COMPARISON OF IMMUNO-PHENOTYPES OF STEM CELLS FROM HUMAN DENTAL PULP AND PERIODONTAL LIGAMENT

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It has been established that human dental pulp and periodontal ligament contain a population of mesenchymal stem cells (MSCs). However, the phenotypic analysis in terms of putative stem cell markers expressed by these stem cell populations is incomplete. It is relevant to understand whether stem cells derived from closely related tissues are programmed differently. The aim of the present study is to analyze whether these stem cells depict distinct characteristics by gaining insight into differences in their immunophenotype. Dental pulp and periodontal ligament tissue samples were obtained from extracted impacted wisdom teeth. Cell cultures were analyzed for surface and intracellular markers by indirect immunoflourescence. Detailed immunophenotype analysis was carried out by flow cytometry using relevant markers. The present study data shows dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) expressed embryonic stem (ES) cell markers Oct-4, Nanog and mesodermal marker Vimentin by indirect immunoflourescence. PDLSCs, however, had a weak expression of Nanog. Immunophenotyping revealed strong expression of MSC markers (CD73, CD90) in DPSCs and PDLSCs. Differences were observed in expression of stemness-related markers. DPSCs displayed increased percentages of SSEA4, CD13 and CD166 and decreased CD9 expression compared to PDLSCs. Both stem cells express common MSC markers, different levels of expression suggests there might be more than one stem cell population existing within these tissues which differ in their embryonic status, and DPSCs are a more primitive stem cell population in comparison to PDLSCs.

Mesenchymal stem cells (MSCs) were first identified in aspirates of adult bone marrow (1). In recent years, stem cells from dental tissues have provided an alternate source of MSCs with characterization of stem cells within the dental pulp (DPSCs) (2), periodontal ligament (PDLSCs), (3-5) deciduous teeth (6), dental follicle (7), stem cells from apical papilla (SCAP) (8) and recently from gingival connective tissue (9). Various experiments have compared the characteristics of dental stem cells (10). Differences have been noted between dental stem cells and BMMSCs, wherein DPSCs were shown to share a similar pattern of protein expression with BMMSCs *in vitro* (11). Stem cells from human dental pulp, dental follicle and root apical papilla from wisdom teeth have been analyzed for the expression of transcription factors (Oct-4, Nanog and Sox-2) and cell surface markers (CD44, CD90

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and CD105) (12). It was observed that there was high expression of transcription factors in root apical papilla stem cells and osteogenic differentiation was less in dental pulp stem cells compared to the apical papilla stem cells. The PDLSCs have been observed to express the antigens CD90, CD29, CD44, CD166, CD105, CD13 that are also identified as stromal precursors of the bone marrow (13). There have been no systematic comparisons of the phenotypic characteristics in terms of putative stem cell markers expressed by DPSCs and PDLSCs. Dental pulp and periodontal ligament are closely related tissues and share a common developmental pathway. It is relevant to understand whether stem cells derived from these closely related tissues are programmed differently. Thus, the present study compares the phenotypes of these two stem cell populations by analyzing differences in expression of various cell-surface markers used to identify putative MSCs as well as gaining insight into the immunophenotypes of these stems cells. Selection of these markers was based on their expression in human dental pulp and periodontal ligament (11, 14-15).

MATERIALS AND METHODS

Cell cultures

Dental pulp and periodontal ligament tissue samples were obtained from four healthy subjects who had to undergo extraction of impacted wisdom teeth. The subjects were aged 18-22 years. The experimental protocol was approved by the Ethics committee of Manipal University, and written informed consent was obtained from all the subjects. Periodontal ligament tissue was removed from the surface of the extracted tooth root with a sterile scalpel #15, after which the tooth was sectioned longitudinally and pulp tissue was obtained. Both the tissues were minced separately into tiny pieces of 0.5 mm^3 sections as previously described (14). Both the tissues were processed separately, and digested in a solution of 2 mg/ml of collagenase type I and 4mg/ml of dipase for 1 h at 37°C. The tissue pellet thus obtained was re suspended in Dulbecco's modified Eagle's Medium (DMEM) containing penicillin G (100 U/ml) and streptomycin (100 µg/ml) supplemented with 15% (weight/volume) fetal bovine serum (FBS), placed into culture flasks, and cultured at 37°C in a humidified atmosphere of 5% CO_2 as previously described (3). Dental pulp and periodontal ligament cell cultures from the third passage were used for the experiments.

Indirect immunoflourescence

Analysis of cell surface and intracellular markers was carried out by using monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) and phycoeryhtrin (PE). The cells were fixed using 4% paraformaldehyde + 5% sucrose in phosphate buffered saline (PBS) for five minutes. Cell surface markers were analyzed after incubation with 3% bovine serum albumin (BSA) supplemented with 0.5 Mm (milli Mole) ethylene diamine tetra acetic acid (EDTA) in 1 M (mole) PBS for 30 minutes. For intracellular markers cells were permeabilised with 0.1% Triton supplemented with 3% BSA in PBS for 30 minutes. The cells were first incubated with primary antibodies that were in empirically optimized quantities, in 0.5% BSA overnight at 4°C followed by the respective fluorescent conjugated secondary antibodies for half an hour in a dark room. Microscopic analysis was performed with laser confocal microscopy. The embryonic stem cell markers Octamer-4 (Oct-4), Nanog and mesodermal marker Vimentin were analyzed in all four dental pulp and periodontal ligament stem cells at passage three.

Flow Cytometric Analysis (FACS)

Flow Cytometry was carried out on a BD FacsCalibur cytometer, and data were processed with Cell Quest software. A total of 0.5 $\times 10^6$ dental pulp and periodontal ligament cells at passage three were incubated with specific individual monoclonal antibodies, conjugated with fluorescence isothiocyanate (FITC), phycoerythrin (PE) in 250 µl phosphate buffered saline for 30 minutes in the dark at room temperature. The expression of the isotype controls (conjugated with Phycoerythrin and Fluorescence Isothiocyanate) was analyzed to rule out any nonspecific binding of the antibodies. The primary antibodies used for mesenchymal markers were: CD73, CD90, CD105, CD106 and CD166; immune markers like human leukocyte antigen (HLA-ABC), HLA-DR, co stimulatory markers CD80 and CD86; hematopoietic markers CD34, CD45 and putative stem cell markers, stage specific embryonic antigen-4 (SSEA4), CD9 and CD13 were analyzed. Using the Mann Whitney 'U' test, the mean values and standard deviation were calculated for each marker, in the dental pulp stem cells (Group 1; n=4) and periodontal ligament stem cells (Group 2; n=4) for comparison of their immunophenotype.

RESULTS

The dental pulp and periodontal ligament MSCs were successfully isolated by adherence separation, reaching 80% confluency by 2-3 weeks. Under optic microscopy, the initial culture showed small,

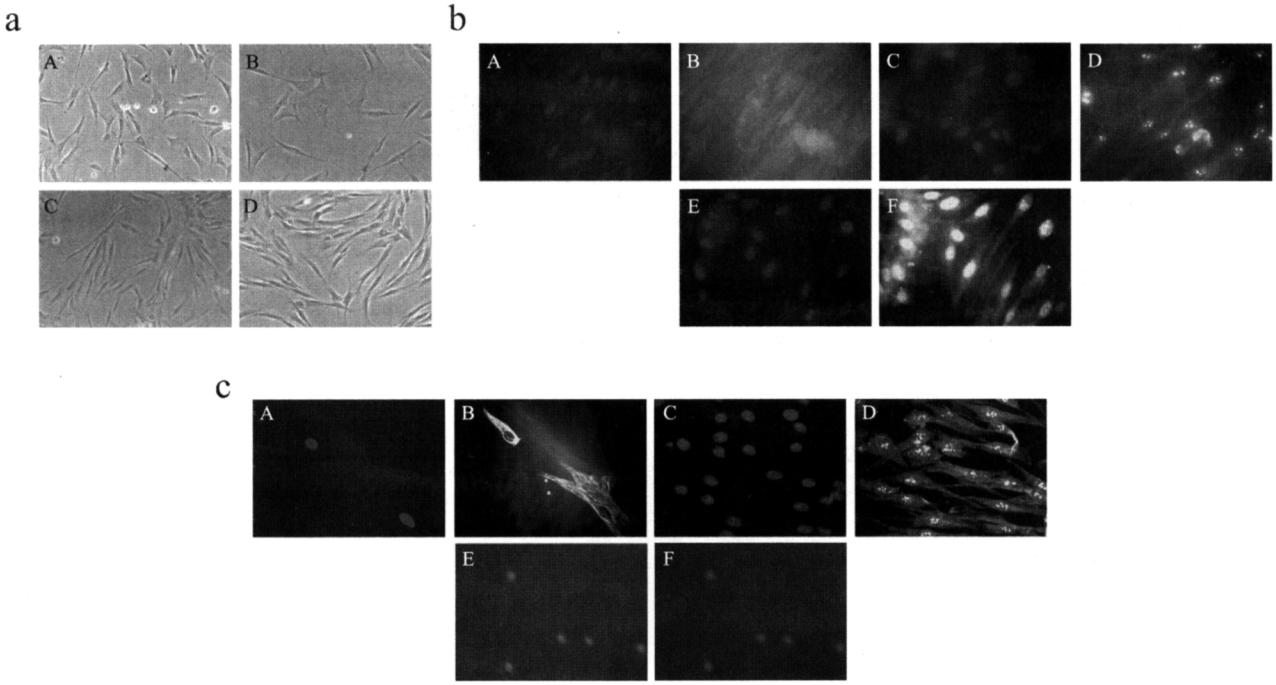

Fig. 1. *Phase-contrast images of dental pulp (A and B) and periodontal ligament stem cells (C and D). **A)** Morphology of the DPSCs at day four passage 0. **B)** Morphology of DPSCs on the 20th day of culture. The presence of homogenous fibroblast-like population of cells was evident during the in vitro expansion (40 × magnification). One representative experiment is shown. **C)** Phase-contrast image of PDLSCs. Morphology of the cells at day four passage 0. **D)** Morphology of periodontal ligament cells on the 20th day of culture. The presence of homogenous elongated fibroblast-like population of cells was evident during the in vitro expansion (40 × magnification). One representative experiment is shown. **b)** Epifluorescence images of DPSCs, on 20-day-old culture. Cultures were examined and photographed by fluorescence microscopy and appropriate filters. Expression of Vimentin (A and B), Oct 4 (C and D) and Nanog (E and F). **A)** Represents DAPI fluorescence in the nucleus. **B)** Vimentin fluorescence expressed on the surface. **C)** DAPI fluorescence depicting the nucleus. **D)** Depicting strong expression of Oct 4 fluorescence intracellularly. **E)** DAPI fluorescence depicting the nucleus. **F)** Expression of Nanog florescence on the surface of the MSCs. The antibodies were FITC Conjugated Results are representative of four independent experiments (Original magnification×40). **c)** Epifluorescence images of PDLSCs, on 20-day-old culture. Expression of Vimentin (A and B), Oct 4(C and D) and Nanog (E and F). **A)** DAPI fluorescence exhibited by the nuclei. **B)** Expression of Vimentin on the surface of the cells. **C)** DAPI fluorescence depicted by the nuclei. **D)** Strong expression of Oct 4 intracellularly. **E)** DAPI fluorescence depicted by the nuclei. **F)** Note the weak expression of Nanog on PDLSCs compared to DPSCs. The antibodies were FITC Conjugated. Cultures were examined and photographed by fluorescence microscopy and appropriate filters. Results are representative of four independent experiments (Original magnification×40).*

rounded cells and spindle-shaped cells. From the first passage, cultivated cells homogenously showed a fibroblast-like spindle shape. The periodontal ligament cells had a more elongated spindle-shaped morphology compared to the dental pulp cells (Fig. 1a).

Indirect immunoflourescence

The MSCs derived from dental pulp and periodontal ligament were analyzed for embryonic stem (ES) cell markers such as Oct-4, Nanog along with mesodermal marker Vimentin. Both stem cell populations were positive for Vimentin. Differences were observed in ES cell markers. DPSCs demonstrated a strong expression of Oct 4 and Nanog (Fig. 1b); however, PDLSCs had a weak expression of Nanog (Fig. 1c).

Immunophenotype characterization

In each of the four cultivated specimens of

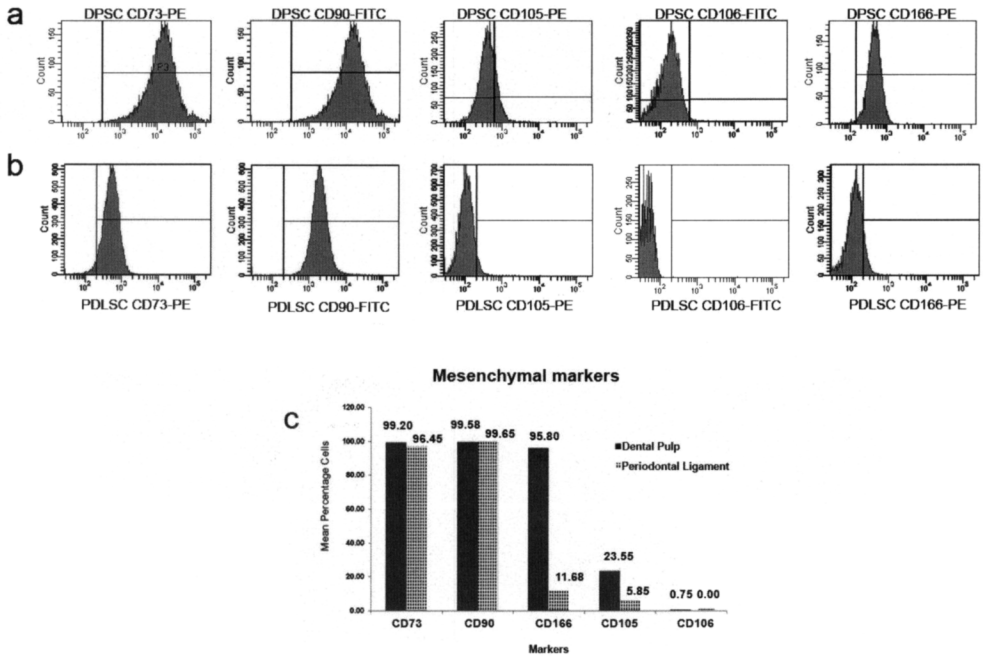

Fig. 2. *Expression of Mesenchymal markers in Dental pulp and Periodontal ligament stem cells by flow Cytometry.* ***a)*** *Histograms showing surface marker expression of DPSCs.* ***b)*** *Histograms of surface marker expression of PDLSCs. Relative cell count (y-axis) and fluorescence intensity (x-axis).* ***c)*** *Column diagram on flow cytometry data indicating expression of mesenchymal markers in DPSCs and PDLSCs. Columns illustrate mean surface marker expression levels ± standard deviation. The results are representative of four independent experiments.*

both DPSCs and PDLSCs a negative expression of typical hematopoietic markers CD34, CD45 and co-stimulatory markers CD80 and CD86 (Fig. 3) was consistently obtained. Immunostaining for mesenchymal markers, stem cell markers and immune markers revealed significant differences between DPSCs and PDLSCs. The expression of CD73 in the dental pulp was 99.2±0.08% in pulp and 96.4±0.28% in periodontal ligament ($p = 0.02$). However, with regard to CD90 no significant expression was observed in either of the groups ($p=0.36$). CD105 and CD106 were negligibly expressed in dental pulp and periodontal ligament (Fig. 2). The expression of CD166 was statistically significant in the dental pulp with a mean of (95.8±0.08%) compared to the periodontal ligament (11.6±0.2%) ($p=0.02$). Immune marker HLA-ABC expression was high in dental pulp 97.7±0.25% compared to 22.6±0.29% in periodontal ligament. There was negligible expression of HLA-DR in both the tissues (Fig 4). The expression of stem cell marker CD13 was also statistically significant in the dental pulp compared to the periodontal ligament with a mean of 99.4±0.29% and 89.1±0.44% ($p=0.02$), respectively. SSEA4 expression in dental pulp was 57.5± .5% compared to 3.45±0.12% in periodontal ligament. CD9 expression was high in periodontal ligament 96.2±0.46% compared to 58.2±0.28% in periodontal ligament. High variations were seen in surface marker expression levels for CD166, HLA-ABC; stemness related markers SSEA4 and CD9 were consistent in all four samples of DPSCs and PDLSCs (Table I).

DISCUSSION

The results of the present study demonstrate that DPSCs and PDLSCs are positive for Oct-4

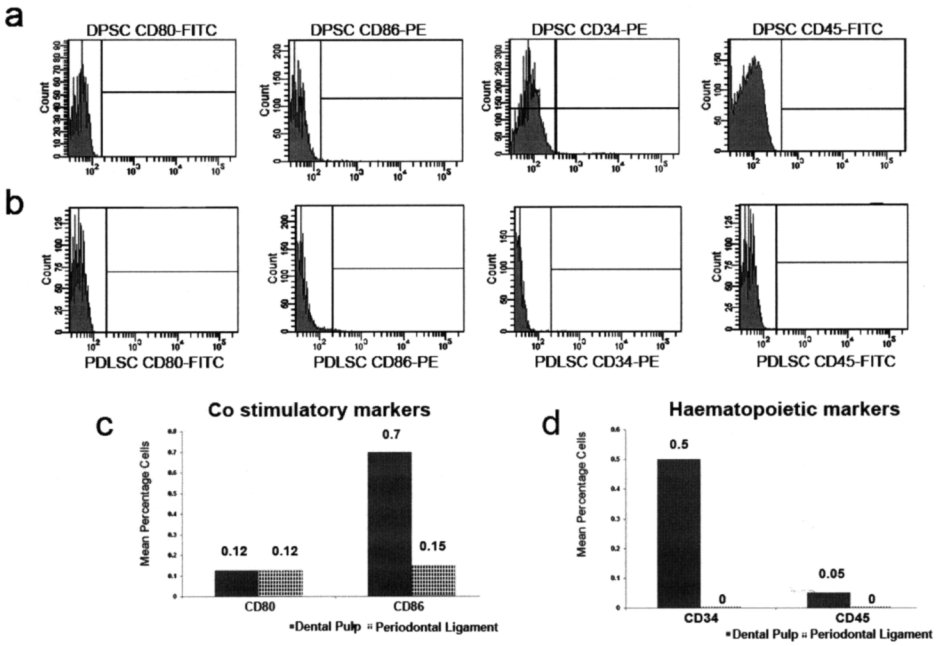

Fig. 3. *Analysis of Co-stimulatory and hematopoietic markers on Dental pulp and Periodontal ligament stem cells by flow Cytometry.* ***a)*** *Histogram from analysis of DPSCs.* ***b)*** *Histogram from analysis of PDLSCs. Relative cell count (y-axis) and fluorescence intensity (x-axis).* ***c)*** *Column diagram showing difference in expression of Co-stimulatory markers in DPSCs and PDLSCs.* ***d)*** *Column diagram depicting comparison of Hematopoietic markers in DPSCs and PDLSCs. The results are representative of four independent experiments. Data results shown correspond to average ± standard deviation. Passage 3 cells were analyzed by Flow Cytometry after staining with fluorescence isotiocyanate (FITC) or Phycoeryhtrin (PE) control isotype IgG.*

and Vimentin, but PDLSCs had weak expression of Nanog compared to DPSCs. This is in contrast to previous observation (16) wherein a positive expression of Nanog was observed in PDLSCs.

Phenotypic profile of surface markers using flow cytometry showed significant differences between DPSCs and PDLSCs. The expression of mesenchymal markers CD73 and CD90 was high in both DPSCs and PDLSCs. Endodermal markers CD105 (endoglin) and CD106 were weakly expressed in both the stem cells which could be attributed to the ectomesenchymal origin of tooth (17). CD166 expression on DPSCs was high compared to PDLSCs. This is in contrast to previous observations in PDLSCs (12, 18). These results indicate that DPSCs include a more primitive stem cell population whereas PDLSCs might consist of a heterogeneous mixture of stem cells together with committed progenitor cells.

There was a strong expression of MHC Class I molecule HLA-ABC in DPSCs compared to PDLSCs. These results are in accordance with previous observations (2, 19-20). Such high expression of MHC class I markers could be due to the possibility that DPSCs are capable of stimulating mixed lymphocyte cultures, as observed previously (21).

CD13, a stromal precursor of bone marrow, was high in both the stem cells but expression of SSEA4 was negligible in PDLSCs as compared to DPSCs (Fig. 4). This is similar to previous observations (18) wherein a minor proportion of PDLSCs exhibited SSEA4 positivity. However, CD9, a protein highly expressed in cells that differentiate into osteogenic precursors (22), was high in PDLSCs compared to DPSCs in the present study. Such a finding has not been observed in any previous studies. The difference in expression of CD9 could be attributed

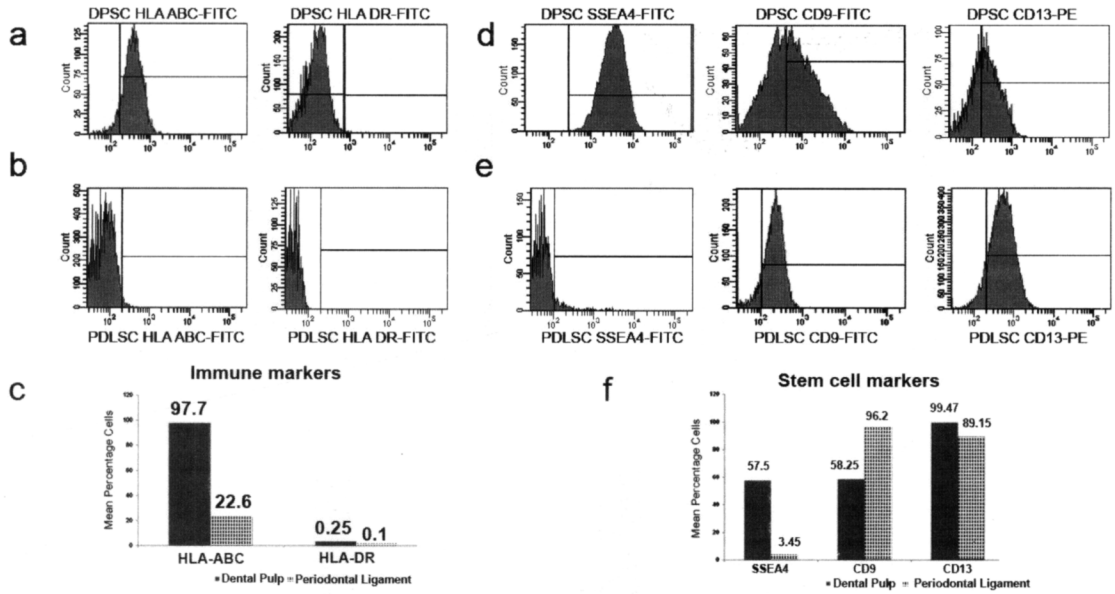

Fig. 4. *Expression of Immune markers and Stem cell markers in Dental pulp and Periodontal ligament stem cells by flow Cytometry.* ***a)*** *Histograms depicting expression of Immune markers in DPSCs.* ***b)*** *Histograms depicting expression of Immune markers in PDLSCs.* ***c)*** *Column diagram depicting the differences in expression of Immune markers in DPSCs and PDLSCs.* ***d)*** *Histogram depicting expression of Stem cell markers in DPSCs.* ***e)*** *Histogram depicting expression of stem cell markers in PDLSCs.* ***f)*** *Column diagram depicting difference in expression of Stem cell markers in DPSCs and PDLSCs. The results are representative of four independent experiments. Data results shown correspond to average ± standard deviation. Passage three cells were analyzed by flow Cytometry after staining with fluorescence isotiocyanate (FITC) or Phycoeryhtrin (PE) control isotype IgG.*

Table I. *Surface marker expression profile of dental pulp and periodontal ligament stem cells by flow cytometry analysis of 0.5 ×10⁶ cells.*

Surface Protein	Antigen	Dental Pulp (1)	Periodontal Ligament (2)
CD 73	Ecto -5'-nucleotidase	99.2 ± 0.08	96.4 ± 0.28
CD90	Thy-1	99.5 ± 0.09	99.6 ± 0.12
CD105	endoglin	23.2 ± 0.34	5.8 ± 0.12
CD106	VCAM-1	0.75 ± 0.12	0.0 ± 0.0
CD166	ALCAM	95.8 ± 0.08	11.6 ± 0.22
HLA-ABC	MHC-I	97.7 ± 0.25	22.6 ± 0.29
HLA-DR	MHC-II	0.25 ± 0.05	0.10 ± 0.11
CD80	Leukocyte common antigen	0.12 ± 0.09	0.12 ± 0.09
CD86	Leukocyte common antigen	0.25 ± 0.05	0.10 ± 0.11
CD34	Leukocyte common antigen	0.05 ± 0.06	0.00 ± 0.0
CD45	Leucocyte common antigen	0.05 ± 0.05	0.00 ± 0.0
SSEA4	Stage specific embryonic antigen	57.5 ± 0.5	3.45 ± 0.12
CD13	Aminopeptidase N	58.2 ± 0.28	96.2 ± 0.46
CD9	Tetraspanin receptor	99.4 ± 0.29	89.1 ± 0.44

Results are displayed as means of the surface marker expression in percent ± standard deviation. VCAM- vascular cells adhesion molecule. ALCAM - activated leucocyte cell adhesion molecule. Thy-1 - T cell surface glycoprotein. MHC – major histocompatibility complex. SSEA4 - embryonic stem cell marker. Group 1, n = 4. Group 2, n = 4.

to the location of PDLSCs and interaction with different surrounding cells. It can be speculated that PDLSCs are more likely to differentiate into osteogenic precursors. Observing the location of adult stem cell niches and the types of neighboring cells would help us understand whether progenitor cells arise from blood vessels in the periodontal ligament or surrounding bone stroma (23, 10).

The reported results highlight that DPSCs and PDLSCs possess stem cell qualities, as observed by expression of mesenchymal stem cell markers on their surface. The present study provides an insight into the understanding of how MSCs from related tissue could depict distinct characteristics. Understanding that different stem cells express distinct phenotypes may have further implications in understanding the factors that regulate the formation of mineralized matrices and other associated connective tissues. This would ultimately lead to novel tissue engineering strategies in future. However, definitive experiments like *in situ* hybridization of specific markers and further clonal studies need to be performed to highlight the heterogeneous nature of stem cells from these two sources.

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