration of cementum is not always predictable for the available regenerative procedures. Thus, in this study, to create the optimal niche of PDLSCs differentiation, we used APTG-CM, which is likely to contain multiple molecular signals or growth factors necessary for PDLSCs proliferation and differentiation, to induce PDLSCs towards the cementoblast phenotype. Also, as expected, the induced PDLSCs exhibited several crucial characteristics of cementoblast-like cells.

First, the morphological changes from spindle-shaped fibroblasts towards round cells could be observed in monolayer PDLSCs cultures treated with APTG-CM. This shorter spindle or round appearance is similar to what is seen with cemento/osteoblasts. While morphology alone is not necessarily indicative of phenotype, it does distinguish these cells from untreated fibroblastic cells, which do not undergo such differentiation in morphology and tend to maintain an elongated shape even when very confluent. Second, analysis by flow cytometry and BrdU incorporation showed that many PDLSCs tend to escape G0G1 arrest and traverse the cell cycle in the presence of APTG-CM. This result is consistent with a previous report from Yokokoji (46), in which it was shown that cementum matrix may permit human fibroblasts to escape cell cycle arrest and complete cell division and differentiation, and indicates that APTG-CM may provide a proper microenvironment which contains multiple molecular signals necessary for the proliferation and differentiation of PDLSCs. In parallel with determining the effects of APTG-CM on morphological appearance and cell proliferation, our study also provided evidence that PDLSCs exposed to APTG-CM showed high ALP activity, calcified nodule formation and the expression of mineralization-related genes, including genes for COLI, ALP, OCN, BSP and CP-23. In contrast, untreated cells showed low ALP and mineralization activity, and did not express OCN and BSP. The above characteristics of induced cells are essentially identical to those of cementoblasts. Using immunocytochemistry and *in situ* hybridization, D'Errico *et al*. demonstrated that OCN and BSP are selectively expressed by root-lining cells (47). It should be noted that expression of OCN and BSP is highly specific for mineralizing tissues. Besides cementum, bone, mineralizing cartilage, dentine and even ameloblasts also express OCN and BSP, although they have been implicated as markers that are specific for mature differentiation of cementoblasts (48–50). Recently, a novel human cementum-derived protein (CP-23), which has once been reported as a local regulator of cementoblast differentiation and cementoid matrix mineralization (33), has been proposed to be a marker of cementum or cementum/periodontal ligament (51). In this study, both co-cultured cells and control cells expressed CP-23 mRNA. Therefore, we believe that APTG-CM promoted cementoblastic differentiation.

The results of *in vivo* transplantation provided direct evidence that co-cultured PDLSCs possess the tissue-regenerative capacity to produce cementum/PDL-like structures, characterized by a layer of cementum-like mineralized tissues and associated PDL-like collagen fibers connecting with the newly formed cementum-like deposits. It is generally believed that mesenchymal stem cells are inclined to develop tissues compatible with their origin in an ectopic site. Many experiments have demonstrated that in conditions devoid of any influence derived from relevant cell types and matrix normally present in bone organ or in periodontium *in situ*, bone marrow stromal cells (BMSCs) and PDLSCs recapitulate most of the basic features of the respective tissue formation when transplanted into immunodeficient mice (7,9,34). There are no exceptions in the present study. While the absence of a suitable marker for cementum hampers the precise identification of the mineralized tissues as cementum, mRNA expression of markers of

cementum tissue (OCN, BSP and CP 23), together with several histological features attributed to cementum, such as mineralized matrix with irregular inserted collagen bundles like Sharpey's fibers, lack of hematopoietic tissues and relatively low cellularity, strongly implies that this tissue is of a periodontal nature (7,9,36,52). Compared with the above observation, deposition of cementum-like structures was hardly detected within the untreated PDLSCs transplants. Previous reports have described that PDLSCs had a heterogeneous nature and about 60% of single-colony-derived PDLSCs clones possessed a capacity to generate cementum/PDL-like structures with variable amounts of cementum, which could range from a total absence of any cementogenesis to levels comparable with those in multi-colony-derived populations (7,8). Similarly, in the present study, of five selected single-colony strains of PDLSCs, only one colony formed small amounts of cementum-like structures in the control transplants, suggesting that the putative PDLSCs remained constrained within the periodontal tissues. Although five single-colony strains of PDLSCs represent a limited number of cultures, the marked difference between treated and untreated PDLSCs transplants is a somewhat unexpected result. It has been generally recognized that the periodontal ligament contains heterogeneous fibroblastic populations and harbors progenitor cells that can differentiate into periodontal ligament fibroblasts, osteoblasts and cementoblasts. The evidence supporting this notion came exclusively from large numbers of *in vitro* and *in vivo* studies (53–55). If our findings in the present study prove to be valid through more extended research, then the following explanations may be possible.

First, there are committed osteogenic/cementogenic precursors present in normal PDL, while prolonged passaging during the course of clone selection (over 35 days) may result in the loss of such commitment. A recent study from Mohyeddin Bonab *et al*. indicates that mesenchymal stem cells (MSCs) enter senescence almost undetectably from the moment of *in vitro* culturing and lose their multipotential nature with a long period of culture (56). Vacanti *et al*. also showed that the phenotype of porcine MSCs can be dramatically altered by prolonged passaging and, at the same time, the adipogenic potential of long-term MSC cultures was greatly enhanced at the expense of the osteochondrogenic potential (57). In the present study, a similar phenomenon was observed. With extended passaging, some of the culture dishes derived from the cell strains presented he signs of senescence and exhibited a longer spindle-shaped fibroblastic appearance (Fig. 1F), which is distinct from the round or shorter spindle shape of cementoblasts. Therefore, it is likely that committed cementogenic precursors may be under negative regulation by other fibroblastic populations which lose the cementogenic commitment in long-term culture and receive a suppressive influence *in vivo*. In contrast, APTG-CM successfully provided a microenvironment with multiple molecular signals necessary for the proliferation and differentiation of PDLSCs and maximally avoided the loss of cementogenic commitment. Second, the putative PDLSCs in normal conditions may not be committed, but may require an inducing stimulus (e.g. enamel matrix derivative, EMDs) and will form cementum-like tissues only when this stimulus is applied. As far as the present study is concerned, APTG-CM served this role.

Our findings may have some important implications for developing new strategies aimed at periodontal regeneration via PDLSCs transplantation-based therapies. First, the fact that PDLSCs can be induced to a cementoblastic phenotype and form cementum/PDL-like tissues offers the possibility for developing a more radical treatment of cases where there has been a massive loss of cementum *in situ* as well as adjacent soft tissues owing to periodontal disease. The application of growth factors with apparent cementum-promoting activities, such as enamel matrix derivative (EMD), seems more logical in cases with only a partial cementum loss. In principle, cementogenic cells can be isolated from small fragments of root cementum, expanded *in vitro* and subsequently retransplanted *in vivo* (58,59). However, this approach has a limitation because of the requirement that healthy teeth be extracted for the cultures to be established. Also, it remains unclear whether cementogenic cells can be reproducibly obtained in this way, from teeth of aged and/or diseased patients. Therefore, cementum may not be a practical cell source. Such a scenario would undoubtedly be more feasible if cells that can be induced towards cementoblastic differentiation could be obtained from sources other than cementum. Thus, PDLSCs that contain a pool of less differentiated (or even non-committed) cementogenic precursors are the most likely candidates in this setting. Second, at present, no defined molecular factor specifically inducing the cementoblastic phenotype is known. We hope the combined *in vitro*/*in vivo* model system developed in present study (i.e. the APTG-CM co-culture system) could be an alternative to establish a microenvironment in which PDLSCs would differentiate into cells committed to formation of cementum. As to the sources of tooth germ cells, recent studies have shown that human periodontal ligament cells were able to contribute to periodontal tissue repair in an immunocompromised rat model (60); combined with the findings in the present study, it is feasible to obtain tooth germ cells from sources other than humans, i.e. rats or pigs.

In summary, the lines of evidence accumulated here suggest that APTG-CM, which includes multiple signals and molecules, is able to create a highly cementogenic microenvironment and induce differentiation of PDLSCs along the cementoblastic lineage. This may have general implications for periodontal engineering. Further studies are required to address the underlying mechanisms involved in the APTG-CM and PDLSCs-mediated cementogenesis.

Acknowledgements

This work was supported by grants from the Nature Science Foundation

of China (project nos 30572046 and 30725042). The authors acknowledge the kind assistance rendered by the staff of the Research and Development Center for Tissue Engineering, Fourth Military Medical University.

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