# The differentiation potential of gingival mesenchymal stem cells induced by apical tooth germ cell-conditioned medium

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**Abstract.** Gingival-derived mesenchymal stem cells (GMSCs) have recently been harvested; however, the use of GMSCs in periodontal tissue engineering requires further study. The present study established an indirect co-culture system between rat apical tooth germ-conditioned medium (APTG-CM) and GMSCs, in order to determine the effects on periodontal tissue differentiation *in vitro* and *in vivo*. Using the limiting dilution technique, single-colony derived human GMSCs and periodontal ligament stem cells (PDLSCs) were isolated and expanded to obtain homogeneous populations. PDLSCs were used as a positive control group. Cell cycle distribution, alkaline phosphatase (ALP) activity, mineralization behavior, expression of genes associated with a cementoblast phenotype (osteocalcin, bone sialoprotein, ALP, type I collagen, cementum-derived protein 23), and *in vivo* differentiation capacities of GMSCs/PDLSCs co-cultured with APTG-CM were evaluated. Flow cytometry indicated that GMSCs and PDLSCs were positive for STRO-1 and CD105, whereas CD45 expression was negative. The cell types were capable of forming colonies, and of osteogenic and adipogenic differentiation in response to appropriate stimuli. The induced GMSCs and PDLSCs exhibited numerous characteristics associated with cementoblast lineages, as indicated by increased proliferation and ALP activity, and upregulated expression of cementum-associated genes *in vitro*. *In vivo*, cementum/periodontal ligament-like structures were shown to form along the dentin surface and ceramic bovine bone in GMSCs and PDLSCs induced by APTG-CM group. Conversely, vertical fibers could not insert in the control group, which was not co-cultured with APTG-CM. In conclusion, GMSCs are likely to have a role in periodontal tissue regeneration. In addition, APTG-CM was able to provide a cementogenic microenvironment and promote differentiation of GMSCs along the cementoblastic lineage.

## Introduction

Periodontitis is a common inflammatory disease, which induces destruction of the tooth-supporting apparatus and may lead to tooth loss. However, current or emerging treatment options for periodontitis have limited and variable outcomes, or have yet to be developed for clinical use (1). The use of tissue engineering may be a possible therapeutic strategy for periodontal regeneration (2).

Periodontal ligament stem cells (PDLSCs) are effective and readily available cells for periodontal regeneration (3). However, due to the limitations in acquiring these cells, their application in clinical therapy is limited. The gingiva, which is an important component of periodontal tissue, exhibits promising regenerative ability and is able to rapidly repair injury. In addition, almost no scars remain after healing (4), it is available in large quantities and is easy to acquire. Previous studies have identified the presence of mesenchymal stem cells (MSCs) in human gingival connective tissue, and have reported on their morphological and functional characteristics (4-6). Therefore, gingival-derived MSCs (GMSCs) have recently received increased attention (5,7,8).

Cell sheet technology refers to a scaffold-free tissue reconstruction technique. Based on a unique temperature responsive culture dish, cultured cells can be non-invasively harvested as intact contiguous sheets along with their deposited extracellular matrix (9,10). Cell sheets may be used to avoid unforeseeable complications, including biomaterial degradation, inflammation and limited cell-seeding efficiency (9,10). It has the natural reaggregation potential of monodispersed cells and the advantage of an affluent natural matrix (11-13).

It is well-known that tooth generation depends on the interaction between dental epithelium and MSCs (14); this interaction is also important in root and periodontal tissue formation (15-17). Therefore, it has being suggested that the interaction between dental epithelium and MSCs may be important in periodontal regeneration. During tooth development, the periodontium is formed by the apical mesenchyme of the tooth germ, which is associated with the expression of several signaling molecules, including transforming growth

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factor-β, Notch-1, fibroblast growth factors or bone morphogenetic proteins (18-22).

The present study used apical tooth germ-conditioned medium (APTG-CM) to induce differentiation of GMSCs and PDLSCs; subsequently, transplant complexes containing dentin slices, ceramic bovine bone (CBB), and GMSC and PDLSC sheets were generated. The complex was subcutaneously transplanted into athymic mice for 8 weeks to observe periodontal regeneration.

**Materials and methods**

*Isolation and characterization of PDLSCs and GMSCs.* Gingival connective tissue samples were obtained from 10 healthy male donors (age, 15-25 years) at the Stomatology Hospital Affiliated to Tongji University (Shanghai, China). Under local anesthetic a ~2x2x1 mm sample of keratinized gingival tissue was obtained from the maxillary tuberosity. In addition, human periodontal ligaments were obtained from healthy premolars extracted from 10 male donors (age, 13-25 years) undergoing orthodontic treatment at the Stomatology Hospital Affiliated to Tongji University (Shanghai, China). Sample collection conformed to a protocol approved by the ethical authorities at the School of Stomatology, Tongji University (Shanghai, China) and all donors provided informed consent.

Human GMSCs and PDLSCs were isolated as previously described (3,6). Using the limiting dilution technique, single-colony derived human GMSCs and PDLSCs were isolated and expanded to obtain homogeneous populations. The cells were confirmed by fluorescence-activated cell sorting analysis using antibodies against CD45 (cat. no. FAB1430A), CD105 (cat. no. FAB10971P) and STRO-1 (cat. no. MAB1038; all obtained from R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. The two types of stem cell were stimulated with osteogenic conditioned medium [100 nM dexamethasone, 5 mM β-glycerophosphate, 50 mg/ml ascorbate phosphate and 5% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA)] and adipogenic conditioned medium [1 mM dexamethasone, 1 mg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and 5% FBS]. The conditions used for the induction of calcium accumulation and adipogenesis were the same as previously reported (12). Normal medium was used in the blank control group. Calcium accumulation was detected using 2% Alizarin Red (pH 4.2) staining (Sigma-Aldrich; Merck Millipore). Adipogenic cultures were stained with fresh Oil Red O solution (Sigma-Aldrich; Merck Millipore). Staining was observed by light microscopy (DX51; Olympus Corporation, Tokyo, Japan).

*APTG-CM and co-culture system.* All experiments involving the use of animals were reviewed and approved by the Animal Care Committee of Tongji University. Five-day postnatal Sprague-Dawley rats (n=30; male and female; weight, 10±2 g; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were bred in plastic cages (5 mice/cage) under standard laboratory conditions with a 12 h light/dark cycle, at 20°C with 48% humidity. Food and water were provided. The rats

were sacrificed by decapitation under isoflurane anesthesia. Developing mandibular first molar tooth germs were then dissected from the jaws. The apical portion of the tooth germ was separated as previously reported (14), and was cultured in alpha-minimum essential medium (alpha-MEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The primary APTG culture medium was changed every 48 h until 100% confluence for supernatant collection was reached. The cells were then centrifuged at 2,000 x *g* for 15 min at 4°C. The supernatants were mixed with an equal volume of fresh alpha-MEM supplemented with 10% FBS and 50 μg/ml ascorbic phosphate (Sigma-Aldrich; Merck Millipore), which was used as APTG-CM for PDLSCs and GMSCs co-culture. Alpha-MEM supplemented with 10% FBS and 50 μg/ml ascorbic phosphate was used as a control medium.

*Cell cycle analysis.* Following 10 days of co-culture at 37°C with APTG-CM, single-cell suspensions of GMSCs and PDLSCs were harvested and were washed twice with phosphate-buffered saline (PBS). Subsequently, the cells were fixed in cold 70% dehydrated alcohol at 4°C for 24 h. After further washing, cell suspensions were treated with RNase A and then stained with propidium iodide (PI; Sigma-Aldrich; Merck Millipore) at 4°C for 30 min. The content of DNA was assayed by flow cytometry to confirm the cell cycle analysis and subjected to cell cycle analysis using MultiCycle software (Beckman Coulter, Inc., Brea, CA, USA. One million cells were counted per sample.

*Alkaline phosphatase (ALP) activity assay.* An ALP activity assay was performed to determine the influence of APTG-CM co-culture. Single-cell suspensions of GMSCs and PDLSCs were seeded at a density of $1x10^3$ cells/well in 96-well plates. After 3, 6, 9, 12 and 15 days of co-culture with APTG-CM, the ALP activity of GMSCs and PDLSCs was detected using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China.). The results were measured at 405 nm in a spectrophotometer using a multiplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.* The co-cultured GMSCs and PDLSCs, and the control cells, were harvested and RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The total RNA concentration was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA (1.0 mg) was reversed transcribed into cDNA using PrimeScript First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocols. Subsequently cDNA synthesis was conducted using a Superscript II First-strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.), and RT-qPCR was performed using a MyiQ2 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 sec, 56°C for 30 sec and extension at 72°C for 1 min. Relative quantities of mRNA were calculated using the $2^{-\Delta\Delta Cq}$ method (23) and normalized to housekeeping gene β-actin. The experiment was repeated ≥3 times. Sequences for the sense

Table I. Oligonucleotide primer sequences used in polymerase chain reaction.

| Gene | Primer | Sequence |
|---|---|---|
| Osteocalcin | Forward | 5'-GGGTCAGGAGGAGAATCGT-3' |
| | Reverse | 5'-GTGTCTTAGCAGGCAGGGA-3' |
| Bone sialoprotein | Forward | 5'-CTGCTTCCTCACTCCAGGAC-3' |
| | Reverse | 5'-ATTGAGAAAGCACAGGCCAT-3' |
| Alkaline phosphatase | Forward | 5'-GCCAAGAAAGCAGGAAAGTC-3' |
| | Reverse | 5'-GAGTACCAGTTGCGGTTCAC-3' |
| Type I collagen | Forward | 5'-CTGACCTTCCTGCGCCTGATGTCC-3' |
| | Reverse | 5'-GTCTGGGGCACCAACGTCCAAGGG-3' |
| Cementum-derived protein 23 | Forward | 5'-ATGGGCACATCAAGCACTGA-3' |
| | Reverse | 5'-CCCCATTAGTGTCATCCTGC-3' |
| β-actin | Forward | 5'-CAGGCTGTGCTATCCCTGTA-3' |
| | Reverse | 5'-CATACCCCTCGTAGATGGGC-3' |
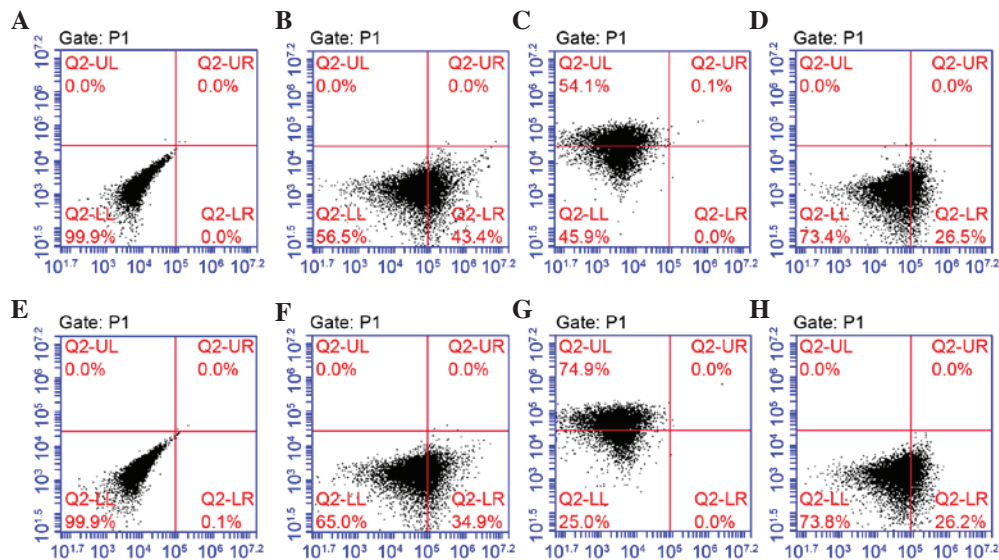


Figure 1. Flow cytometric analysis of gingival-derived mesenchymal stem cells (GMSCs) and periodontal ligament stem cells (PDLSCs). (A) Blank. (B) STRO-1, (C) CD105 and (D) CD45 markers identified in GMSCs. (E) Blank. (F) STRO-1, (G) CD105 and (H) CD45 markers identified in PDLSCs. STRO-1 and CD105 are surface epitopes of mesenchymal cells, and were positively expressed. CD45 is a surface epitope of hematopoietic stem cells, and was negatively expressed.

and anti-sense primers (Sangon Biotech Co., Ltd., Shanghai, China) used in the present study are listed in Table I.

*GMSC and PDLSC sheets for the regeneration of cementum/periodontal ligament (PDL)-like complexes in mice.* Single colony-derived GMSCs and PDLSCs wee cultured for 2 weeks to a density to form cell sheets, which were then detached from the bottom of the culture plates using a cell scraper. Cell sheets were used to wrap dentin slices (from root of tooth; 2x3x0.5 mm) and CBB (Research and Development Center for Tissue Engineering, Fourth Military Medical University, Xi'an, China) was placed on either side. Surgical sutures were used to fasten the complex, and transplanted into the subcutaneous tissue of the upper backs of six-week-old immunodeficient mice (n=30; weight, 20±2 g; Shanghai SLAC Laboratory Animal Co., Ltd.). A total of 30 complexes (10x3 groups) were implanted. Each immunodeficient mouse received one of three different complexes (APTG-CM GMSC sheet + dentin + CBB; GMSC sheet + dentin + CBB; and APTG-CM PDLSC sheet + dentin + CBB). The implants were recovered 8 weeks post-transplantation following decapitation under isoflurane anesthesia, were fixed in 4% paraformaldehyde for 2 days and were then decalcified for a further 28 days in 10% EDTA (pH 8.0), prior to embedding in paraffin. For histological analysis, 5-$\mu$m implant sections were prepared and stained with hematoxylin and eosin (H&E; Sigma-Aldrich; Merck Millipore), according to the manufacturer's protocol (24). Staining was evaluated by light microscopy (DX51; Olympus Corporation).

*Statistical analyses.* Data are presented as the mean ± standard deviation. Experiments were repeated three times. Statistical significance was analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by Tukey's test was used to determine the significant differences among the groups. P<0.05 was considered to
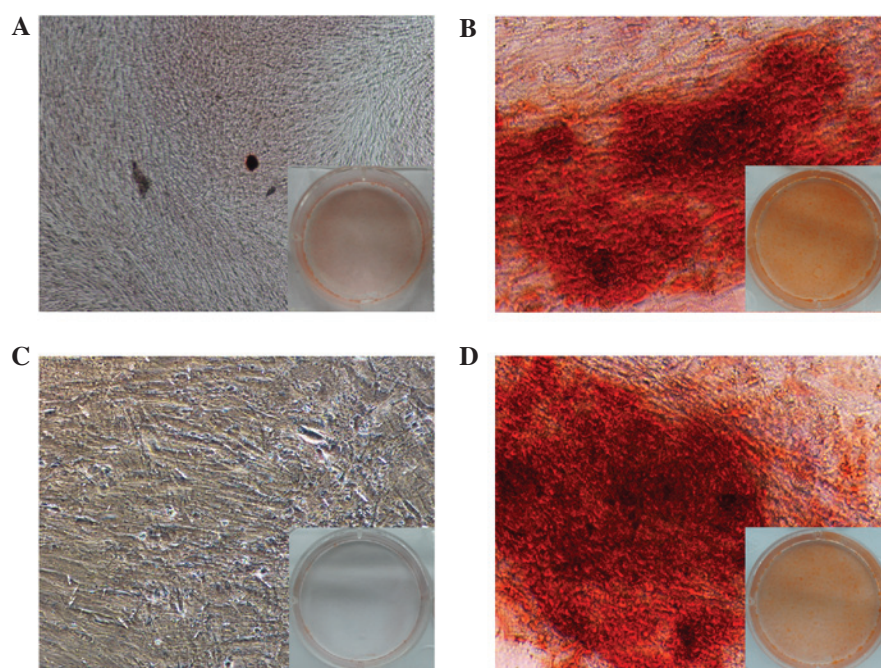
Figure 2. Osteogenic differentiation of (A and B) gingival-derived mesenchymal stem cells (GMSCs), and (C and D) periodontal ligament stem cells (PDLSCs). (A and C) Control groups; magnification, x40. (B and D) GMSCs and PDLSCs cultured in osteogenic induction conditions for 3 weeks, mineralized nodules were detected by Alizarin Red staining; magnification, x100.
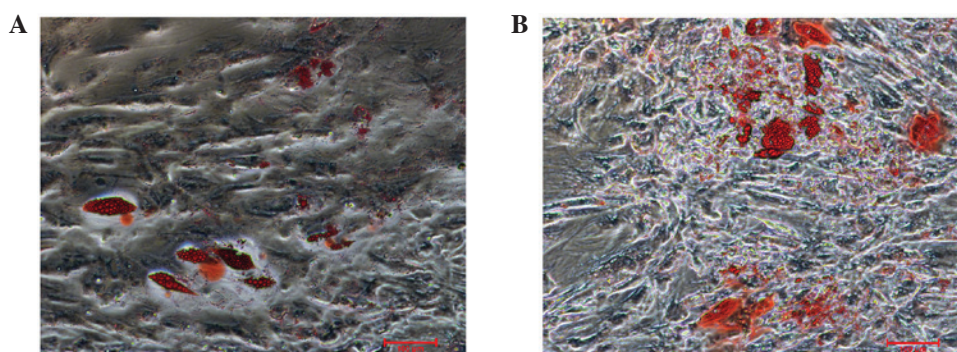


Figure 3. Adipogenic differentiation of (A) gingival-derived mesenchymal stem cells (GMSCs), and (B) periodontal ligament stem cells (PDLSCs); magnification, x100. Cultured GMSCs and PDLSCs formed lipid clusters that were positively stained for Oil Red O following 3 weeks of adipogenic differentiation.

indicate a statistically significant difference. All procedures were performed blinded.

## Results

*Characterization of PDLSCs and GMSCs.* Using the limiting dilution technique, putative clonogenic GMSCs and PDLSCs were isolated and purified. They were identified by the positive expression of STRO-1 and CD105, and negative expression of CD45 (Fig. 1). To investigate the differentiation potential of GMSCs and PDLSCs, the osteogenic and adipogenic potential of the cells was investigated. Osteogenesis was confirmed by the presence of red-stained mineral nodules (Fig. 2), and adipogenesis was confirmed by intracellular lipid droplet accumulation (Fig. 3).

*Cell cycle analysis of GMSCs and PDLSCs co-cultured with APTG-CM.* Cell cycle analysis was performed to investigate whether APTG-CM was able to affect cell proliferation *in vitro*. In GMSCs and PDLSCs co-cultured with APTG-CM a markedly higher percentage of cells were present in S and $G_2/M$ phases (GMSCs, 35.1%; PDLSCs, 26.3%) whereas a lower percentage of cells were present in $G_0/G_1$ phase (Fig. 4) compared with in the untreated cells. These results suggest that APTG-CM may exert a stimulating effect on GMSCs and PDLSCs proliferation.

*ALP activity assay.* ALP is an early marker of dental MSC differentiation towards the cementoblastic/osteoblastic phenotype (25). In the present study, ALP activity of GMSCs and PDLSCs cultured with or without APTG-CM was measured. ALP activity was markedly higher in the co-culture groups. The effects of APTG-CM on GMSCs and PDLSCs increased in a time-dependent manner, up to day 12, during the 15-day culture period. As presented in Fig. 5 (P<0.05), ALP activity had steadily increased in the co-culture group by day 3, and
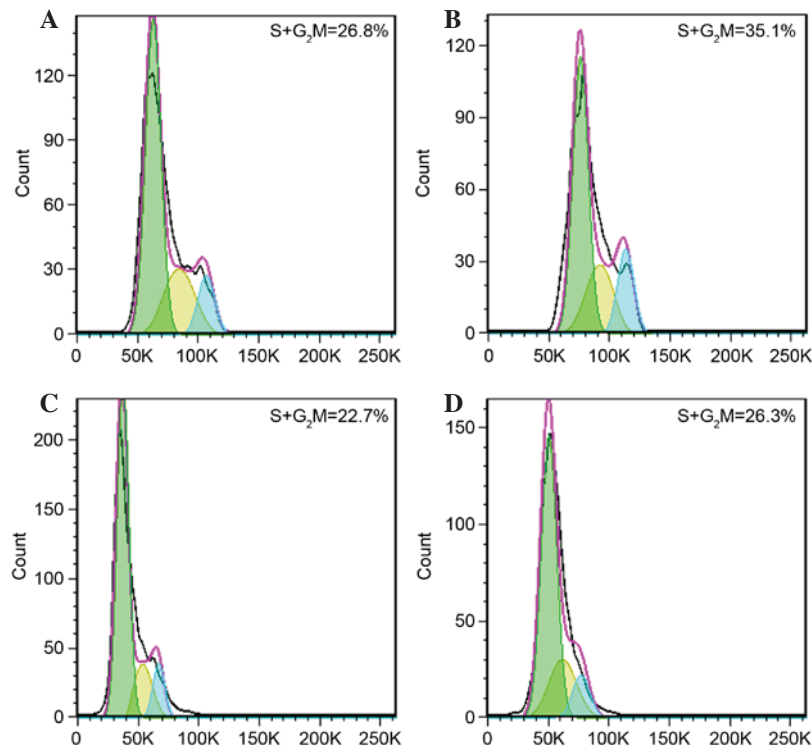
Figure 4. Cell cycle analysis of (A and B) gingival-derived mesenchymal stem cells (GMSCs) and (C and D) periodontal ligament stem cells (PDLSCs). (A and C) Representative cell cycle distribution of untreated GMSCs and PDLSCs. (B and D) Representative cell cycle distribution of GMSCs and PDLSCs co-cultured with apical tooth germ-conditioned medium (APTG-CM). APTG-CM-induced GMSCs and PDLSCs appeared to escape $G_0/G_1$ arrest and progressed to S and $G_2$/M phases.
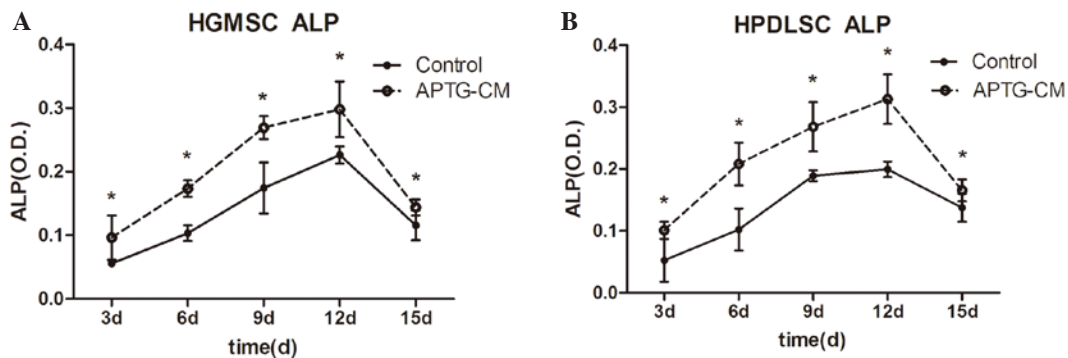


Figure 5. Alkaline phosphatase (ALP) activity of (A) gingival-derived mesenchymal stem cells (GMSCs) and (B) periodontal ligament stem cells (PDLSCs) co-cultured with apical tooth germ-conditioned medium (APTG-CM). ALP activity was increased in the APTG-CM groups compared with in the control group. *P<0.05. O.D, optical density.

continued to increase until it reached a peak at day 12, thus indicating that co-cultured cells continually differentiate into hard tissue-forming cells. After day 12, ALP activity gradually decreased until day 15, which may be correlated with increasing mineral deposition.

*Gene expression in GMSCs and PDLSCs co-cultured with APTG-CM.* RT-PCR analysis was conducted following co-culture of the two cell types with APTG-CM. The expression levels of bone- and extracellular matrix-associated genes (Table I) were upregulated in GMSCs (Fig. 6A) and PDLSCs (Fig. 6B) co-cultured with APTG-CM compared with cells in the control group (P<0.05). The mRNA expression patterns of bone-related genes suggest that GMSCs and PDLSCs

co-cultured with APTG-CM exhibit some molecular properties of cementoblast-like cells.

*Differentiation of GMSCs and PDLSCs following heterotopic transplantation.* A total of 30 complexes were harvested after 8 weeks of transplantation and evaluated by light microscopy following H&E staining (Fig. 7). In the experimental group (APTG-CM GMSC sheet + dentin + CBB), a cementum/PDL-like structure was regenerated on dentin surfaces, and the collagen content and orientation of PDL-like tissues were markedly obvious (Fig. 7B and C); similar characteristics were observed in the positive control group (APTG-CM PDLSC sheet + dentin + CBB; Fig. 7D and E). Furthermore, distinctive dental cementum-like matrices were
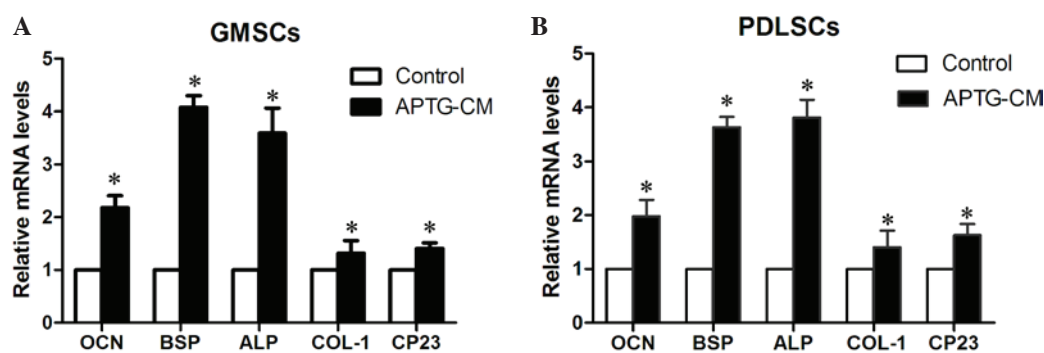
Figure 6. Bone-associated and extracellular matrix-associated gene expression was detected using reverse transcription-quantitative polymerase chain reaction before and after co-culturing gingival-derived mesenchymal stem cells (GMSCs) and periodontal ligament stem cells (PDLSCs) with apical tooth germ-conditioned medium (APTG-CM). The results revealed that (A) GMSCs and (B) PDLSCs exhibited upregulated osteocalcin (OCN), bone sialoprotein (BSP), alkaline phosphatase (ALP), type 1 collagen (COL-1) and cementum-derived protein 23 (CP23) expression following APTG-CM co-culture (n=9 each). $^*$P<0.05 vs. the control.
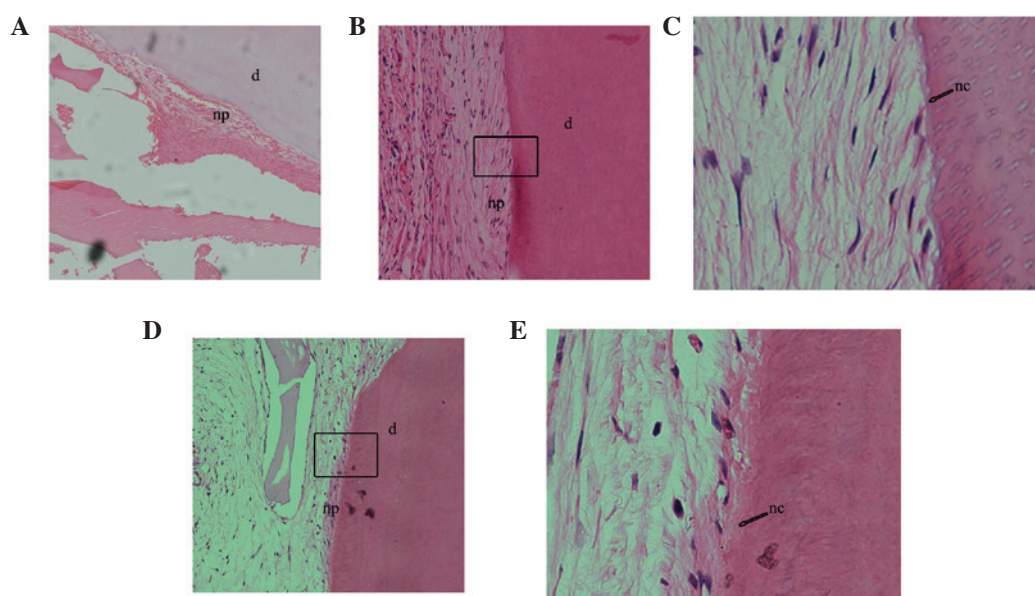


Figure 7. Following subcutaneous transplantation for 8 weeks, the differentiation potential of gingival-derived mesenchymal stem cells (GMSCs) and periodontal ligament stem cells (PDLSCs) co-cultured with apical tooth germ-conditioned medium (APTG-CM) were determined in vivo after hematoxylin and eosin staining. (A) Non-co-cultured GMSCs formed a small amount of periodontal ligament (PDL)-like tissue but no cementum-like deposits; magnification, x100. (B) GMSCs co-cultured with APTG-CM exhibited tissue-regenerative capacity, were able to produce cementum-like mineralized deposits on the surface of dentin slices, and PDL-like collagen fibers connected with the newly formed cementum-like tissues; magnification, x100x. (C) Magnification of the rectangular area in (B) (magnification, x400). (D) PDLSCs co-cultured with APTG-CM group also formed cementum and PDL-like structures; magnification, x100. (E) Magnification of the rectangular area in (D) (magnification, x400). np, new PDL-like collagen fibers; nc: new cementum-like tissues; d, dentin.

regenerated and clearly observed on dentin after transplantation for 8 weeks. Conversely, non-co-cultured GMSC sheet transplants (GMSC sheet + dentin + CBB) formed small amounts of PDL-like tissue but no cementum-like deposits (Fig. 7A). The images were captured on a compound microscope.

## Discussion

Ideal periodontal reconstruction would involve the development of Sharpey's fibers, which consist of collagen fibers. Therefore, generation of a suitable regenerative periodontal environment is of great importance. Furthermore, a mineralized matrix is essential for periodontal regeneration. Certain osteoinductive technologies, including the use of conditioned medium from developing apical tooth germ cells, periapical follicle stem cells and Hertwig's epithelial root sheath cells, as well as osteoinductive medium, have previously been applied to create a regenerative microenvironment (4,26-29). The present study developed a periodontal complex using APTG-CM-induced GMSC sheets, dentin slices and CBB for periodontal regeneration. As hypothesized, transplantation of immunodeficient mice with this periodontal complex resulted in the generation of a dental cementum/PDL-like complex. These results indicated that the development of this periodontal complex may provide an alternative clinical approach for tooth reconstruction in future therapeutic strategies.

In the present study, the APTG-CM used may contain several molecular signals and growth factors that are necessary for GMSC and PDLSC proliferation and differentiation,

thus inducing differentiation of GMSCs and PDLSCs towards a cementoblast phenotype. As expected, the induced GMSCs exhibited several crucial characteristics of cementoblast-like cells. Firstly, flow cytometric cell cycle analysis demonstrated that cells co-cultured with APTG-CM presented a higher percentage of cells in S and $G_2$/M phases. These results suggested that DNA synthesis was enhanced and APTG-CM may provide an appropriate microenvironment, which is necessary for the proliferation and differentiation of GMSCs and PDLSCs. Secondly, ALP activity of GMSCs and PDLSCs co-cultured with APTG-CM was increased. It is well-known that when odontogenic mesenchymal cells are differentiated towards cementum-like and osteogenic phenotypes, ALP activity is an early marker (30). The increase in ALP activity indicated that the mineralization ability of GMSCs and PDLSCs co-cultured with APTG-CM was enhanced. ALP is considered to be a prerequisite, which has a major role in the formation of mineral tissue. Furthermore, several important bone-associated genes, including osteocalcin, bone sialoprotein, ALP, type I collagen and cementum-derived protein 23 were upregulated in GMSCs and PDLSCs co-cultured with APG-CM, which may enhance PDL-like tissue regeneration. These bone-associated genes were also important markers associated with mineral extracellular matrix (31). These changes may be considered the mechanistic basis for altering the fates of GMSCs and PDLSCs, and may contribute to the regeneration of periodontal tissue. The results of an *in vivo* study detected similar changes in GMSCs and PDLSCs induced by APTG-CM.

The results of the *in vivo* heterotopic transplantation were consistent with the results of the *in vitro* study. In the experimental and positive control groups, PDL-like structures were regenerated on the dentin surfaces and novel cementum matrix generation was detected. These findings were the most important in the present study; to the best of our knowledge, there are no reports regarding the use of GMSCs to replace PDLSCs in periodontal regeneration, and the subsequent regeneration of a PDL-like structure. However, it is worth noting that the formation of PDL-like structures was continuous, this may be due to the stability and compactness between cell sheets and dentin slices due to the surgical sutures used, and may be associated with novel cementum matrix generation, it is well-known that acellular cementum regeneration is the gold standard of periodontal regeneration (32). Still, it was related to the environmental factors where the complex located. In the present study, only heterotopic transplantation was used to simulate periodontal regeneration. A previous study demonstrated that the alveolar bone environment differs from that of other areas (33) and is suitable for periodontal regeneration (34). If the complexes were orthotopic transplants into jaws, the microenvironment would be better suited to periodontal regeneration, this could be a target of future investigation. Angiogenesis may also be important. In the present study regeneration of the PDL-like structure was accompanied by angiogenesis. Restoration of vascular supply was considered to be a critical factor in successful tissue engineering (35,36). Additional studies are required to confirm the differences between periodontal tissue formed by GMSCs and PDLSCs.

In conclusion, the results of the present study suggested that GMSCs induced by APTG-CM have the potential for periodontal tissue regeneration. This may have general implications for periodontal engineering. Future research is required to confirm the differences between periodontal tissue formed by GMSCs and PDLSCs.

## Acknowledgements

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