

Research Article
Periodontal Science



Identification of stemness and differentially expressed genes in human cementum-derived cells

EunHye Lee ¹, Young-Sung Kim ², Yong-Moo Lee ³, Won-Kyung Kim ²,
Young-Kyoo Lee ⁴, Su-Hwan Kim ^{2,*}

¹Dental Research Institute, Seoul National University, Seoul, Korea

²Department of Periodontics, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

³Department of Periodontology and Dental Research Institute, Seoul National University School of Dentistry, Seoul, Korea

⁴Department of Dentistry, Uijeongbu Eulji Medical Center, Eulji University, Uijeongbu, Korea



Received: Apr 23, 2021

Revised: Jul 5, 2021

Accepted: Jul 9, 2021

*Correspondence:

Su-Hwan Kim

Department of Periodontics, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 05505, Korea.

E-mail: shkimperio@amc.seoul.kr

Tel: +82-2-3010-5797

Fax: +82-2-3010-6967

Copyright © 2021. Korean Academy of Periodontology

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0/>).

ORCID iDs

EunHye Lee

<https://orcid.org/0000-0001-9530-1508>

Young-Sung Kim

<https://orcid.org/0000-0003-2674-3649>

Yong-Moo Lee

<https://orcid.org/0000-0002-5619-3847>

Won-Kyung Kim

<https://orcid.org/0000-0003-2557-1646>

Young-Kyoo Lee

<https://orcid.org/0000-0001-7065-282X>

Su-Hwan Kim

<https://orcid.org/0000-0002-3431-453X>

Funding

This study was supported by a grant (2013-541) from the Asan Institute for Life Sciences, Seoul, Korea.

ABSTRACT

Purpose: Periodontal treatment aims at complete regeneration of the periodontium, and developing strategies for periodontal regeneration requires a deep understanding of the tissues composing the periodontium. In the present study, the stemness characteristics and gene expression profiles of cementum-derived cells (CDCs) were investigated and compared with previously established human stem cells. Candidate marker proteins for CDCs were also explored.

Methods: Periodontal ligament stem cells (PDLSCs), pulp stem cells (PULPSCs), and CDCs were isolated and cultured from extracted human mandibular third molars. Human bone marrow stem cells (BMSCs) were used as a positive control. To identify the stemness of CDCs, cell differentiation (osteogenic, adipogenic, and chondrogenic) and surface antigens were evaluated through flow cytometry. The expression of cementum protein 1 (CEMP1) and cementum attachment protein (CAP) was investigated to explore marker proteins for CDCs through reverse-transcription polymerase chain reaction. To compare the gene expression profiles of the 4 cell types, mRNA and miRNA microarray analysis of 10 samples of BMSCs (n=1), PDLSCs (n=3), PULPSCs (n=3), and CDCs (n=3) were performed.

Results: The expression of mesenchymal stem cell markers with a concomitant absence of hematopoietic markers was observed in PDLSCs, PULPSCs, CDCs and BMSCs. All 4 cell populations also showed differentiation into osteogenic, adipogenic, and chondrogenic lineages. CEMP1 was strongly expressed in CDCs, while it was weakly detected in the other 3 cell populations. Meanwhile, CAP was not found in any of the 4 cell populations. The mRNA and miRNA microarray analysis showed that 14 mRNA genes and 4 miRNA genes were differentially expressed in CDCs vs. PDLSCs and PULPSCs.

Conclusions: Within the limitations of the study, CDCs seem to have stemness and preferentially express CEMP1. Moreover, there were several up- or down-regulated genes in CDCs vs. PDLSCs, PULPSCs, and BMSCs and these genes could be candidate marker proteins of CDCs.

Keywords: Dental cementum; Microarray analysis; MicroRNAs; RNA, messenger; Stem cells

Author Contributions

Conceptualization: Young-Kyoo Lee, Yong-Moo Lee, Su-Hwan Kim; Formal analysis: Young-Sung Kim, EunHye Lee; Investigation: EunHye Lee, Su-Hwan Kim; Methodology: EunHye Lee, Su-Hwan Kim, Yong-Moo Lee; Project administration: EunHye Lee, Su-Hwan Kim; Writing - original draft: EunHye Lee, Su-Hwan Kim; Writing - review & editing: Young-Sung Kim, Won-Kyung Kim, Su-Hwan Kim.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

INTRODUCTION

The ultimate goal of stem cell research may be to achieve the formation or development of desired tissues or organs. Since periodontitis results in destruction of the periodontium, periodontal therapy ultimately aims at regeneration of destroyed periodontal tissues. In the 1980s, guided tissue regeneration (GTR) was introduced to regain periodontal tissues [1,2]. The goal of GTR was to flood periodontal wounds with slowly migrating periodontal ligament (PDL) cells and osteoblasts to form the corresponding tissues. New cementum formation was usually measured as proof of successful periodontal regeneration resulting from GTR therapy. However, GTR did not always accomplish complete regeneration of the 4 components of the periodontium (i.e., the gingiva, PDL, alveolar bone, and cementum) and the outcome of GTR treatment was technique-sensitive [3,4]. In recent years, many groups have researched the application of stem cells for periodontal regeneration [5-8].

Cementum is a mineralized layer covering the root surface that provides a bed for PDL attachment through Sharpey's fiber insertion. The principal cells associated with the cementum are cementoblasts and cementocytes. Cementoblasts line the root surface, whereas cementocytes are embedded in the cementum [9]. Cementoblasts are similar to osteoblasts. Both cementoblasts and osteoblasts produce type I collagen and non-collagenous proteins such as osteocalcin, osteopontin, and bone sialoprotein (BSP). New markers for cementum or cementum-PDL have recently been reported, including cementum protein 1 (CEMP1) and cementum attachment protein (CAP) [10-12]. At present, the uniqueness of the cementoblast cell type has been well identified [13-16]. However, the detailed mechanisms regulating the proliferation and differentiation of human cementum-derived cells (CDCs) have yet to be fully elucidated [17,18].

It was reported that stem cells could be isolated from the PDL (periodontal ligament stem cells [PDLSCs]) [19] and tooth pulp (pulp stem cells [PULPSCs]) [20], and these stem cells were collectively named dental stem cells (DSCs). In light of the need to harvest adult stem cells, DSCs are easily obtained with minimum tissue damage, especially in cases of intact third molars extracted for orthodontic treatment [21,22].

The microRNA (miRNA) is a type of short non-coding RNA that consists of approximately 22 base sequences and functions as a post-transcriptional regulator of gene expression. The miRNA can suppress the translation of target mRNA by complementary binding. Through miRNAs, it is possible to identify the gene expression profiles of certain cells. Numerous miRNAs have recently been widely investigated in studies of cancer, other adult diseases, development, differentiation, and stem cells. At present, only a few studies have investigated the gene expression patterns of DSCs using complementary DNA (cDNA) microarray analysis [19,23,24].

The aim of this study was to evaluate the stemness characteristics of human CDCs and to determine the specific markers for CDCs by comparing the gene expression patterns of CDCs with those of PULPSCs, PDLSCs, and bone marrow stem cells (BMSCs) through mRNA and miRNA microarray analyses.

MATERIALS AND METHODS

In this study, PDLSCs, PULPSCs, CDCs, and BMSCs were investigated. PDLSCs, PULPSCs, and CDCs were collected from human third molars extracted simply without any damage, while BMSCs were obtained from purchased established cell lines. This study was approved by the Institutional Review Board of Asan Medical Center (2012-0427).

Subjects

Teeth were collected from 3 patients (a 21-year-old man, a 24-year-old woman, and a 25-year-old woman) who were scheduled for third molar extraction for orthodontic reasons, were generally healthy, did not have periodontal disease or dental caries on the tooth to be extracted, and were non-smokers [25]. Two third molars per patient were collected for the study. The extraction was performed at the Department of Dentistry, Asan Medical Center by 1 investigator (SHK), who took particular care not to damage the teeth, especially the PDL. The teeth were kept in Hank's Balanced Salt Solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 5% antibiotics (Gibco) processed immediately after extraction.

Cell cultures

PDLSCs

The PDL from the middle portion of the tooth root was scraped with a surgical scalpel, digested in a solution of 4 mg/mL dispase (Roche, Basel, Switzerland) and 3 mg/mL collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ, USA) for 1 hour at 37°C. The medium was collected and centrifuged at 1,200 rpm for 3 minutes, and the cells were washed 3 times with medium. After the final wash, the cells were plated in alpha-minimal essential medium (α -MEM, Gibco) containing 100 U/mL penicillin, 100 μ g/mL streptomycin (Gibco), and 15% fetal bovine serum (FBS, Gibco). The cells were incubated in a humidified atmosphere with 95% air and 5% CO₂ at 37°C. The medium was changed every second day. Under the conditions employed, the PDL cells grew without forming discrete colonies. When the cultures became semiconfluent, cells were passaged with trypsin-ethylene-diamine-tetraacetic acid (EDTA) (Gibco). PDLSCs from the second to fifth passages were used for the experiments described below [19,21].

PULPSCs

The tooth surfaces were cleaned and cut around the cemento-enamel junction with sterilized dental fissure burs to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root and then digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 hour at 37°C. Single-cell suspensions were obtained by passing the cells through a 70- μ m strainer (Falcon, Thermo Fisher Scientific). The medium was collected and centrifuged at 1,200 rpm for 3 minutes, and the cells were washed 3 times with medium. After the final wash, the cells were plated in α -MEM containing 100 U/mL penicillin, 100 μ g/mL streptomycin, and 15% FBS. The cells were incubated in a humidified atmosphere with 95% air and 5% CO₂ at 37°C. The medium was changed every second day. Under the conditions employed, PULPSCs grew without forming discrete colonies. When the cultures became semiconfluent, cells were passaged with trypsin-EDTA. PULPSCs from the second to fifth passages were used for the experiments described below.

CDCs

The cultures of CDCs were established from the same teeth as the PDL cells using a modification of a method previously reported for the establishment of osteoblastic cell cultures. After the

PDL and pulp were manually dissected from the tooth root with a surgical scalpel, each tooth was washed with Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (Gibco) and incubated in 10 mL of medium containing 100 mU/mL collagenase P (Roche) for 1 hour at 37°C with rotation. The medium with released cells was discarded and the teeth were washed 3 times with fresh medium. Using a sterile surgical scalpel, cementum and a thin layer of underlying dentin were dissected and collected. Cementum-dentin fragments were thoroughly washed with medium 5 times and then minced with scissors until small fragments (<0.5 mm in diameter) were obtained. The fragments were washed again with medium 5 times and then digested with collagenase P (100 mU/mL; 4 mL total volume/0.1–0.2 mL of the settled bed volume of the fragments) for 1 hour at 37°C. The medium with released material was discarded. The fragments were washed thoroughly 5 times with medium and then placed in 60-mm cell culture dishes containing 5 mL of growth medium (DMEM/F12 supplemented with penicillin/streptomycin and 10% FBS). The cultures were incubated in a humidified atmosphere with 95% air and 5% CO₂ at 37°C for at least 5 weeks. The medium was changed every second day [9,20,26]. CDCs from the third to fifth passages were used for the experiments described below.

BMSCs

BMSCs were purchased from Lonza (cat. PT-2501; Basel, Switzerland). The cells were plated in DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. The cells were incubated in a humidified atmosphere with 95% air and 5% CO₂ at 37°C. The medium was changed every second day [27-29].

Flow cytometry

The percentage of surface markers indicating the stem cell immunophenotype was analyzed by flow cytometry. The PDLSCs, PULPSCs, BMSCs, and CDCs from each group were harvested in PBS, washed once with PBS, fixed in 3.7% paraformaldehyde (Wako, Tokyo, Japan), and then incubated with anti-Stro-1, anti-CD44, anti-CD90, and anti-CD146, antibodies (R&D Systems, Minneapolis, MN, USA) at a dilution of 1:100 (volume/volume) in PBS for 1 hour at room temperature in the dark. Immunostaining with an antibody against the hematopoietic marker anti-CD19 and anti-CD45 was conducted as a negative control. The cells were then incubated with secondary anti-mouse antibodies—allophycocyanin-conjugated antibody for CD44, CD90, CD146, CD19 and CD45 or phycoerythrin-conjugated antibody for STRO-1—at a dilution of 1:100 (volume/volume) in PBS for 30 minutes at 4°C in the dark, and the staining was finally analyzed with a flow-cytometry cell-sorting device (CANTO II, Becton Dickinson, Franklin Lakes, NJ, USA) [30].

***In vitro* differentiation assay**

Adipogenic differentiation

Adipogenic differentiation was induced by culturing confluent cells for 2 weeks in complete medium supplemented with StemXVivo™ Human Adipogenic Supplement (R&D Systems). The cells were maintained in the inducing medium for 2 weeks. The medium was changed every 2 days. Adipogenic-differentiated cells were stained with Oil Red O (Sigma, St. Louis, MO, USA) [21,28].

Osteogenic differentiation

Osteoblastic differentiation was induced by culturing confluent cells for 2 weeks in complete medium supplemented with StemXVivo™ Human Osteogenic Supplement (R&D Systems). The cells were maintained in the inducing medium for 2 weeks. The medium was changed every 2 days. Osteogenic-differentiated cells were stained with Alizarin Red S (Sigma) [31].

Chondrogenic differentiation

To induce chondrocyte differentiation, harvested cells were rinsed once by centrifugation, resuspended in chondrogenic medium, and finally centrifuged for 3 minutes at 1,200 rpm in a 15-mL polypropylene Falcon tube to form a pellet. The chondrogenic medium constituted of Chondrogenic Basal Medium and StemXVivo™ Human/Mouse Chondrogenic Supplement (R&D Systems). The medium was changed every 2 days. Chondrogenic-differentiated cells were stained with Safranin O (Sigma) [28].

RNA expression of cementum-related proteins by reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The preparations were quantified and their purity was determined by nanodrops. The cDNA was synthesized from 1 µg of total RNA using the Thermo Fisher Scientific RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). The oligonucleotide RT-PCR primers for CEMP1, CAP, and glyceraldehydes-3-phosphate were purchased from Cosmogenetech (Seoul, Korea): CEMP1, forward 5'-ATGGGCACATCAAGCAVCTGA-3' and reverse 5'-CCCCATTAGTGTTCATCCTGC-3'; CAP, forward 5'-TCCAGACATTTGCCTTGCTT-3' and reverse 5'-TTACAGCAATAGAAAAACAGCATGA-3'. Aliquots of total cDNA were amplified with Taq polymerase (RBC Bio, Taipei, Taiwan), and the amplifications were performed in a thermal cycler for 35 cycles after an initial 5-minute denaturation step at 95°C, annealed for 30 seconds at 56°C and extended for 1 minute at 72°C. The amplification reaction products were resolved on 1.2% agarose/TBE gels, electrophoresed at 100 mV, and visualized by ethidium bromide staining [17,32,33].

The mRNA microarray

Total RNA was extracted using TRIzol (Invitrogen Life Technologies, Thermo Fisher Scientific) and purified using RNeasy columns (Qiagen) according to the manufacturers' protocols. After processing with DNase digestion and clean-up procedures, the RNA samples were quantified, aliquoted, and stored at -80°C until use. For quality control, RNA purity and integrity were evaluated using denaturing gel electrophoresis with the optical density 260/280 ratio analyzed using a Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) to yield biotinylated complementary RNA (cRNA) according to the manufacturer's instructions. Then, 750 ng of labeled cRNA samples were hybridized to each human HT12 expression v.4 bead array for 16–18 hours at 58°C, according to the manufacturer's instructions (Illumina Inc., San Diego, CA, USA). All data analyses and visualization of differentially expressed genes were conducted using R 2.15.3 (R Foundation for Statistical Computing, Vienna, Austria).

The miRNA microarray

The miRNA Microarray System with miRNA complete labeling and hybridization Kit (Agilent Technologies) was used according to the manufacturer's recommended protocol. The Agilent microRNA Spike-In Kit was used for in-process control to measure the efficiency of labeling and hybridization. Briefly, 100 ng of total RNA was dephosphorylated at 37°C for 30 minutes with calf intestinal phosphatase and denatured using 100% DMSO at 100°C for 7 minutes. The samples were labeled with pCp-Cy3 using T4 ligase by incubation at 16°C for 2 hours. The labeled RNA samples were dried in a vacuum concentrator for 1 hour. Once the samples were completely dried, they were prepared for hybridization by adding nuclease-free water, Hyb

Spike-In solution, 10× Gene Expression Blocking Agent, and 2× Hi-RPM Hybridization Buffer (Agilent Technologies). The arrays were hybridized in 45 µL of the mixture, and then rotated at 20 rpm for 20 hours at 55°C. Agilent Gene Expression Wash Buffers 1 and 2 (37°C) were used after hybridization as recommended for the Agilent miRNA Microarray System and were scanned on an Agilent Technologies G4900DA SureScan (Agilent Technologies) at a 3-µm resolution. Data were acquired using Agilent Feature Extraction Software version 11.0.1.1 (Agilent Technologies). All data analyses and visualization of differentially expressed miRNA were conducted using R version 3.0.2 (R Foundation for Statistical Computing).

RESULTS

Cell culture

The PDLSCs, PULPSCs, BMSCs, and CDCs adhered well to the bottom of the culture plates and exhibited typical fibroblastic morphology (**Figure 1**). On the first day after plating, CDC adhesion as a small population of polygonal or spindle-shaped cells was established. The CDCs propagated rapidly *in vitro* and showed a homogeneous fibroblast-like morphology.

Flow cytometry

All the cells were positive for CD44, CD90, and CD146 surface markers and negative for CD19 and CD45. A partially positive result for STRO-1 was observed, although there were slight differences among cells (**Figure 2**). Among the CDCs that were investigated, 99% of the cells were positive for CD44, CD90, and CD146, while only 3% of the cells were positive for STRO-1.

In vitro differentiation assay

The PDLSCs (**Figure 3A-C**), PULPSCs (**Figure 3D-F**), BMSCs (**Figure 3G-I**), and CDCs (**Figure 3J-L**) all showed adipogenic, osteogenic, and chondrogenic differentiation. Of particular note, to test the multipotential differentiation capability of CDCs, cells from passage 3 were induced to differentiate towards adipocyte-, osteocyte- and chondrocyte-like cells. For adipogenic differentiation, the CDCs were cultured in DMEM/F12 supplemented with StemXVivo™ Human Adipogenic Supplement.

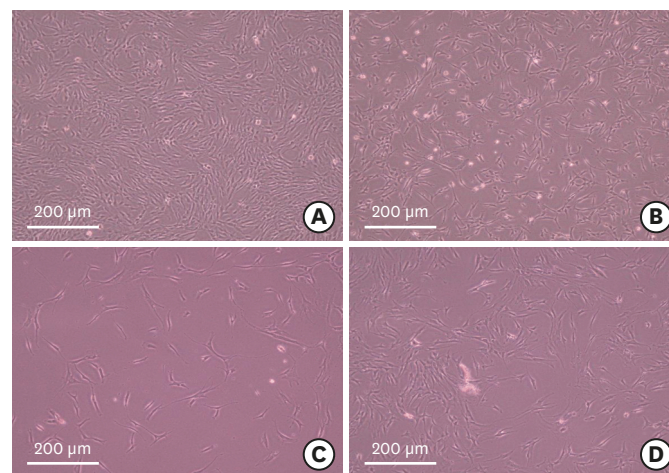


Figure 1. Microscopic images showing the cultured cell shapes of PDLSCs (A), PULPSCs (B), BMSCs (C), and CDCs (D). All the cells exhibited typical fibroblastic morphology. Active cell migration and proliferation were still observed at the edges of colonies.
PDLSC: periodontal ligament stem cell, PULPSC: pulp stem cell, BMSC: bone marrow stem cell, CDC: cementum-derived cell.

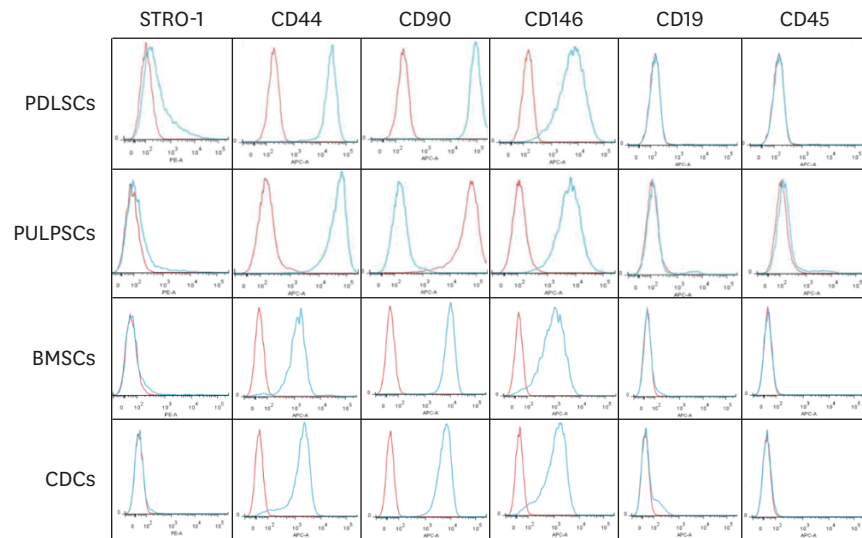


Figure 2. Phenotypic identification of stemness through fluorescence activated cell sorting. Dental cells (PDLSCs, PULPSCs, and CDCs) showed similar expression patterns to BMSCs. In detail, all dental cells expressed mesenchymal markers (STRO-1, CD44, CD90 and CD146) and did not express hematopoietic markers (CD19 and CD45), as was also shown in BMSCs. Red line, control cells labeled without primary antibodies; blue line, cells labeled with primary antibodies conjugated with PE or APC. PDLSC: periodontal ligament stem cell, PULPSC: pulp stem cell, BMSC: bone marrow stem cell, CDC: cementum-derived cell, PE: phycoerythrin, APC, allophycocyanin.

After 2 weeks, the cells were stained with Oil Red O, and lipid droplet accumulation was clearly detected in the cells (**Figure 3J**). For osteogenic differentiation, CDCs from passage 3 were cultured in α -MEM supplemented with StemXVivo™ Human Osteogenic Supplement, and after 2 weeks, the cells were stained with Alizarin Red S (**Figure 3K**). For chondrogenic differentiation, CDCs from passage 3 were cultured in DMEM/F12 supplemented with StemXVivo™ Human Chondrogenic Supplement, and after 3 weeks, the cells were positively stained with Safranin O (**Figure 3L**).

RNA expression of cementum-related proteins by RT-PCR

CEMP1 expression was markedly observed in CDCs, although it was weakly detected in PDLSCs, PULPSCs, and BMSCs, whereas CAP was not expressed in any of the cell types (**Figure 4**).

The mRNA microarray

In total, 1,469, 94, and 94 genes were up- or down-regulated in CDCs compared to BMSCs, PDLSCs, and PULPSCs, respectively. Among those genes, 10 (CDCs vs. PDLSCs) and 9 (CDCs vs. PULPSCs) showed more than 4-fold differences in the transcription level and a *P* value of less than 1×10^{-7} between CDCs and PDLSCs/PULPSCs, which were set as the cut-off values (**Table 1**). Up-regulation of ribosomal protein S4, Y-linked 1 (RPS4Y1), eukaryotic translation initiation factor 1A, Y-chromosomal (EIF1AY) and multiple EGF-like-domains 6 (MEGF6), and down-regulation of chromosome 13 open reading frame 15 (C13orf15) and pregnancy specific beta-1-glycoprotein 5 (PSG5) were common in CDCs compared to both PDLSCs and PULPSCs. Moreover, MEGF6 were commonly up-regulated and PSG5 were down-regulated in CDCs compared to BMSCs, PDLSCs, and PULPSCs.

The miRNA microarray

In total, 22, 8, and 9 genes were up- or down-regulated in CDCs compared to BMSCs, PDLSCs, and PULPSCs, respectively. Among those genes, hsa-miR-3653, hsa-miR-342-3p,

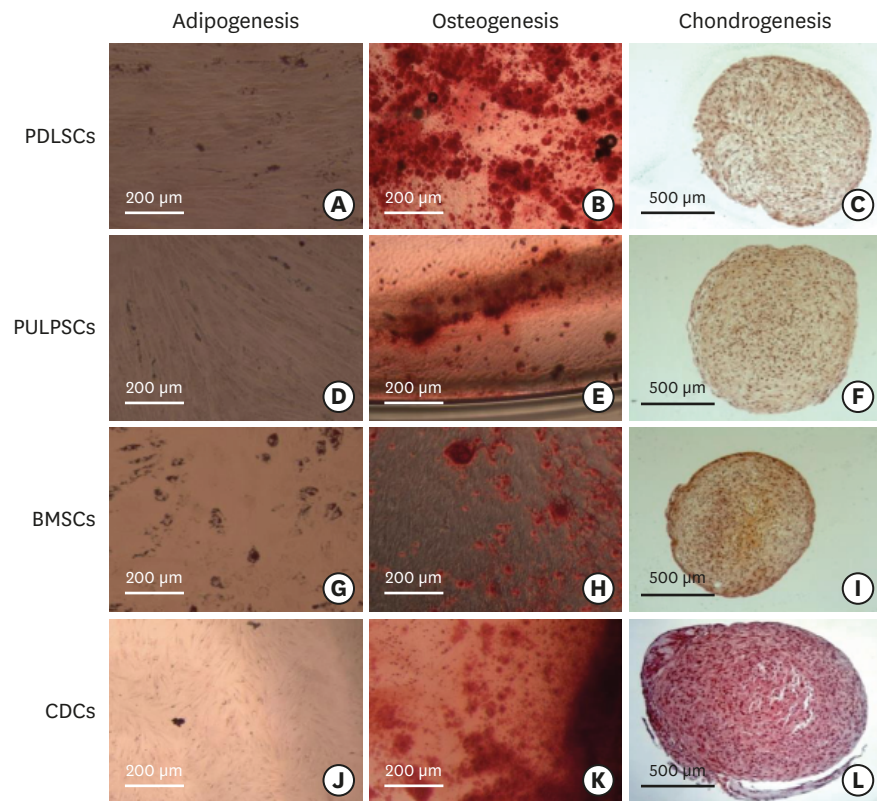


Figure 3. Microscopic images from *in vitro* differentiation assays. CDCs (J-L), as well as PDLSCs (A-C), PULPSCs (D-F), and BMSCs (G-I), were each shown to differentiate appropriately to the adipogenic, osteogenic, and chondrogenic lineages. Adipogenesis was indicated by the accumulation of neutral lipid vacuoles stained with Oil Red O. Osteogenesis was identified by the increase in calcium deposition and alkaline phosphatase, which was stained with Safranin Red S. Chondrogenesis was shown by Safranin O staining. PDLSC: periodontal ligament stem cell, PULPSC: pulp stem cell, BMSC: bone marrow stem cell, CDC: cementum-derived cell.

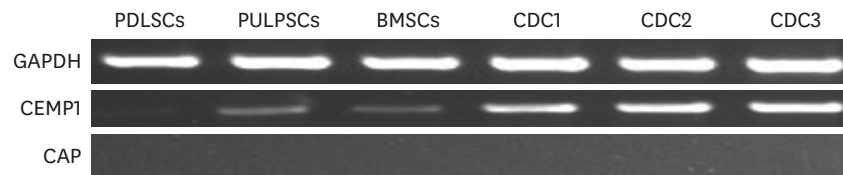


Figure 4. The mRNA expression of cementum-related proteins. CEMPI was strongly expressed in CDCs, however, it was also weakly expressed in PDLSCs, PULPSCs, and BMSCs. Meanwhile, CAP expression was not detected in any cell.

GAPDH: glyceraldehyde 3-phosphate dehydrogenase, CEMPI: cementum protein 1, CAP: cementum attachment protein, PDLSC: periodontal ligament stem cell, PULPSC: pulp stem cell, BMSC: bone marrow stem cell, CDC: cementum-derived cell.

hsa-miR-455-3p, and hsa-miR-193b-3p remained following the selection of miRNA genes with more than 2-fold differences and *P* values of less than 0.05 (**Table 2**).

Up-regulation of hsa-miR-3653 and down-regulation of hsa-miR-455-3p were common in CDCs compared to BMSCs, PDLSCs, and PULPSCs.

Table 1. mRNA genes differentially expressed in CDCs

Gene expression	Gene symbol	Gene name	Fold change	P value	Reference
Up	<i>RPS4Y1</i>	Ribosomal protein S4, Y-linked 1	24.14	0	PDLSCs
			22.36	0	PULPSCs
	<i>EIFIAY</i>	Eukaryotic translation initiation factor 1A, Y-chromosomal	9.40	0	PDLSCs
			9.33	8.41E-12	PULPSCs
	<i>MEGF6</i>	Multiple EGF-like-domains 6	5.38	2.37E-08	PDLSCs
			4.11	1.89E-08	PULPSCs
	<i>LAMA5</i>	Laminin subunit alpha-5	8.46	3.29E-08	PDLSCs
	<i>FAM43A</i>	Family with sequence similarity 43, member A	13.49	0	PULPSCs
	<i>HSPB7</i>	Heat shock 27kDa protein family, member 7	6.87	8.41E-10	PULPSCs
	<i>POSTN</i>	Periostin, osteoblast specific factor	5.79	1.40E-08	PULPSCs
Down	<i>C13orf15</i>	Chromosome 13 open reading frame 15	-5.85	1.04E-08	PDLSCs
			-20.37	7.50E-13	PULPSCs
	<i>PSG5</i>	Pregnancy specific beta-1-glycoprotein 5	-5.22	5.66E-10	PDLSCs
			-8.14	7.74E-08	PULPSCs
	<i>EFEMP1</i>	EGF-containing fibulin-like extracellular matrix protein 1	-27.89	3.95E-10	PDLSCs
	<i>PENK</i>	Proenkephalin	-14.15	5.30E-20	PDLSCs
	<i>MME</i>	Membrane metallo-endopeptidase	-8.08	3.95E-10	PDLSCs
	<i>HLA-A29.1</i>	Human leukocyte antigen serotype within HLA-A	-6.16	2.03E-08	PDLSCs
	<i>IGFBP5</i>	Insulin-like growth factor binding protein 5	-9.59	4.17E-08	PULPSCs

Differential expression was considered to be present if a distinct transcript met the fold change cut-off of more than 4 and the P value cut-off of less than 1×10^{-7} . CDC: cementum-derived cell, PDLSC: periodontal ligament stem cell, PULPSC: pulp stem cell, EGF: epidermal growth factor, HLA: human leukocyte antigen.

Table 2. miRNA genes differentially expressed in CDCs

Gene expression	Probe ID	Fold change	P value	Reference
Up	hsa-miR-3653	2.71	0.00935	PDLSCs
		2.49	0.04207	PULPSCs
	hsa-miR-342-3p	2.87	0.00048	PULPSCs
Down	hsa-miR-455-3p	-4.87	0.01187	PDLSCs
		-3.06	0.04340	PULPSCs
	hsa-miR-193b-3p	-2.06	0.01289	PDLSCs

Differential expression was considered to be present if a distinct transcript met the fold change cut-off of more than 2 and the P value cut-off of less than 0.05.

CDC: cementum-derived cell, PDLSC: periodontal ligament stem cell, PULPSC: pulp stem cell.

DISCUSSION

In the present study, PDLSCs, PULPSCs, and CDCs were successfully isolated from human teeth and their stemness was confirmed through phenotypic characterization and an *in vitro* differentiation assay. In addition, it seemed that CDCs showed a specific association with the expression of CEMP1, but not CAP. Moreover, 14 mRNA and 4 miRNA genes were identified as being expressed differently in CDCs vs. PDLSCs and PULPSCs, meaning that both mRNAs and miRNAs could be candidate marker genes for CDCs.

As the periodontium is composed of gingiva, the PDL, alveolar bone, and cementum, the regeneration of destroyed periodontium should include the respective reformation of its 4 components. To achieve regeneration of a certain tissue, it is necessary to understand the characteristics and behavior of related cells. The cementum and its related cells are the least investigated of the 4 components of the periodontium. The cementum is a special mineralized tissue that covers the surface of roots and provides a bed for PDL fiber insertion. Since cementum-related cells are attached to or embedded in the cementum, it is difficult to separate and culture CDCs. Thus, few studies have investigated the primary culture of CDCs. In the present study, we were able to successfully isolate and expand human CDCs according to the method described by Grzesik et al. [9].

The stemness of CDCs was demonstrated through fluorescence activated cell sorting and a differentiation assay. CDCs, as well as PULPSCs, PDLSCs, and MSCs, were positive for mesenchymal stromal cell markers such as CD44, CD90, and CD146 and negative for the hematopoietic markers CD 19 and CD45. Weak positivity was observed for STRO-1, the initial expression marker, and this expression pattern is similar to the results of previous DSC research [29]. The weak positive pattern of STRO-1 may be explained by the results of Gronthos et al. [34,35], who found that STRO-1 expression was initially high in MSCs and then became low as passages increased. In differentiation assays, CDCs showed the capability for multipotential differentiation, which was also found in other cells.

Cementum-specific markers have not been clearly established before the present study. Although some specific markers, such as BSP [36], CAP, and CEMP1, were proposed as cementum-specific markers, they are not expressed only in the cementum. BSP and CEMP1 were proposed by Kitagawa et al., but these proteins were also observed only weakly in other cells while being expressed more in the cementum [17]. In another study, CAP was present in the mature cementum matrix of adult periodontium. When CAP bound to cementoblasts or PDL progenitor cells, they showed ALP activity and mineralized tissue formation [10]. Based on this fact, CAP seems to be associated with progenitors of mineralized tissue formation in the PDL. Furthermore, both selective binding of CAP to PDL cells and CAP support for PDL cell attachment to root surfaces were observed [37]. This means that cementoblast progenitors migrate to the root surface through selective chemotaxis and attachment mediated by CAP [38]. CAP was not expressed in this study, which may have been because the origin of CDCs trapped in the lacunae after cementum formation was complete; alternatively, the limited number of samples may be an explanation for this finding. The expression of CEMP1 was found to be limited to cementoblasts in a recent study [12]. In addition, CEMP1 and protein tyrosine phosphatase-like member A/cementum attachment protein (PTPLA/CAP) were found at high levels in cementoblast-like cells, as well as in PDL cells, from which CEMP1 and PTPLA/CAP could be considered potential local regulators of cementum metabolism and key markers of the cementoblast phenotype [34]. It was observed, in an *in vitro* study, that almost all of the cementoblastoma-derived cell population was CEMP1-positive, whereas only 6% of PDL cells stained positive for CEMP1. The small proportion of CEMP1-positive PDL cells may be considered as cementoblast precursors, and this also suggests that CEMP1 could be a marker of the cementoblastic lineage [38,39]. In the present study, CEMP1 was markedly expressed in CDCs, and it was also found weakly in PDLSCs, PULPSCs and BMSCs. This result is in accordance with previously reported results for CEMP1, and it can be inferred that the CDCs used in this study also originated from the same ancestors.

Both mRNA and miRNA microarray investigations were performed to explore uniquely transcribed or suppressed genes in CDCs. There were differentially expressed 14 mRNAs and 4 miRNAs in CDCs. Although the differently expressed genes were identified through microarray analysis, the current pilot experiment has some limitations. The differential expression of the mRNAs and miRNAs was not confirmed by RT-PCR, and the detailed functions of the respective genes have not been well elucidated. The *RPS4Y1* and *EIF1AY* genes, which were found to be up-regulated, are known to be related to eukaryotic translation initiation. Another limitation associated with microarray experiments is the inconsistency of the results among samples or subjects, which makes the results more difficult to interpret. As data from microarray investigations are gathered in the future, a more precise interpretation of the results of the present study may be feasible.

Within the limitations of this study, it can be concluded that CDCs have stemness, as has previously been established for PULPSCs and PDLSCs. In addition, CDCs may have genetically distinct expression patterns from other DSCs and CEMP1 expression in CDCs was confirmed. Further studies are needed to understand the molecular mechanisms of cementogenesis and the characteristics of CDCs.

REFERENCES

1. Gottlow J, Nyman S, Lindhe J, Karring T, Wennström J. New attachment formation in the human periodontium by guided tissue regeneration. Case reports. *J Clin Periodontol* 1986;13:604-16.
[PUBMED](#) | [CROSSREF](#)
2. Nyman S, Lindhe J, Karring T, Rylander H. New attachment following surgical treatment of human periodontal disease. *J Clin Periodontol* 1982;9:290-6.
[PUBMED](#) | [CROSSREF](#)
3. Tonetti MS, Cortellini P, Suvan JE, Adriaens P, Baldi C, Dubravec D, et al. Generalizability of the added benefits of guided tissue regeneration in the treatment of deep intrabony defects. Evaluation in a multi-center randomized controlled clinical trial. *J Periodontol* 1998;69:1183-92.
[PUBMED](#) | [CROSSREF](#)
4. Tonetti MS, Prato GP, Cortellini P. Factors affecting the healing response of intrabony defects following guided tissue regeneration and access flap surgery. *J Clin Periodontol* 1996;23:548-56.
[PUBMED](#) | [CROSSREF](#)
5. Park JY, Jeon SH, Choung PH. Efficacy of periodontal stem cell transplantation in the treatment of advanced periodontitis. *Cell Transplant* 2011;20:271-85.
[PUBMED](#) | [CROSSREF](#)
6. Liu Y, Zheng Y, Ding G, Fang D, Zhang C, Bartold PM, et al. Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine. *Stem Cells* 2008;26:1065-73.
[PUBMED](#) | [CROSSREF](#)
7. Park JY, Park CH, Yi T, Kim SN, Iwata T, Yun JH. rhBMP-2 pre-treated human periodontal ligament stem cell sheets regenerate a mineralized layer mimicking dental cementum. *Int J Mol Sci* 2020;21:3767.
[PUBMED](#) | [CROSSREF](#)
8. Cho H, Tarafder S, Fogge M, Kao K, Lee CH. Periodontal ligament stem/progenitor cells with protein-releasing scaffolds for cementum formation and integration on dentin surface. *Connect Tissue Res* 2016;57:488-95.
[PUBMED](#) | [CROSSREF](#)
9. Grzesik WJ, Kuzentsov SA, Uzawa K, Mankani M, Robey PG, Yamauchi M. Normal human cementum-derived cells: isolation, clonal expansion, and *in vitro* and *in vivo* characterization. *J Bone Miner Res* 1998;13:1547-54.
[PUBMED](#) | [CROSSREF](#)
10. Liu HW, Yacobi R, Savion N, Narayanan AS, Pitaru S. A collagenous cementum-derived attachment protein is a marker for progenitors of the mineralized tissue-forming cell lineage of the periodontal ligament. *J Bone Miner Res* 1997;12:1691-9.
[PUBMED](#) | [CROSSREF](#)
11. Alvarez-Pérez MA, Narayanan S, Zeichner-David M, Rodríguez Carmona B, Arzate H. Molecular cloning, expression and immunolocalization of a novel human cementum-derived protein (CP-23). *Bone* 2006;38:409-19.
[PUBMED](#) | [CROSSREF](#)
12. Komaki M, Iwasaki K, Arzate H, Narayanan AS, Izumi Y, Morita I. Cementum protein 1 (CEMP1) induces a cementoblastic phenotype and reduces osteoblastic differentiation in periodontal ligament cells. *J Cell Physiol* 2012;227:649-57.
[PUBMED](#) | [CROSSREF](#)
13. Bosshardt DD. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? *J Dent Res* 2005;84:390-406.
[PUBMED](#) | [CROSSREF](#)
14. Foster BL. Methods for studying tooth root cementum by light microscopy. *Int J Oral Sci* 2012;4:119-28.
[PUBMED](#) | [CROSSREF](#)
15. Matthews BG, Roguljic H, Franceschetti T, Roeder E, Matic I, Vidovic I, et al. Gene-expression analysis of cementoblasts and osteoblasts. *J Periodontal Res* 2016;51:304-12.
[PUBMED](#) | [CROSSREF](#)

16. Salmon CR, Tomazela DM, Ruiz KG, Foster BL, Paes Leme AF, Sallum EA, et al. Proteomic analysis of human dental cementum and alveolar bone. *J Proteomics* 2013;91:544-55.
[PUBMED](#) | [CROSSREF](#)
17. Kitagawa M, Tahara H, Kitagawa S, Oka H, Kudo Y, Sato S, et al. Characterization of established cementoblast-like cell lines from human cementum-lining cells *in vitro* and *in vivo*. *Bone* 2006;39:1035-42.
[PUBMED](#) | [CROSSREF](#)
18. Wu D, Ikezawa K, Parker T, Saito M, Narayanan AS. Characterization of a collagenous cementum-derived attachment protein. *J Bone Miner Res* 1996;11:686-92.
[PUBMED](#) | [CROSSREF](#)
19. Kim SH, Kim KH, Seo BM, Koo KT, Kim TI, Seol YJ, et al. Alveolar bone regeneration by transplantation of periodontal ligament stem cells and bone marrow stem cells in a canine peri-implant defect model: a pilot study. *J Periodontol* 2009;80:1815-23.
[PUBMED](#) | [CROSSREF](#)
20. Gronthos S, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 2000;97:13625-30.
[PUBMED](#) | [CROSSREF](#)
21. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahimi J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;364:149-55.
[PUBMED](#) | [CROSSREF](#)
22. Estrela C, Alencar AH, Kitten GT, Vencio EF, Gava E. Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. *Braz Dent J* 2011;22:91-8.
[PUBMED](#) | [CROSSREF](#)
23. Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, et al. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod* 2008;34:166-71.
[PUBMED](#) | [CROSSREF](#)
24. Shi S, Robey PG, Gronthos S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. *Bone* 2001;29:532-9.
[PUBMED](#) | [CROSSREF](#)
25. Kim SH, Kim YS, Lee SY, Kim KH, Lee YM, Kim WK, et al. Gene expression profile in mesenchymal stem cells derived from dental tissues and bone marrow. *J Periodontol Implant Sci* 2011;41:192-200.
[PUBMED](#) | [CROSSREF](#)
26. Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? *Arch Oral Biol* 2012;57:1439-58.
[PUBMED](#) | [CROSSREF](#)
27. Taha MF, Hedayati V. Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells. *Tissue Cell* 2010;42:211-6.
[PUBMED](#) | [CROSSREF](#)
28. Tropel P, Noël D, Platet N, Legrand P, Benabid AL, Berger F. Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. *Exp Cell Res* 2004;295:395-406.
[PUBMED](#) | [CROSSREF](#)
29. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143-7.
[PUBMED](#) | [CROSSREF](#)
30. Carnes DL, Maeder CL, Graves DT. Cells with osteoblastic phenotypes can be explanted from human gingiva and periodontal ligament. *J Periodontol* 1997;68:701-7.
[PUBMED](#) | [CROSSREF](#)
31. Torii D, Konishi K, Watanabe N, Goto S, Tsutsui T. Cementogenic potential of multipotential mesenchymal stem cells purified from the human periodontal ligament. *Odontology* 2015;103:27-35.
[PUBMED](#) | [CROSSREF](#)
32. Hoz L, Romo E, Zeichner-David M, Sanz M, Nuñez J, Gaitán L, et al. Cementum protein 1 (CEMP1) induces differentiation by human periodontal ligament cells under three-dimensional culture conditions. *Cell Biol Int* 2012;36:129-36.
[PUBMED](#) | [CROSSREF](#)
33. Nuñez J, Sanz M, Hoz-Rodríguez L, Zeichner-David M, Arzate H. Human cementoblasts express enamel-associated molecules *in vitro* and *in vivo*. *J Periodontol Res* 2010;45:809-14.
[PUBMED](#) | [CROSSREF](#)
34. Paula-Silva FW, Ghosh A, Arzate H, Kapila S, da Silva LA, Kapila YL. Calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of mesenchymal periodontal ligament cells in a CEMP1- and ERK-dependent manner. *Calcif Tissue Int* 2010;87:144-57.
[PUBMED](#) | [CROSSREF](#)

35. Gronthos S, Zannettino AC, Graves SE, Ohta S, Hay SJ, Simmons PJ. Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. *J Bone Miner Res* 1999;14:47-56.
[PUBMED](#) | [CROSSREF](#)
36. Bronckers AL, Farach-Carson MC, Van Waveren E, Butler WT. Immunolocalization of osteopontin, osteocalcin, and dentin sialoprotein during dental root formation and early cementogenesis in the rat. *J Bone Miner Res* 1994;9:833-41.
[PUBMED](#) | [CROSSREF](#)
37. Pitaru S, Narayanan SA, Olson S, Savion N, Hekmati H, Alt I, et al. Specific cementum attachment protein enhances selectively the attachment and migration of periodontal cells to root surfaces. *J Periodontol* 1995;30:360-8.
[PUBMED](#) | [CROSSREF](#)
38. Arzate H, Zeichner-David M, Mercado-Celis G. Cementum proteins: role in cementogenesis, biomineralization, periodontium formation and regeneration. *Periodontol* 2000 2015;67:211-33.
[PUBMED](#) | [CROSSREF](#)
39. Arzate H, Jiménez-García LF, Alvarez-Pérez MA, Landa A, Bar-Kana I, Pitaru S. Immunolocalization of a human cementoblastoma-conditioned medium-derived protein. *J Dent Res* 2002;81:541-6.
[PUBMED](#) | [CROSSREF](#)