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Periodontal tissue engineering with stem cells from the periodontal ligament of human retained deciduous teeth

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Background and Objective: Periodontal ligament stem cells from human permanent teeth (PePDLSCs) have been investigated extensively in periodontal tissue engineering and regeneration. However, little knowledge is available on the periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs). This study evaluated the potential of DePDLSCs in periodontal tissue regeneration.

Material and Methods: DePDLSCs were isolated and purified by limited dilution. The characteristics of DePDLSCs were evaluated and compared with PePDLSCs both *in vitro* and *in vivo*.

Results: DePDLSCs presented a higher proliferation rate and colony-forming capacity than PePDLSCs *in vitro*. During the osteogenic induction, alkaline phosphatase (ALP) activity, mineralized matrix formation and expression of mineralization-related genes, including runt-related transcription factor 2 (*RUNX2*), *ALP*, collagen type I (*COLI*) and osteocalcin (*OCN*) were significantly enhanced in DePDLSCs compared with PePDLSCs. Furthermore, DePDLSC cell sheets showed a stronger synthesis of collagen type I in the extracellular matrix than did PePDLSC cell sheets. After *in vivo* transplantation, DePDLSC cell sheets recombined with human dentin blocks were able to generate new cementum/periodontal ligament-like tissues.

Conclusion: Our findings suggest that DePDLSCs can be used as a promising candidate for periodontal tissue engineering.

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The dental attachment apparatus consists of two mineralized tissues – cementum and alveolar bone – with an interposed fibrous, cellular and vascular soft connective tissue termed the periodontal ligament (PDL) (1). The main function of the PDL is to serve as a supporting tissue by connecting teeth to alveolar bone (2). However, periodontal diseases affect more than 10% of adults and are the main cause of tooth loss (3–5). Regeneration of a healthy periodontium that has been destroyed by periodontal disease is the major goal of periodontal therapy. Recent advances in stem cell biology and tissue engineering have presented opportunities for periodontal therapy

(6). It can be imagined that expanded stem cells available in sufficient quantities and possessing the potential to regenerate alveolar bone, cementum and PDL could be used with appropriate biomaterials to engineer living tissues *in vitro* for subsequent transplantation into periodontal defect sites (7).

In fact, during the past few years, the discovery of stem cells in the PDL of permanent teeth has raised the intriguing possibility of using periodontal ligament stem cells from permanent teeth (PePDLSCs) for bio-root engineering and periodontal tissue regeneration (8–11). As already known, the transition from deciduous teeth to permanent teeth is a very unique and dynamic process in which the development and eruption of permanent teeth coordinates with the resorption of the roots of deciduous teeth (12). In the dental pulp, stem cells from human exfoliated deciduous teeth (SHED) and dental pulp stem cells from permanent teeth have been isolated and applied in dental tissue regeneration (13–21). Furthermore, SHED that also expressed the embryonic stem cell markers were termed immature human dental pulp stem cells. Thus, we speculate that there might be a population of stem cells residing in the PDL of deciduous teeth that has similar characteristics to PePDLSCs.

Retention of primary teeth beyond their expected exfoliation date is encountered relatively frequently, in about 16.6% of individuals (22,23). Normally, retained deciduous teeth should be extracted surgically as soon as they are discovered, as such teeth may cause many dental problems, such as plaque build-up, overcrowding in the mouth and malocclusion (22). A recent report has suggested that stem cells from the PDL of deciduous teeth isolated by $CD105^+$-expressing magnetic cell sorting showed mesenchymal stem cell-like qualities (24). However, to date, no research has been performed on the possibility of isolating periodontal ligament stem cells from retained deciduous teeth (DePDLSCs) and their potential application in periodontal regeneration *in vivo*.

In this study, we successfully isolated a population of multipotent stem cells from the PDL of retained deciduous teeth and further used DePDLSC cell sheets for successful ectopic periodontal regeneration. The significance of this study is that it provides evidence that the PDL of retained deciduous teeth may offer an ideal stem cell resource for clinical applications.

Material and methods

Cell isolation and culture

The study was approved by the Institutional Review Board of the Fourth Military Medical University. Human retained deciduous teeth ($n = 16$) were collected from 16 donors, 6–9 years of age, undergoing extraction of deciduous incisors. There was no sign of root resorption in any tooth collected. Normal human premolars ($n = 16$) were obtained from four donors, 12–19 years of age, for orthodontic treatment purposes.

The PDLs from the deciduous and the permanent teeth were gently scraped from the middle part of the root surface and then digested in a solution containing 3 mg/mL of collagenase type I and 4 mg/mL of dispase (Sigma, St Louis, MO, USA) at 37°C for 20 min and 1 h, respectively. Upon completion of digestion, cells from different individuals were pooled and single-cell suspensions were obtained by passing the cells through a 70-mm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA). To further isolate and purify the putative stem cells, single-cell suspensions from the PDLs of human retained deciduous and permanent teeth were cloned using the limiting-dilution technique, as previously reported (8,25). All colonies were then pooled and expanded to obtain DePDLSCs or PePDLSCs. The DePDLSCs and PePDLSCs were cultured separately in basal medium consisting of Dulbecco's modified Eagle's medium containing 15% fetal bovine serum (FBS) (Gibco, Life Technologies, Grand Island, NY), 100 units/mL of penicillin and 100 mg/mL of streptomycin. The cells were cultured in 75-cm^2 flasks (Costar, Cambridge, MA, USA) at 1×10^4 cells/mL. The cultures were incubated at 37°C in 5% carbon dioxide. For each experiment, cells at passages three to five were used.

Immunohistochemistry of DePDLSCs

To identify the characteristics of DePDLSCs, their cell phenotypes were determined by immunocytochemical techniques. DePDLSCs at passage five were seeded onto coverslips at a density of 2×10^5 cells/mL, maintained for another 24 h in Dulbecco's modified Eagle's medium containing 10% FBS and then fixed with 4% paraformaldehyde. Immunocytochemical analysis was performed with the streptavidin–biotin complex or using immunofluorescence methods. The antibodies used were as follows: mouse anti-STRO-1 (1 : 100 dilution; R&D Systems, Minneapolis, MN, USA), rabbit anti-CD146 (1 : 100 dilution; Abcam, Cambridge, MA, USA), rabbit anti-CD90 (1 : 200 dilution; Abcam, Cambridge, MA, USA), mouse anti- alkaline phosphatase (ALP, 1 : 100 dilution; R&D Systems), glial fibrillary acidic protein (GFAP, 1 : 100 dilution; Abcam, Cambridge, MA, USA), nestin (1 : 100 dilution; Abcam), light neurofilament (NFL, 1 : 100 dilution; Abcam, Cambridge, MA, USA) and stage-specific embryonic antigen-4 (SSEA-4, 1 : 100; Abcam, Cambridge, MA, USA). Phosphate-buffered saline (PBS), instead of primary antibodies, was used as the negative control.

Flow cytometric analysis

DePDLSCs were harvested and cell-surface antigen expression was analyzed by flow cytometric analysis. Approximately 1×10^6 DePDLSCs (passage five) were washed in PBS and incubated with mouse anti-human CD146, STRO-1 (R&D Systems, Minneapolis, MN, USA), CD29, CD90 and CD34 (eBioscience, San Diego, CA, USA) IgG or IgM for 30 min at 4°C. Cells were washed twice with cold PBS containing 2% FBS and incubated with 1 mg of fluorescein isothiocyanate-conjugated goat anti-mouse IgG or IgM (Santa Cruz

Biotechnology, Santa Cruz, CA, USA) for 30 min at 4°C. Mouse isotype antibodies (BD Bioscience, San Jose, CA, USA) served as a control. Labeled cells were analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Multiple differentiation of DePDLSCs *in vitro*

To investigate the osteogenic ability, DePDLSCs at passage 3 were seeded at a density of 3×10^4 cells per well in 12-well plates and were cultured in basal medium supplemented with 100 nM dexamethasone, 0.2 mM ascorbic acid-2-phosphate and 10 mM β-glycerophosphate (all from Sigma) for 3 wk to induce mineralization. Then, the cultures were fixed with 4% paraformaldehyde for 0.5 h. Alizarin red staining was performed to assess mineralization ability, as previously reported (26).

To investigate the adipogenic ability, DePDLSCs at passage 3 were seeded at a density of 3×10^4 cells per well in 12-well plates and cultured for 3 wk in the basal medium supplemented with 0.5 mM methylisobutylxantine, 0.5 μM hydrocortisone, 60 μM indomethacin and 10 μg/mL of insulin. Then, the cultures were fixed with 4% polyoxymethylene for 0.5 h and stained with fresh Oil Red O solution (Sigma), as previously described (26).

The chondrogenic medium contained the following components in addition to the basal medium: 1 × insulin-transferring-selenium supplement (ITS+; Collaborative Biomedical, Becton Dickinson, Bedford, MA, USA), 0.15 mM ascorbic acid-2-phosphate (Sigma), 100 nM dexamethasone (Sigma) and 10 ng/mL of recombinant human transforming growth factor-beta1 (R&D Systems). Chondrogenesis was assayed by staining with toluidine blue after 3 wk of chondrogenic induction (27).

Colony-forming assay

To assess the colony-forming efficiency, single-cell suspensions (1×10^3 cells) of DePDLSCs and PePDLSCs at passage 2 were separately seeded into 10-cm culture dishes (8). After 14 d of culture, the cells were fixed with 4% paraformaldehyde and then stained with 0.1% toluidine blue. Aggregates of 50 or more cells were scored as colonies.

Cell cycle analysis

DePDLSCs and PePDLSCs at passage 3 were separately collected and washed twice with 1 × PBS. The cells were then fixed in cold 70% alcohol at 4°C for 24 h. Afterwards, the cells were washed twice with PBS, stained with propidium iodide (100 μg/mL; Sigma) at 4°C for 30 min and subjected to Elite ESP flow cytometry (Beckman Coulter Inc.). Cell cycle analysis was performed on at least three independent synchronization experiments (28).

Proliferation assay

To compare the growth rates of DePDLSCs and PePDLSCs at passage 3, the cells were seeded at a density of 2×10^3 cells per well into 96-well plates. MTT assays were carried out on days 1, 3, 5 and 7, respectively, according to the cell proliferation kit protocol (Sigma). Attenuance values for each well were measured using a spectrophotometer at 490 nm, and the assay was repeated three times.

ALP staining and Alizarin red staining

DePDLSCs and PePDLSCs at passage 5 were seeded separately at a density of 3×10^4 cells per well in 12-well plates and were cultured in osteogenic induction medium, as described previously. The cultures were fixed after 1 wk with absolute alcohol, for at least 0.5 h, for alkaline phosphatase (ALP) staining and, after 4 wk, the cultures were fixed with 4% paraformaldehyde for 0.5 h for Alizarin red staining. ALP staining was performed using an ALP kit, according to the manufacturer's protocol (Napthol AS-MX phosphate alkaline solution, Sigma 855; Fast Violet B salt, Sigma 201596-5G). Alizarin red staining was performed as previously described. The stained area of mineralized nodules per well was measured quantitatively using an image-analysis system (IMAGE-PRO plus 5.0; Media Cybernetics, Silver Spring, MD, USA).

ALP specific activity

DePDLSCs and PePDLSCs at passage 5 were seeded separately at a density of 2×10^5 cells per well in six-well plates and were cultured in basal medium and osteogenic induction medium for 7 d, as described previously. Four groups of cells (three wells per group) were then washed with 1 × PBS and stored at −80°C until required. To each well, 0.7 mL of 0.1% (v/v) Triton X-100 was added before freeze–thawing twice. Total cell lysis in Triton X-100 was confirmed by light microscopy. ALP activity was measured spectrophotometrically using *p*-nitrophenylphosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1.5 M, pH 10.3, at 25°C) the absorbance was read using a microplate reader (Dynex Technologies, Chantilly, Virginia, USA). The DNA content was measured using the PicoGreen fluorescence reagent according to the manufacturer's instructions (Molecular Probes). Sample fluorescence was measured using a CytoFluor micro-plate reader (Fluoroskan Ascent; Thermo Scientific, Waltham, MA, USA). ALP activity was then expressed as nanomoles of *p*-nitrophenol/h/μg of DNA.

Real time RT-PCR

DePDLSCs and PePDLSCs at passage 5 were seeded separately at a density of 2×10^6 cells per T75 flask and were cultured in the basal medium and in the osteogenic induction medium for different periods of time. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA, USA) at days 7, 14 and 21 for determination of the expression of runt-related transcription factor 2 (*RUNX2*), *ALP*, collagen type I (*COLI*) and osteocalcin (*OCN*). First-strand cDNA syntheses were performed according to the manufacturer's protocol. The Quantitect SYBR Green Kit (Toyobo,

Osaka, Japan) and the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) were used for real-time PCR. The primer sequences used were as follows: (i) *RUNX2* (GenBank accession no. NM004348), sense, 5′-CACTGGCG CTGCAACAAGA-3′ and antisense, 5′-CATTCCGGAGCTCAGCAGAA TAA-3′; (ii) *ALP* (GenBank accession no. NM000478), sense, 5′-GGACCA TTCCCACGTCTTCAC-3′ and antisense, 5′-CCTTGTAGCCAGGCCC ATTG-3′; (iii) *COLI* (GenBank accession no. NM000088), sense, 5′-CCCG GGTTTCAGAGACAACTTC-3′ and antisense, 5′-TCCACATGCTTTATT CCAGCAATC-3′; (iv) *OCN* (GenBank accession no. NM000711), sense, 5′-CCCAGGCGCTACCTGTATCA A-3′ and antisense, 5′-GGTCAGC CAACTCGTCACAGTC-3′; and (v) β-actin (GenBank accession no. NM001101), sense, 5′-TGGCACCC AGCACAATGAA-3′ and antisense, 5′-CTAAGTCATAGTCCGCCTAGA AGCA-3′. β-actin was used as an internal control. Amplification was performed under the following conditions: denaturation at 95°C for 30 s followed by 35 cycles at 95°C for 10 s and 64°C for 30 s. This experiment was repeated three times, and the gene-expression levels were normalized according to the β-actin expression level.

Cell sheet preparation and transplantation

DePDLSCs and PePDLSCs at passage 3 were seeded in six-well plates at a cell density of 3×10^5 cells/well, and were cultured in basal medium supplemented with 50 μg/mL of ascorbic acid-2-phosphate for 7 d. During the monolayer culture, the DePDLSCs and PePDLSCs secrete white substances on the bottom of the dish, and a cell sheet forms and can be easily detached from the bottom of the culture plate using a cell scraper (29). The cell sheets were fixed in 10% neutral-buffered formalin, dehydrated in ethanol, embedded in paraffin and cut into 5-μm sections. After deparaffinization, the sections were evaluated using routine hematoxylin and eosin (H&E) staining and immunohistochemistry staining with antibodies against extracellular matrix (ECM)-related proteins, including collagen I, fibronectin and laminin. For immunohistochemical studies, sections were rinsed with PBS and then blocked in 20% normal goat serum for 5 min at room temperature. The primary antibodies used were as follows: collagen I (1 : 100 dilution, Abcam ab6308) overnight, and fibronectin (1 : 100 dilution, Abcam ab6328) and laminin (1 : 100 dilution, Abcam ab49726) for 1 h at room temperature. Sections were detected using the Dako REAL EnVision detection system (Dako, Carpinteria, CA, USA). Then, sections were counterstained with haematoxylin. PBS instead of primary antibodies was used as the negative control. For *in vivo* transplantation, human dentin blocks were used as the scaffold for periodontal regeneration. For preparation of human dentin blocks, PDL tissues were carefully scraped away. The crowns of teeth were removed to obtain the root. The outer cementum and the inner dental-pulp tissues were removed. The root was ground into the dentin block and cut into sections of about 2 mm × 2 mm × 5 mm. The remaining steps were performed according to the previous study (30,31). For a single transplantation, the dentin block was wrapped in DePDLSC sheets and embedded in a diffusion chamber (Millipore, Billerica, MA, USA) and implanted into the peritoneal cavities of 8-wk-old nude mice. Dentin block wrapped in PePDLSC sheets was used as the control. All animal procedures complied with the guidelines provided by the Animal Care Committee of the Fourth Military Medical University. Grafts were recovered after 8 wk, fixed in 4% paraformaldehyde for 1 d, decalcified with buffered 10% EDTA (pH 8.0) and processed for routine histological examination (H&E staining and Masson's trichrome staining).

Statistical analysis

Statistical analysis was performed using an independent samples *t*-test with SPSS v12.0 software (SPSS, San Rafael, CA, USA). $p < 0.05$ was judged to be statistically significant.

Results

Characterization of DePDLSCs

Similarly to PePDLSCs, human DePDLSCs were capable of forming adherent clonogenic cell clusters of fibroblast-like cells (Fig. 1A) with a typical fibroblastic spindle shape (Fig. 1B). DePDLSCs expressed STRO-1, CD146 and CD90, which have been considered as markers of mesenchymal stem cells (Fig. 1C–F) and of a variety of neural cell markers, including nestin, GFAP and NFL, as measured by immunocytochemical staining (Fig. 1G–I). Further immunohistotypic analysis showed that cultured DePDLSCs expressed the stromal- and embryonic-related markers ALP and SSEA-4 (Fig. 1J–L).

Cell-surface markers of DePDLSCs

The mesenchymal cell nature of DePDLSCs was further confirmed by flow cytometry by expression of mesenchymal cell markers, including STRO-1, CD146, CD29 and CD90, but lack of expression of the hematopoietic cell marker, CD34 (Fig. 2).

Multipotentiality of DePDLSCs *in vitro*

The multipotentiality of DePDLSCs was verified by their induction in the osteogenic, adipogenic and chondrogenic media *in vitro*. When DePDLSCs were cultured in osteogenic medium, the cell morphology changed at day 14 from an elongated fibroblastic appearance to a rounder, more cuboidal, shape. The mineralization assay showed that many calcified nodules appeared in the cultures after 3 wk of induction, as indicated by Alizarin red staining (Fig. 3A and 3D). Similarly, after same period of culture in adipogenic medium, intracellular lipid vacuoles appeared in DePDLSC cultures, which were confirmed by Oil Red O staining (Fig. 3B and 3E). Chondrogenesis was examined using toluidine blue staining to detect the sulfated proteoglycans.

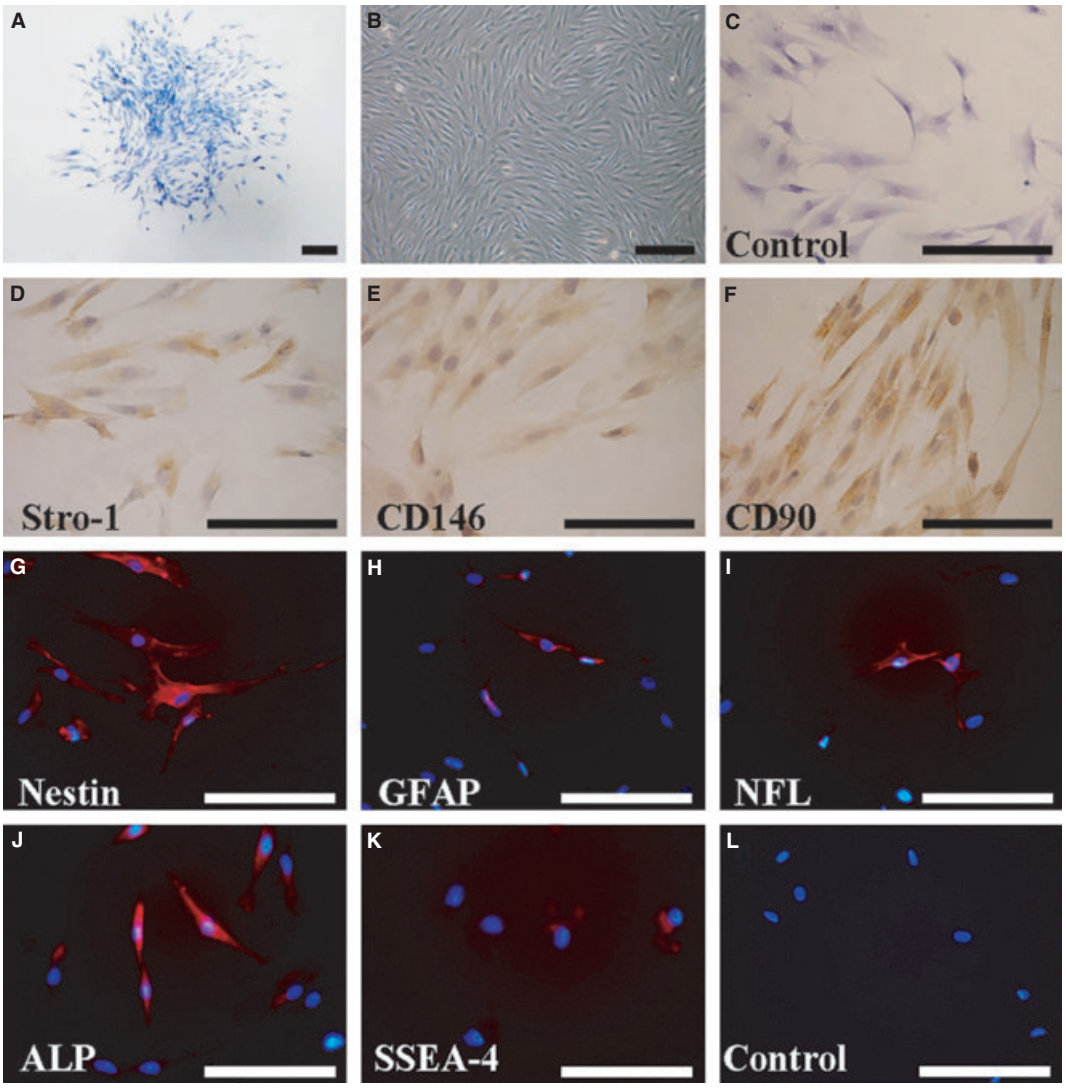

Fig. 1. Identification of stem cells from the periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs). (A) Single colonies of DePDLSCs showed typical fibroblast-like morphology under light microscopy. (B) Morphological appearance of DePDLSCs at passage 5. (C–L) Immunocytochemical or immunofluorescence analysis of DePDLSCs was performed. For the negative control, the first antibody was omitted. STRO-1 (D), CD146 (E), CD90 (F), alkaline phosphatase (ALP) (J) and stage-specific embryonic antigen-4 (SSEA-4) (K) were expressed in DePDLSCs. Several neural cell markers, including nestin (G), glial fibrillary acidic protein (GFAP) (H) and light neurofilament (NFL) (I) were also detected in DePDLSCs. Scale bar = 50 μm.

DePDLSCs stained positive for toluidine blue (Fig. 3C and 3F).

Colony-forming assay of DePDLSCs

The ability of DePDLSCs to form adherent clonogenic cell clusters of fibroblast-like cells was significantly higher than that of PePDLSCs ($p < 0.05$), and was shown by the formation of approximately 260 single colonies that were generated from 1000 single cells cultured at low density (Fig. 4A–C).

Proliferation ability of DePDLSCs

To test the proliferation ability of DePDLSCs, cell cycle analysis and the MTT assay were performed. Cell-cycle distribution (G0G1, S and G2M phases) was analyzed by flow cytometry and the results were expressed as the proliferation index (percentage of cells in the S and G2M phases) (32). It was shown that DePDLSCs had a significantly higher percentage of cells in S + G2M phases (51.22%) than did PePDLSCs (37.21%), indicating a more active cell proliferation in cultured DePDLSCs ($p < 0.05$) (Fig. 4D and 4E). Similar results were also found in the MTT assay. DePDLSCs grew faster than PePDLSCs at days 3, 5 and 7 ($p < 0.05$) (Fig. 4F).

Osteogenic potential of DePDLSCs

DePDLSCs and PePDLSCs were induced in the osteogenic medium. After 1 wk, ALP staining (an early marker of bone formation) showed that ALP is expressed more strongly in

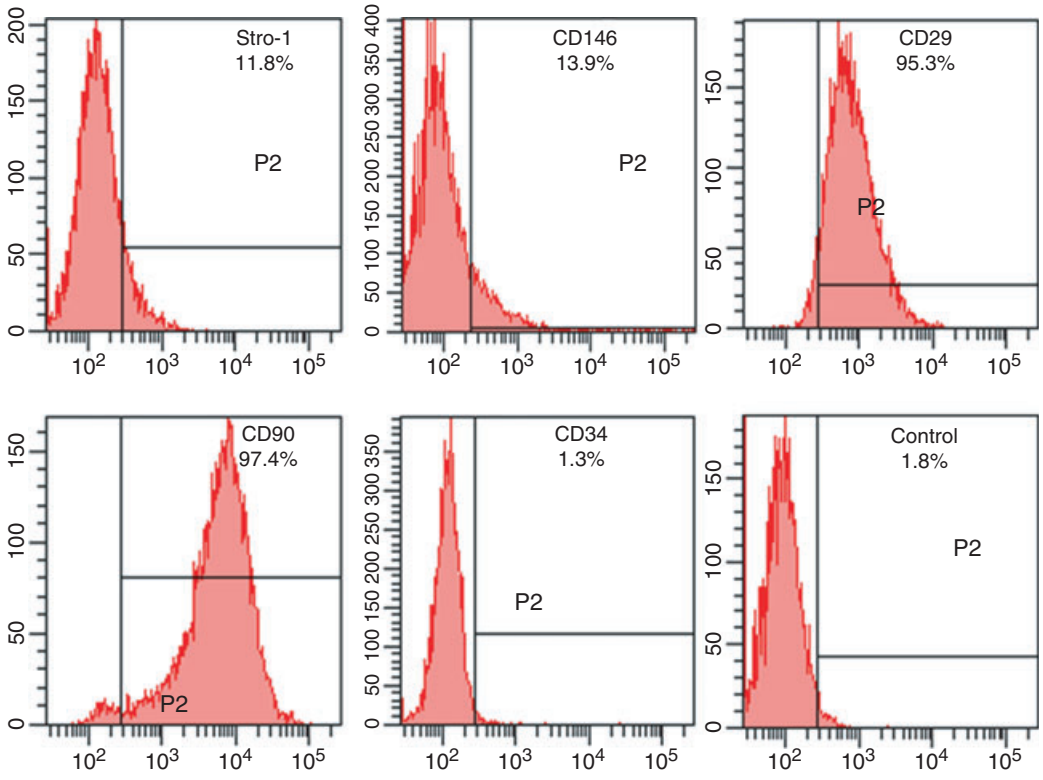

Fig. 2. Expression of cell-surface markers of periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs) at passage 5. Flow cytometric analysis of *ex vivo*-expanded DePDLSCs revealed the expression of STRO-1 (11.8%), CD146 (13.9%), CD29 (95.3%), CD90 (97.4%) and CD34 (1.3%).

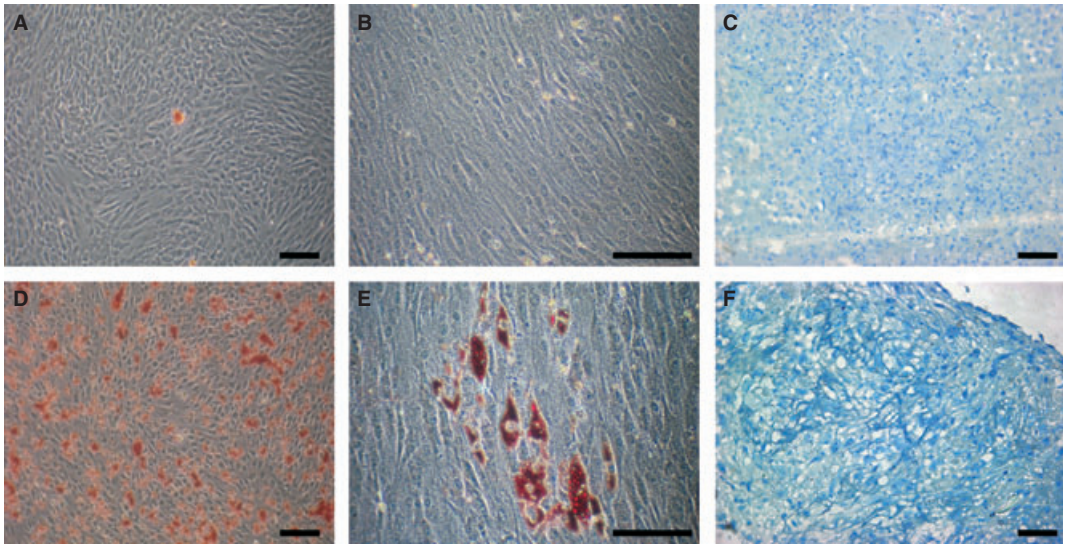

Fig. 3. Multilineage differentiation of periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs). (A) No Alizarin red-positive nodule was present in the standard culture system. (B) Cultured DePDLSCs formed calcified nodules after 3 wk of induction in the osteogenic medium. (C) No Oil Red O-positive lipid clusters appeared in the standard culture medium. (D) Cultured DePDLSCs formed the Oil Red O-positive lipid clusters after 3 wk of induction in the adipogenic medium. (E) After 3 wk, control cell pellets were negative for toluidine blue stain. (F) After 3 wk, induced cell pellets were stained positively for toluidine blue. Scale bar = 50 μm.

DePDLSCs (Fig. 5A and 5C) than in PePDLSCs (Fig. 5B and 5D) after osteogenic induction. In addition, the ALP activity of induced DePDLSCs is significantly higher than that of induced PePDLSCs ($p < 0.05$) (Fig. 5I). The ALP activities of DePDLSCs and PePDLSCs were enhanced significantly after the osteogenic induction

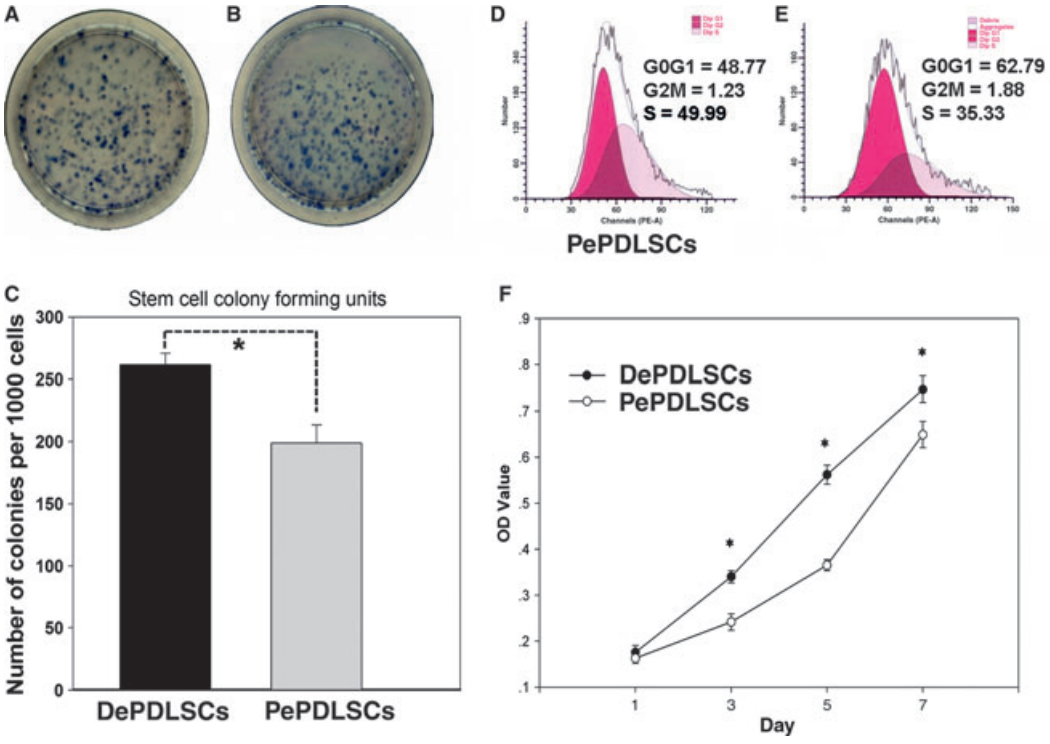

Fig. 4. Colony-forming capability of periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs) and periodontal ligament stem cells from human permanent teeth (PePDLSCs). Colonies were formed when DePDLSCs (A) and PePDLSCs (B) were plated separately at low density. On day 14 after plating, DePDLSCs and PePDLSCs were separately fixed with 10% formalin and stained with toluidine blue. An aggregate containing 50 or more cells was considered as a colony. (C) There was a statistically significant difference in the total colony number between DePDLSCs and PePDLSCs. (D–F) Proliferative ability of DePDLSCs and PePDLSCs. Representative cell cycle distributions of DePDLSCs (D) and PePDLSCs (E). (F) The graph showed a statistically significant difference in the proliferative ability of DePDLSCs compared with PePDLSCs as determined by the MTT assay. DePDLSCs had a higher optical density (OD) value than did PePDLSCs at 3, 5 and 7 d (*$p < 0.05$).

($p < 0.001$) (Fig. 5I). After 4 wk of osteogenic induction, Alizarin red staining showed that the mineralization ability of DePDLSCs (Fig. 5E and 5G) was significantly higher than that of PePDLSCs (Fig. 5F and 5H). The areas of mineralized nodules in the DePDLSC osteogenic group were larger than those in the PePDLSC osteogenic group ($p < 0.01$) (Fig. 5J). Real-time PCR analysis further indicated an increased expression of *RUNX2*, *ALP, COLI* and *OCN* in induced DePDLSC and PePDLSC populations, compared with noninduced controls (Fig. 6). There was a 1.5- to 2-fold increase in the mRNA levels of *RUNX2* and *ALP* in the cells obtained from deciduous teeth vs. permanent teeth during the osteogenic induction (Fig. 6A and 6B). Furthermore, the level of *COLI* mRNA was higher in DePDLSCs than in PePDLSCs after 2 wk of osteogenic induction (Fig. 6C). Moreover, the level of *OCN* mRNA was higher in DePDLSCs than in PePDLSCs after 3 wk of osteogenic induction (Fig. 6D).

Histological analysis of DePDLSC and PePDLSC cell sheets

H&E staining revealed that the harvested DePDLSCs cell sheet was four- or five-layered (Fig. 7A) and that the PePDLSCs cell sheet was two- or three-layered (Fig. 7E). Histological assessment of DePDLSC and PePDLSC cell sheets revealed a uniform tissue structure with connective tissue-like morphology that contained cells entrapped within the ECM (Fig. 7A and 7E). Although the ECM was abundant, collagen fibrils were arranged randomly into the matrices and the cells embedded in the matrices were also not oriented in any particular manner. Immunohistochemical staining showed that collagen I was expressed more strongly in the DePDLSC cell sheet (Fig. 7C) than in the PePDLSC cell sheet (Fig. 7G). DePDLSCs cell sheets also expressed fibronectin (Fig. 7D) and laminin (major proteins in the basal lamina) (Fig. 7B). However, there was no significant difference in the expression of fibronectin and laminin between PePDLSC (Fig. 7F and 7H) and DePDLSC cell sheets. The expression levels of protein in the different types of cell sheets them were measured using an automated image-analysis system (Image-Pro Plus; Media Cybernetics).

Periodontal regeneration *in vivo* using DePDLSC cell sheets

To examine whether DePDLSCs cell sheets have the potential to regenerate the periodontium complex, we trans-

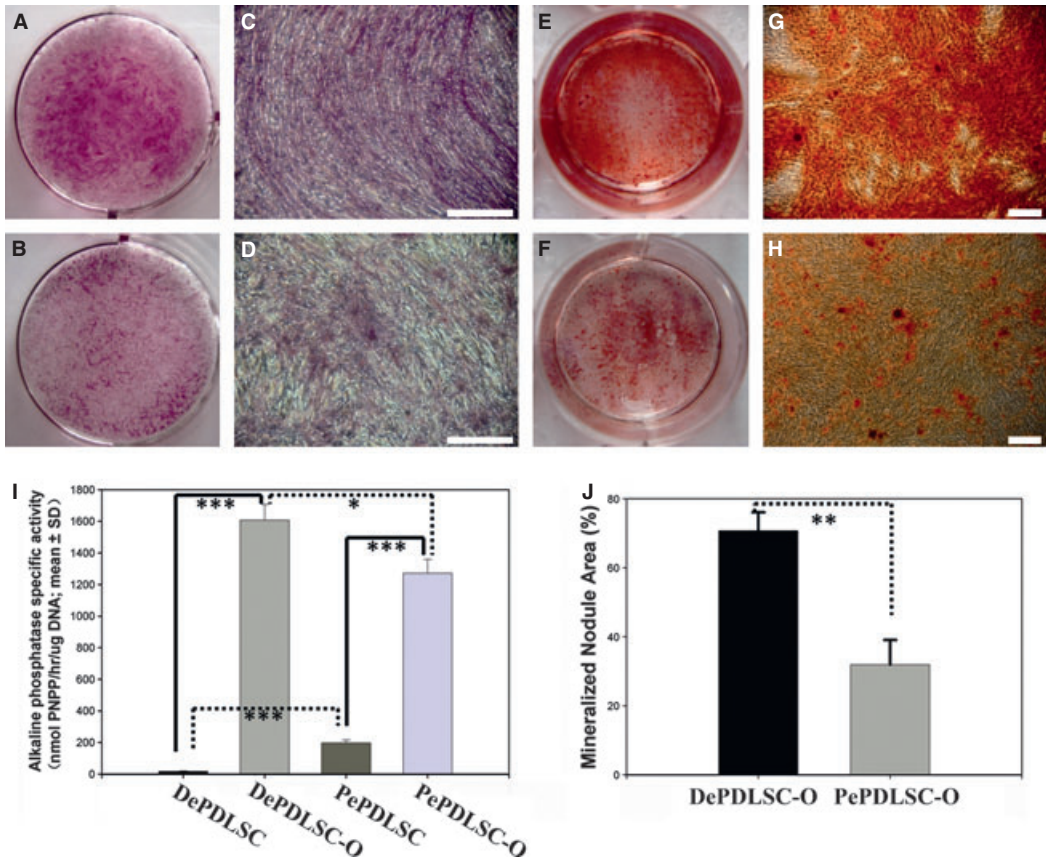

Fig. 5. Osteogenic differentiation of periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs) compared with periodontal ligament stem cells from human permanent teeth (PePDLSCs). DePDLSCs after 1 wk of induction in the osteogenic medium (A, C), stained more strongly for alkaline phosphatase (ALP) than did PePDLSCs (B, D). DePDLSCs, after 4 wk of induction in the osteogenic medium (E, G), showed more mineralized matrix deposition than did PePDLSCs (F, H), as indicated by Alizarin red staining. (I) The ALP activity of DePDLSCs and PePDLSCs was enhanced after 7 d of osteogenic induction. The ALP activity of DePDLSCs was significantly higher than that of PePDLSCs after induction in the osteogenic induction. $^{***}p < 0.001$, $^{*}p < 0.05$. (J) The graph showed a statistically significant difference in the mineralized nodule areas of DePDLSCs compared with PePDLSCs after 4 wk of osteogenic induction. $^{**}p < 0.01$. DePDLSCs-O and PePDLSCs-O represent the induced DePDLSCs and PePDLSCs, respectively. Scale bar = 50 μm.

planted DePDLSCs cell sheet–dentin block constructs into nude mice. A total of six specimens were available for the subsequent analysis. A cementum-like tissue was regenerated on the dentin surfaces in two of six (33.3%) samples in the experimental group, 8 wk post-transplantation. Representative examples of the light microscopy and histology of DePDLSC cell sheet–dentin block recombinants at 8 wk post-transplantation are illustrated (Fig. 8A, 8C, 8E and 8G). Histological observations revealed that a typical regularly arranged cementum/PDL-like structure was regenerated on the dentin surfaces (Fig. 8A). Significantly, higher magnification revealed cementum-like mineralized tissues lining along the dentin surface, and PDL-like fibrous tissues interfaced with these newly deposited cementum-like structures (Fig. 8C). Masson's trichrome staining further confirmed the collagen content and the orientation of PDL-like tissues (Fig. 8E). Higher magnification revealed that a few collagen bundles were inserted perpendicularly into the new cementum-like tissue and that this orientation closely resembled the native periodontal fibers (i.e. Sharpey's fibers) (Fig. 8G). By contrast, transplantation of dentin blocks with PePDLSC cell sheets resulted in a PDL-like structure attached to the dentin surface. However, there was no cementum formation on the dentin surface throughout the observation periods (Fig. 8B, 8D, 8F and 8H). Higher magnification revealed that collagen bundles were inserted perpendicularly into the dentin surface (Fig. 8D and 8H).

Discussion

The delayed exfoliation of deciduous teeth among children is a common and frequent dental problem whose most cited cause is misalignment of the crown of the successional permanent tooth with the root apex of the primary tooth (22). Treatment is often extraction of the over-retained primary teeth. Until now, there has been no research on the PDL of retained deciduous teeth, which presumably contain a population of mesenchymal stem cells. In the present study, using the limiting-dilution technique, multicol-

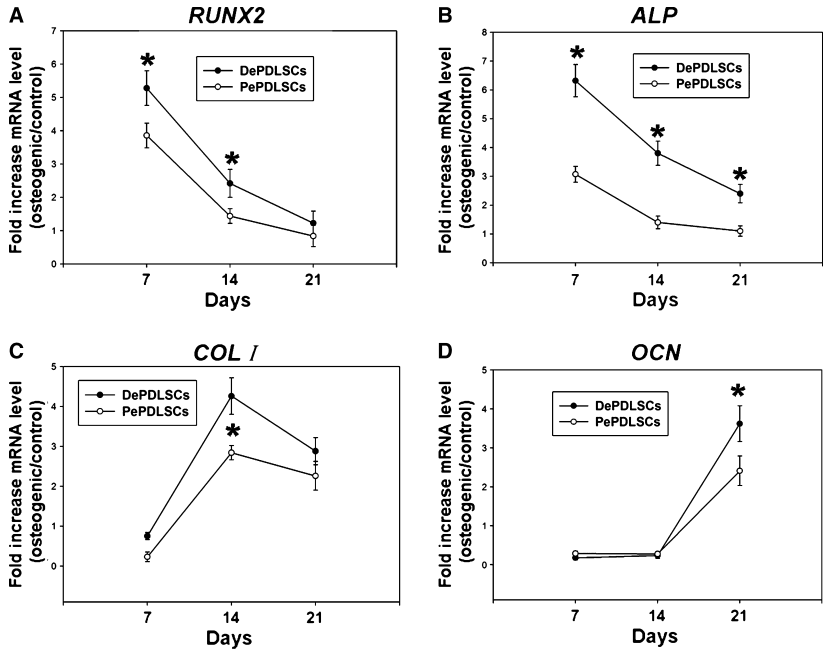

Fig. 6. Expression of osteoblast/cementoblast phenotypes in periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs) and in periodontal ligament stem cells from human permanent teeth (PePDLSCs). (A–D) Real-time PCR analysis presented the upregulated expression of runt-related transcription factor 2 (*RUNX2*) (A), alkaline phosphatase (*ALP*) (B), collagen type I (*COLI*) (C) and osteocalcin (*OCN*) (D) in both DePDLSC and PePDLSC populations after the osteogenic induction. β-actin was used as an internal control. The histogram showed the fold differences between the induced group and the control. Expression of *RUNX2, ALP, COLI* and *OCN* was detected in both DePDLSCs and PePDLSCs on days 7, 14 and 21. (A) *RUNX2* expression in induced DePDLSCs was significantly higher than in induced PePDLSCs on days 7 and 14. (B) *ALP* expression in induced DePDLSCs was significantly higher than in induced PePDLSCs on days 7, 14 and 21. (C) *COL I* expression in induced DePDLSCs was significantly higher than in induced PePDLSCs on day 14. (D) *OCN* expression in induced DePDLSCs was significantly higher than in induced PePDLSCs on day 21. Statistical significance was determined using the Student's *t*-test (*$p < 0.05$).

ony-derived stem cells isolated from the PDL of retained deciduous teeth manifested mesenchymal stem cell-like properties. Importantly, DePDLSCs have a stronger ability to proliferate and to differentiate than do PePDLSCs. Furthermore, our study demonstrated the feasibility of using a cell sheet cultivation system for human DePDLSCs to obtain the complete reconstruction of the physiological architecture of a cementum–PDL complex.

Stem cells are defined by their self-renewal and multidifferentiation abilities *in vivo* and/or *in vitro* (33). We provide evidence here that the PDL of retained deciduous teeth contains a multipotent mesenchymal stem-cell population – DePDLSCs. DePDLSCs have self-renewal ability, confirmed by a colony-forming assay, and can differentiate into osteoblast-like cells, adipocyte-like cells and chondrocyte-like cells under different conditions *in vitro*, similarly to other stem cells. Previous experiments have shown that stem cells from the PDL of deciduous teeth can be isolated by $CD105^{+}$-expressing magnetic cell sorting (24). Alternatively, in the present study, we isolated and purified DePDLSCs by using the limiting-dilution techinique. It was found that DePDLSCs expressed neuronal and glial cell markers (nestin, GFAP and NFL), which may be related to the neural crest origin of the PDL

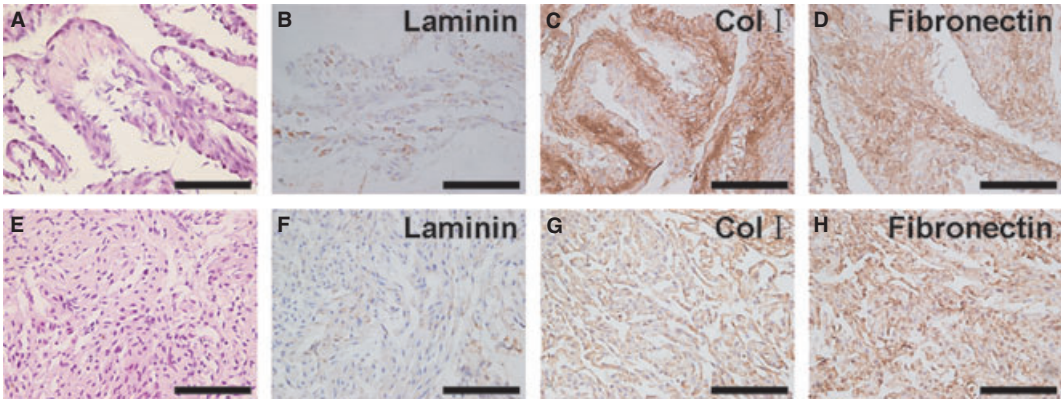

Fig. 7. Histology and immunohistochemistry of periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs) (A–D) and of periodontal ligament stem cells from human permanent teeth (PePDLSCs) (E–H) cell sheets. Hematoxylin and eosin (H&E) staining revealed that the DePDLSCs cell sheet was four- or five-layered (A), whereas the PePDLSCs cell sheet was two- or three-layered (E). DePDLSC (B–D) and PePDLSC (F–H) cell sheets stained positive for laminin, collagen I and fibronectin by immunohistochemical staining. Collagen was strongly expressed in the DePDLSC cell sheet (C) compared with the PePDLSC cell sheet (G). Scale bar = 100 μm.

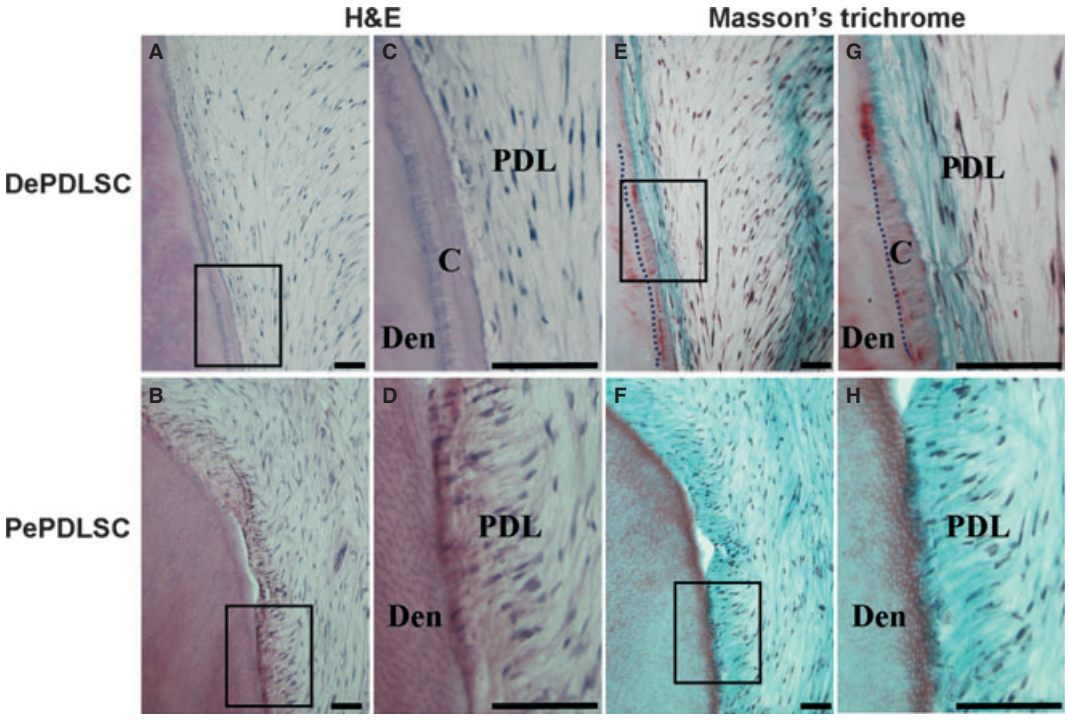

Fig. 8. Periodontal ligament stem cells from human retained deciduous teeth (DePDLSCs) or periodontal ligament stem cells from human permanent teeth (PePDLSCs) cell sheet-based cementum/PDL-like tissue regeneration *in vivo*. Morphology of representative DePDLSCs (A,C,E,G) and PePDLSCs transplants (B,D,F,H) were harvested 8 wk post-transplantation and stained with hematoxylin and eosin and Masson's trichrome. The box represents an area of higher magnification. (A) DePDLSCs produced cementum-like mineralized deposits on the dentin surface and PDL-like collagen fibers connecting with the newly formed cementum-like tissues. Higher magnifications of panels A and B are shown in panels C and D. (G) At the higher magnification of Masson's trichrome staining, collagen bundles were inserted into the cementum-like tissues, similarly to the physiological attachment of natural Sharpey's fibers in normal periodontal ligament. (B,D,F,H) PDL-like tissues without mineralized tissue were observed within the PePDLSCs and dentin block recombinants. Higher magnifications of panels E and F are shown in panels G and H. Scale bar = 50 μm. C, cementum; Den, dentin; PDL, periodontal ligament.

(25), and they also expressed ALP and SSEA-4, early-stage markers of stem cells.

Deciduous teeth are significantly different from permanent teeth with regard to their developmental processes, tissue structure and function (13). When the deciduous teeth exfoliate, the PDL of deciduous teeth has been absorbed (34). Therefore, it is not a surprise to find that DePDLSCs have some properties different from PePDLSCs. The proliferative ability is significantly different between these two cell populations, as indicated by the ratio of S + G2M phase cells in the cell cycle and MTT assays. There is currently no consensus regarding the effect of donor age on postnatal mesenchymal stem cell function. The proliferative ability of DePDLSCs is significantly higher than that of PePDLSCs, which may be related to the donor age. Clinically, DePDLSCs can rarely be isolated from a donor of the same age as a donor of PePDLSCs.

Interestingly, DePDLSCs were more apt than PePDLSCs to differentiate into osteoblasts under the osteogenic induction *in vitro,* which differs from the results of a previous report (24). A possible reason was that different cell-isolation methods were used. In the present study, we used the limiting-dilution technique to isolate stem cells from the PDL of retained deciduous teeth, which can ensure that real stem cells are obtained with self-renewal capacity. As a consequence, the ability of DePDLSCs to form adherent clonogenic cell clusters is significantly higher than that of PePDLSCs. ALP activity has been extensively used as an indicator of stem cell osteogenic differentiation (35–38). In this study, ALP activity of DePDLSCs increased significantly in comparison with that of PePDLSCs after 1 week of osteogenic induction. A similar scenario was also found in SHED and dental pulp stem cells (39). Real-time PCR analysis indicated that the mRNA levels of *RUNX2*, *ALP* (an early marker of osteogenesis), *COLI* (a mid-stage marker of osteogenesis) and *OCN* (a late marker of osteogenesis) were much higher in DePDLSCs than in PePDLSCs at different time-points after osteogenic induction. Besides, DePDLSCs were more apt than PePDLSCs to differentiate into adipocytes *in vitro* (data not shown). These differences may result from a number of factors, including the colony-forming ability, proliferation rate and immature properties of DePDLSCs vs. PePDLSCs.

The primary goal of periodontal therapy is to re-establish the attachment of connective tissues between newly formed supporting bone and the tooth root by the PDL. Mesenchymal stem cells isolated from the PDL of

human permanent teeth (8,10,29,40) have been considered as one of the most effective cell sources and the cell sheet technique has been developed and applied for periodontal regeneration (41–43). In this study, the matrix assay for cell sheets of DePDLSCs and PePDLSCs indicated that sufficient collagen I, fibronectin and laminin proteins were present in the ECM of DePDLSC and PePDLSC cell sheets. The role of fibronectin is to attach cells to a variety of extracellular matrices, except for fibronectin type IV, which uses laminin as the adhesive molecule. The integrity of the fibronectin molecule in the matrix is important for formative or anabolic phases of periodontal regeneration or wound healing (44). Furthermore, fibronectin matrix adhering to the basal side of each harvested cell sheet can function as a natural adhesive to attach cell sheets to other surfaces. Collagen I is the major protein comprising the ECM of the PDL. Cell sheets of DePDLSCs showed stronger collagen I staining than those of PePDLSCs, suggesting that expression of the collagen gene in DePDLSCs may be more sensitive to vitamin C than that in PePDLSCs (45). Periodontal regeneration requires new connective tissue attachment to the root surface, a process that involves the formation of new cementum on previously exposed root surfaces, the synthesis of Sharpey's fibers and their insertion into newly formed cementum (25). In the present study, DePDLSC cell sheets combined with dentin blocks *in vivo* generated regularly arranged PDL-like fibrous tissue that interfaced with new cementum-like tissue formed on the surface of the dentin block. In contrast, there was only PDL-like tissue regeneration, without cementum formation, in transplanted PePDLSC cell sheets. From a biological perspective, the occurrence of predictable periodontal regeneration depends upon two crucial factors: the availability of appropriate cell types and a favorable local environment (40). The explanation for no cementum formation in PePDLSCs may be that PePDLSCs should be induced under some conditions before transplantation, or transplanted with osteoinductive materials (25,43). Alternatively, the experiment should be repeated with a large sample size to fully investigate the *in vivo* regenerative capacity of PePDLSCs as only a small number of subjects were used in the current study. Cementum/PDL-like tissues can be regenerated *in vivo* by DePDLSC cell sheets combined with dentin blocks, which may be related to the high osteogenic potential of DePDLSCs.

In summary, the lines of evidence accumulated here show that DePDLSCs are highly proliferative cells capable of regenerating the cementum/PDL-like tissues. Therefore, DePDLSCs can be used in cell-based periodontal therapy. Patients with periodontitis can be treated with banked DePDLSCs for periodontal regeneration. Further studies are required to focus on DePDLSCs used in periodontal defect repair *in situ*.

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