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# Stem cell properties of human periodontal ligament cells

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*Background and Objective:* Stem cells have been used for regenerative therapies in various fields. The proportion of cells that possess stem cell properties in human periodontal ligament (PDL) cells is not yet well understood. In this study, we quantitatively characterized human PDL cells to clarify their stem cell properties, including self-renewal, multipotency, and stem cell marker expression.

*Material and Methods:* PDL cells were obtained from extracted premolar or wisdom teeth, following which a proliferation assay for self-renewal, a differentiation assay for multipotency, immunostaining for STRO-1, and fluorescence-activated cell sorter (FACS) analysis for stem cell markers (including CD105, CD166, and STRO-1) were performed.

*Results:* Approximately 30% of 400 PDL cells were found to possess replicative potential and formed single-cell colonies, and 30% of these colonies displayed positive staining for STRO-1, 20% differentiated into adipocytes and 30% differentiated into osteoblasts. FACS analysis revealed that PDL cells, including cell populations, expressed the stem cell markers CD105, CD166, and STRO-1.

*Conclusion:* The findings of this study indicated that PDL cells possess crucial stem cell properties, such as self-renewal and multipotency, and express the mesenchymal stem cell markers CD105, CD166, and STRO-1 on their cell surface, although there were some variations. Thus, PDL cells can be used for periodontal regenerative procedures.

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Periodontitis is an infectious disease involving periodontal ligament and bone, which is characterized by gingival inflammation and periodontal attachment loss, eventually leading to tooth loss. The periodontal ligament (PDL) is an unmineralized connective tissue that connects the alveolar bone to teeth. PDL cells consist of heterogeneous cell populations with fibroblastic or osteoblastic properties (1–6). Previous studies indicate that PDL cells possess the potential to form mineralized nodules *in vitro* (7), to express bone-related markers, such as bone sialoprotein and osteocalcin (8), and to express characteristic bone regulatory hormone responses (9). Thus, it is very likely that PDL cells include cell populations at different stages of differentiation and lineage commitment. The isolation and characterization of cell phenotypes that are effective for periodontal regeneration in the PDL cell population is definitely needed. Recently, Seo *et al.* reported that PDL cells contain stem cells that have the potential to form cementum/PDL-like tissue *in vivo* and that the application of PDL stem cells may be effective for periodontal regenerative therapy (10). However, the properties of PDL stem cells are not fully understood.

A number of studies have shown that bone marrow-derived mesenchymal stem cells (MSCs), which possess characteristics of self-renewal and multilineage differentiation potential, are suitable for regenerative therapies such as the treatment of osteogenesis imperfecta or bone/cartilage defects (11). Kawaguchi *et al.* reported that the transplantation of bone marrow-derived MSCs into partial periodontal defects was effective for periodontal regeneration (12).

MSCs can proliferate rapidly and differentiate into osteoblasts, adipocytes, chondrocytes, and (possibly)

muscle cells (13–15). MSCs express stem cell markers, including CD105 and CD166, on their cell surface (15,16). STRO-1 was originally identified as a colony-forming osteogenic precursor cell isolated from bone marrow (17,18). Previous observations by many investigators suggested that human bone marrow-derived MSCs generated single-cell derivative colonies if plated at extremely low densities (13,14,19). Digirolamo *et al.* (19) showed that the replicative potential of the cells in culture was best predicted by a simple colony-forming assay when cells were plated at low densities, and the samples with the highest colony-forming efficiency also exhibited the greatest replicative potential. In addition, single cell-derived colonies obtained by low-density plating were able to differentiate into mineralizing cells, adipocytes, and chondrocytes (14).

Although the existence of periodontal ligament stem cells has been reported (10), the characterization of those cells, including their proportion in PDL cell populations, is not fully understood.

In this study, in order to investigate the proportion of cells in PDL cell populations that possess stem cell properties, we quantitatively analyzed the surface epitopes of PDL cells along with replicative potential, STRO-1 staining, and the multilineage differentiation potential.

## Material and methods

### Cell culture

PDL cells were obtained from healthy premolar or wisdom teeth extracted from 17, 19, and 22-year-old-subjects ($n = 3$), according to the method reported by Sommerman *et al.* (20). The experimental protocol was approved by the Ethics committee of Tokyo Medical and Dental University, and informed consent was obtained from all the subjects.

PDL tissue was removed from the middle-third of the root by using a sterile scalpel, rinsed five times with growth medium [modified α-minimal essential medium (α-MEM); Invitrogen, Carlsbad, CA, USA] supplemented with 20% (v/v) fetal bovine serum (FBS; Invitrogen) and 1% (v/v) antibiotic-antimicotic (Invitrogen), and cultured. The cells that grew out from the tissue were cultured in growth medium at 37°C in a humidified atmosphere of 5% $CO_2$. The cells were subcultured, after reaching confluency, by using 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA (Invitrogen).

### Colony-forming assay

PDL cells were cultured until confluent, and detached by 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA. Then, the cells were confirmed, by microscopy, to be dispersed. About 8 ml of growth medium was added to with 400 single cells and those cells were replated in 60-cm$^2$ dishes. The cultures were incubated for 14 d, and the dishes were stained with 0.5% (v/v) Crystal Violet (Sigma, St Louis, MO, USA) in methanol for 5 min. The cells were washed twice with distilled water and the number of colonies was counted. Colonies less than 2 mm in diameter and faintly stained colonies were not counted. The percentage colony-forming efficiency was calculated by dividing the number of total colonies by 400 and multiplying by 100.

### Cell proliferation assay

As described above, 400 PDL cells were plated in 60-cm$^2$ dishes and cultured. After 14 d, the cells were detached with 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA. The total number of cells was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

### Staining of STRO-1 and alkaline phosphatase (ALP)

STRO-1 cells were originally identified as colony-forming osteogenic precursors isolated from bone marrow (17).

PDL colonies were cultured for 14 or 21 d. The colonies were fixed in 4% (v/v) paraformaldehyde for 15 min, blocked at room temperature for 30 min, and then incubated with a 1 : 10 dilution of mouse anti-human STRO-1 immunoglobulin (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) at 4°C overnight. The samples were subsequently incubated with rabbit anti-mouse immunoglobulin M (IgM) (Nichirei, Tokyo, Japan) for 1 h at 37°C. ALP staining was then performed for 30 min with napthol AS-MIX phosphate (Sigma) and fast blue BB salt (Sigma) in 0.1 M Tris-HCl, pH 8.5, containing 10 mM $MgCl_2$. After staining, the number of STRO-1-positive/ALP-positive colonies, STRO-1-positive/ALP-negative colonies, STRO-1-negative/ALP-positive colonies and STRO-1-negative/ALP-negative colonies was counted, the same cultures were subsequently stained with Crystal Violet, and the total number of colonies was counted.

### Adipogenesis in a colony-forming assay

PDL cells were plated at a density of 400 cells per 60-cm$^2$ dish in growth medium, and cultured for 14 d under conditions described previously by Sakaguchi *et al.* (21). The medium was switched to adipogenic differentiation medium: α-MEM supplemented with 10% (v/v) FBS, 0.5 mM isobutyl-1-methyl xanthine (Sigma), $10^{-7}$ M dexamethasone (Sigma), and 50 μM indomethacin (Sigma) and the cells were cultured for another 21 d. As control, α-MEM containing 10% FBS (v/v) only was used. The cells were stained with fresh Oil Red-O solution (Sigma) and then the number of Oil Red-O-positive colonies was counted. The same adipogenic cultures were subsequently stained with Crystal Violet, and the total number of colonies was counted.

### Calcification in a colony-forming assay

PDL cells were plated at a density of 400 cells per 150-cm$^2$ dish in growth medium and cultured for 14 d, according to Sakaguchi *et al.* (21). The medium was switched to osteogenic differentiation medium [α-MEM

supplemented with 10% (v/v) FBS, 50 μg/ml ascorbate-2 phosphate (Nacalai, Kyoto, Japan), $10^{-9}$ M dexamethasone and 20 mM β-glycerophosphate (Sigma)] and the cells were cultured for another 21 d. As control, α-MEM containing 10% (v/v) FBS was used. The cells were stained with fresh Alizarin Red solution (Wako, Osaka, Japan) and then the number of Alizarin Red-positive colonies was counted. The same calcification cultures were subsequently stained with Crystal Violet, and the total number of colonies was counted.

### Quantitative assay of calcium content

To examine the amount of calcification in PDL colonies, we measured calcium intake into the cell culture. PDL cells were plated at a density of 400 cells per 150-$cm^2$ dish and cultured for 14 d. Then, the medium was replaced with osteogenic differentiation medium and the cells were cultured for another 21 d. As a control, α-MEM containing 10% (v/v) FBS only was used. The cells were dissolved in 0.5 N hydrochloric acid (Wako). The calcium content of the resulting solution was measured by Calcium C-test kits (Wako). The absorbance at 570 nm was read on a plate reader.

### Epitope profile by fluorescence-activated cell sorter (FACS) analysis

PDL cells were cultured by the ordinary cell-culture technique, which basically consisted of seeding the cells. When the cells reached confluency, they were detached with 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA. The cells were counted in a haemocytometer, and $1 \times 10^6$ cells were taken for FACS analysis.

The isolated and expanded PDL cells were characterized by flow cytometric analysis of specific surface antigens, as described previously (15). One million cells were resuspended in 200 μl of phosphate-buffered saline (PBS) containing 20 μl/ml of antibodies. The cells were incubated for 30 min at 4°C, then washed with PBS and resuspended in 1 ml of PBS for FACS analysis. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-coupled antibodies against CD34 and CD45 (hematopoietic stem cell markers) were from Becton Dickinson (Franklin Lakes, NJ, USA). CD31 (an endothelial cell marker) and CD117 (a hematopoietic stem cell marker) (22) were from eBioscience (San Diego, CA, USA); CD105 (SH-2) (13,23,24) and CD166 [activated leukocyte cell-adhesion molecule (ALCAM), SB-10] (23,25) were from Ancell Corporation (Minneapolis, MN, USA); and STRO-1 (17) was from Genzyme-Techne (Minneapolis, MN, USA). For isotype control, FITC- or PE- coupled nonspecific mouse immunoglobulin G (IgG) (Becton Dickinson) was substituted for the primary antibody. Cell fluorescence was evaluated by flow cytometry using a FACS Calibur instrument (Becton Dickinson).

The positive fraction was sorted by FACSVantage (Becton Dickinson). The data were analyzed using CELLQUEST software (Becton Dickinson). Positive expression was defined as the level of fluorescence greater than 99% of the corresponding isotype-matched control antibodies.

### Statistical analysis

To assess the differences, the Student's *t*-test was used to detect statistical significance. A *p*-value of $< 0.05$ was considered significant.

## Results

### Colony-forming efficiency and cell proliferation assay

All trials of single cell-derived colony-forming efficiency and cell proliferation assay were successfully performed, although some variation of cell proliferation was observed. Crystal Violet staining revealed that 15–35% of 400 PDL cells possessed replicative potential (Fig. 1A) and the first loading of 400 cells resulted in 28,442–76,789 cells, a proliferation of between 71- and 191-fold (data not shown). The PDL colony-forming efficiency varied, and the largest number of colonies was 3–4 mm in diameter. (Fig. 1B).

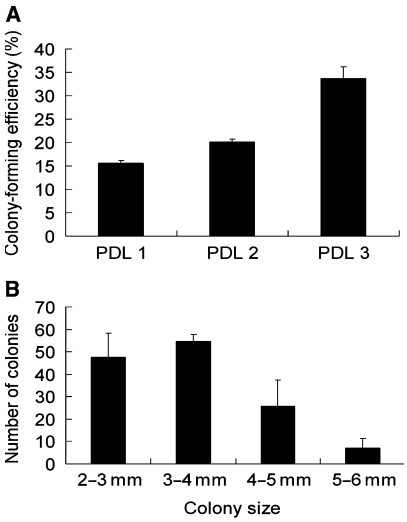


*Fig. 1.* Proliferative potential of periodontal ligament (PDL) cells. The cultures were incubated for 14 d, after which the dishes were stained with 0.5% (v/v) Crystal Violet. (A) After staining, the number of colonies was counted. The colony-forming efficiency of PDL cells was calculated ($n = 3$). PDL 1, PDL 2, and PDL 3 represent samples from three different patients. (B) Size distribution of colonies was obtained in a colony-forming assay. Values represent the largest diameters of the colonies. PDL colony-forming efficiency varied in size and the largest number of colonies had a diameter of 3–4 mm.

### Staining of STRO-1 and ALP

We examined how many colonies deriving from a single cell were STRO-1 positive. The results were consistent in all trials. The STRO-1-positive/ALP-negative colony-forming efficiency was 63% and 29% after 14 and 21 d, respectively.

Only a few colonies were positive on ALP staining after 14 d of culture (data not shown); however, the number of STRO-1-positive/ALP-positive colonies and STRO-1-negative/ALP-positive colonies did increase by almost fivefold and twofold, respectively, after 21 d of culture, as shown in Fig. 2.

### Differentiation potential of PDL cells

To determine the adipogenic differentiation potential, adipogenesis in a colony-forming assay was performed. The number of colonies accumulating

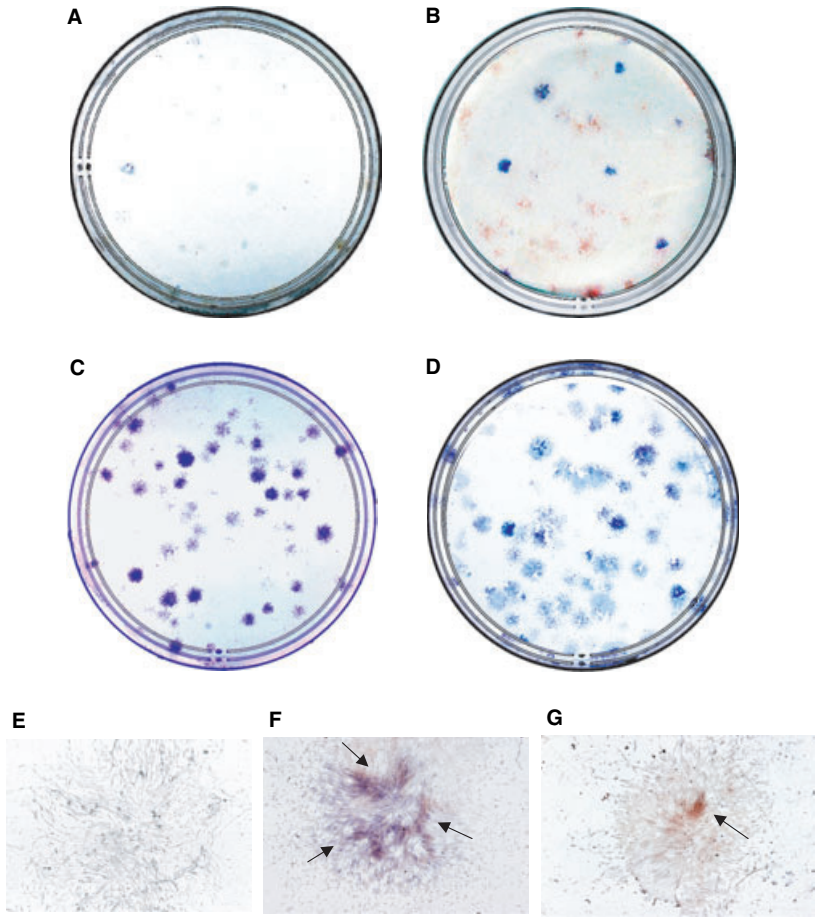


*Fig. 2.* Staining of STRO-1 and alkaline phosphatase (ALP). Periodontal ligament (PDL) colonies were cultured for 21 d and immunostained with (B) and without (A) STRO-1 antibody, followed by ALP staining. STRO-1-positive colonies were stained with pale pink and ALP-positive colonies with purple (C,D). After staining, the same cultures were subsequently stained with Crystal Violet. (E) STRO-1-negative/ALP-negative colony at a higher magnification (×3.3). (F) STRO-1-negative/ALP-positive colony (arrowhead) at a higher magnification (×3.3). (G) STRO-1-positive/ALP-negative colony (arrowhead) at a higher magnification (×3.3).

lipids was evaluated 21 d after the induction of adipogenesis. In control cells, there were no Oil Red-O-positive colonies (Fig. 3A, panels a and e). Adipogenic induction was apparent by the accumulation of lipid-rich vacuoles within the cells cultured in adipogenic differentiation medium (Fig. 3A, panels b and f). The Oil Red-O-positive colony-forming efficiency was 20.6 ± 6.1% (Fig. 3B). The differentiation occurred primarily at the center of the colonies, and a few adipocytes that formed near the periphery migrated towards the center.

To evaluate the calcification potential of PDL cells, calcification in a colony-forming assay was also performed. When PDL cells were stained with Alizarin-Red after 21 d of culture in the presence or absence of osteogenic differentiation medium, a number of mineralized nodules were distinctly stained in the calcification medium as compared with the control (Fig. 4A). The Alizarin Red-positive colony-forming efficiency was 27.8 ± 4.8% in PDL cells cultured in calcification-differentiation medium (Fig. 4B). Quantitative assays revealed that the calcium content stimulated by the calcification medium was almost threefold higher than the control (Fig. 4C).

### Analysis of surface epitopes by FACS

Analysis of surface epitopes by FACS using $1 \times 10^6$ PDL cells is shown in Table 1. Of the seven antibodies examined, the percentage positivity for CD105, CD166, and STRO-1 (mesenchymal stem cell markers) was 27.4 ± 11.8%, 59.8 ± 20.1%, and 1.2 ± 0.1%, respectively. FACS analysis of PDL cells demonstrated that the expression of STRO-1 was very low. This result was different from that obtained by STRO-1 staining during the single cell-derived colony-forming assay, which showed ≈30% positive total colonies, as described previously. The reason for this is discussed below.

The percentage positivity for CD34, CD45, CD117 (hematopoietic stem cell markers), and CD31 (an endothelial cell marker) was less than 2%.

## Discussion

PDL cells play an important role, not only in the maintenance of the periodontium but also in promoting periodontal regeneration. Recent studies have shown that proper manipulation of PDL cells is essential for tissue engineering (26,27). However, PDL cells are heterogeneous and include cells at different stages of differentiation and lineage commitment. In the present study, PDL cells cultured at low densities exhibited replicative potential and formed colonies. This finding suggests that PDL cells possess the ability both to proliferate and to produce colonies from a single cell. Digirolamo *et al.* (19) showed that the replicative potential of the cells in culture was best predicted by a simple colony-forming assay when cells were plated at low densities, and the samples with the highest colony-forming efficiency also exhibited the greatest replicative potential. Seo *et al.* reported that PDL stem cells (PDLSCs) are more proliferative than bone marrow-derived MSCs (10). They showed that PDLSCs have a relatively low colony-forming efficiency, whereas our study showed a much higher proliferation. This may be explained by the different method of cell culture, collagenase

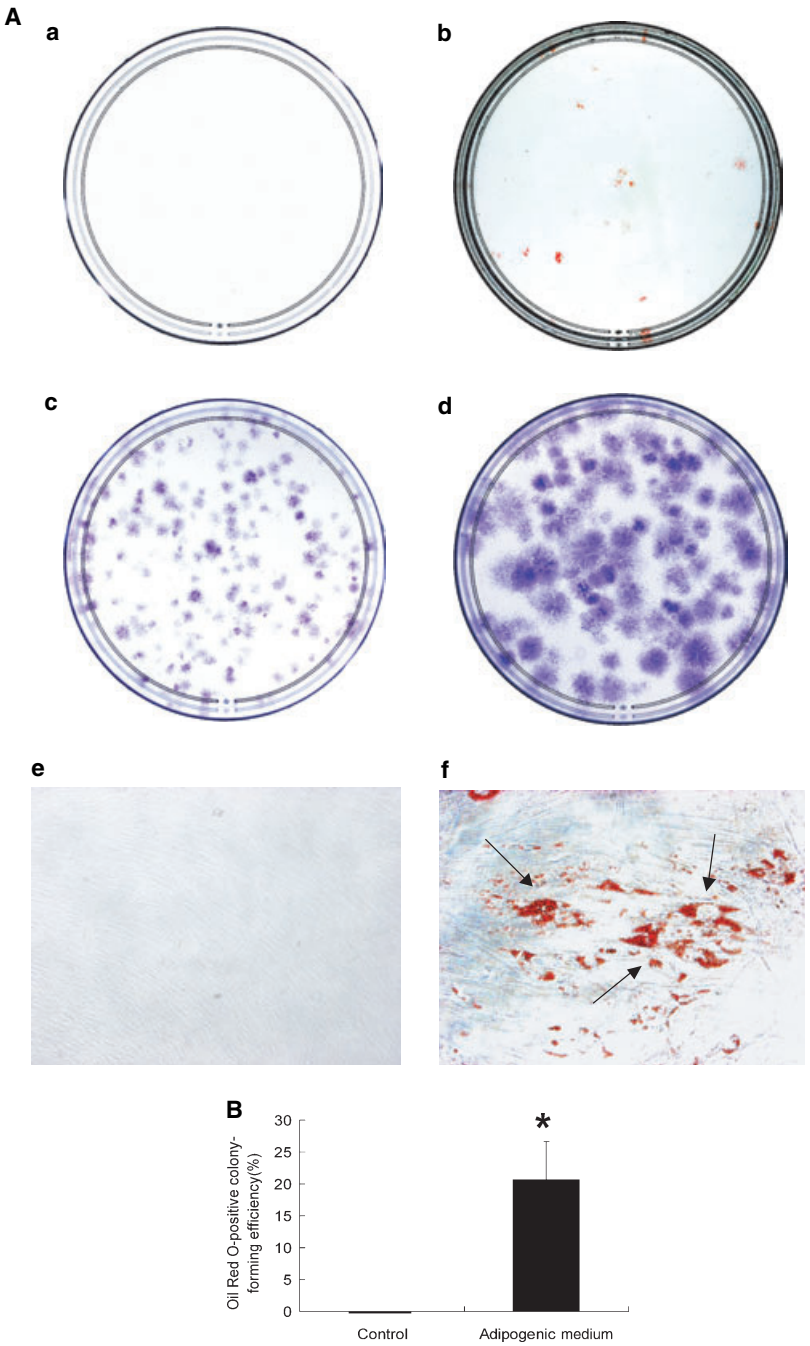


*Fig. 3.* Adipogenesis in a colony-forming assay. Periodontal ligament (PDL) cells were harvested at 400 cells/60-cm$^2$ dish and cultured for 14 d in growth medium. Then, the medium was switched to adipogenic differentiation medium for another 21 d. (A) PDL colonies were stained with Oil Red-O solution at the end of the experiment. (a) In control cells, there were no Oil Red-O-positive colonies. (b) Adipocyte colonies stained with Oil Red-O are shown as red colonies. (c, d) The same dishes were then stained with Crystal Violet and the total number of colonies was counted. (e) Control and (f) the Oil Red-O-positive colony (arrowheads) are shown at higher magnification (×4). (B) The ratio of Oil Red-O positive colonies to the total number of colonies was calculated for each dish, and the Oil Red O-positive colony-forming efficiency is shown in comparison with control. The data are expressed as mean ± standard deviation (SD) ($n = 3$). *Significantly different from the control ($p < 0.0001$).

digestion or outgrowth methods. Previous reports indicate that bone marrow-derived MSCs are morphologically heterogeneous, as well as for features such as surface epitopes (16). In addition, several antibodies have been used to routinely characterize the expanded mesenchymal cell populations (13,28).

Sakaguchi *et al.* (15) previously investigated the surface epitopes of bone marrow-derived MSCs. Although PDL cells are considered to be heterogeneous, it is still not clear how PDL cells express mesenchymal stem cell markers on their cell surface.

FACS analysis of PDL cells demonstrated that the percentage positivity for CD34, CD45, CD117 (hematopoietic stem cell markers) and CD31 (endothelial cell marker) was less than 2%. This value was the same as in bone marrow-derived MSCs (15). These results indicate that the PDL cells were not contaminated by hematopoietic stem cells or endothelial cells.

CD105 (endoglin, SH-2) – a stem cell marker (24) – was detected in 27% of PDL cells, less than previously reported in bone marrow-derived MSCs (15). This may be explained by the results of previous studies, where SH-2 expression was reported to decline towards the osteogenic differentiation process (29). CD166 (SB-10) antigen, which is also known as ALCAM (23), is reported to be expressed on human bone marrow-derived MSCs and to be lost during their development into differentiated phenotypes (29). The positive rate for CD166 on the PDL cell surface was 59 ± 20%, which is similar to that reported in MSCs (15,30). These results indicate that PDL cells include mesenchymal stem cells, together with more differentiated progenitor cells (29).

STRO-1 cells were originally identified as colony-forming osteogenic precursors isolated from bone marrow (17,18). It has been reported that STRO-1-positive cells are present in human PDL tissues (10). In the present study, we found that the STRO-1-positive/ALP-negative colony-forming efficiency decreased from 60% to 30% after 21 d of culture. On the other hand, the number of STRO-1-negative/ALP-positive colonies

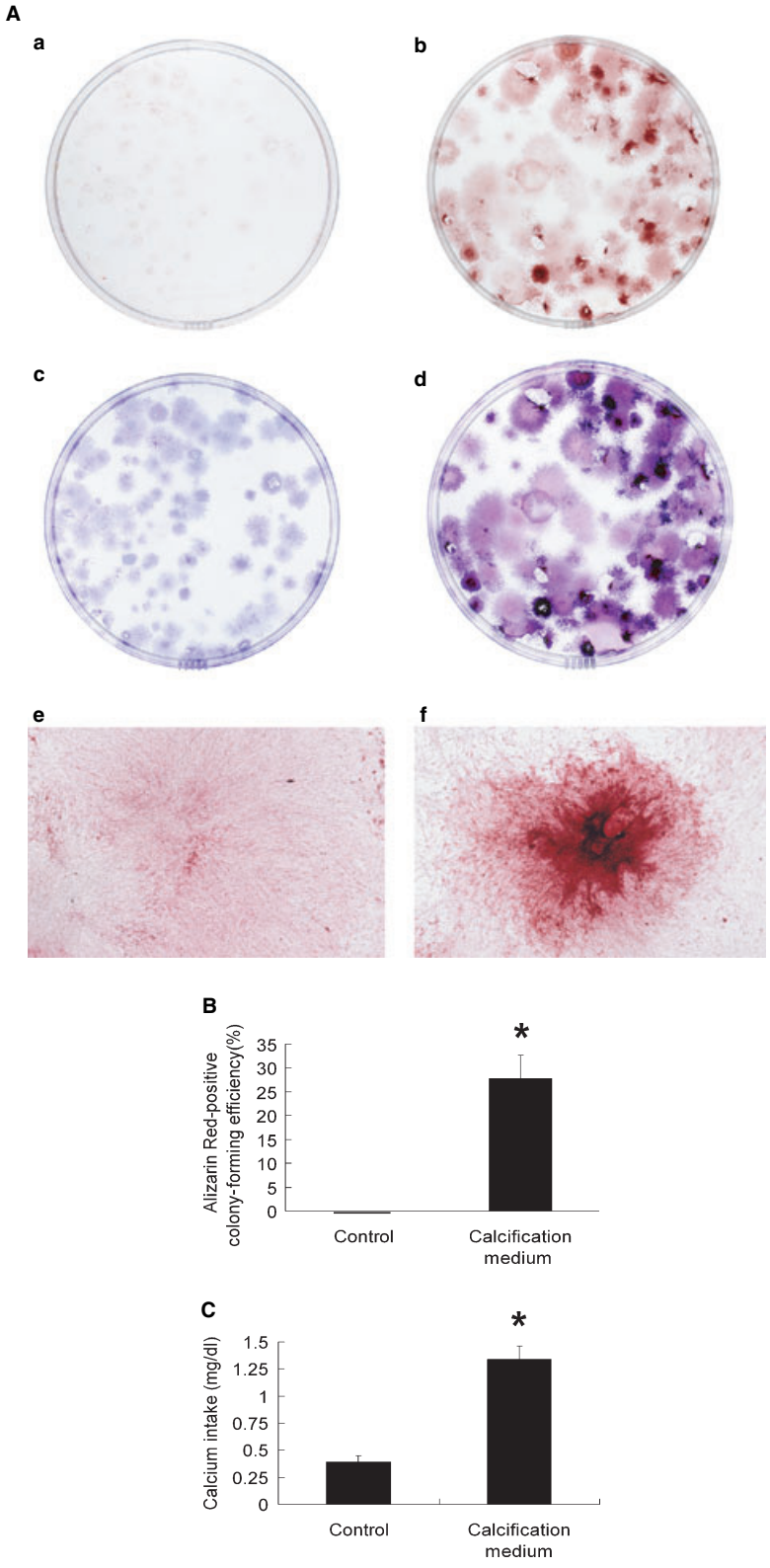


*Fig. 4*. Calcification in a colony-forming assay. Periodontal ligament (PDL) cells were harvested at 400 cells/150-cm$^2$ dish and cultured for 14 d in growth medium. Then, the medium was switched to calcification medium for another 21 d. (A) PDL colonies were stained with Alizarin Red at the end of the experiment. (a) In control cells, there were no Alizarin Red-positive colonies. (b) Alizarin Red staining revealed limited amounts of mineralized nodule formation in PDL colonies. (c, d) Then, the same dishes were stained with Crystal Violet. (e) an Alizarin Red-negative colony and (f) an Alizarin Red-positive colony are shown at higher magnification (×2.3). (B) The ratio of Alizarin Red-positive colonies to the total number of colonies was calculated for each dish, and Alizarin Red-positive colony-forming efficiency is shown in comparison with the control. The data are expressed as mean ± standard deviation (SD) ($n = 3$). *Significantly different from control ($p < 0.0001$). (C) PDL colonies were cultured in calcification medium for another 21 d and the calcium content was measured ($n = 3$). *Significantly different from the control ($p < 0.0003$).

increased almost twofold during the same time-period. These observations suggest that the number of STRO-1-positive colonies decrease as a result of an increase in ALP-positive colonies after 21 d of culture, and may be explained by the fact that the addition of ascorbic acid to the medium increases the ALP activity in PDL cells (31), and STRO-1 expression disappears during maturation of the osteoblastic cell lineage (29). FACS analysis of PDL cells demonstrated that the expression of STRO-1 was very low and much less than bone marrow-derived MSCs (21). These results are in accordance with the report by Trubiani *et al*. (32). However, we found that the STRO-1-positive single-cell-derived colony-forming efficiency was 30%. This discrepancy may be explained by the different method of cell culture, colony-forming assay or cell-culture technique, and that the STRO-1-positive PDL cells possess high replicative potential and formed colonies readily in the colony-forming assay.

PDL cells have been shown to form mineralized matrix in the presence of ascorbic acid, dexamethasone, and β-glycerophosphate, which suggests that PDL cells possess osteogenic potential (33). Seo *et al*. have shown that PDL cells have the potential to differentiate into other cell lineages, such as adipocytes and osteoblastic cells (10). In

*Table 1.* Epitope profile of periodontal ligament (PDL) cells

| | Marker | Percentage positivity |
|---|---|---|
| Mesenchymal stem cell markers | CD105 | 27.5 ± 11.8% |
| | CD166 | 59.3 ± 20.1% |
| | STRO-1 | 1.2 ± 0.1% |
| Endothelial cell marker | CD31 | 0.8 ± 0.3% |
| Hematopoietic stem cell markers | CD34 | 1.0 ± 0.1% |
| | CD45 | 0.6 ± 0.3% |
| | CD117 | 0.8 ± 0.2% |

One million PDL cells were analyzed with antibodies against the human antigens CD31, CD34, CD45, CD105 (endoglin, SH2), CD117 (c-kit), CD166 (ALCAM, SB-10), and STRO-1 by flow cytometry. Positive expression was defined as the level of fluorescence greater than 99% of the corresponding isotype-matched control antibodies. All analysis was performed on three donors. Percentage positivity (%) is displayed as mean ± standard deviation (SD) ($n = 3$).

the present study, the Alizarin-Red-positive colony-forming efficiency was 27% in the presence of osteogenic differentiation medium. Furthermore, the Oil Red-O-positive colony-forming efficiency was 20% in the presence of adipogenic differentiation medium. The differentiation occurred primarily at the center of the colonies, and the few adipocytes that formed near the periphery migrated towards the center (34). It may be, for this reason, that the cells in the center of the colony shifted to a differentiation phase earlier than those in the periphery of the colony.

In this study, ≈30% of 400 PDL cells possessed replicative potential and 30% of total colonies displayed multipotency. Although it has been reported that MSCs in human bone marrow exist at a rate of ≈1 per 100,000 cells, Sakaguchi *et al.* (15) showed that cultured MSCs possessed ≈27% colony-forming efficiency, and 40–50% multipotential on young donors. This may be the result of a high adherence potential to the culture dish and a high proliferative potential of MSCs.

It is still not clear how many stem cells are present in PDL tissues; however, identification of cells that possess stem cell properties in the cultured PDL cell population, is definitely important for clinical application in the near future.

As human bone marrow-derived MSCs transplantation is recognized as a possible option for treatment of periodontal defects (12), our results indicate that human PDL cells will be a useful source for periodontal regenerative therapies. Further investigation will be necessary in order to clarify the relevance of the different stem cell properties in donors.

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