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Isolation and characterization of multipotent human periodontal ligament stem cells

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Structured Abstract

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Backround – Periodontal ligament (PDL) repair is thought to involve mesenchymal progenitor cells capable of forming fibroblasts, osteoblasts and cementoblasts. However, full characterization of PDL stem cell (SC) populations has not been achieved.

Objective – To isolate and characterize PDLSC and assess their capability to differentiate into bone, cartilage and adipose tissue.

Methods – Human PDL cells were stained for STRO-1, FACS sorted and expanded in culture. Human bone marrow SC (BMSC) served as a positive control. PDLSC and BMSC were cultured using standard conditions conducive for osteogenic, chondrogenic and adipogenic differentiation. Osteogenic induction was assayed using alizarine red S staining and expression of alkaline phosphatase (ALP) and bone sialoprotein (BSP). Adipogenic induction was assayed using Oil Red O staining and the expression of PPARγ 2 (early) and LPL (late) adipogenic markers. Chondrogenic induction was assayed by collagen type II expression and toluidine blue staining.

Results – Human PDL tissue contains about 27% STRO-1 positive cells with 3% strongly positive. In osteogenic cultures ALP was observed by day-7 in BMSC and day-14 in PDLSC. BSP expression was detectable by day-7; with more intense staining in PDLSC cultures. In adipogenic cultures both cell populations showed positive Oil Red O staining by day-25 with PPARγ 2 and LPL expression. By day-21, both BMSC and PDLSC chondrogenic induced cultures expressed collagen type II and glycosaminoglycans.

Conclusions – The PDL contains SC that have the potential to differentiate into osteoblasts, chondrocytes and adipocytes, comparable with previously characterized BMSC. This adult PDLSC population can be utilized for potential therapeutic procedures related to PDL regeneration.

Key words: adult stem cells; gene expression; periodontal ligament; periodontal tissue engineering; STRO-1 antibody

Introduction

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). The PDL provides anchorage and support to the functional tooth, dispersing the mechanical forces associated with mastication. The PDL cell population is heterogeneous, consisting of two major mesenchymal lineages, fibroblastic and mineralizing tissues, further divided into osteoblastic and cementoblastic subsets (1–4). In the adult PDL, the progenitors for both cell lineages originate in the paravascular zones (5). This progenitor stem cell (SC) population is enriched by precursors derived from the endosteal spaces of the alveolar bone (6). Cell kinetic studies suggest that some of these progenitors migrate toward alveolar bone and cementum where they terminally differentiate into osteoblasts, cementoblasts and fibroblasts (5, 7). Cell migration and differentiation pathways are the basis for remodeling and healing in periodontal tissues, and it is likely that some form of signaling has evolved to orchestrate, in a temporally and spatially appropriate manner, these repopulation and differentiation responses (8). However, a detailed understanding of the biochemical and molecular events associated with the remodeling process in the periodontum has not been determined because of the diversity of cells and tissue types. Methods that can identify specific PDL cell populations and PDL stem cells (PDLSC) are of central importance for our understanding of remodeling and healing in these tissues.

By definition, a stem cell has the ability to proliferate in culture for indefinite periods and give rise to specialized cells. Embryonic stem cells derived from the inner cell mass of the embryo are termed pluripotent because they can give rise to many cell types but not all the cells necessary for fetal development. In addition, multipotent SC have been identified in young and adult tissues, and are capable of further differentiation. Examples are blood and skin SC that play a key role in homeostasis and tissue repair by continually replenishing our blood cells and skin layers throughout life.

Adult SC, once thought able to develop only the same type of tissue, have recently been shown to be able to develop into many types of specialized cells (9). These data suggest that adult SC have outstanding potential for the development of novel therapies such as tissue transplantation and regeneration. It is well documented that adult bone marrow (BM) contain a population of SC that retain the capacity to differentiate along different lineages including reticular, adipogenic, fibroblastic, osteogenic, and chondrogenic (10–13). In addition, BMSC can interact with other cell phenotypes in bone marrow microenvironments to influence and regulate a variety of physiological pathways including bone remodeling (14, 15), hematopoiesis (16–18) and angiogenesis (19). Effective techniques have been developed to isolate BMSC using specific antibodies to cell surface markers with fluorescence-activated cell sorting (FACS) to enrich cell populations.

Stem cell differentiation processes have been successfully demonstrated *in vitro* by exposing undifferentiated stem cells to a variety of microenvironments and tissue culture conditions (20). Adipocyte differentiation is accompanied not only by gross changes in cellular morphology, but also by the transcriptional activation of many genes. A member of the nuclear receptor superfamily of ligand-activated transcription factors identified as peroxisome proliferators-activated receptor gamma 2 (PPARγ2) has been shown to be expressed early in the adipocyte differentiation program. It acts synergistically with CCAATT enhancer-binding protein α to coordinate the adipocyte differentiation cascade. Importantly, the peroxisome proliferator response element has been identified in the promoter regions of several enzymes associated with triglyceride metabolism (21). LPL encodes lipoprotein lipase, which is expressed in heart, muscle, and adipose tissue. LPL functions as a homodimer and has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake (22).

Cells isolated from postnatal mammalian BM have also the potential for differentiation into cartilage when implanted *in vivo* (23). However, attempts to develop *in vitro* conditions in which mesenchymal stem cells, derived from postnatal mammalian bone marrow, will progress down the chondrogenic lineage has been less successful. A system that cultures cells in aggregates has been described (24, 25) and utilized in studies of terminal differentiation of growth plate chondrocytes (26). This culture system allows cell–cell interactions analogous to the ones that occur in pre-cartilage condensation during embryonic development. However, this cell configuration is not sufficient for the induction of chondrogenesis; it requires defined medium and bioactive factors such as TGF-β (27). Evidence of chondogenic differentiation includes the appearance of toluidine blue metachromasia and immunohistochemical detection of type II collagen (28).

Recent findings show that mesenchymal progenitors can differentiate into osteoblast-like cells when challenged with dexamethasone, β-glycerophosphate and ascorbic acid *in vitro*. These cells express terminal phenotype identification markers such as Runx2 promoter activity, alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OP), osteocalcin (OC), and collagen type I, as well as mineral deposits in the extracellular matrix (29).

In this study, we report that PDL tissue contains undifferentiated progenitor cells, adult SC, that are capable of differentiation into bone, cartilage and adipose-like tissues comparable with the well-defined BMSC. Also, we have demonstrated PDLSC capability to express gene products compatible with differentiated cell types. This, therefore, clearly demonstrates the existence of progenitor undifferentiated STRO-1 positive cells that could potentially be used for PDL regenerative therapies as well as the possible formation of a true PDL apparatus associated with a titanium implant surfaces.

Materials and methods

Cell isolation, FACS sorting and cell culture

Disease free impacted third molars were collected from 10 patients, 18–26 years old, at the Oral and Maxillofacial Surgery Department at the University of Texas Health Science Center at San Antonio (UTHSCSA). Individuals were selected on the basis of good general health and elective surgery procedures, following the approved guidelines of the UTHSCSA Institutional Review Board.

Periodontal ligament cells were scraped from third molars, enzymatically digested for 1 h at 37°C in a solution of 3 mg⁄ml collagenase type I (Worthington Biochem, Freehold, NJ, USA) and 4 mg⁄ml of dispase (Worthington Biochem, Freehold, NJ, USA). PDL cells from different individuals were pooled and single cell suspensions were obtained through a 70 μm cell strainer (Falcon BD, Franklin Lakes, NJ, USA). Samples were centrifuged at 400 *g* for 10 min and resuspended in blocking buffer (HBSS, 20 mM HEPES, 1% normal human serum, 1% bovine serum albumin, 5% FCS) for 20 min on ice and stained with STRO-1 IgM antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) (2 μg⁄ml) for 1 h on ice. A goat anti-mouse IgM FITC secondary antibody (Molecular Probes, Eugene, Carlsbad, CA, USA) (2 μg⁄ml) was incubated for 1 h. Cell surface-marker STRO-1 positive cells were analyzed and sorted by flow cytometry. This procedure was performed on a FACScan with automated cell deposition unit (Becton Dickinson, Parsippany, NJ, USA) equipped with an argon laser and data analyzed with CellQuest software (Becton Dickinson) at the Institutional Flow Cytometer Core Facility (UTHSCSA, San Antonio, TX, USA).

Cells were expanded with Dulbecco's modified Eagle's medium containing 10% FCS and 1% antibiotics. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. Human BMSC kindly provided by Dr A Caplan (Case Western Reserve University, Cleveland, OH, USA) were grown under the same conditions as PDLSC and used as a positive control. Cultures at second or third passage were utilized for all experiments, unless otherwise stated.

Cell growth rate assay

To compare cell growth capabilities we determined the growth rates on both cell populations (BMSC and PDLSC). Cells were plated at a density of 5×10^3 cells per cm^2 in 12 well plates and cultured with DMEM containing 20% FCS and 1% antibiotics for 0, 48 and 96 h. At harvest, cells were washed twice with phosphate-buffered saline (PBS) and released from the culture surface by the addition of 200 μl of trypsin-EDTA 1× for 10 min at 37°C. The cell suspension was transferred to a vial containing 9.8 ml of 0.9% NaCl. Cell number was determined with a Coulter Counter (Coulter Electronics Inc, Hialeah, FL, USA). Cells harvested in this manner exhibited >95% viability on the basis of Trypan blue exclusion.

Clonogenic assays

Colony forming units were evaluated for the BMSC and PDLSC using DMEM (GIBCO⁄BRL) with 20% FBS (Equitech-Bio, Kerrville, TX, USA) under standard culture conditions at 37°C. At day 7 and 14, cultures were fixed with 10% formalin (Fischer Scientific, Fair Lawn, NJ, USA), stained with 0.1% toluidine blue (Sigma-Aldrich, Milwaukee, WI, USA) and observed under an inverted microscopy. Aggregates of 50 cells or more were scored as colonies, results were

recorded and compared between PDLSC and BMSC cultures.

Differentiation culture conditions

Osteogenic induction

Periodontal ligament stem cells and BMSC were plated at 5×10^3 cm^2 in 24 well plates. At 24 h the media was changed to an osteogenic inducing media (Cambrex Bio Science, Walkersville, MD, USA). Control groups were treated with DMEM containing 10% FBS and 1% antibiotics only. Cells were assayed at 7, 14, 21, 28, 35, and 42 days for ALP expression and calcium deposits using alizarine red S staining.

Chondrogenic induction

Bone marrow stem cell and PDLSC populations (2.5×10^5 cells) were centrifuged in 15 ml polypropylene tubes (Corning Incorporated Life Sciences, Acton, MA, USA). Cell pellets were treated with chondrogenic differentiation media (Cambrex Bio Science) supplemented with 10 ng⁄ml of TGF-β3 (Cambrex Bio Science). Pellets were left free floating for 14 and 21 days and fixed with 10% phosphate buffered formalin.

Adipogenic induction

Periodontal ligament stem cells and BMSC were plated at 5×10^3 cm^2 in six well plates. At 100% confluence, three alternated cycles of induction⁄maintenance were performed utilizing Cambrex adipogenic induction medium and Cambrex maintenance medium (Cambrex Bio Science). Control cultures were treated with Cambrex maintenance medium. At day 25, after induction, cultures were fixed with 10% formalin (Fischer Scientific, Fair Lawn, NJ, USA) and stained with Oil Red O.

Culture histochemical staining

Osteogenic cultures were stained with 2% alizarine red S (Sigma-Aldrich), (pH 4.2) according to the manufactures instructions. Chondrogenic culture pellets were embedded in 2% agarose followed by paraffin. Serial histological sections were stained with 0.2% toluidine blue (Sigma-Aldrich) according to suppliers instructions. Adipogenic cultures were stained with 3% Oil Red O (Sigma-Aldrich) for 5 min and counterstained with Harris hematoxylin (Sigma-Aldrich) for 1 min using standard techniques.

In situ ALP Staining

For localization of ALP activity, cultures were washed twice with PBS, fixed with 70% ethanol for 5 min, and washed for 5 min in buffer (100 mM⁄L Tris-HCL, ph 9.5; 100 mM⁄L NaCl; 50 mM⁄L $MgCl_2$). *In situ* ALP staining was performed according to the supplier's instructions (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

Chondrogenic culture samples were immunostained for collagen type II expression using a commercial collagen type II antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution for a 1-h incubation according to the supplier's instructions. The secondary antibody used was a rabbit anti goat IgG FITC (Molecular Probes) (2 μg⁄ml) incubated for 1 h. Images were obtained at the optical facility, UTHSCSA under the same parameters using an Olympus Wide Field microscope.

RT-PCR Analysis

Total RNA was isolated from osteogenic and chondrogenic cultures (PDLSC and BMSC) using RNA STAT-60 (TEL-TEST, Inc. Friendswood, TX, USA). First-strand cDNA was produced from 500 ng of total RNA with random hexamers using MultiScribe reverse transcriptase according to manufacturer's instructions (ABI TaqMan Kit; Applied Biosystems, Foster City, CA, USA). After mRNA conversion to cDNA, 500 ng of target cDNA was amplified with 1 Unit of Thermus aquaticus (Taq) polymerase (Perkin-Elmer Cetus, Boston, MA, USA) using various specific primer sets.

Osteogenic marker primer sets and PCR conditions

Specific primer sets (10 pmol) included ALP (accession number NM_000478: antisense 5′-atgcaggctgcaatacgccat-3′, sense 5′-atctttggtctggcccccatg-3′) and BSP (accession number AH_006462: antisense 5′-ttcctcctc ctcttctgaactg-3′, sense 5′-atggcctgtgctttctaat-3′). PCR amplification for each primer included denaturation at 95°C for 1′, annealing at 58–60°C for 1′ and an elongation step of 72°C for 1′ for a total of 40 cycles. GAPDH (accession number AL_136219: antisense 5′-gcaaagttgtcatggatgacc-3′, sense 5′-ccatggagaaggctgggg-3′)

was used as an internal control for cDNA integrity. PCR products were separated by agarose gel electrophoresis (2–4%) in 1× Tris-borate buffer (0.01 M Tris-base, 0.1 M boric acid, and 00.2 M EDTA, pH 8.3).

Adipogenic marker primer sets and real-time PCR conditions

To verify gene expression in the adipogenic induction cultures amplification reactions were analyzed in real-time on an ABI 7500 (Applied Biosystems) using SYBR Green chemistry and the threshold values calculated using SDS2 software (Applied Biosystems) according to the supplier's instructions. Thermal cycling parameters were 95°C for 30 s and 60°C for 1 min for 40 cycles. Reactions were performed in quadruplicate and threshold cycle numbers were averaged. A single melt curve peak was observed for each sample used in data analysis, confirming the purity and specificity of all amplified products. Expression fold was calculated according to the formula $2^{(Rt-Et)}/2^{(Rn-En)}$ where Rt is the threshold cycle number for the housekeeping gene (18S) from non-induced PDLSC and Et is the threshold number for the experimental gene observed in non-induced PDLSC. Rn is the threshold value for the housekeeping gene in induced PDLSC (18S) and En is the threshold cycle number for the experimental gene in induced PDLSC (30). Real-time PCR primers were designed to the following human genes: PPARγ2 (accession number D83233: antisense 5′-caggaaagac-aacagacaaatca-3′, sense 5′-ggggtgatgtgtttgaacttg-3′); LPL (accession number NM_000237: antisense 5′-gtg-gccgagagtgagaacat-3′, sense 5′-gaaggagtaggtcttatttgtg-gaa-3′), and 18S (accession number NM_022551: antisense 5′-agacctggagcgactgaaga-3′, sense 5′agaagt-gacgcagccctcta-3′).

Statistical analysis

Each individual value represents the mean ± SEM of six individual sample measurements, performed in triplicate. The data were analyzed by using Student's *t*-test. Significance level was set at $p < 0.05$ for all tests.

Results

To identify the existence of potential stem cells within the PDL cell population single cell suspension of the PDL was plated on four chamber slides and cultured for 24 h to promote cell attach. As a positive control, purified BMSC were plated in the same manner. Immunohistochemistry was performed using an antibody directed against a known stem cell marker STRO-1. Experiments were performed in parallel on the PDL and BMSC cultures to avoid possible time-related biological fluctuation. The BMSC, an enriched population, served as a positive control due to their well-characterized stem cell properties. Fig. 1 shows the immunohistochemistry of third passage human BMSC from a 31-year-old male and primary PDL cells obtained from a pool of patients (18- to 26-year olds). The BMSC stained positive for STRO-1 confirming their stromal stem cell status (Fig. 1B and C). The PDL cell heterogeneity was confirmed showing a low number of STRO-1 positive cells. Fig. 1H shows a single STRO-1

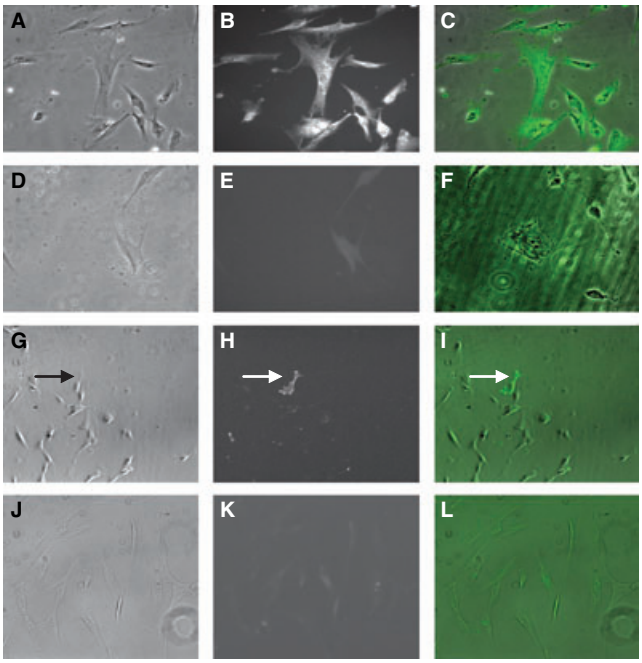

Fig. 1. Immunohistochemistry of human bone marrow stem cell (BMSC) and periodontal ligament cells with mesenchymal stem cell marker STRO-1. Panels A–F represent images from BMSC and PDLSC cultures bright field (A, D, G and J), fluorescence (B, E, H and K) and overlay of both images (C, F, I and L). Panels B and C show positive cell staining for STRO-1 of the BMSC population (20×). Panels E and F represent the negative control results lacking the primary antibody (20×). Panel G represents light microscopy of the heterogeneous population of PDL. Panel H and I show the STRO-1 staining with a single positive cell observed in the visible field (10×), in contrast to the negative controls with no staining shown in panels K and L. Panel J represents light microscopy of the heterogeneous population of PDL that will be used as control and will lack primary antibody. Images were obtained with an Olympus Wide Field Microscope at the Imaging Core Facility University of Texas Health Science Center at San Antonio (UTHSCSA). Details of the experiments are described under *Materials and methods* section.

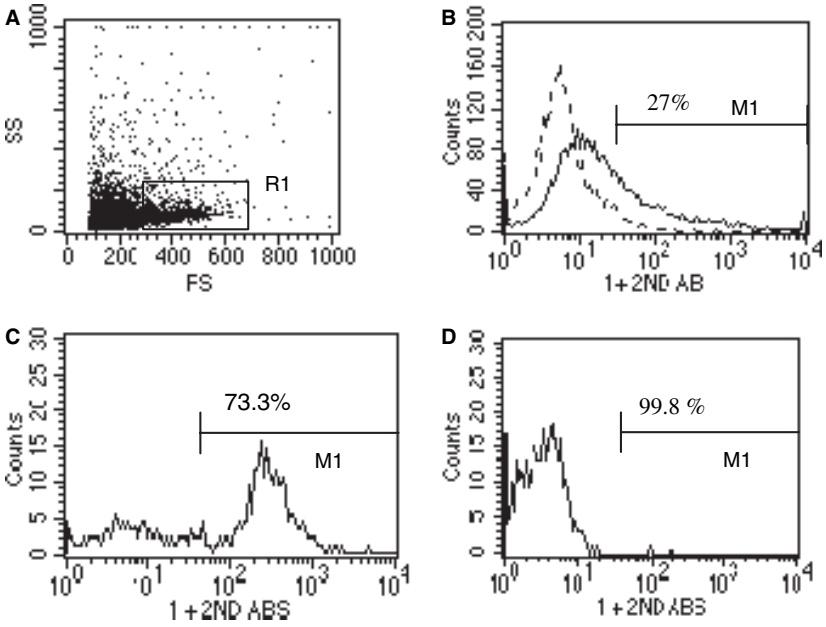

Fig. 2. Fluorescence-activated cell sorting (FACS) of STRO-1 Positive periodontal ligament (PDL) Cells. Primary digested pooled adult human PDL cells were immunostained with STRO-1 antibody and cells analyzed on a FACScan with automated cell deposition unit (Becton Dickinson, Parsippany, NJ, USA) equipped with an argon laser and data analyzed with CellQuest software (Becton Dickinson) at the UTHSCSA Institutional Flow Cytometry Core Facility. (A) The gated cell population. (B) The percent of the STRO-1 positive cell population identified (27%). (C) A 73.3% enrichment of STRO-1 positive PDL cells at second cell sorting. Strongly positive STRO-1 cells represent around 3% of this total cell population. (D) Analysis of the negative STRO-1 cell population with a 99.8% purity.

positive cell observed in the field of the PDL mixed cell population, The negative controls lacking the primary antibody for both cell populations shown no staining (Fig. 1E,F,K and L).

Next, PDL single cell suspensions obtained from the 38 molars were stained with STRO-1 antibody. Surface-marker STRO-1 positive cells were analyzed and sorted by flow cytometry. A total of 27% of the PDL cell population was positive for STRO-1 and of that population 3% were strongly positive. This enriched population had several intensity ratios to the mesenchymal stem cell antibody as demonstrated by various peaks within the M1 region after sorting was performed a second time and the population re-analyzed (Fig. 2C). The PDL STRO-1 negative cell population analysis demonstrated 99.8% purity as seen in Fig. 2D.

To investigate the potential of the enriched PDLSC to undergo mitosis compared with the BMSC, cell suspensions from both cell types were plated and counted at 0, 48, and 96 h using a Coulter Counter. At 96 h, PDLSC demonstrated a statistically significant ($p < 0.05$) nearly double number of cells as compared to the BMSC, whereas at 48 h there was no significant difference between the two cell populations (Fig. 3).

The ability of PDLSC to form characteristic stem cell adherent clonogenic cell clusters of fibroblast-like cells was compared with BMSC. Both PDLSC and BMSC showed the ability to form multi-cell clusters (Fig. 4A and B). PDLSC showed the formation of about 50 colonies (Fig. 4C) generated from single cells cultured at

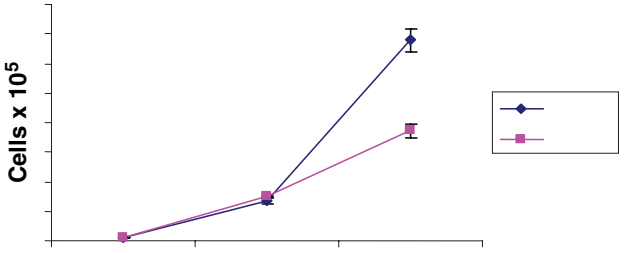

Fig. 3. Periodontal ligament stem cell (PDLSC) and bone marrow stem cell (BMSC) proliferation rates. PDLSC and BMSC were cultured for 0, 48 and 96 h in 12 well plates with media changes every other day. The cells were harvested by trypsinization and counted with a Coulter Counter. The data are from one of three representative experiments each yielding similar results. Values are the mean ± SEM for six cultures ($p < 0.05$). Pink squares correspond to BMSC and blue diamonds correspond to PDLSC.

low density (5×10^3 cm^2) for 7 days; whereas BMSC generated 35 colonies. These results showed statistically significant difference with PDLSC having an increased colony forming unit behavior as compared to BMSC.

To investigate the potential of PDLSC to differentiate into mineralizing osteoblast or cementoblast-like lineages, we induced the formation of mineralized matrix containing calcium deposits by the addition of culture media containing L-ascorbate-2-phosphate, dexamethasone and β-glycerophosphate (osteogenic media). After 48 days in culture, PDLSC showed calcium deposits, whereas at day 35, BMSC demonstrated calcium deposits as visualized by alizarine red stain (Fig. 5). Staining of the PDLSC cultures was associated with cell clusters whereas staining within the BMSC cultures was more diffuse seen throughout the culture.

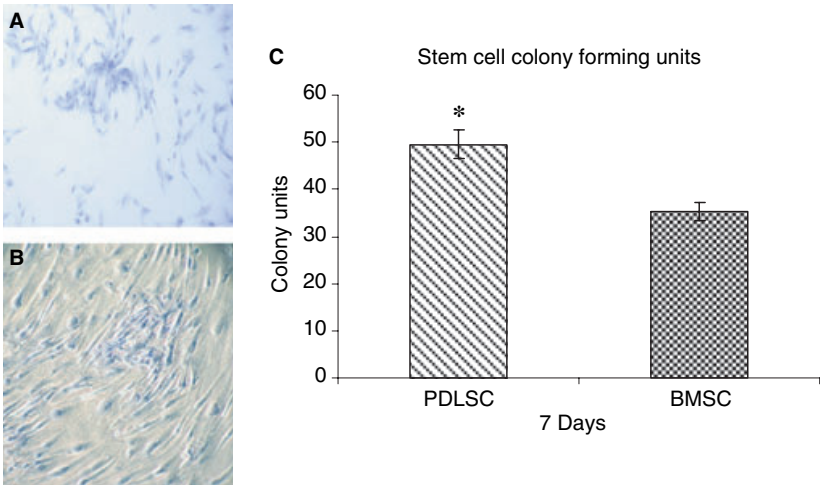

Fig. 4. Periodontal ligament stem cells (PDLSC) and bone marrow stem cells (BMSC) Colony Forming Units. At day 7 after plating, PDLSC and BMSC were fixed with 10% formalin and stained with toluidine blue. Aggregates of 50 or more cells were considered colonies and counted. (A) PDLSC representative colony (10×). (B) BMSC representative colony (10×). (C) Graph represents a statistically significant difference in total colony number between PDLSC (n = 50) and BMSC (n = 35) cultures. Values are the mean ± SEM for six cultures ($p < 0.05$). *The result is statistically significant as compared to BMSC which is the positive control.

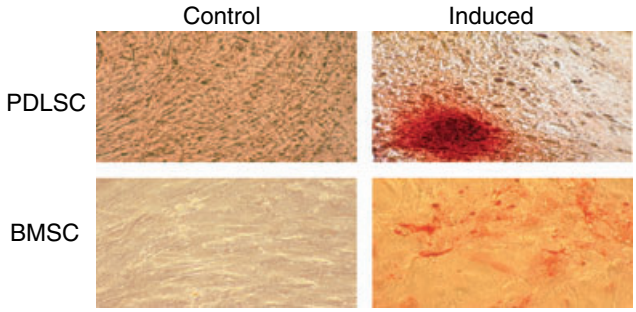

Fig. 5. Expression of mineral deposits within periodontal ligament stem cells (PDLSC) and bone marrow stem cells (BMSC) long-term cultures. At day 35 BMSC show mineral deposits as is evidenced by alizarine red stain. Those deposits are distributed throughout the tissue culture well. In contrast, PDLSC cultures demonstrate calcium deposits at day 48 and the distribution was seen as dense foci associated with cell clusters (20×).

To confirm the differentiation of PDLSC toward an osteoblast/cementoblast-like tissue, we investigated the presence of ALP involved in the early mineralization events and BSP a gene expression product known as a late mineralized tissue marker (Fig. 6). Immunohistochemical evaluation (Fig. 6A–C) and RT-PCR analysis (Fig. 6D) showed that osteogenic induced PDLSC cultures express known mineralized tissue markers ALP and BSP. The positive control samples BMSC cultures were positive for both proteins. *In situ* staining for ALP activity was detectable by 14 days of culture in the PDLSC as compared with 7 days for the BMSC cultures. However, by 21 days no detectable difference in level of ALP activity was seen between the two cell populations. ALP expression was confirmed by RT-PCR analysis as well as the expression of a late mineralized tissue marker BSP. Expected amplification products (ALP 288 bp and BSP 204 bp) were detectable in the PDLSC and BMSC cultures as early as 7 days.

GAPDH, a housekeeping gene, served as a positive control for the PCR reaction. Control samples were negative for all tested markers at all time points.

We next assessed whether PDLSC, like BMSC, had the potential to differentiate into other cell lineages such as adipocytes. After 25 days of culture with an adipogenic inducing media, PDLSC as well as BMSC developed oil red O positive lipid-laden droplets (Fig. 7A and C) whereas control cultures grown in control media failed to produce similar results (Fig. 7B and D). This finding clearly correlate with an up-regulation in the expression of two adipocyte specific early/late differentiation transcripts LPL and PPARγ2, respectively. As shown in Fig. 8E quantitative RT-PCR normalized against the pseudogene 18 S (31) showed increase in the two adipocyte specific gene markers in both cell populations. While the two transcripts increased in the PDLSC cultures, the change in the later marker PPARγ2 was greater in the BMSC culture showing a nearly 10-fold increase.

Another well-charcterized feature of undifferentiated mesenchymal stem cells such as BMSC is their capability to differentiate into chondrocytes, the process of chondrogenesis. After 14 days of exposure to chondrogenic differentiation media containing 10 ng/ml of TGF-β3, BMSC express metachromasia with toluidine blue stain as well as positive immuno histochemistry for collagen type II a gene marker for chondrocyctes. PDLSC failed to express metachromasia at day 14, although they were positive for collagen type II expression. At 21 days both cell populations show chondrocyte-like cell morphology as

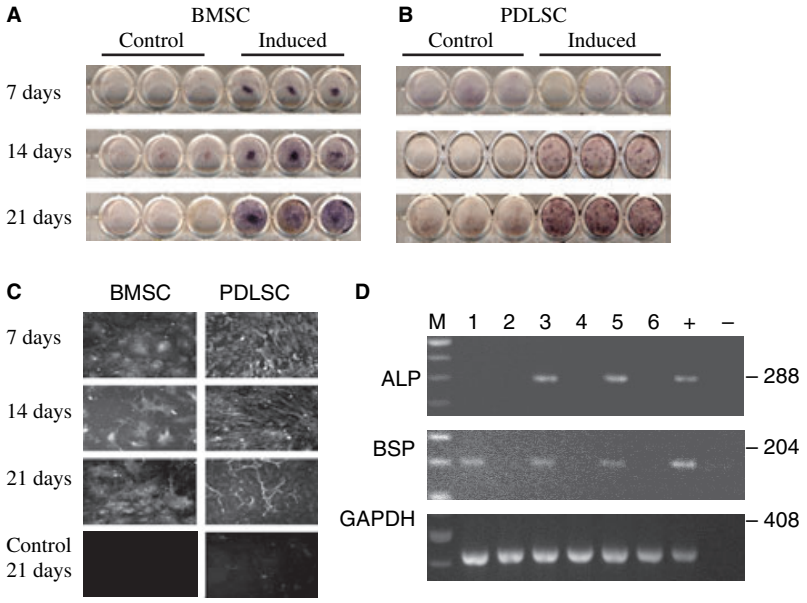

Fig. 6. BSP and ALP gene expression profiles of osteogenic bone marrow stem cell (BMSC) and periodontal ligament stem cell (PDLSC) cultures. BMSC and PDLSC were grown in osteogenic (experimental) or normal (control) media for 7, 14 and 21 days. ALP staining increased temporally in the experimental induced groups whereas the control groups failed to express detectable enzyme activity (A & B). Immunohistochemistry images reveal the presence of BSP positive cells after 7 days of culture (10×) in both cell populations. Staining was more intense in the PDLSC and continued through day 21. Negative controls, lacking primary antibody showed no staining at all days tested (C). RT-PCR image for ALP, BSP and the housekeeping gene GAPDH on PDLSC cells. At day 7 there is no detectable ALP expression. Starting at day 14, ALP expression increases throughout whereas BSP remains as a constant expression from day 7 through day 21 in culture (D). Lanes were loaded as follows: (1) PDLSC 7 days induced; (2) PDLSC 7 days control; (3) PDLSC 14 days induced; (4) PDLSC 14 days control; (5) PDLSC 21 days induced; and (6) PDLSC 21 days control.

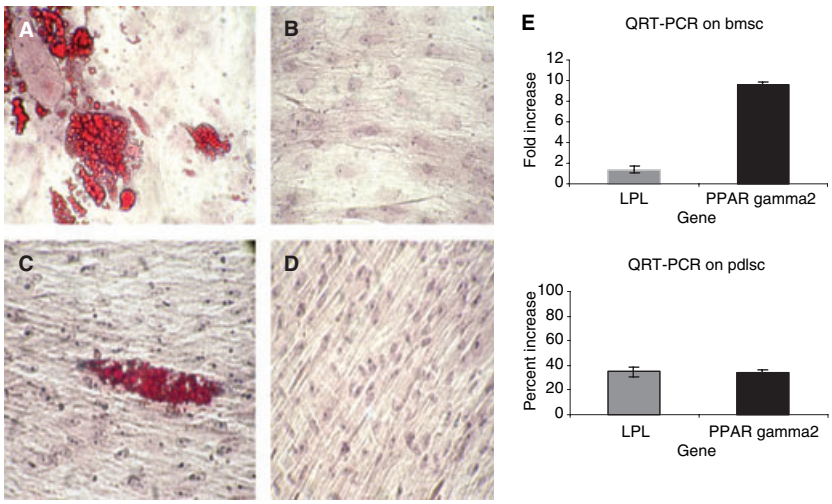

Fig. 7. Characterization of adipogenic differentiation of periodontal ligament stem cell (PDLSC) and bone marrow stem cell (BMSC) cultures. Panel A shows BMSC induced cultures at 25 days with visible positive oil red O staining lipid droplets. This is in contrast to the control cultures grown for the same period in normal media (B). Adipogenic Induced cultures of PDLSC also formed positive lipid droplets after 25 days in culture (C), These droplets were not seen in the negative control cultures (D). Magnification 20×. Panel E shows the RT-PCR analysis of adipogenic markers demonstrating statistical significant upregulation of PPARγ2 and LPL in BMSC. Direct comparison between BMSC and PDLSC demonstrates increased production of both gene expression markers and lipid accumulation in BMSC. Values are the mean ± SEM for six cultures ($p < 0.05$).

well as the expression of collagen type II and toluidine blue metachromasia. Staining with touidine blue demonstrates the accumulation of glycosaminoglycans in the extracellular matrix (Figs 8 and 9). Negative controls exposed to control media were not possible to obtain due to the fact that stem cells fail to form aggregates unless they are exposed to differentiation media.

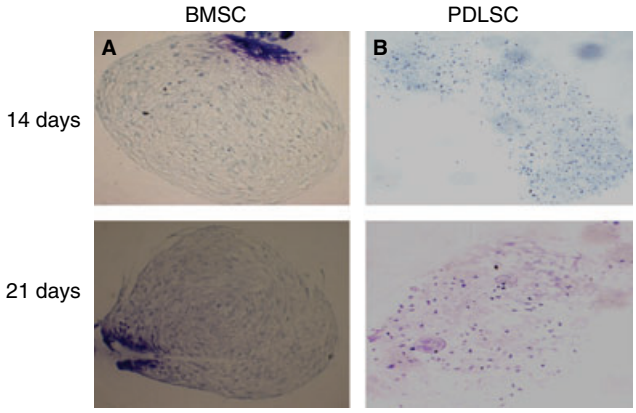

Fig. 8. In vitro chrondogenic differentiation of periodontal ligament stem cells (PDLSC) and bone marrow stem cells (BMSC). Panels A and C show BMSC cultures after 14 and 21 days, respectively treated with chondrogenic differentiation media (10×). Panels B and D show PDLSC cultures after 14 and 21 days, respectively treated with chondrogenic differentiation media (10×). In both cell populations metachromasia and cell morphology correspond with embryonic stages of cartilage formation.

Discussion

Our findings clearly demonstrate the successful isolation and characterization of undifferentiated progenitor cells contained in human PDL that can be expanded *in vitro*, providing a unique reservoir of stem cells obtained with minimally invasive procedures. Therefore, the PDL apparatus represents a viable alternative to obtain potentially high numbers of cells for regenerative procedures without the necessity of bone marrow aspiration or more invasive procedures that will result in increased morbidity.

Our experiments show PDLSC multipotential capability to differentiate *in vitro* toward osteogenic, adipogenic and chondrogenic tissues. These results positively correlate with the well-characterized BMSC when exposed to appropriate culture conditions. In this study we found that PDLSC are similar to other mesenchymal stem cells such as BMSC (11, 32, 33) and dental pulp derived stem cells (34–37) with respect to their expression of the STRO-1 marker. The development of a series of monoclonal antibodies raised toward BMSC surface antigens, along with other antibodies developed to characterize BM stromal cells has been crucial for the immunophenotyping of these cells. Results have shown that the antigenic profile of BMSC is not unique, presenting features of mesenchymal, endothelial, epithelial, and muscle cells (29). Several research laboratories have joined efforts to find the potential 'gold standard' antibody for stem cell identification. From these, STRO-1 IgM antibody has shown consistent, promising results (38–40). In our laboratory, other surface antibodies, such as SH2, SH3, SH4, endoglin (CD105), and CD44 (41–44) yielded 100% positive results which would seem to indicate that all PDL cells are multipotent (data not shown). These results are not compatible with heterogeneous PDL cell population that contains fibroblasts, osteoblasts, cementoblasts and progenitor cells. After exhaustive surface marker testing we were able to isolate and purify a PDLSC population derived from the PDL using the STRO-1 antibody.

Periodontal ligament stem cells demonstrated by 48 h the same growth rate as BMSC. At 96 h PDLSC cell numbers were almost double those of BMSC cells. Similar findings have been reported by other authors

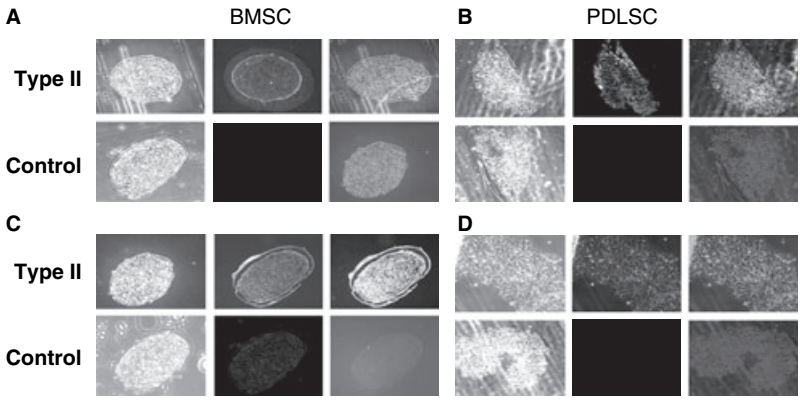

Fig. 9. Immunohistochemistry for collagen type II expression in periodontal ligament stem cell (PDLSC) and bone marrow stem cell (BMSC) chrondogenic induced cultures. BMSC and PDLSC were cultured as aggregates in a differentiation media for 14 days (A & B) and 21 days (C & D), Top rows show images correspond to samples exposed to primary collagen type II antibody; whereas bottom rows show negative control images lacking collagen type II antibody (10×). Both BMSC and PDLSC induced cultures were strongly positive for type II collagen expression at the time points tested.

for other dental tissue-derived stem cells such as those isolated from permanent tooth pulp (34–37). The exact mechanism for this increased cell growth remains obscure. The nature of this difference may arise from several determinants, including the procedure used to harvest the cells, the age difference of the PDLSC donors (18–26 years) vs. the BM donor (37 years). Another possibility is that dental tissue-derived stem cells exhibit higher mitotic properties than cells derived from BM aspirations, specifically BMSC. As a consequence of faster cell growth, the clonogenic capabilities of PDLSC were superior as compared with BMSC.

The osteogenic potential of the heterogeneous population of PDL cells has been demonstrated earlier with *in vitro* procedures as well as the ability of such cultures to form a mineralized matrix (45). In our series of experiments PDLSC fail to produce such results at all time points tested. Mineralization was induced after 14 days of exposure to differentiating media. Our data and other published data (34–37, 46) shows the potential of PDLSC to form mineralized deposits *in vitro* as previously has been shown with other mesenchymal cell populations (47). However, PDLSC formed sparse calcified focal nodules that took longer to form as compared with their BMSC counterparts. These results are supported by the gene expression profiles and protein expression patterns as determined by RT-PCR analyses, *in situ* expression, and immunohistochemistry.

A possible explanation for the delay in differentiation seen in the PDLSC cultures is the presence of terminally differentiated cell types in the enriched population. By FACS sorting techniques, we were able to obtain a 73.3% enrichment of STRO-1 positive cells, thus it is possible that differentiated cell types such as fibroblasts interfered with the mineralization process. In other words, it is possible that PDLSC used in our experiments represent a more heterogeneous SC enriched population mixed with a greater subpopulation of terminally differentiated cell types. Another possible explanation for this finding is that our PDLSC population is at a different stage of commitment as compared with the BMSC.

Recent *in vivo* data has shown that SC differentiation may be due to cell fusion with other differentiated cell types (48, 49). Although it is known that several various cell types reside in PDL tissue, adipocytes have not been reported as a native component. In our series of experiments, we were able to demonstrate that an inductive adipogenic tissue culture media can induce PDLSC and BMSC to form characteristic oil red O positive lipid laden droplets that are comparable with those found in adipocyte cells. This phenotypic conversion was also correlated with the expression of the early adipogenic transcription factor PPARγ2 and the later marker LPL. Similar results for dental pulp SCs and PDLSC have been published (34–37, 46).

A differentiation pathway that has not been reported for PDLSC is their ability to form chondrocyte-like tissues when exposed to chondrogenic media with the addition of TGF-β3. We successfully induced not only a shift in cell morphology toward a chondrocyte cell morphology, but also the expression of collagen type II and proteoglycans as indicated by metachromasia with toluidine blue stain seen at 21 days. The resulting differentiated tissue shows characteristics representing a prechondroyd differentiation stage. Cartilage is not a normal tissue type associated with the periodontal apparatus or surrounding areas, which further demonstrates the multipotent potential of PDLSC to differentiate into multiple cell types.

The specific cues to initiate the proliferation and differentiation of PDLSC *in vivo* are not known. The ability to isolate, expand in culture and direct the differentiation of PDLSC *in vitro* to particular lineages provides the opportunity to study events associated with commitment and differentiation. From the various unique culture conditions required for terminal differentiation, we conclude that basal nutrients, cell density, spatial organization, growth factors, and cytokines present in the culture media have a profound influence on PDLSC differentiation. PDLSC cultured in each of the differentiation conditions may also produce autocrine and paracrine factors that are essential for specific lineage progression. The PDLSC described here have the ability to proliferate extensively and maintain their capability to differentiate into multiple cell types *in vitro*, establishing their SC characteristics. Their cultivation and selective differentiation should provide further understanding of this important progenitor of multiple tissue types.

We are currently evaluating the behavior of these cells *in vivo* on an osteoconductive scaffold environment as well as their interaction with SLA titanium surfaces for the possible formation of a natural PDL on the surface of dental implants. Therefore, PDLSC can potentially be isolated and expanded *in vitro* for their use in PDL regenerative therapies, with minimal dis-

comfort due to invasive procedures commonly performed.

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