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Multiple Differentiation Capacity of STRO-1$^+$/CD146$^+$ PDL Mesenchymal Progenitor Cells

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Although mesenchymal progenitor cells can be isolated from periodontal ligament (PDL) tissues using stem cell markers STRO-1 and CD146, the proportion of these cells that have the capacity to differentiate into multiple cell lineages remains to be determined. This study was designed to quantify the proportions of primary human PDL cells that can undergo multilineage differentiation and to compare the magnitude of these capabilities relative to bone marrow-derived mesenchymal stem cells (MSCs) and parental PDL (PPDL) cells. PDL mesenchymal progenitor (PMP) cells were isolated from PPDL cells using the markers STRO-1 and CD146. The colony-forming efficiency and multilineage differentiation potential of PMP, PPDL, and MSCs under chondrogenic, osteogenic, and adipogenic conditions were determined. Flow cytometry revealed that on average 2.6% of PPDL cells were STRO-1$^+$/CD146$^+$, whereas more than 63% were STRO-1$^-$/CD146$^-$. Colony-forming efficiency of STRO-1$^+$/CD146$^+$ PMP cells (19.3%) and MSCs (16.7%) was significantly higher than that of PPDL cells (6.8%). Cartilage-specific genes, early markers of osteoblastic differentiation, and adipogenic markers were significantly upregulated under appropriate conditions in PMP cells and MSCs compared to either their noninduced counterparts or induced PPDL cells. Consistent with these findings, immunohistochemistry revealed substantial accumulation of cartilaginous macromolecules, mineralized calcium nodules, and lipid vacuoles under chondrogenic, osteogenic, or adipogenic conditions in PMP and MSC cultures, respectively, compared to noninduced controls or induced PPDL cells. Thus STRO-1$^+$/CD146$^+$ PMP cells demonstrate multilineage differentiation capacity comparable in magnitude to MSCs and could potentially be utilized for regeneration of the periodontium and other tissues.

Introduction

The functional periodontal attachment apparatus anchors the teeth to the alveolar bone and consists of periodontal ligament (PDL) connective tissue fibers running between the bone and cementum that lines the root surface [1,2]. These fibers help the tooth withstand pathophysiological forces that occur during mastication [3]. The PDL contains several cell types, including fibroblasts, which are the most numerous, epithelial cells, endothelial cells, inflammatory cells, and neural and lymphatic cells. These cells are important in fulfilling the multiple roles of the PDL including the elaboration of an extracellular matrix and collagen fiber formation for load bearing and structural requirements. Since the PDL has one of the highest metabolic turnover rates in the body [4,5], PDL cells must also participate and contribute substantially to its renewal. Finally, PDL cells are intricately involved in repair and regeneration of these tissues following surgical wounding and after its breakdown in inflammatory periodontal disease. Thus the PDL is a complex structure of connective tissue collagen fibers, other extracellular matrix proteins, and a heterogenous group of cells necessary for the many functions of the PDL tissues.

Periodontitis is a collective name for inflammatory diseases affecting the tissues that surround and support teeth. If left untreated, periodontitis often causes progressive bone loss around teeth, mobility of teeth and eventually tooth loss [3]. Although it is highly prevalent, the precise mechanisms that govern this disease and the regenerative processes that recapitulate the lost tissues following disease have not been fully characterized. Precipitating factors, such as microorganisms and their byproducts, initiate an inflammatory process that leads to the dissolution of tissue components in a susceptible host. Furthermore, the complex structures

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of the periodontium, which consists of the soft connective tissues of the gingiva and PDL and the mineralized tissues, cementum, and bone, make periodontal regeneration a very complicated and unique process. Periodontal regeneration requires the formation of new periodontal fibers, the orchestrated and simultaneous formation of new bone, and the coordinated insertion of the newly formed fibers into the regenerating cementum and bone. Consequently, both the complexity of the PDL attachment apparatus and the heterogeneity of its cell population complicates efforts in dissecting out the precise mechanisms of tissue breakdown and in identifying the cells necessary for the complete regeneration of these tissues following disease. As a result, currently the complete regeneration of all these periodontal tissues is clinically unpredictable, resulting at best in only partial regeneration of some of the tissues [2].

The heterogeneity of the PDL cell population and the spatial and temporal processing of these cells to differentiate into appropriate cell types remains one of the key factors in implementing optimal approaches to periodontal regeneration. From a cell-therapy standpoint, future prospects for improved periodontal regeneration will rely on the ability to identify specific subtypes of PDL cells that are capable of proliferation and differentiation into various tissues by specific spatially and temporally regulated exogenous cues [6,7]. Given this, previous studies have isolated cells termed multipotent postnatal stem cells from the PDL by single colony selection and magnetic activated cell sorting using one of the early mesenchymal stem cell (MSC) surface markers STRO-1 [8]. Similarly, the ability of putative stem cells derived from human PDL for multidifferentiation potential have been determined by using a mixed population of STRO-1^+/CD146^- and STRO-1^+/CD146^+ cells [9]. No previous attempts have been made to determine the capability and potency of STRO-1^+/CD146^+ double positive PDL cells to differentiate into different lineages. The successful isolation of such cells that we refer to as periodontal mesenchymal progenitor (PMP) cells from parental PDL (PPDL) cells is likely to augment both the purity of this unique cell population and their ability for multilineage potential possibly mimicking that of bone marrow-derived MSCs. Therefore, the purpose of this study was to determine the proportion of primary PDL cells that are STRO-1^+/CD146^+ and can undergo multilineage differentiation, and to compare the magnitude of these capabilities relative to MSCs and PPDL cells. This work will help lay a substantial foundation toward understanding the fundamental molecular mechanisms in the regeneration of periodontal and craniofacial structures. Also knowing the relative abundance of these cells present in any given parental population of PDL cells will enhance our knowledge for improved clinical outcomes.

Materials and Methods

Subjects, tissue collection, and cell culture

PDL cells were retrieved from healthy premolars extracted for orthodontic reasons ($n = 8$) and impacted third molars ($n = 4$) from 6 individuals (16–25 years old) under informed consent at the University of Michigan School of Dentistry. PDL tissues were carefully dissected and retrieved from the middle third of the root surfaces of extracted teeth under sterile conditions as previously described [10]. The explants were minced and covered with sterile glass coverslips in 35-mm tissue culture dishes in α-minimal essential media (α-MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and fungizone (Invitrogen Corp., Carlsbad, CA, USA) in a humidified atmosphere (37°C, 5% CO_2). After the outgrowth of PDL cells reached confluency, they were expanded twice until sufficient cells were obtained for subsequent procedures.

Flow cytometric analysis of stem cell surface markers on PDL cells

Single cell suspensions were obtained by detaching monolayers of PDL cells with cell dissociation buffer (Invitrogen Corp.) and filtering the cell suspension through 40 μm nylon cell strainers (BD Biosciences, Bedford, MA, USA). Cells (1×10^6) were incubated with a mouse antihuman monoclonal STRO-1 primary antibody (R&D Systems Inc., Minneapolis, MN, USA) in 2% FBS/PBS for 1 h. After three washes with 2% FBS/PBS, the cells were incubated with FITC-conjugated goat antimouse IgM secondary antibody (BioFX Laboratories Inc., Owings Mills, MD, USA) for 30 min. Cells were washed, incubated with R-Phycoerytherin (PE)-conjugated mouse antihuman CD146 monoclonal (BD Biosciences) antibody for 30 min, fixed in BD Cytofix buffer (BD Biosciences) for 30 min, washed, and resuspended in 500 μL PBS. For negative controls, PE-conjugated nonspecific mouse IgG (BD Biosciences) or nonspecific mouse serum (Oncogene™, Cambridge, MA, USA) were substituted for the primary antibodies. All the above procedures were performed in the dark at 4°C. The expression profile of STRO-1 and CD146 on PDL cells was examined using flow cytometry (Becton-Dickinson, San Jose, CA, USA) and analyzed with Cell-Quest 3.1 software (Becton-Dickinson). To characterize the proportion of MSCs that are positive for STRO-1 and/or CD146, these procedures were repeated on MSCs.

Immuno-magnetic Dynabead cell sorting

To isolate the PMP cells from the PPDL cell population, the CELLection Biotin Binder kit and the Dynal MPC-1 (Invitrogen Corp.) system were employed as per the manufacturer's instructions. Detached PDL cells (1×10^7) were incubated with mouse antihuman monoclonal STRO-1 primary antibody for 60 min on ice, while the biotin conjugated goat antimouse IgM secondary antibody (BioFX Laboratories) was mixed with the streptavidin-bound magnetic Dynabeads for 30 min at room temperature. PDL cells were then thoroughly washed and resuspended in 1 mL Buffer 2 (PBS with 0.1% BSA and 0.6% Na-citrate) and mixed well with the appropriate volume of Dynabead-bound biotin conjugated goat antimouse IgM secondary antibody for 20 min at 4°C with gentle mixing. Subsequently, the Dynabead-bound cells were washed three times with Buffer 1 and resuspended in 200 μL preheated (37°C) Buffer 3 (RPMI 1640 with 1% fetal calf serum, 1 mM $CaCl_2$, and 4 mM $MgCl_2$, pH 7.0–7.4). Following this, 4 μL of Releasing Buffer were added and incubated with the Dynabead-bound cells for 15 min at room temperature with gentle mixing. The mixture was then transferred to the Dynal MPC-1 magnet for 2 min. The supernatants containing the released STRO-1 positive cells were collected and plated in MSC growth media for approximately 1 week after

which the STRO-1 positive cells were detached and subjected to a second isolation with a biotinylated mouse antihuman CD146 antibody using the procedures described above. The isolated STRO-1 and CD146 double positive PMP cells were then plated for up to 3 weeks prior to subsequent experimentation. In addition, cells from three individuals were similarly isolated and cultured for subsequent flow cytometric anaylsis to determine the proportion of cells that remained double positive for STRO-1$^+$/CD146$^+$ at the beginning of the differentiation experiments.

Colony-forming efficiency assay

Four-hundred Dynabead-sorted STRO-1$^+$/CD146$^+$ PMP cells and the same number of corresponding PPDL cells and bone marrow MSCs (Cambrex Bio Science, Walkersville, MD, USA) were plated in 100 mm tissue-culture plates in MSC growth media for 10 days. The newly formed colonies were visualized using 0.1% (w/v) toluidine blue (Sigma-Aldrich, St. Louis, MO, USA) staining following 1% paraformaldehyde fixation. Aggregates of more than 50 cells were scored as colonies [11]. Colony-forming efficiency was determined by the number of colonies relative to the total number of seeded cells in each plate.

Chondrogenic, osteogenic, and adipogenic cultures of PPDL cells, PMP cells, and MSCs

To characterize the ability of PMP cells to undergo multilineage differentiation in comparison to that of the PPDL cells and MSCs, all cells were subjected to chondrogenic-, osteogenic-, and adipogenic-induction, as previously described [12–16].

A pellet culture system was used for chondrogenic differentiation. Approximately 2×10^5 PPDL cells, PMP cells, or MSCs were placed in a 15-mL conical polypropylene tube and pelleted under centrifugation at 500G for 10 min. The pellet was cultured in 500 µL chemically defined growth medium with L-gluatamine, penicillin–streptomycin, and 10% FBS or chemically defined serum-free chondrogenic induction medium containing dexamethasone, ascorbate, insulin–transferrin–sodium selenite (ITS), sodium pyruvate, proline, L-glutamine, and TGF-β_3, purchased from Cambrex Bio Science. The medium was replaced every 3 days for up to 21 days.

For osteogenic and adipogenic differentiation, each of the cell types were initially plated in monolayers at $3 \times 10^3/cm^2$ (osteogenic) or $2 \times 10^4/cm^2$ (adipogenic) in growth media containing L-glutamine, penicillin/streptomycin, and 10% FBS. For the osteogenic assays, cells were cultured in either the growth media (controls) or in the osteogenic induction media containing L-glutamine, ascorbate, dexamethasone, and β-glycerophosphate for 2–3 weeks according to manufacturer's instructions. For adipogenesis, cells grown to confluence were subjected to three cycles of induction and maintenance for optimal adipogenic differentiation following manufacturer's instruction. Each cycle consisted of feeding cells with the adipogenic induction media for 3 days followed by 3 days of culture in the maintenance media. The adipogenic induction media contained recombinant human insulin, dexamethasone, indomethacin, L-glutamine, and 3-isobutyl-1-methylxanthine, whereas the adipogenic maintenance media contained only recombinant human insulin and L-glutamine. Cells in the control group were maintained only in the maintenance media on the same schedule. All growth media, osteogenic induction media, adipogenic maintenance media, and adipogenic induction media were purchased from Cambrex Bio Science.

Total RNA extraction and qRT-PCR analyses

RNeasy Mini kit (Qiagen Corp., Valencia, CA, USA) was used to extract total RNA from PPDL cells, PMP cells, and MSC monolayer cultures on day 5 for osteogenic and day 7 for adipogenic gene assays. For the chondrogenic gene expression studies, total RNA was extracted on day 7 from high-density pellet cultures with Trizol reagent (Invitrogen Corp.). Total RNA (1 µg) from each condition was reverse-transcribed using the Omniscript RT Kit (Qiagen Corp.). A 1:100 dilution of the resulting cDNA was utilized as a template to quantify the relative content of mRNA by qRT-PCR (Applied Biosystems, Carlsbad, CA, USA). Primers specific for human collagen type II (forward primer: 5′-GGA AGA GTG GAG ACT ACT GGA TTG AC-3′, reverse primer: 5′-TCC ATG TTG CAG AAA ACC TTC AT-3′), cartilage oligomeric matrix protein (COMP; forward primer: 5′-GAC AGT GAT GGC GAT GGT ATA GG-3′, reverse primer: 5′-TCA CAA GCA TCT CCC ACA AAG T-3′), and 18s rRNA (forward primer: 5′-CGC CGC TAG AGG TGA AAT TC-3′, reverse primer: 5′-CAT TCT TGG CAA ATG CTT TCG-3′) were designed with Primer Express 2.0 (Applied Biosystems). Primers specific for osteogenic- and osteoblast-related markers (osterix and alkaline phosphatase) and adipogenic markers [lipoprotein lipase (LPL) and peroxisome proliferation-activated receptor-γ2 (PPAR-γ2)] were purchased from Applied Biosystems. PCR reactions were performed with the SYBR green PCR master mix kit (Applied Biosystems). As an internal control, 18s rRNA was used. Cycle threshold values of the genes of interest and the quantitative gene expression levels relative to 18s rRNA in the samples were determined as per the manufacturer's instructions (Applied Biosystems).

Alkaline phosphatase activity assays

An alkaline phosphatase activity assay was used to assess osteogenic differentiation in day 7 cultures of all cell types. This activity was measured in cell lysates by enzymatic conversion of *p*-nitrophenylphosphate (*p*NPP; Sigma-Aldrich) to *p*-nitrophenol as previously described [10]. The amount of *p*-nitrophenol produced was measured spectrophotometrically (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 405 nm against a standard curve. The results were standardized by the total amount of protein in the cell lysates. The total amount of protein in the cell lysates was determined using the BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Histochemistry and immunohistochemistry

The chondrogenic pellets were rinsed in PBS, fixed with 4% paraformaldehyde/PBS, embedded in paraffin, and cut through the center to obtain 5-µm sections. Newly synthesized glycosaminoglycans (GAGs) were evaluated by Safranin-O (Sigma-Aldrich) staining and imaged by

photomicrography (Nikon TS100, Tokyo, Japan) as described elsewhere [17].

To localize collagen type II and aggrecan in the chondrogenic pellets, sections were rehydrated prior to immunostaining with the Histostain-SP kit (Invitrogen Corp.). To expose epitopes, sections were pretreated with either chondroitinase ABC for aggrecan staining or pepsin for collagen type II staining. Endogenous peroxidase was quenched, sections were incubated with a blocking solution for 10 min, followed by incubation with primary antibodies specific for human collagen type II (Chemicon International Inc., Temecula, CA, USA) or aggrecan (Abcam Inc., Boston, MA, USA) for 3 h at 37°C. Negative control sections were incubated with nonimmune serum. Sections were washed, incubated with biotinylated secondary antibody, washed again, and incubated with a streptavidin–peroxidase conjugate. Following further washing, sections were incubated with a diaminobenzidine solution and counterstained with hematoxylin. Specimens were observed and imaged under a microscope (Nikon TS100).

Osteogenic monolayer cultures were stained for matrix mineralization with Alizarin red S (Sigma-Aldrich) staining on day 14 as described previously [18]. In brief, cultures were fixed with 70% ethanol for 10 min and stained with 2% Alizarin red S solution (pH 4.0) for 5 min at room temperature. To quantify the degree of calcium accumulation in the mineralized extracellular marix, Alizarin red S–stained cultures were incubated with 100 mM cetylpyridinium chloride (Sigma-Aldrich) for 1 h to release calcium-bound dye into solution. The absorbance of the released dye was measured at 570 nm using a spectrophotometer (Molecular Devices) and standardized by the total amount of protein in the culture.

Lipid vacuole formation in the adipogenic cultures was determined by Oil Red O (Sigma-Aldrich) staining on day 18. The cells were gently rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 1 h, rinsed with ddH_2O, and incubated with 60% isopropanol for 5 min, and stained with the Oil Red O working solution (30 mL of 3 mg/mL Oil Red O/isopropanol stock solution plus 20 mL ddH_2O) for 5 min at room temperature. After rinsing with water, the cultures were counterstained with haematoxylin for 1 min at room temperature, rinsed again, and observed with phase contrast microscopy (Nikon TS100).

Statistical analysis

The mean ± standard deviation of data from experiments performed in triplicate for three to six independent samples were plotted as histograms for each quantitative variable. The statistical significance of any differences was determined by one-way analysis of variance (ANOVA) with the significance set at $P < 0.05$.

Results

A small proportion of PDL cells are presumptive PMP cells

Among the MSC surface markers available [8,9,16,19], STRO-1 and CD146 were selected for identification of the putative PMP cells in the PDL, because these markers have been used successfully in the past. Flow cytometric data showed a wide interindividual variability in the proportion of cells that were positive for both markers, one of the two markers, or double negative for both markers. On average 2.6% of PPDL cells were STRO-1^+/CD146^+ or presumptive PMP cells, whereas more than 63% were double negative for these markers (Fig. 1A and B). For the remainder of the cells, more than 31% were STRO-1^-/CD146^+ whereas only 2.6% were STRO-1^+/CD146^- suggesting a potentially mixed phenotype and limited multilineage differentiation potential in these cells. Based on these findings and previous reports [8,20,21], the putative PMP cells present within the PDL represent only a small subset of the whole population of PDL cells. We also found that more than 12% of the MSCs were STRO-1^+/CD146^+, whereas STRO-1^+/CD146^-, and STRO-1^-/CD146^+ cells comprised more than 13% and 1% of MSCs, respectively, which are higher than reported previously for general bone marrow stromal cells [22–24] (Fig. 1A).

To assess the stability of the STRO-1/CD146 makers on the PMP cells, flow cytometry was performed on cells purified for STRO-1^+/CD146^+ from three individuals and cultured for 3 weeks. These experiments demonstrated that on the average more than 67% (SD ± 23%) of the isolated PMP cells remained STRO-1^+/CD146^+ before we commenced our experiments for multilineage induction (Fig. 1C). These data indicate that the double positive PMP cells retain relatively stable stem cell surface marker expression over the short term in culture and with passage.

PMP cells form clonogenic cell colonies with equivalent efficiency to that of MSCs

To determine whether the isolated STRO-1^+/CD146^+ cells or presumptive PMP cells possess the characteristics of MSCs, they were examined for their ability to form stem cell-like adherent clonogenic cell clusters in colony-forming assays. PMP cells were also compared to their PPDL counterparts and to bone marrow-derived MSCs. PMP cells showed slightly, but not significantly, higher levels of colony forming efficiency (19.3%) relative to MSCs (16.7%) (Fig. 1D). Both these cell types had significantly greater ($P < 0.01$) colony-forming efficiency than the PPDL cells (6%).

PMP cells exhibit osteoblastic marker expression, alkaline phosphatase activity, and mineralization similar to MSCs and higher than PPDL cells

qRT-PCR analyses on RNA from PMP cells, the corresponding PPDL cells, and MSCs placed in osteogenic induction media revealed that gene expression of both osterix and alkaline phosphatase, the early osteoblastic differentiation and mineralization markers, respectively, were significantly upregulated ($P < 0.01$) in osteogenically induced PMP cells and MSCs compared to their noninduced counterparts (Fig. 2A and B). Specifically, osterix expression levels were 2.3-fold and 2.6-fold greater while alkaline phosphatase levels were 22.5-fold and 38-fold greater in PMP cells and MSCs, respectively, than in their noninduced controls. Moreover, the levels of these two genes were also significantly higher ($P < 0.01$) in the induced PMP cells and MSCs than that in the noninduced and osteogenically induced PPDL cells.

A

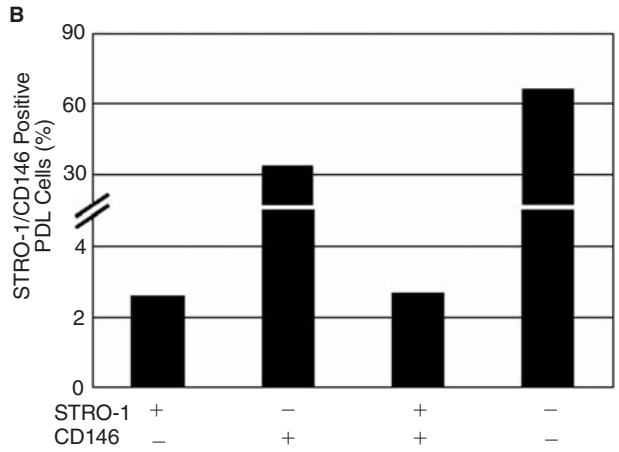
STRO-1 and CD146 Expression on PDL and hMSC Cells (%)

Subject Gender/Age	STRO-1+/ CD146−	STRO-1−/ CD146+	STRO-1+/ CD146+	STRO-1−/ CD146−
F 18	2.06	59.28	1.14	37.52
M 24	6.89	34.69	0.13	58.29
M 16	0.5	32.23	7.13	60.14
F 23	4.26	38.18	2.33	55.23
F 16	0.99	7.98	1.35	89.68
M 25	0.81	18.1	3.67	77.42
hMSC	13.29	1.02	12.07	71.64

C

STRO-1 and CD146 Expression on PMP Cells (%)

Subject Gender/Age	STRO-1+/ CD146−	STRO-1−/ CD146+	STRO-1+/ CD146+	STRO-1−/ CD146−
F 18	1.9	19.43	58.77	19.91
M 24	7.14	0	50.00	42.86
M 16	0.34	1.59	93.38	4.69



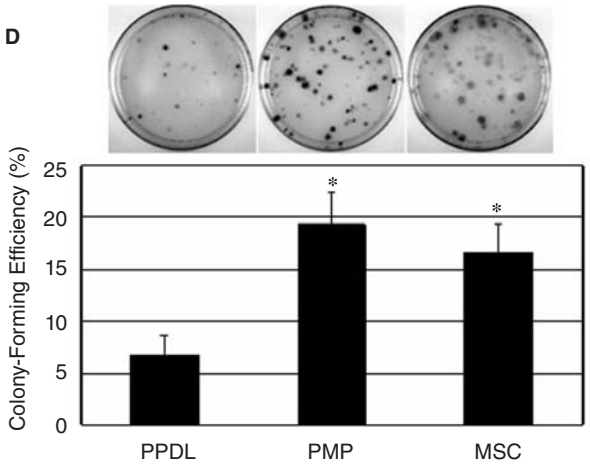


FIG. 1. Isolation and characterization of PDL mesenchymal progenitor (PMP) cells and evaluation of their stem cell-like self-renewal ability. (**A**) The proportions of STRO-1 and CD146 double positive, single positive, and double negative PDL cells from six individuals was determined by examining the surface expression profiles of these stem cell surface markers using flow cytometry. Female (F) and male (M) subjects ranged from 16 to 25 years of age. The proportions of STRO-1 and CD146 double positive, single positive, and double negative mesenchymal stem cells (MSCs) was determined by examining the surface expression profiles of these stem cell surface markers using flow cytometry. (**B**) The mean percent of STRO-1 and CD146 double positive, single positive, and double negative cells was calculated and depicted graphically. (**C**) The stability of STRO-1 and CD146 double positive PMP cells prior to commencing the differentiation experiments was determined from a subset of three individuals. The STRO-1 single positive cells were isolated, expanded for 1 week before the CD146+ cells were secondarily isolated, and expanded for further 3 weeks before being assayed by flow cytometry. (**D**) Assays performed to assess stem cell-like adherent clonogenic cell clusters formed in colony-forming assay plates by the presumptive PMP cells, the counterpart PPDL cells, and bone marrow-derived MSCs showed a high number of colonies for PMP cells and MSCs but not for PPDL cells. Newly formed colonies were stained with toluidine blue. Histogram depicting the colony-forming efficiency for cells were quantified from colony-forming assays. The colony-forming efficiency was determined as the number of colonies standardized by the total number of seeded cells in each plate. Aggregates of more than 50 cells were scored as colonies. Data, expressed as mean ± SD, were obtained from experiments performed in triplicate for three independent cultures (*$P \leq 0.01$ for PMP or MSC compared to PPDL).

The upregulation of alkaline phosphatase gene expression in osteogenically induced PMP cells and MSCs was paralleled by a similar increase in alkaline phosphatase activity in these cells (Fig. 2C). Osteogenically induced PMP cells exhibited approximately 15-fold and 7-fold increase in alkaline phosphatase activity compared to noninduced and induced PPDL cells, respectively. Similarly, osteogenically induced MSCs exhibited ~60-fold and 30-fold increase in alkaline phosphatase activity compared to noninduced and induced PPDL cells, respectively. Alkaline phosphatase activity in osteogenically induced PPDL cells was increased slightly relative to that in noninduced PPDL cells.

Alizarin red S staining corroborated the findings that PMP cells have a greater osteoblastic potential than their PPDL counterparts. Both PMP and MSC cultures exhibited obvious accumulation of calcium-containing mineralized nodules after 2 weeks of osteogenic induction, whereas the accumulation of mineralized nodules in the induced PPDL cell cultures was barely discernible (Fig. 3A). The released calcium-bound Alizarin red S was more than 2.5-fold and

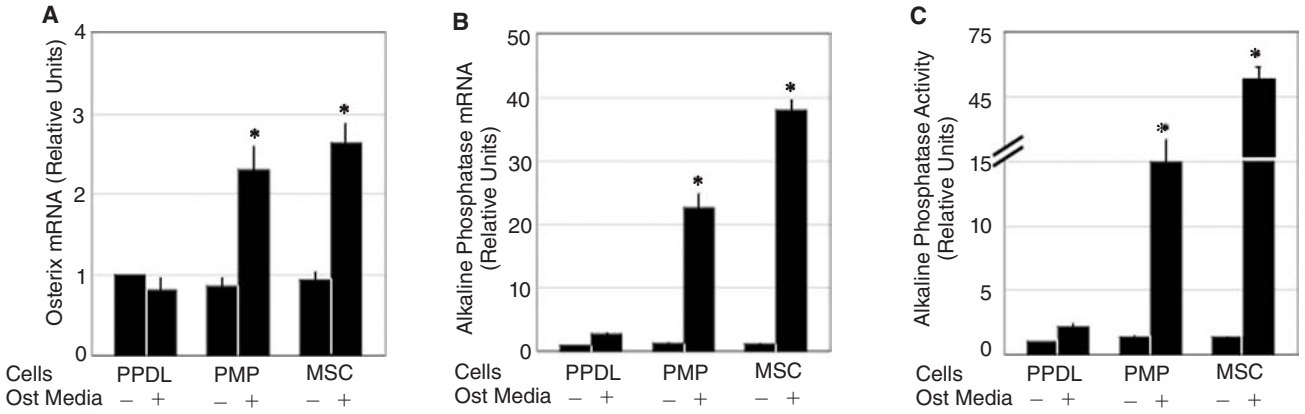


FIG. 2. Osteoblastic gene expression and alkaline phosphatase activity is induced in PMP cells and MSCs by osteogenic media (Ost Media). (**A**) Mean ± SD relative expression levels of osterix (**A**) and alkaline phosphatase (**B**) mRNA measured by qRT-PCR in noninduced (−) and induced (+) PPDL cells, PMP cells, and MSCs on Day 5 of culture. (**C**) Histograms of mean ± SD alkaline phosphatase activity in noninduced and osteoblastically induced PPDL cells, PMP cells, and MSCs on Day 7 of culture. Alkaline phosphatase activity was quantified spectrophotometrically and standardized by the total amount of protein in the cell lysate. Data were obtained from experiments performed in triplicate on three independent cultures (*$P \leq 0.01$ for osteoblastically induced PMP cells or MSCs compared to noninduced PMP cells, MSCs, or PPDL cells, or osteoblastically induced PPDL cells).

12-fold higher in induced PMP cell and MSC cultures, respectively, compared to that in noninduced or induced PPDL cell cultures (Fig. 3B).

Stimulated PMP cell and MSC pellets exhibit increased cartilage-specific gene and protein expression

To examine the potential for the PMP cells to differentiate down a chondrogenic lineage, the STRO-1$^+$/CD146$^+$ cells were studied for their ability to upregulate their expression of the cartilage-specific genes, collagen type II, and COMP. Gene expression for both cartilage specific markers was significantly higher in chondrogenically induced PMP cells and MSCs cells than their corresponding noninduced controls on day 7 (Fig. 4A and B). However, PPDL cells cultured in chondrogenic media showed minimal increase in these genes relative to noninduced controls. Specifically, the increase in collagen type II was approximately 2.6-fold and 4.3-fold greater in chondrogenically induced PMP cells and MSCs, respectively, relative to both noninduced and induced PPDL cells. Similarly, COMP was 7-fold and 35-fold higher in chondrogenically stimulated PMP cells and MSCs compared to noninduced PPDL cells.

Safranin-O staining for newly synthesized GAGs, which is a component of cartilage-specific proteoglycans was observed in both chondrogenically induced PMP cell and MSC pellets (Fig. 5A). In contrast, PPDL cell pellets from both the induced and noninduced cells displayed no staining of these cartilage specific ECM macromolecules. Consistent with these findings, immunohistochemical examination of the cartilage specific ECM proteins aggrecan and collagen type II in cell pellets showed substantial staining for both macromolecules in chondrogenically induced PMP cell and MSC pellets, whereas neither collagen type II nor aggrecan was detected in sections of noninduced PPDL, PMP, or MSC pellet cultures (Fig. 5B and C). Interestingly, minimal positive staining for aggrecan and collagen type II was found in chondrogenically induced sections of PPDL cell pellets.

PMP cells and MSCs but not PPDL cells express adipocytic markers and accumulate lipid vacuoles under adipogenic conditions

PMP cells stimulated with adipogenic media showed a 450-fold increase in LPL and a 7-fold increase in PPAR-γ2 compared to their noninduced counterparts or PPDL cells (Fig. 6A and B). Similarly, MSCs exhibited a 15,000-fold and 60-fold increase in LPL and PPAR-γ2, respectively, relative to their noninduced counterparts or PPDL cells.

Oil red O staining to visualize the accumulation of lipid vacuoles within the cytoplasm revealed staining in adipogenically induced PMP cells and MSCs but not the induced or noninduced PPDL cells (Fig. 6C). It is also notable that the adipogenically induced MSCs had much higher adipogenic marker gene expression for both LPL and PPARγ2 and stronger Oil red O staining than that of induced PMP cells, demonstrating much more robust adipogenic ability in MSCs relative to both PMP and PPDL cells.

Discussion

In this study, we found that on the average 2.6% of PDL cells present within all patient and tooth samples were positive for the stem cell surface markers STRO-1 and CD146. On the basis of these findings and those of others [8,20,21], we used STRO-1 and CD146 as selection markers for isolation of the putative PMP cells from parental pools of primary PDL cell cultures. This small subset of PMP cells was indeed shown to possess a stronger self-renewal ability and more potent multilineage differentiation potential under

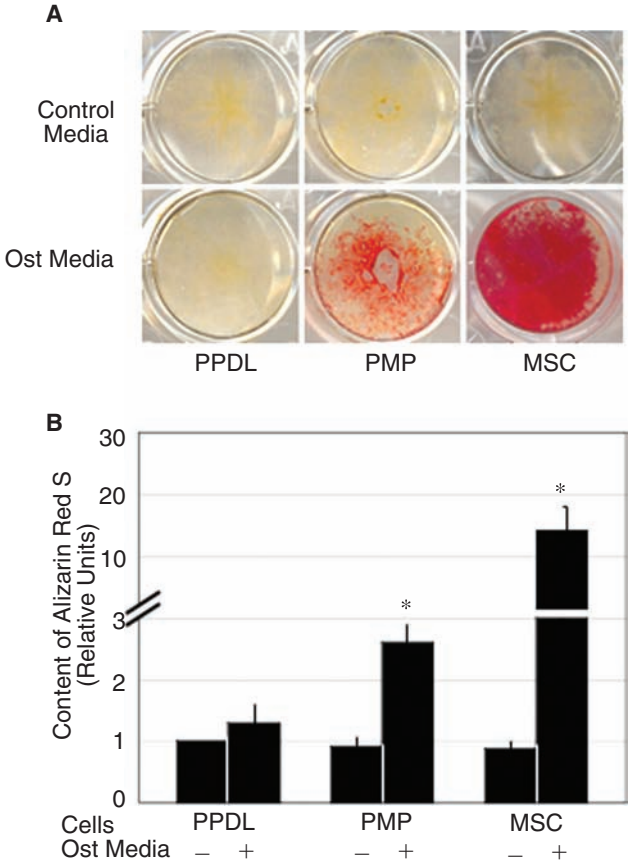


FIG. 3. PMP cells and MSCs but not PPDL cells cultured in osteogenic media show increased deposition of mineralized ECM. (**A**) Images of monolayer cultures of PPDL cells, PMP cells, and MSCs incubated in control or osteogenic media (Ost Media) for 14 days stained with Alizarin red S staining shows substantial staining in the induced PMP cells and MSCs but not PPDL cells. (**B**) The amount of calcium deposition in the mineralized ECM was quantitated by releasing the calcium-bound alizarin red into solution, measured spectrophotometrically, and standardized by the total amount of protein in each culture. The mean ± SD for experiments performed in triplicate on three independent cultures is presented as a histogram (*$P \leq 0.01$ for osteoblastically-induced PMP cells or MSCs vs. noninduced PMP cells, MSCs, or PPDL cells, or osteoblastically induced PPDL cultures).

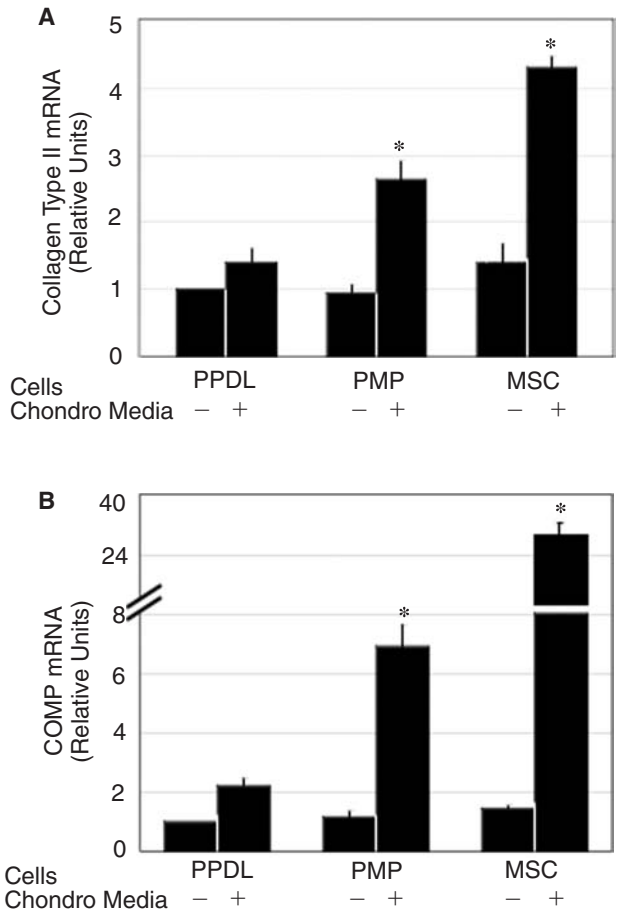


FIG. 4. Chondrogenic media enhances the expression of cartilage-specific genes in PMP cells and MSCs but not in PPDL cells. Cells were cultured in pellet, exposed to control or chondrogenic media (Chondro Media) for 7 days, and the RNA retrieved and assayed for collagen type II (**A**) and COMP (**B**) by qRT-PCR. Data, expressed as mean ± SD, were obtained from experiments performed in triplicate on three independent cultures (*$P \leq 0.01$ for chondrogenically-induced PMP cells or MSCs vs. noninduced PMP, MSC, or PPDL cultures, or chondrogenically induced PPDL cultures).

osteogenic, chondrogenic, and adipogenic conditions beyond that of the PPDL cells. Furthermore, relative to MSCs these PMP cells have largely comparable capabilities of self-renewal and osteogenic differentiation and somewhat lower but still robust capabilities to differentiate into chondrocytes and adipocytes. Overall, our findings suggest that these PMP cells selected with a limited number of stem cell markers represent a unique subpopulation of cells within the PDL that possess several MSC-like properties.

Attempts to isolate and characterize mesenchymal progenitor cells from tooth structures including dental pulp, periapical follicle, and PDL have spanned several years. These attempts have mimicked methodologies used for isolation of stem cells from the bone marrow. Previous studies have employed different single stem cell surface markers such as STRO-1, CD29, CD44, CD105, CD106, CD146, or CD166 to isolate dental pulp and PDL stem cells (PDLSCs) [11,25,26]. Stem cell surface marker expression profile has been used to isolate human dental pulp stem cells, which were then shown to also express CD44, CD146, integrin β1, and STRO-1 [25]. These investigators further isolated ovine PDLSCs by using MSC-associated antigen CD106. These ovine PDLSCs exhibited a high proliferation rate in vitro and expressed a phenotype (CD44+, CD166+, CBFA-1+, collagen-I+, bone sialoprotein+) consistent with that of human-derived PDLSCs [11]. Similarly, postnatal stem cells from human dental tissues (dental pulp, PDL, and periapical follicle) isolated using STRO-1 as a stem cell marker have been shown to also express other MSC markers including CD29 and CD44 [26].

Given the fact that a single specific stem cell marker for MSCs is not currently available and the isolated dental stem cells often express multiple stromal cell markers at varied levels, it is reasonable to utilize a combination of multiple

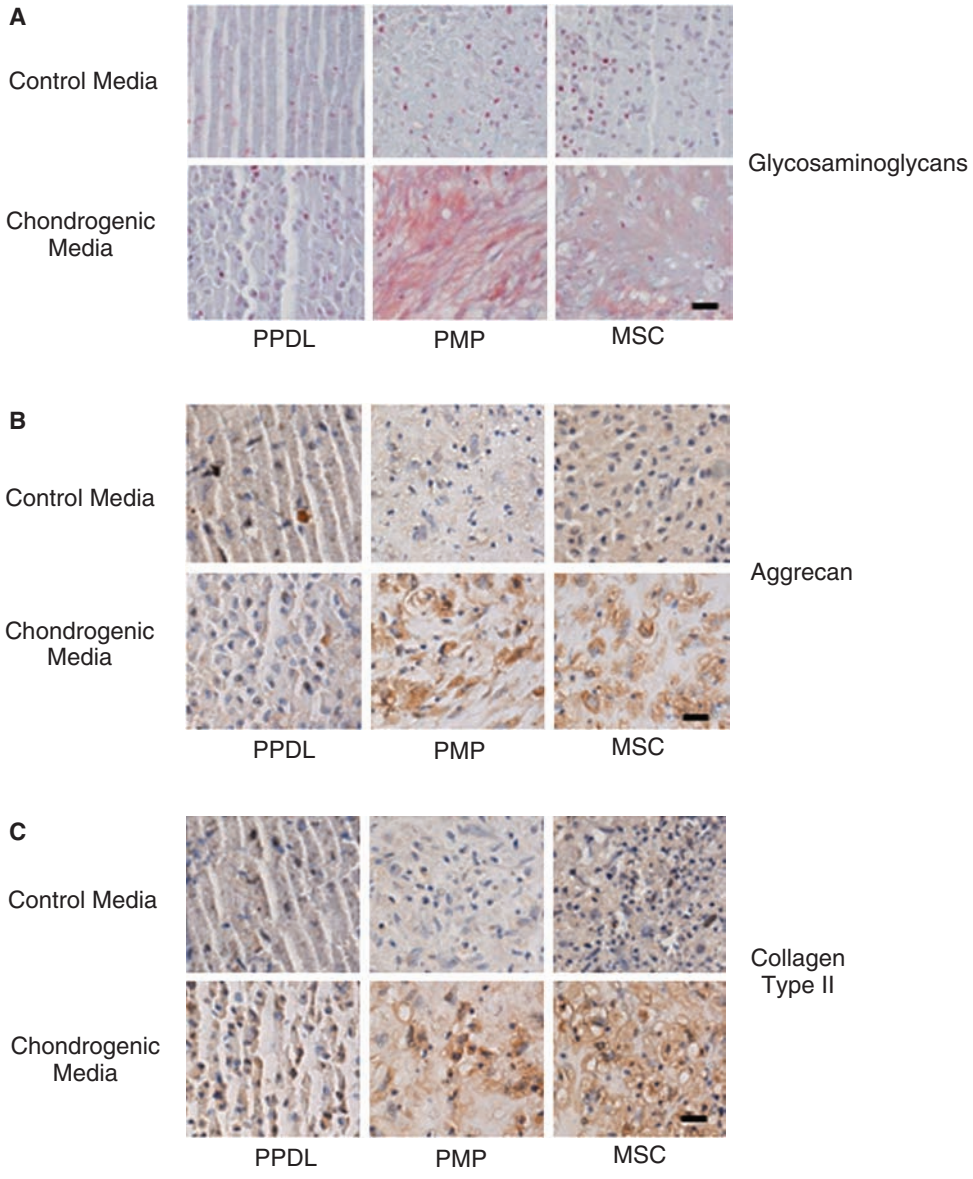


FIG. 5. Chondrogenic conditions increase the levels of cartilaginous ECM in PMP cell and MSC pellets. Cell pellets were cultured as described in Figure 4 for 21 days, the pellets fixed, sectioned, and stained. (**A**) Representative photomicrographs of sections stained with Safranin-O show substantial amounts of staining for newly synthesized glycosaminoglycans in induced PMP and MSC pellets but not in noninduced PMP, MSC, or PPDL pellets, or chondrogenically-induced PPDL pellets. Sections of the pellets immunostained for aggrecan (**B**) and collagen type II (**C**) also demonstrate high levels of staining for these macromolecules in chondrogenically-induced PMP and MSC pellets but not in any noninduced pellets. Images were captured under 40× magnification (Bar represents 50 μm).

stem cell markers for the isolation of more specific PMP cells. Indeed, multipotent stem cells from human PDL isolated for early stem cell surface marker STRO-1 were also shown to strongly express CD146 and the isolated cells were able to differentiate into cementoblast-like cells, adipocytes, and fibroblast-like cells [8]. Transplantation of these cells into artificially created periodontal defects in rats showed that these cells had the capacity to generate a thin layer of cementum-like tissue on the surface of hydroxyapatite or tricalcium phosphate ceramic particles along with the condensed collagen fibers resembling Sharpey's fibers. However, the stem cell properties of isolated PMP cells relative to their PPDL cell pools, and their comparative ability for multilineage differentiation relative to the available gold-standard of MSCs have not been fully delineated. Thus this became the goal of the current investigation.

In our study, PMP cells were isolated from their parental pools of PDL cells using the combined selection markers STRO-1 and CD146, but the temporal stability of these cell surface markers was not known. Therefore, we sought to determine the short-term stability of STRO-1 and CD146 in our PMP cells. We found that a high proportion of the isolated STRO-1$^+$/CD146$^+$ PMP cells retain their surface markers over the short time in culture and with passage. Thus our data indicate a relatively stable stem cell surface marker expression in PMP cells over the short term, which is in agreement with the known continuous growth and passage ability of pluripotent progenitor cells.

The colony-formation assay data indicate that the isolated PMP cells possess a significantly higher colony-forming ability than their PPDL cells providing evidence for their mesenchymal progenitor cell-like quality. This colony-forming ability was similar to that of MSCs. Our data further confirmed the stem cell-like properties of the isolated PMP cells by examining their their multilineage differentiation potential. In agreement with previous studies [19,27], our isolated PMP cells were able to undergo chondrogenic and osteogenic differentiation as evidenced by a significant upregulation of cartilage marker genes

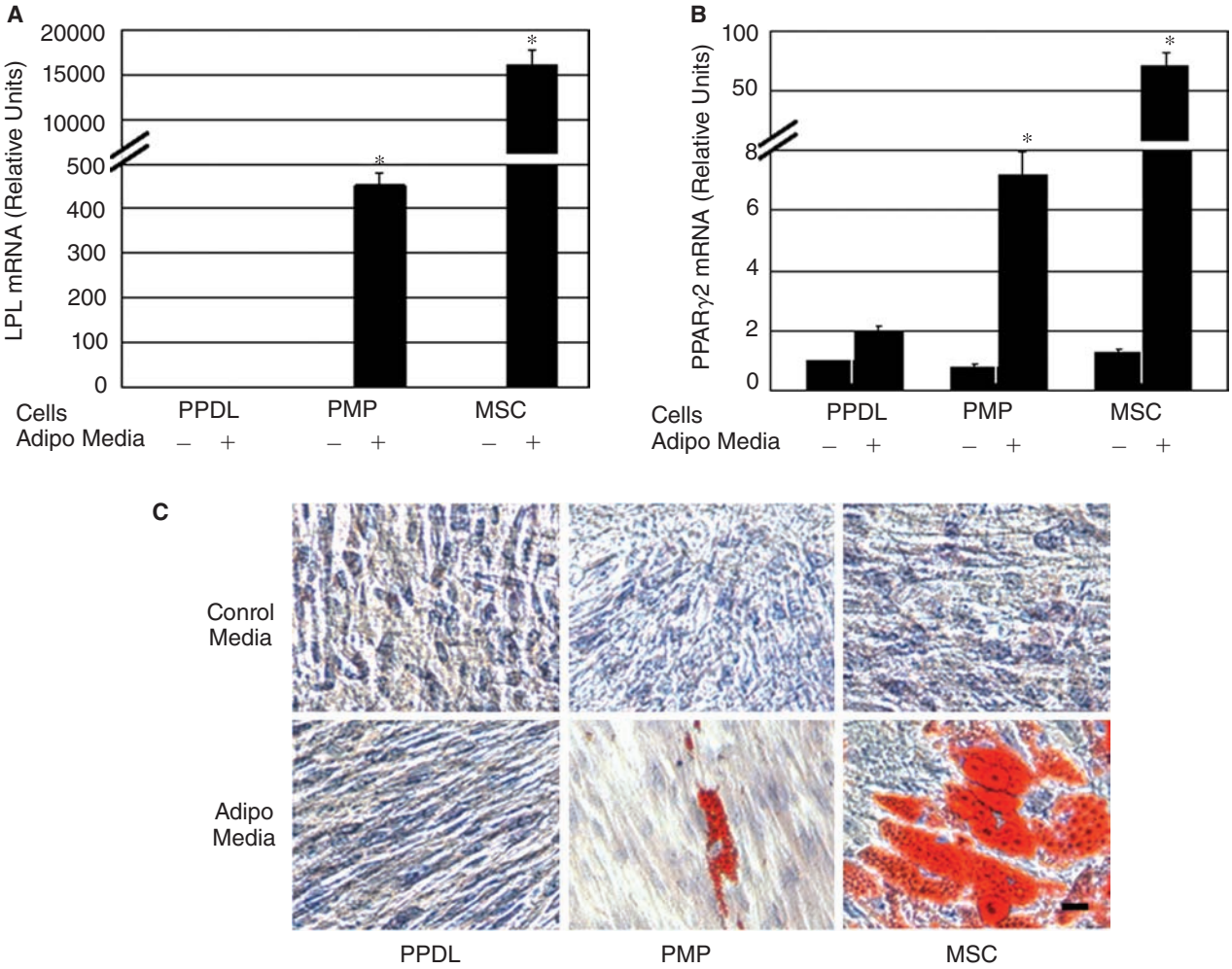


FIG. 6. Adipogenic-specific genes are upregulated and lipid vacuoles accumulate in adipogenically-induced PMP cells and MSCs but not in PPDL cells. (**A**) Cells were cultured in control media or adipogenic media (Adipo Media) for 7 days, the RNA extracted and subjected to qRT-PCR for LPL (**A**) and PPARγ2 (**B**), and the relative mean ± SD expression of these genes for experiments performed in triplicate on three samples was plotted as histograms. (**C**) Cells from similar experiments cultured for 18 days were stained with Oil Red O staining and photomicrographed to show accumulated lipid vacuoles. Images were captured under 10× magnification (*$P \leq 0.01$ for adipogenically-induced PMP cells or MSCs vs. noninduced PMP cells, MSCs, or PPDL cells, or adipogenically induced PPDL cultures; Bar represents 100 μm).

(collagen type II, COMP, aggrecan and glycosaminoglycan) and osteoblastic differentiation markers (osterix, alkaline phosphatase, and matrix mineralization). The chondrogenic potential of PMP cells, although lower than that of MSCs, was still very substantial. In contrast, although PMP cells exhibited a higher adipocyte differentiation ability than their corresponding PPDL cells, the potential for adipogenic differentiation in PMP cells was notably lower than that of MSCs. Thus this predilection of PMP cells toward a more osteogenic and chondrogenic differentiation phenotype indicates that the cells selected are further along in their differentiation pathway towards skeletal tissue cell types rather than being highly undifferentiated MSCs with equal capacity to differentiate into all mesenchymal cell types. These findings also reflect that the former differentiation pathways toward differentiation into skeletal cell phenotypes are more critical than adipogenesis to the physiologic functioning, repair, and regeneration needs of the PDL than differentiation pathways that favor adipogenesis.

In summary, using the combined stem cell surface markers STRO-1 and CD146 and the culture and isolation methods described here, one can expect that a small proportion of primary human PDL cells will be mesenchymal progenitor cells. Using these markers, we successfully isolated mesenchymal progenitor cells from human PDL and characterized their multilineage differentiation potential toward a chondrogenic, osteoblastic, and adipogenic phenotype under defined conditions. Similar to MSCs, human primary PMP cells have a high self-renewal ability and potent chondrogenic and osteogenic differentiation potential that is greater than their PPDL cells. However, their adipogenic differentiation potential is considerably less than that of MSCs. These findings may help future inquiries for improved regenerative methodologies and improved mechanistic studies on PDL mesenchymal progenitor cells.

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