Characteristics of stem cells derived from the periodontal ligament of human deciduous and permanent teeth

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Characteristics of stem cells derived from the periodontal ligament of human deciduous and permanent teeth

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감사의 글

박사학위 과정을 들어온 지가 엊그제 같은데 벌써 학위를 마칠 때가 되었습니다. 그동안 돌아보면 정말 많은 분들의 도우심이 있었음을 고백하지 않을 수 없습니다. 우선 내 삶을 인도하시는 주님께 감사를 드리며, 키워주시고 기도로 지원해 주신 부모님, 어려운 여건 가운데서도 내조해 준 아내와 박사과정을 들어올 수 있도록 물질적으로 지원해 주신 장인어른과 장모님께 감사를 드립니다. 소아치과학을 가르쳐주시고 지도해주신 이제호 지도교수님을 비롯하여 아낌없는 조언을 주셨던 손흥규 교수님, 최병재 교수님, 최형준 교수님, 김성오 교수님, 이승일 교수님께 감사를 드리며 같이 연구실을 지켜준 김승혜 선생에게도 감사를 드립니다. 무엇보다 연구실 장비와 공간을 사용할 수 있도록 배려해 주시고 물심양면으로 지원을 아끼지 않아 주신 구강생물학 교실 정한성 교수님과 조성원 교수님, 곽성욱, 이종민, 신정오, 이민정, 김은정, 권혁제 선생님들에게 깊이 감사드립니다. 또한 이식실험을 알려주신 치주과 김창성 교수님과 연구원들에게도 감사를 드리며 분화실험을 알려주신 차정헌 교수님과 연구원들에게 감사를 드립니다. 골수줄기세포를 제공해 주신 연세대학교 의과대학 의공학 교실 서활 교수님과 조언을 아끼지 않아주셨던 경희대학교 치과대학 생화학교실 김정희 교수님과 연세대 신경과 연구실 조경주 선생님께도 감사를 드립니다. 마지막으로 연구를 도와주었던 최인영, 박지현 선생을 비롯한 의국원들과 김수인 직원을 비롯한 소아치과 직원들에게도 감사를 드립니다.

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Abstract

Characteristics of stem cells derived from the periodontal ligament of human deciduous and permanent teeth

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In many studies, adult stem cells have been found in human periodontal ligament (PDL), but in most cases they were found in the permanent teeth, and seldom in the deciduous teeth. The aim of the present study was to characterize stem cells from the PDL of deciduous teeth (dPDLSCs) and compare them with those from the PDL of permanent teeth (pPDLSCs). First, we investigated the proliferation rates and cell cycles of the stem cells. Stem-cell markers were examined by flow cytometric analysis and reverse transcriptase–polymerase chain reaction (RT-PCR). The results of in vitro differentiation into adipogenic, osteogenic, and chondrogenic lineages were analyzed by histochemical staining and quantitative RT-PCR. The results of in vivo transplantation into immunodeficient mice were analyzed by histological staining, immunohistochemical staining, and quantitative RT-PCR. There were no difference in proliferation rate, cell-cycle distribution, and expression of stem-cell markers such as Oct-4, Nanog, Nestin, Stro-1, CD146, CD105, and CD90 between the stem cells. The

potential for adipogenic differentiation was greater in the pPDLSCs than in the dPDLSCs, but that of osteogenic and chondrogenic differentiation was similar in the two cell types. The pPDLSC transplants made more structural cementum/PDL-like tissues than the dPDLSC transplants, in which the expression of cementum/PDL-related genes was also low (CP23, scleraxis, and collagen XII). Together these results suggest that dPDLSCs resemble pPDLSCs with regard to proliferation rate and the presence of stem-cell markers. The dPDLSCs could be a good stem-cell source for use in hard tissues or in cartilage regeneration as well as cementum/PDL complex.

Keywords: periodontal ligament, stem cells, deciduous teeth, permanent teeth, differentiation, regeneration

I. Introduction

There are several kinds of adult stem cells in teeth and tooth-related tissues such as dental pulp stem cells (DPSCs) (Gronthos et al. 2000, 13625-30), stem cells from the apical papilla (SCAP) (Kerkis et al. 2006, 105-16), dental follicle precursor cells (DFPCs) (Yao et al. 2008, 767-71), periodontal ligament stem cells (PDLSCs) (Seo et al. 2004, 149-55), and stem cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003, 5807-12). Most of these originate from permanent teeth or related tissues; however, SHED originate from the dental pulp of deciduous teeth.

Because deciduous teeth differ from permanent teeth with respect to their morphology, constituents, and life cycle, it is reasonable to assume that cells originating from deciduous and permanent teeth will behave differently. Some investigators have reported that SHED differ from DPSCs with regard to their proliferation rate (that of the former being greater than that of the latter) and differentiation pattern (unlike DPSCs, SHED are unable to reconstitute a complete dentin/pulp-like complex in vivo) (Koyama et al. 2009, 501-6, Miura et al. 2003, 5807-12, Nakamura et al. 2009, 1536-42). It was recently reported that the periodontal ligament (PDL) of deciduous teeth also contains adult stem cells (Silverio et al. 2010, 1207-15, Song et al. 2010, 575-82) and it was found that the proliferation rate and potential to differentiate into adipogenic and osteogenic lineages of these stem cells were superior to those from permanent teeth (Silverio et al. 2010, 1207-15). However, in vitro differentiation to other lineages and in vivo transplantation have not yet been studied in this cell type.

There have been many attempts to use PDLSCs for tissue reconstruction, not only to

replace destroyed periodontium in animal and human models (Akizuki et al. 2005, 245-51, Feng et al. 2010, 20-8, Liu et al. 2008, 1065-73, Park, Jeon, and Choung 2010, Washio et al. 2010, 397-404), but also for other applications such as the formation of bone around prosthetic implants (Kim et al. 2009, 1815-23), and even plastic reconstruction (Fang et al. 2007, 1021-8). However, the application of stem cells from the PDL of deciduous teeth to tissue engineering has not yet been reported.

Stem cells obtained from deciduous teeth have some advantages as a source of stem cells in regenerative medicine. This is not because deciduous teeth can be obtained easily and noninvasively; rather, it is because the proliferation and differentiation activities are higher for cells isolated from patients at a younger age (Nakamura et al. 2009, 1536-42, Zheng et al. 2009, 2363-71). Therefore, the aim of the present study was to determine the characteristics of stem cells from the PDL of deciduous teeth and how they differ from those of permanent teeth, and thus to establish whether they would be suitable for use in regeneration medicine.

II. Materials and Methods

1. Cell Cultures

PDL tissues were obtained from healthy permanent premolars (*n*=4) or anterior deciduous teeth (*n*=4) extracted for orthodontic reasons or space management from seven healthy persons (four males and three females, aged 5–13 years). The experimental protocol was approved by the Institutional Review Board of the Dental Hospital, Yonsei University, and informed consents to participate were obtained from all of the subjects and their parents (#2-2010-0011). PDL stem cells were obtained by explant culture from the tissues. In brief, the tissues were gathered from the middle area of the root. The explants were covered with cover glass and incubated with growth medium comprising α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), and 10 mM L-ascorbic acid (Sigma, St. Louis, MO, USA) at 37℃ in a humid atmosphere containing 5% CO_2. The cells grown out from explants were labeled as either stem cells from the PDL of permanent teeth (pPDLSCs) or stem cells from the PDL of deciduous teeth (dPDLSCs). The same number of cells from the same type were blended at the second passage (*n*=4, respectively), and cultures at passages 2–4 were utilized for all experiments except where stated otherwise. Human bone marrow-derived mesenchymal stem cells (BMMSCs), which were kindly provided by Prof. Hwal Suh (Department of Medical Engineering, Yonsei University, Korea), were grown under the same conditions as the pPDLSCs and dPDLSCs, and used

as positive controls.

2. Proliferation Assay and Cell Cycle Analysis

Proliferation Assay: The proliferation of the cells was measured using the Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The cells were plated in 96-well culture plates (BD Falcon, Franklin Lakes, NJ, USA) at a density of 500 cells/well. At the test time points (1, 3, 5, 7, and 9 days), 10 μl of the CCK-8 solution was added and the cells incubated for a further 4 hours. The absorbance at 450 nm was measured using a Benchmark Plus Microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) to estimate the number of vital cells in each well.

Cell Cycle Analysis: The cells were harvested by trypsinization and then fixed in cold 70% ethanol for 1 hour at 4℃. After washing twice with phosphate-buffered saline (Invitrogen), samples were incubated in 0.2 mg/ml RNase A (LaboPass, Sapporo, Japan) for 1 hour at 37℃. The cells were stained with propidium iodide (40 μg/ml; Sigma) at 4℃ for 30 min, and then subjected to FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) for cell cycle analysis. The findings were analyzed with FCSExpress V3 software (De Novo Software, Los Angeles, CA, USA).

3. Colony Forming Unit-Fibroblast (CFU-F) Assay

Single-cell suspensions of the dPDLSCs, pPDLSCs, and BMMSCs were seeded into 6-well culture plates (480 cells/well) and incubated therein for 10 days. Cultures were then fixed with 10% buffered formalin (Sigma) for 1 hour, and stained with 0.3% crystal violet

(BD Biosciences) for 5 min. The number of colonies containing over 50 cells was counted with the aid of a light microscope.

4. Flow Cytometry Analysis

Single-cell suspensions were obtained by detaching monolayers of the cells with cell dissociation buffer (Invitrogen). The cells were resuspended in flow cytometry staining buffer (eBiosciences, San Diego, CA, USA) and incubated with 20 μl of mouse monoclonal antihuman antibodies [fluorescein isothiocyanate-conjugated CD146, R-phycoerythrin (PE)-conjugated CD90, PE-conjugated CD105, and PE-conjugated CD31; all supplied by eBiosciences] or 5 μg of antihuman Stro-1 (IgM, R&D Systems, Minneapolis, MN, USA) per 1×10^6 cells for 1 hour. For antihuman Stro-1 staining, the cells were additionally incubated with PE-conjugated goat antimouse antibody (0.1 μg/1×10^6 cells; IgM, SouthernBiotech, Birmingham, AL, USA) for 30 min. For negative controls, primary antibodies were omitted. All of the aforementioned procedures were performed in the dark at 4℃. The expression profiles were examined using a FACSCalibur flow cytometer and analyzed with FCSExpress V3 software. Positive expression was defined as a level of fluorescence greater than 99% of the corresponding control.

5. Gene Expression Analysis Using Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

When the cells reached subconfluency (i.e., they occupied 70–80% of the culture dish),

total cellular RNA was isolated using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was eluted in 30 ml of water, and the integrity and concentration were evaluated using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Waltham, MA, USA). One microgram of total RNA was reverse transcribed with a Maxime RT premix kit (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Briefly, total RNA was reverse transcribed using an oligo $d(T)_{15}$ primer for 1 hour at 45℃, and the reaction was stopped by incubation for 5 min at 95℃. The PCR amplifications were performed in a 20-ml reaction volume using a Maxime PCR PreMix Kit (Intron Biotechnology) and gene-specific primers (as listed in Table 1) in a thermal cycler (Swift MaxPro, ESCO, Singapore). The PCR conditions were 95℃ for 2 min, followed by the appropriate number of cycles of 94℃ for 20 sec, 60℃ for 10 sec, 72℃ for 20 sec, and a final 5-min extension at 72℃. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplifications were carried out as positive controls to assure the quality of the cDNAs used for this experiment. All PCR reactions were performed within the exponential amplification range. The PCR products were mixed with LoadingStar (DyneBio, Sungnam, Korea), separated on 2% agarose gels by electrophoresis, and then photographed under ultraviolet excitation with ChemiDoc XRS (Bio-Rad Laboratories).

6. Quantitative RT-PCR

Total cellular RNA extraction and cDNA synthesis were performed as described above. A quantitative PCR assay was performed by monitoring in real time the increase in fluorescence of SYBR Green dye on a Thermal Cycler Dice real-time system (Takara Bio,

Otsu, Japan) according to the manufacturer's instructions. Each PCR assay was carried out in duplicate in a 20-μl volume using SYBR Premix Ex Taq (Takara Bio) for 10 sec at 95℃ for the initial denaturing step, followed by 45 cycles at 95℃ (denaturation) for 5 sec, 60℃ (annealing) for 15 sec, and 72℃ (amplification) for 10 sec. Amplification specificity was confirmed by visualizing PCR products on 1.5% agarose gels and by melting-curve analysis (from 60℃ to 95℃) after the completion of 45 cycles. The values for each gene were normalized to the expression levels of GAPDH, and relative quantification of studied genes was calculated by using the formula $2^{-\Delta\Delta Ct}$ (Livak, and Schmittgen 2001, 402-8). Specific primer sequences and product sizes for each gene are listed in Table 1.

Table 1. RT-PCR and quantitative RT-PCR primers used in this study

Genes	Primer sequence (5'-3')	Size (bp)	Cycles
Oct-4	F: CGACCATCTGCCGCTTTGAG	573	28
	R: CCCCCTGTCCCCCATTCCTA		
NANOG	F: TGCAAATGTCTTCTGCTGAGAT	287	28
	R: GTTCAGGATGTTGGAGAGTTC		
Nestin	F: GCCCTGACCACTCCAGTTTA	200	30
	R: GGAGTCCTGGATTTCCTTCC		
GAPDH	F: AGGTGAAGGTCGGAGTCAACG	231	24
	R: GCTCCTGGAAGATGGTGATGG		
CP23	F: AACACATCGGCTGAGAACCTCAC	142	45*
	R: GGATACCCACCTCTGCCTTGAC		
Collagen	F: CGGACAGAGCCTTACGTGCC	180	45*
XII	R: CTGCCCGGGTCCGTGG		
Osteocalcin	F: CAAAGGTGCAGCCTTTGTGTC	150	45*
	R: TCACAGTCCGGATTGAGCTCA		

PPAR γ2	F: ACAGCAAACCCCTATTCCATGCTGT	159	45*
	R: TCCCAAAGTTGGTGGGCCAGAA		
LPL	F: TGGACTGGCTGTCACGGGCT	167	45*
	R: GCCAGCAGCATGGGCTCCAA		
BSP	F: CTGGCACAGGGTATACAGGGTTAG	182	45*
	R: ACTGGTGCCGTTTATGCCTTG		
Aggrecan	F: TTCCTGGTGTGGCTGCTGTC	95	45*
	R: TTCTGGCTCGGTGGTGAACTC		
SOX9	F: CTGAGTCATTTGCAGTGTTTTCT	103	45*
	R: CATGCTTGCATTGTTTTTGTGT		
Osteopontin	F: ACCTGAACGCGCCTTCTG	66	45*
	R: CATCCAGCTGACTCGTTTCATAA		
Scleraxis	F: CTGGCCTCCAGCTACATCTC	210	45*
	R: CTTTCTCTGGTTGCTGAGGC		
GAPDH	F: TCCTGCACCACCAACTGCTT	100	45*
	R: TGGCAGTGATGGCATGGAC		

*Quantitative RT-PCR. References: Oct-4, NANOG and Scleraxis (Tomokiyo et al. 2008, 337-47); Nestin (Honda et al. 2007, 949-58); GAPDH (Lapsys et al. 2000, 4293-7); CP23, Collagen XII, BSP and GAPDH (Fujii et al. 2008, 743-9); Osteocalcin (Garlet et al. 2007, 355-62); PPAR ɣ2 and LPL (Song et al. 2010, 575-82); Aggrecan (Merceron et al. 2010, C355-64); SOX9 (Dehne et al. 2009, R133); Osteonectin (Huang et al. 2009, 809-21)

7. In Vitro Differentiation

Adipogenic Differentiation: The cells were seeded at a density of 1×10^4 cells/cm^2 in growth medium in 12-well culture dishes. When reaching confluence, the cells were treated for 10 days with adipogenic induction medium [α-MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone (Sigma), 10 μg/ml human insulin (Sigma), 100 μM indomethacin (Sigma), and 500 μM 3-isobutyl-L-methylxanthine (Sigma)]. After 10 days, the medium was switched to adipogenic maintenance medium (α-MEM containing 10% FBS, 1% antibiotics, and 10 μg/ml human insulin) for 10 days. As a control, the cells were cultured only in growth medium without differentiation stimuli. After 20 days, intracellular accumulation of lipids was visualized using Oil Red O staining. Briefly, cells were fixed for 30 min with 10% natural-buffered formalin (Sigma) at 4℃ and stained with 0.2% Oil Red O (Sigma) for 10 min at room temperature. Changes in the gene expressions of peroxisome proliferator-activated receptor γ2 (PPARγ2) and lipoprotein lipase (LPL) were evaluated with quantitative RT-PCR.

Cementogenic/Osteogenic Differentiation: The cells were prepared in 12-well culture dishes as described above. When reaching confluence, cultures were treated with osteogenic induction medium [α-MEM containing 10% FBS, 1% antibiotics, 0.1 M dexamethasone, 2 mM β-glycerolphosphate (Sigma), and 50 μM ascorbic acid 2-phosphate] for 5 weeks. As a control, the cells were cultured only in growth medium without differentiation stimuli. After 5 weeks, calcification of the extracellular matrix was visualized using Alizarin Red S staining. Briefly, cells were fixed for 30 min with 10% natural-buffered formalin at 4℃ and stained with 2% Alizarin Red S (pH 4.2; Sigma) for

10 min at room temperature. Changes in the gene expressions of alkaline phosphatase (ALP) and bone sialoprotein (BSP) after cementogenic/osteogenic differentiation were evaluated with quantitative RT-PCR.

Chondrogenic Differentiation: Chondrogenic differentiation was induced by using the "pellet culture" technique (Vunjak-Novakovic, and Freshney 2006, xiii, 512 p.). Briefly, approximately 2.5×10^5 cells were placed in a 15-ml polypropylene tube (BD Falcon), and centrifuged to a pellet at $300\times g$ for 5 min. After aspiration of the medium, the chondrogenic medium from the Stempro chondrogenesis differentiation kit (Invitrogen) supplemented with 10 ng/ml recombinant human TGF-β3 (Peprotech, Rochy Hill, NJ, USA) was added. Pellets cultured in growth medium were used as controls. After 4 weeks, the presence of glycosaminoglycans (GAG) in the cell pellets was determined by Safranin O staining. In brief, the pellets were fixed in 10% neutral buffered formalin, embedded in 2% agarose, dehydrated in an ethanol series, embedded in paraffin, and then sectioned at 5 μm. After deparaffinization, the sections were stained with 0.1% Safranin O (Sigma) and counterstained with hematoxylin–eosin (H&E). Changes in the gene expressions of aggrecan (ACAN) and SRY (sex determining region Y)-box 9 (SOX9) were evaluated by quantitative RT-PCR.

8. In Vivo Transplantation

All animal procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Yonsei University (#10-056). Approximately 3×10^6 in-vitro expanded cells were mixed with 40 mg of macroporous biphasic calcium phosphate (MBCP; Biomatlante, Vigneux de Bretagne, France) and then

incubated at 37℃ for 2 hours. After centrifugation at 500×*g* for 5 min, the supernatant was removed. The cell pellets with MBCP were transplanted into the dorsal subcutaneous pockets of 5-week-old male immunocompromised mice (BALB/c-nu, SLC, Shizuoka, Japan), as described previously (Gronthos et al. 2000, 13625-30). Briefly, midlongitudinal skin incisions were made on the dorsal surface of each mouse, and four subcutaneous pockets were made by blunt dissection. The BMMSC, pPDLSC, and dPDLSC pellets mixed with MBCP, and MBCP particles alone (used as a negative control) were placed in each pocket of the same mouse. After 8 weeks, the mice (*n*=14) were sacrificed and all transplants were retrieved.

Quantitative RT-PCR Analysis: The specimens *(n*=11) for quantitative RT-PCR were homogenized in the RLT buffer from an RNeasy Mini Kit with a homogenizer (Bullet Blender Next Advance, Averill Park, NY, USA) immediately after retrieval. Total RNA was extracted and reverse transcription was performed as described above. The relative gene expressions of human BSP, human collagen XII, human osteopontin, human osteocalcin, human scleraxis, and human CP23 for each transplant were evaluated by real-time quantitative PCR. Values for each gene were normalized to the expression levels of GAPDH, and the expression levels of the genes of concern in each transplant relative to those of carrier transplants (MBCP without any cells) were calculated.

Histology and Immunohistochemical Analysis: Histological and immunohistochemical specimens (*n*=3) were fixed in 10% buffered formalin for 1 day, decalcified with 10% EDTA (pH 7.4; Fisher Scientific, Houston, TX, USA) for 2 weeks, embedded in paraffin, and sectioned at a thickness of 5 μm. Specimens were subjected to H&E and Masson's trichrome staining, as well as immunohistochemical staining with antihuman BSP (rabbit

polyclonal; Abcam, Cambridge, UK), antihuman osteocalcin (rabbit polyclonal; Millipore, Temecula, CA, USA), and antihuman collagen XII (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For antigen retrieval prior to osteocalcin staining, sections were pretreated with proteinase K (Dako, Carpinteria, CA, USA) and for BSP and collagen XII, sections were pretreated by boiling in 1% citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by the addition of 3% hydrogen peroxide. Sections were incubated in 5% bovine serum albumin to block nonspecific binding. The primary antibodies were diluted to give optimal staining (anti-BSP, 1:1500; anti-osteocalcin, 1:2500; and anti-collagen-XII, 1:400), and the sections were incubated overnight. After incubation, a secondary biotinylated goat antimouse IgG (1:100; Vector Laboratories, Burlingame, CA, USA) was applied for 30 min. Color development was performed using labeled streptavidin biotin kits (Dako) according to the manufacturer's instructions. In brief, sections were incubated with streptavidin peroxidase conjugate for 10 min. Color was developed 1 min after the addition of diaminobenzidine substrate. The sections were counterstained with Gill's hematoxylin (Sigma). Control sections were treated in the same manner but without treatment with primary antibodies.

9. Statistical Analysis

All of the experiments were repeated under at least three independent conditions. All data are presented as mean and standard deviation values. Any differences were determined by one-way analysis of variance (ANOVA) followed by Scheffé's F-test using SPSS (version 17.0; Chicago, IL, USA), with the level of statistical significance set at $p<0.05$.

III. Results

1. Proliferation Assay and Cell Cycle Analysis

The cells outgrown from the explants exhibited a typical spindle-shaped fibroblastic morphology; the morphology did not differ between pPDLSCs and dPDLSCs (Fig. 1A–D). In the proliferation assay, the optical density of dPDLSCs was similar to that of pPDLSCs at every time point examined, but was significantly higher than that of BMMSCs (Fig. 1E). The cell cycle analysis also showed that the percentage of G1-phase dPDLSCs (57.1%) was similar to that of dPDLSCs (57.3%), but was lower than that of BMMSCs (70.1%), as seen in Fig. 1F.

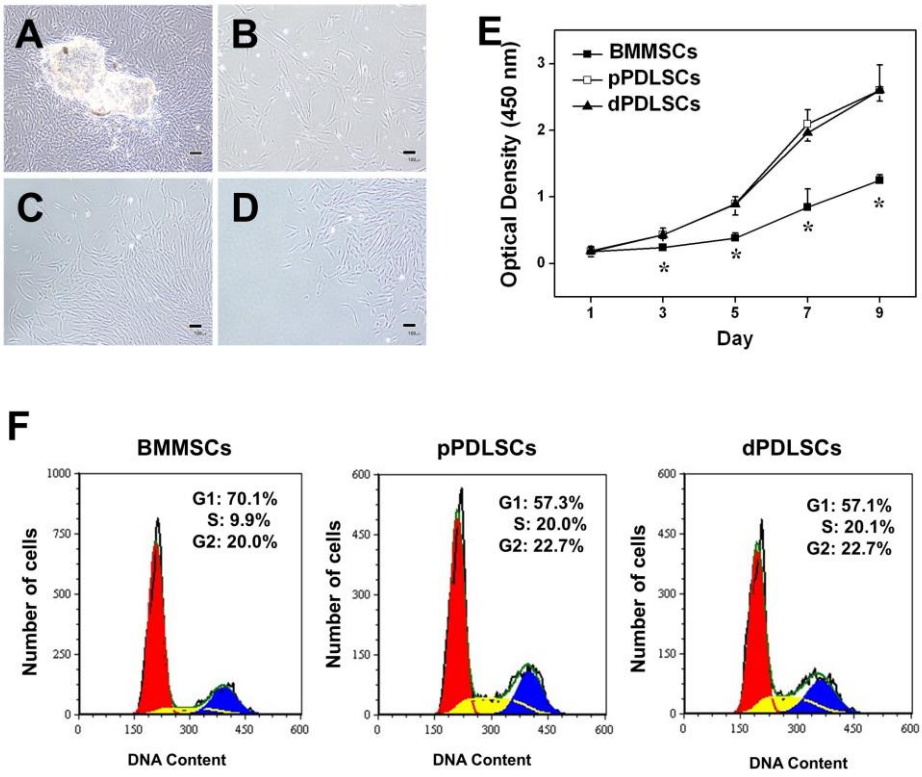

Figure 1. Morphologic characteristics and proliferation of BMMSCs, pPDLSCs, and dPDLSCs. **(A–D)** Morphologic characteristics. Scale bar: 100 μm. **(A)** The cells outgrown from the PDL tissue of deciduous teeth. **(B)** BMMSCs. **(C)** pPDLSCs. **(D)** dPDLSCs. **(E)** Changes in optical density in the proliferation assay. Data are mean and standard deviation values. *One-way ANOVA, $p<0.05$. **(F)** Cell-cycle distribution.

2. CFU-F Assay

All three cell types exhibited colony-forming ability (Fig. 2A). The number of CFU-Fs per 400 cells in dPDLSCs (42.3±7.3) was slightly lower than that in pPDLSCs (51.4±8.9), but the difference was not statistically significant. However, the BMMSCs had the significantly lowest number of CFU-Fs (20.7±2.2; $p>0.05$; Fig. 2B).

3. Flow Cytometry Analysis

The results of flow cytometric analysis of markers related to mesenchymal stem cells are shown in Fig. 2C. Almost all of the pPDLSCs and dPDLSCs expressed CD90 and CD105 (>99.9%). CD146 and Stro-1 were expressed in a considerable number of pPDLSCs and dPDLSCs (>80.4%). However, the expression of CD31 (endothelial stem cell marker) was low in both cell types (<3.5%).

4. Gene Expression Pattern by RT-PCR

The gene expression patterns of BMMSCs, pPDLSCs, and dPDLSCs are shown in Fig. 2D. All three cell types expressed Oct-4 and Nanog, which are pluripotency-related genes expressed in embryonic stem cells (ES cells). The expression of Nestin (a marker for neural crest cells) was higher in both pPDLSCs and dPDLSCs than in BMMSCs.

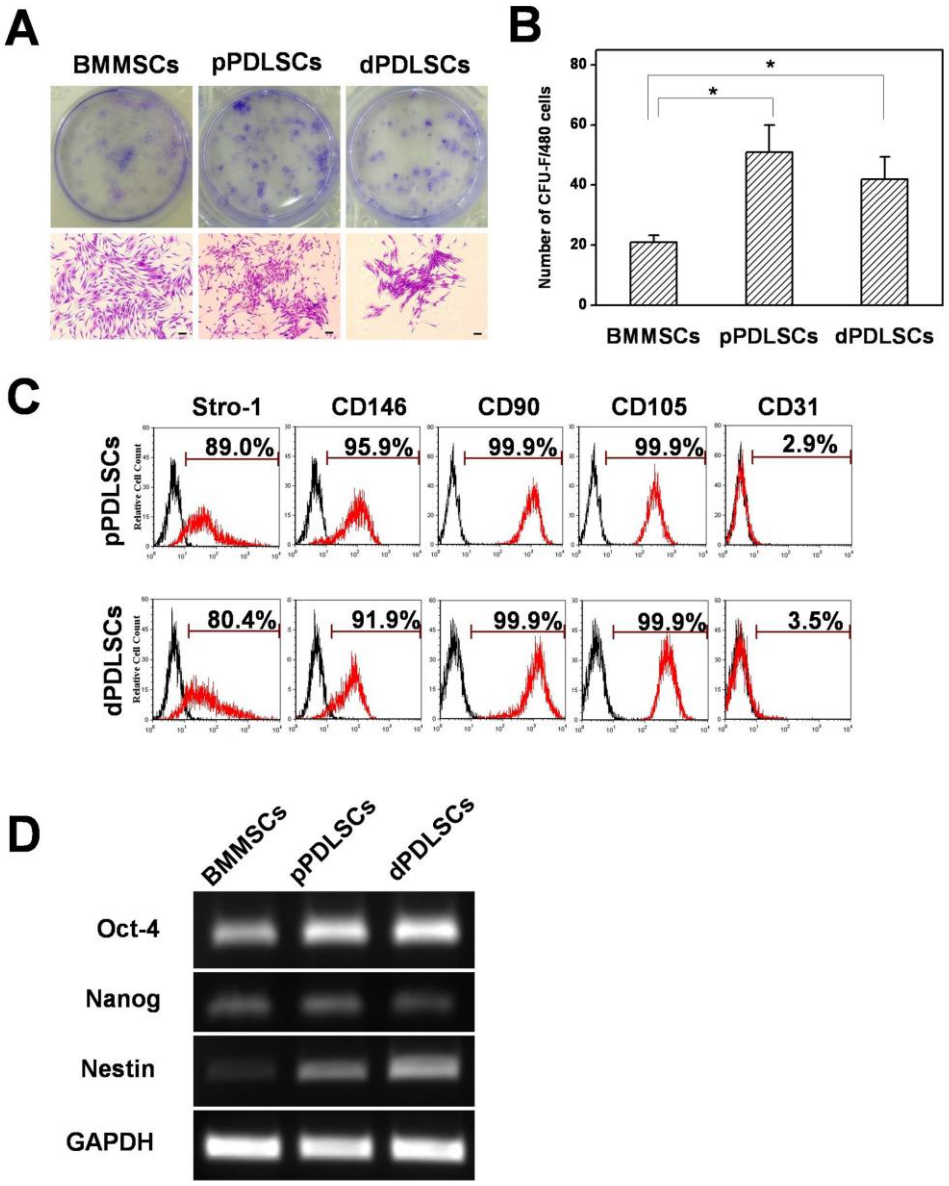

Figure 2. CFU-F assay, flow cytometry analysis, and gene expression of the BMMSCs, pPDLSCs, and dPDLSCs. **(A)** Crystal violet staining. Scale bar: 100 μm. **(B)** The number of colonies per 480 cells. Data are mean and standard deviation values. *One-way ANOVA, $p<0.05$. **(C)** Flow cytometry analysis; black line=controls, red line=tests. Horizontal bars indicate 1% of control samples. **(D)** Gene expression patterns of Oct-4,

Nanog, Nestin, and GAPDH (internal control).

5. In Vitro Differentiation

Under adipogenic stimuli, all three stem cells could differentiate into cells that had vacuoles containing lipids (Fig. 3A). Adipogenic differentiation occurred the most in the BMMSCs, followed (in order) by pPDLSCs and dPDLSCs, as evaluated by Oil Red O staining and quantitative RT-PCR analyses of changes in PPARγ2 and LPL expression (Fig. 3B). Under cementogenic/osteogenic stimuli, all three types of stem cells differentiated into cells that produced mineralized extracellular matrix. These findings were confirmed using Alizarin Red S staining (Fig. 3C) and up-regulation of ALP and BSP gene expressions (Fig. 3D). Under chondrogenic differentiation stimuli, all three stem cells could differentiate into the cells that produced extracellular GAG. These findings were confirmed using Safranin O staining (Fig. 3E) and up-regulation of ACAN and SOX9 gene expressions (Fig. 3F). The extents of cementogenic/osteogenic and chondrogenic differentiation were similar in the three types of stem cell.

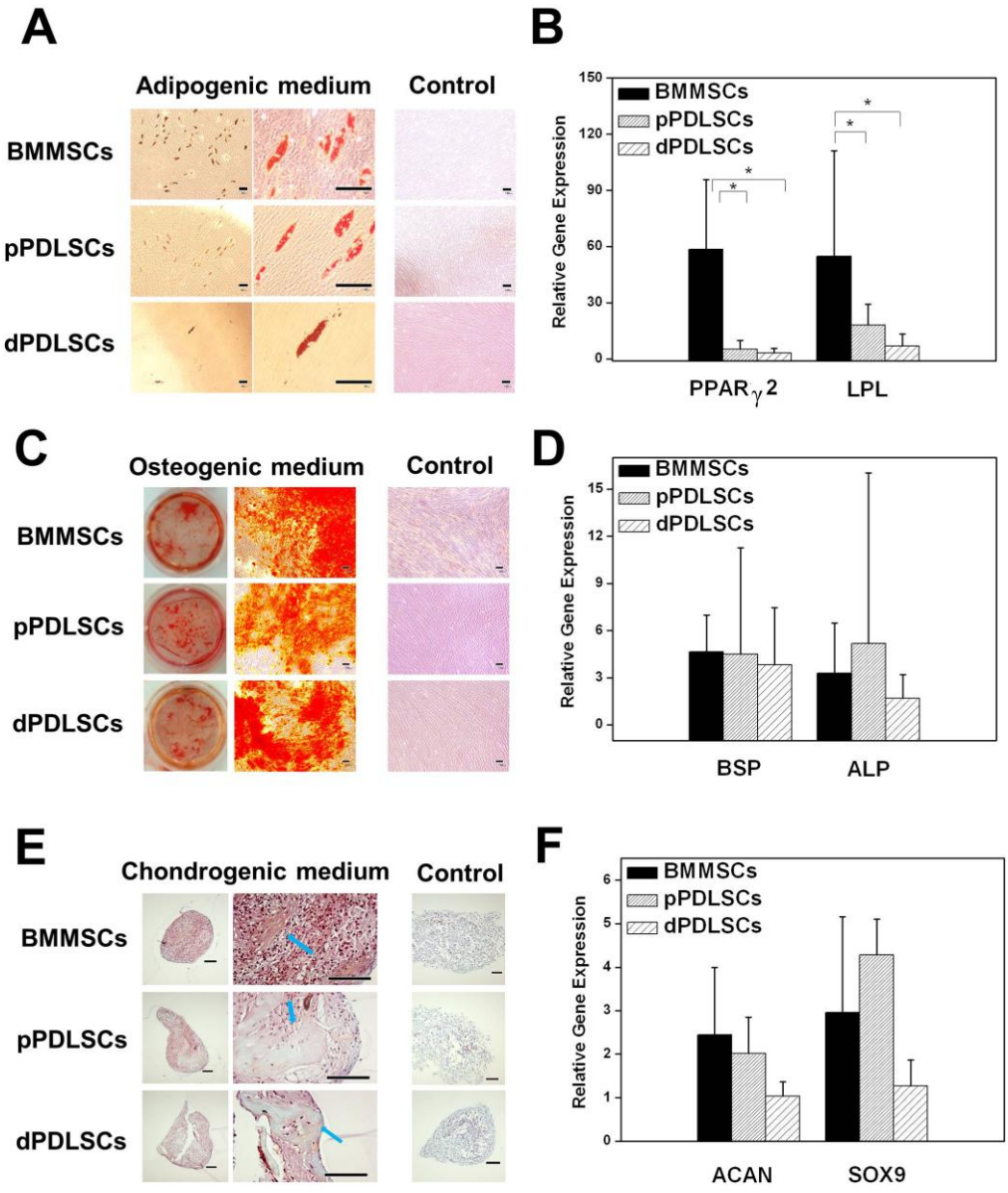

Figure 3. Adipogenic, cementogenic/osteogenic, and chondrogenic differentiation of BMMSCs, pPDLSCs, and dPDLSCs. **(A, B)** Adipogenic differentiation. **(A)** Oil Red O staining. **(B)** Changes in PPARγ2 and LPL gene expression after 3 weeks of culture in adipogenic differentiation medium or control medium. **(C, D)** Cementogenic/osteogenic differentiation. **(C)** Alizarin Red S staining **(D)** Up-regulation of ALP and BSP gene

expressions after 2 weeks of culture in cementogenic/osteogenic differentiation medium or control medium. **(E, F)** Chondrogenic differentiation. **(E)** Safranin O staining. Arrows indicate extracellular GAG. **(F)** Up-regulation of ACAN and SOX9 gene expressions after 3 weeks of culture in the form of pellets in chondrogenic differentiation medium or control medium. Scale bars: 100 μm. Data are mean and standard deviation values. *One-way ANOVA, $p<0.05$.

6. In Vivo Transplantation

Eight weeks after transplantation, all three types of stem cell could produce hard tissues at the periphery of the MBCP, but not in MBCP transplants (Fig. 4A–E). BMMSC transplants made bone-like tissues with a lamellar pattern, whereas pPDLSC and dPDLSC transplants made cementum-like tissues and dense collagen bundles resembling Sharpey's fibers (Fig. 4A, C, and G). The pPDLSCs made more fibrous tissues adjacent to cementum-like tissue than did dPDLSCs (Fig. 4B–D). Immunohistochemical staining revealed that antiosteocalcin and anti-BSP antibodies reacted with the cells on the margin of the bone/cementum-like tissues (Fig. 4I–P), whereas much more collagen XII was found in the pPDLSC transplants than in the dPDLSC transplants (Fig. 4Q–T).

After transplantation, the gene expressions of BSP, osteocalcin, and osteopontin were greater in dPDLSC transplants than in pPDLSC transplants. However, the gene expressions of CP23, collagen XII, and scleraxis, which are related to the cementum/PDL complex, were up-regulated the most in the pPDLSC transplants, followed (in order) by dPDLSC and BMMSC transplants (Fig. 5).

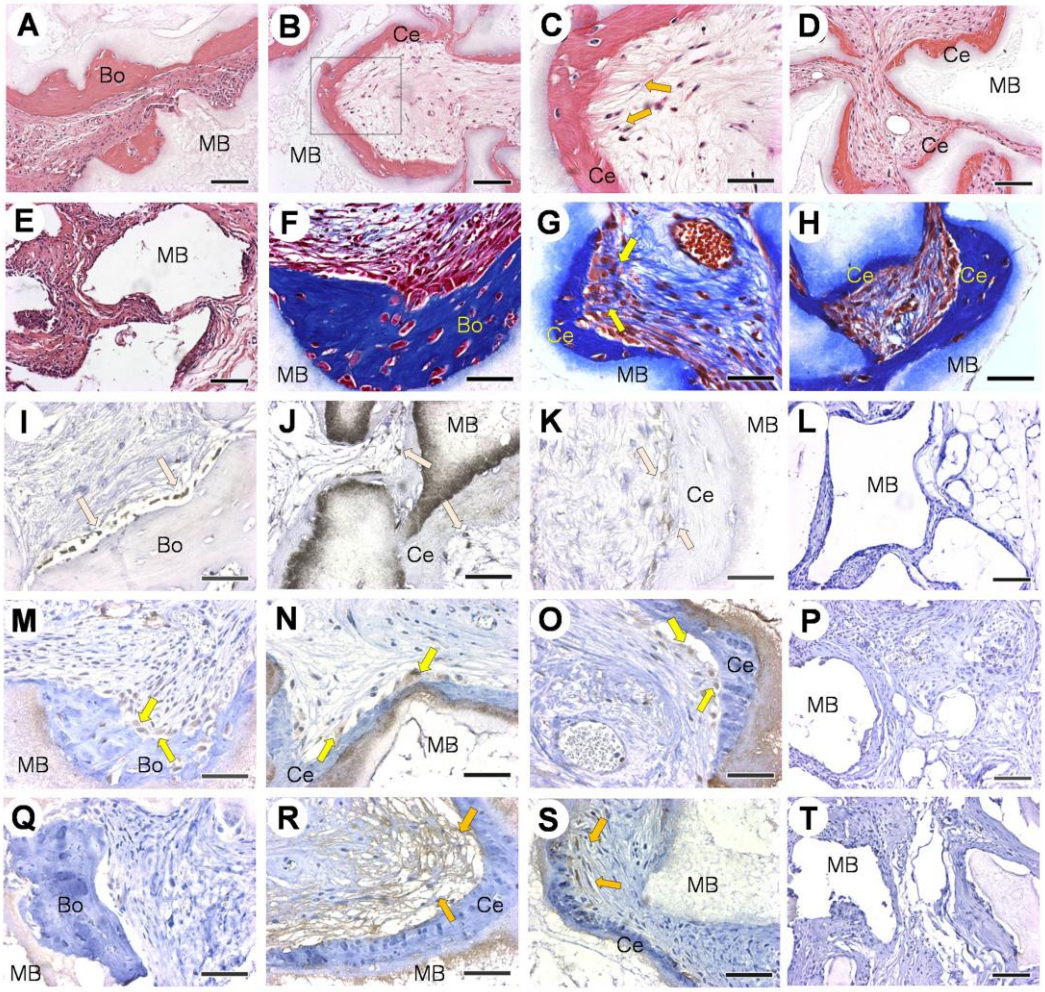

Figure 4. Histological and immunohistochemical staining of BMMSC, pPDLSC, and dPDLSC transplants. **(A–H)** H&E and Masson's trichrome staining. **(A, F)** BMMSCs. **(B, C)** pPDLSCs. **(D, G, and H)** dPDLSCs. **(E)** MBCP transplants. Arrows indicate collagen bundles inserted into cementum-like tissues, resembling Sharpey's fibers. **(I–P)** Immunohistochemical staining for osteocalcin **(I–L)** and BSP **(M–P)**. **(I, M)** BMMSCs. **(J, N)** pPDLSCs. **(K, O)** dPDLSCs. **(L, P)** MBCP transplants. **(Q–T)** Immunohistochemical staining for collagen XII. **(Q)** BMMSC, **(R)** pPDLSC, **(S)** dPDLSC, and **(T)** MBCP transplants. Abbreviations: Bo, bone-like tissue; Ce, cementum-

like tissue; MB, MBCP carrier. Scale bars: 100 μm in **A**, **B**, **D**, **E**, **L**, **P**, and **T**; 50 μm in **C**, **F**, **G**, **H–K**, **M–O**, and **Q–S**.

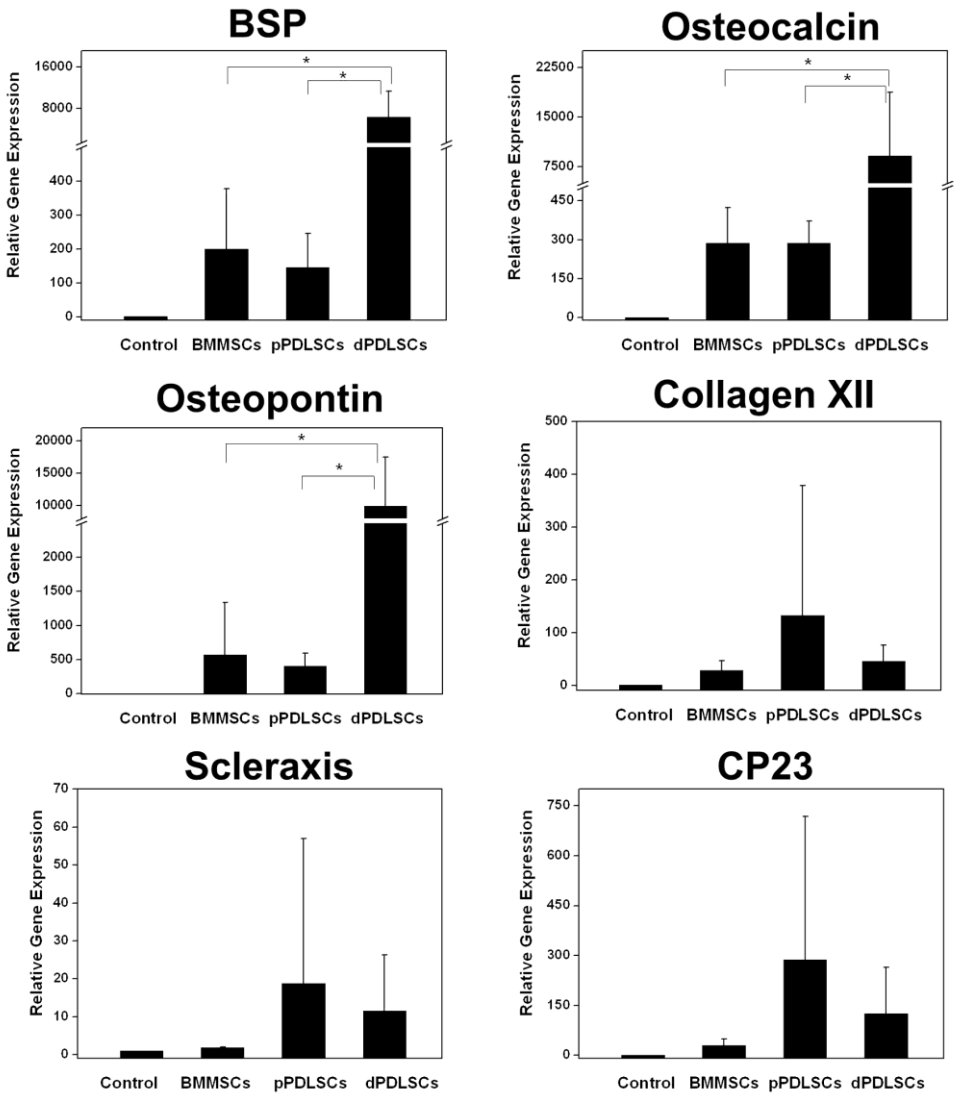

Figure 5. Gene expressions of BSP, osteocalcin, osteopontin, collagen XII, scleraxis, and CP23 in the BMMSC, pPDLSC, and dPDLSC transplants. The gene expression level of MBCP transplants was set as the control (value=1). Data are mean and standard deviation values. *One-way ANOVA, $p<0.05$.

IV. Discussion

Human beings have two kinds of teeth, deciduous and permanent, which differ in morphologic, histological, and developmental characteristics. In addition, the proliferation activities and differentiation patterns differ between the cells isolated from the two types of teeth (Govindasamy et al. 2010, 1504-15, Miura et al. 2003, 5807-12, Nakamura et al. 2009, 1536-42). In the present study, it was found to be difficult to distinguish between pPDLSCs and dPDLSCs simply according to their morphology and proliferation patterns. However, the finding that stem cells from the PDL tissue had a higher proliferation rate than BMMSCs concurred with the findings of some other studies (Gay, Chen, and MacDougall 2007, 149-60, Jo et al. 2007, 767-73). Many reports have stated that the cells isolated from the pulp tissues of deciduous teeth have a higher proliferation rate than those of permanent teeth (Govindasamy et al. 2010, 1504-15, Miura et al. 2003, 5807-12, Nakamura et al. 2009, 1536-42). In the case of the PDL, Silverio et al. reported the PDL cells from deciduous teeth also have a higher proliferation rate [9]. However, they used cells isolated via enzymatic dissociation, and a small population of PDL cells obtained by immunomagnetic cell sorting ($CD105^{+}$, $CD34^{-}$, and $CD45^{-}$ cells). The characteristics of cells isolated by enzyme-digested methods differ from those isolated by the outgrowth method (Spath et al. 2010, 1635-44, Tanaka et al. 2010). This difference in experimental conditions may be an explanation for the disharmony between the obtained results; further investigations are needed to clarify this issue.

CFU-F assays have been used to evaluate self-renewal ability, which is a characteristic of mesenchymal stem cells (Castro-Malaspina et al. 1980, 289-301). In accordance with some previous studies, but not with others, we found that the pPDLSCs exhibited a greater number of CFU-Fs than BMMSCs (Gay, Chen, and MacDougall 2007, 149-60, Singhatanadgit, Donos, and Olsen 2009, 2625-36, Xu et al. 2009, 487-96). Although the dPDLSCs also exhibited a greater number of CFU-Fs than did BMMSCs, it is not possible to compare the findings of the present study to those of previous studies because this is the first study to perform CFU-F assays of dPDLSCs.

The present study revealed the expression of Oct-4 and Nanog – which are transcription factors required to maintain the pluripotency and self-renewal of ES cells (Chambers et al. 2003, 643-55, Loh et al. 2006, 431-40, Niwa, Miyazaki, and Smith 2000, 372-6) – in the pPDLSCs, dPDLSCs, and BMMSCs. However, Nestin, which is a kind of intermediate filament in neuroectodermal stem cells (Lendahl, Zimmerman, and McKay 1990, 585-95, Lobo et al. 2004, 369-76), was observed more in the pPDLSCs and dPDLSCs. These findings may be attributed to dental tissues, including the PDL, developing from ectomesenchyme (Nanci, and Ten Cate 2008, x, 411 p.). In addition, pPDLSCs and dPDLSCs represented mesenchymal stem-cell surface markers such as Stro-1, CD146, CD105, and CD90 (Barry et al. 1999, 134-9, Pittenger et al. 1999, 143-7, Seo et al. 2004, 149-55, Simmons, and Torok-Storb 1991, 55-62), but not CD31, which is an endothelial cell marker (Albelda et al. 1991, 1059-68). Therefore, the pPDLSCs and dPDLSCs in this study appeared to have arisen from the perivascular area, where Stro-1 and CD146 antigen reacted strongly (Lin et al. 2008, 514-23, Song et al.

2010, 575-82).

We found the adipogenic differentiation potentials to be lower for the pPDLSCs and dPDLSCs than for the BMMSCs, which is consistent with the findings of previous studies (Iwata et al. 2010, 1088-99, Singhatanadgit, Donos, and Olsen 2009, 2625-36, Xu et al. 2009, 487-96). The comparison of pPDLSCs and dPDLSCs by Silverio et al. revealed that the adipogenic differentiation of pPDLSCs was superior to that of dPDLSCs (Silverio et al. 2010, 1207-15). This finding does not concur with ours. Furthermore, while Silverio et al. reported that the osteogenic differentiation potential of dPDLSCs was inferior to that of pPDLSCs, we did not. Whether different experimental conditions are responsible for this disagreement requires further investigation.

It has been reported that PDLSC transplants are capable of producing tissues that resemble cementum/PDL complex (Fujii et al. 2008, 743-9, Seo et al. 2004, 149-55, Seo et al. 2005, 907-12). In the present study, both pPDLSCs and dPDLSCs were able to make cementum-like and adjacent PDL-like tissues, but not the lamellar pattern of hard tissue observed in the BMMSC transplants. It was recently reported that stem cells from the PDL adjacent to alveolar bone could make more structural bone-like tissue than those from PDL attached to the root (Wang et al. 2010). Therefore, it is reasonable that stem cells obtained from the PDL attached to extracted teeth might be capable of making cementum/PDL-like tissue rather than bone-like tissue, as shown previously and in our own studies. The pPDLSC transplants were capable of making more structural cementum/PDL complex, and expressed more cementum/PDL-related genes than dPDLSC

transplants: CP23 (identified as a cementoblast marker and regulator of the biomineralization of cementum) (Alvarez-Perez et al. 2006, 409-19, Villarreal-Ramirez et al. 2009, 49-54), collagen XII (which is found in tissues bearing high-tensile stress such as tendons and PDLs) (Berkovitz 1990, 51-76, Chiquet 1999, 417-26, Karimbux, and Nishimura 1995, 313-8, Nanci, and Ten Cate 2008, x, 411 p.), and scleraxis (which is a specific marker for tendons and ligaments) (Schweitzer et al. 2001, 3855-66, Seo et al. 2004, 149-55). However, the dPDLSC transplants expressed more BSP, osteopontin, and osteocalcin genes – which are involved in the mineralization of both cementum and bone (Nanci, and Ten Cate 2008, x, 411 p.) – than cementum/PDL-related genes. In addition, it was reported that cementoblasts of deciduous teeth expressed more BSP and osteopontin, which were thought to be associated with odontoclast adhesion and subsequent root resorption, than those of permanent teeth (Lee et al. 2004, 173-7, Merry et al. 1993, 1013-20, Miyauchi et al. 1993, 132-5, Ross et al. 1993, 9901-7). Therefore, the features of the newly formed hard tissue in the dPDLSC transplants were similar to cementum of deciduous teeth which was prone to be resorbed (Davies et al. 2001, 339-47).

The finding that cells that originate from permanent teeth make more structural tissue resembling the original tissues in vivo than those of deciduous teeth has been reported previously for studies of dental pulp (Batouli et al. 2003, 976-81, Gronthos et al. 2000, 13625-30, Miura et al. 2003, 5807-12). The DPSCs formed vascularized pulp-like tissue surrounded by dentin-like tissues (Batouli et al. 2003, 976-81, Gronthos et al. 2000, 13625-30), but SHED were unable to regenerate a complete dentin/pulp-like complex (Miura et al. 2003, 5807-12), and instead formed bone-like tissues that stained negatively

for anti-dentin sialoprotein and anti-dentin sialophosphoprotein antibodies (Miura et al. 2003, 5807-12, Seo et al. 2008, 428-34). It seems that the cells that produce bone-like tissues originated from both murine cells and transplanted SHED, as shown by their reaction to anti-human-specific mitochondria antibody and the expressions of both mouse mRNAs and human mRNAs (Miura et al. 2003, 5807-12, Seo et al. 2008, 428-34). Transplanted human PDLSCs were revealed to differentiate into cementoblasts and PDL fibroblasts (Fujii et al. 2008, 743-9, Seo et al. 2004, 149-55, Seo et al. 2005, 907-12); however, it is not certain that the dPDLSCs themselves could differentiate into osteoblasts and induce recipient murine cells to differentiate into bone-forming cells, as the SHED could. Although we were unable to determine the lamellar pattern of the newly formed hard tissues and rule out the absence of cementum-specific protein (CP23), it is possible that the dPDLSCs differentiated into bone-forming cells since the dPDLSC transplants exhibited abundant human mineralization-related genes (compared to human CP23).

V. Conclusion

The proliferation rate, expressions of stem-cell markers, and differentiation patterns were similar in dPDLSCs and pPDLSCs. One advantage of dPDLSCs is that they can be obtained easily from younger patients, and may thus be a good source of stem cells for use in the regeneration of cementum/PDL complex, cartilage, or other hard tissues.

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국문요약

유치와 영구치 치주인대에서 유래한 줄기세포의 특성

치주인대에 성체 줄기세포가 존재한다고 보고되어 왔으나 대부분 영구치에서의 보고였으며 유치에서의 보고는 거의 없었다. 본 연구의 목적은 유치의 치주인대로부터 얻은 줄기세포(dPDLSCs)의 특성을 파악하고 이를 영구치의 치주인대로부터 얻은 줄기세포(pPDLSCs)와 비교해 보고자 한다. 세포증식율과 세포주기를 검사하였으며 유세포분석과 역전사 중합효소 연쇄반응(RT-PCR)을 통해 줄기세포 표지자를 확인하였다. 골성, 지방성, 및 연골성 분화를 시행하고 조직화학적 염색법과 정량적 RT-PCR로 분석하였다. 면역 억제된 쥐에 줄기세포를 이식한 후 생성된 조직을 조직학적, 면역조직학적으로 확인하고 정량적 RT-PCR로 분석하였다. 두 세포군 모두 세포증식율, 세포주기 Oct-4, Nanog, Nestin, Stro-1, CD146, CD105, 및 CD90과 같은 줄기세포 표지자의 발현에는 차이가 없었다. 지방세포로의 분화는 pPDLSCs에서 더 많이 일어났으나 골성, 연골성 분화에서는 차이가 없었다. pPDLSC를 이식 후 생성된 조직은 보다 구조적인 백악질/치주인대양 조직이 형성되었으며 관련 유전자 (CP23, scleraxis, 및 collagen XII)의 발현도 높게 나타났다. 결론적으로 dPDLSCs은 세포증식율과 줄기세포 표지자 발현 및 분화양상은 pPDLSCs와 유사하였으며 백악질/치주인대 생성뿐만 아니라 지방조직, 경조직, 그리고 연골조직을 재생하는데 사용될 수 있는 가능성을 제시하였다.

핵심되는 말: 치주인대, 줄기세포, 유치, 영구치, 분화, 재생