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MESENCHYMAL STEM CELLS ISOLATED FROM HUMAN PERIODONTAL LIGAMENT

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Abstract - Mesenchymal stem cells (MSCs) were isolated from human periodontal ligament (hPDL-MSCs) and characterized by their morphology, clonogenic efficiency, proliferation and differentiation capabilities. hPDL-MSCs, derived from normal impacted third molars, possessed all of the properties of MSC, including clonogenic ability, high proliferation rate and multi-lineage (osteogenic, chondrogenic, adipogenic, myogenic) differentiation potential. Moreover, hPDL-MSCs expressed a typical MSC epitope profile, being positive for mesenchymal cell markers (CD44H, CD90, CD105, CD73, CD29, Stro-1, fibronectin, vimentin, α-SMA), and negative for hematopoietic stem cell markers (CD34, CD11b, CD45, Glycophorin-CD235a). Additionally, hPDL-MSCs, as primitive and highly multipotent cells, showed high expression of embryonic markers (Nanog, Sox2, SSEA4). The data obtained provided yet further proof that cells with mesenchymal properties can be obtained from periodontal ligament tissue. Although these cells should be further investigated to determine their clinical significance, hPDL-MSCs are believed to provide a renewable and promising cell source for new therapeutic strategies in the treatment of periodontal defects.

Key words: Periodontal ligament, mesenchymal stem cells, characterization, proliferation, differentiation, tissue regeneration

INTRODUCTION

Stem cells are defined as clonogenic cells capable of both self-renewal and multi-lineage differentiation. Stem cells can be isolated in the earliest stages of embryogenesis (embryonic stem cells) or in different post-natal tissues (adult stem cells) including brain, bone marrow, skin, liver, heart, lungs, kidney and spleen. The most well-characterized source for adult stem cells is bone marrow, which beside hematopoietic stem cells, contains a subset of nonhematopoietic stem cells, called marrow stromal stem cells (Owen, 1988) or mesenchymal stem cells (Caplan, 1991), and more commonly now, mesenchymal stromal cells (MSCs) (Horwitz et al., 2005). These cells have the ability of self-renewal and can differentiate into distinctive end-stage cell types of mesodermal lineages, including osteocytes, chondrocytes, adipocytes, muscle cells, bone marrow stroma and other connective tissues (Pittenger et al., 1999; Jiang et al., 2002; Lee et al., 2004). These properties make MSCs an ideal candidate cell type for tissue engineering and regenerative medicine treatment. In the past ten years, MSCs have been extensively used in many medical disciplines for the repair and/or regeneration of defective tissues and organs such as bone (Horwitz et al., 2002), cartilage (Murphy et al., 2003), heart (Stamm et al., 2003) and spinal cord (Mezey et al. 2003). Except for bone marrow, MSCs were also found in almost all postnatal organs and

tissues, including adipose tissue, the periosteum, synovial membrane, synovial fluid, muscle, dermis, trabecular bone, infrapatellar fat pad, articular cartilage and umbilical cord blood (Bianco et al., 2008; Rebelatto et al., 2008).

The identification and isolation of dental mesenchymal stem cells such as dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED) and stem cells from apical papilla (SCAP) have opened up exciting possibilities for the application of tissue engineering as well as gene-based therapies in reconstructive dentistry (Rodriquez-Lozano et al., 2011). Furthermore, a population of multipotent stem cells, termed periodontal ligament stem cells (PDLSCs), have been found in periodontal ligament (PDL) (Seo et al., 2004). Beside their promising potential for therapeutical application in periodontal regeneration (Shi et al., 2005; Park et al., 2011), data suggest that the proper handling of PDLSCs is of great importance for their use in tissue engineering (Akizuki et al., 2005; Hasegawa et al., 2005). In order to provide valuable insights regarding their future use in periodontal regeneration, the aim of this study was to isolate MSCs from human periodontal ligament (hPDL-MSCs), also known as PDLSCs, and analyze their behavior in cell culture conditions. For this purpose, long-term cultures of hPDL-MSCs were established and the cells morphology, clonogenic efficiency, proliferation rate, immunophenotype and differentiation potential were determined.

MATERIALS AND METHODS

Tissue collection and hPDL-MSCs isolation

Normal impacted third molars were extracted for orthodontic purposes from 3 patients that were 18-25 years old, at the Department of Oral Surgery of the Faculty of Dental Medicine, University of Belgrade, according to the approved ethical guidelines set by the Ethics Committee of the Faculty of Dental Medicine, University of Belgrade. Immediately after extraction, the periodontal ligament was gently separated from the middle third of the tooth root surface, minced into small peaces, transferred to a 25 cm^2 flask with Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories), and cultured at 37°C in a humidified atmosphere containing 5% CO_2 with medium exchange. Adherent cells that morphologically resembled characterized MSCs, as previously described (Seo et al., 2004), could be seen as early as 4 days post-plating, while approximately after 7 days, the first colonies were observed. After 3-4 weeks of culture, when the 80% to 90% confluence was reached, the cells were detached by using 0.05% trypsin with 1 mM EDTA (PAA Laboratories) and the remnants of the tissue fragments were removed. Following the first confluence (P0) cells, the cells were passaged regularly in growth medium (GM – DMEM with 10% FBS). For this study, in all experiments cells from third to sixth passages were used.

Colony-Forming Units-Fibroblastic (CFU-F) and cell growth assays

CFU-F assays were performed by plating hPDL-MSCs in 6-well plates at 100, 500 and 1000 cells/well in GM in two replicas. After 10 days in culture at 37°C in a humidified atmosphere containing 5% CO_2, the cells were washed with Dulbecco's phosphate buffered saline (PBS; PAA Laboratories), fixed with ice-cold methanol and stained with 0.3% crystal violet. The number of visible colonies (comprising of more than 50 cells) was determined and the colony forming efficiency calculated as the percentage of the seeded cells that formed colonies.

Cell growth assay was performed by seeding hPDL-MSCs in GM in triplicate in 96-well plates at 5×10^3 cells/well. After 8 days, the cells were detached and counted by the Trypan blue exclusion test. The population doubling times (PDT) were calculated according to the formula $PDT=(T–T0)\ lg2/(lgNt–lgN0)$, where T0 is the starting time and T is the ending time of the cell culture, while N0 and Nt respectively represent the number of seeded and harvested cells.

Flow cytometry analysis

The mesenchymal stromal phenotype of isolated and *in vitro*-expanded periodontal cells was determined by flow cytometry analysis for expression of positive (CD44H, CD90 – Thy-1/Thy-1.1, CD105 – endoglin, CD 73 – ecto-5'-nucleotidase and CD 29 integrin beta1) and negative (CD45 – leukocyte common antigen/cell marker of hematopoietic origin, CD34 – hematopoietic progenitor cell antigen, CD11b – Mac-1 α, CD235a – glycophorin A) MSC markers. After harvesting with 1 mM EDTA for 10 min, the cells were washed in cold PBS supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, USA), and aliquots of 2×10^5 cells were labeled (30 min in the dark at 4°C) with monoclonal antibodies specific for human markers associated with mesenchymal and hematopoietic lineages including anti-CD34-PE (Dako Cytomation, Glostrup, Denmark), anti-CD11b-FITC (Biosource, Camarillo, CA), anti-CD105-R-PE, anti-CD29-PE (Invitrogen), anti-CD45-FITC, anti-CD235a-PE, anti-CD90-PE, CD44H-PE, anti-CD73-PE (all from R&D Systems, Minneapolis, MN, USA). For the embryonic stem cell (ESC) markers expression analyses, aliquots of 2×10^5 cells were fixed in formaldehyde and permeabilized in 90% methanol. Afterwards, MSCs were labeled with primary antibody for SOX2, SSEA4 and NANOG and with secondary FITC conjugated antibody (all purchased from R&D). The level of nonspecific binding was determined by using the appropriate FITC- and PE-conjugated isotype control antibodies (R&D Systems). Flow cytometry was performed using a CyFlow SL flow cytometer (Partec, Münster, Germany).

Immunocytochemical analysis

For the immunocytochemical analysis, hPDL-MSCs were seeded over rounded coverslips and cultured for 24 h in GM. After being fixed with 4% formaldehyde, cells were treated with PBS-0.1% Triton X for 2 min for cell membrane permeabilization, and then incubated (1 h, room temperature) with primary mouse antibodies against vimentin (Santa Cruz Biotech, Santa Cruz, CA, USA), α-SMA (Sigma-Aldrich, St. Louis, MO, USA), fibronectin (Chemicon, Temecula, CA, USA), and Stro-1 (R&D Systems), followed by incubation (1 h in the dark) with corresponding FITC-labeled secondary antibody and nuclear dye DAPI (Sigma-Aldrich) to counterstain. The samples were analyzed and photographed using an Axioskop 2 plus epifluorescence microscope and AxioCam MRc5 camera (Carl Zeiss GmbH, Göttingen, Germany).

Multilineage differentiation

To determine the differentiation potential of hPDL-MSCs, cells were seeded in a 24-well plate (2×10^4 cells per well) cultured in GM until reaching subconfluent stage. At that point, GM was replaced with specific differentiation medium in order to induce differentiation into diverse mesenchymal lineages, while cells cultured in GM only were utilized as controls. Cells were photographed using a light microscope with a digital camera.

To induce osteogenic differentiation, cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 nM dexamethasone (AppliChem), 200 μM ascorbic acid-2-phosphate (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma-Aldrich), and the media were replaced three times a week. On day 7, osteogenic differentiation was analyzed via assessment of alkaline phosphatase (ALP) activity by staining with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, BCIP/NBT (Sigma–Aldrich, Saint Louis, MO, USA). Visualization of calcium deposition and extracellular matrix mineralization was obtained by Alizarin red staining assay performed after three weeks of osteogenic treatment.

Chondrogenic medium containing DMEM supplemented with 2 ng/ml transforming growth factor-β1 (TGF-β1; R & D Systems, Minneapolis, MN, USA), 200 μM ascorbic acid-2-phosphate, 10 nM dexamethasone, 100 U/ml penicillin/streptomycin and 5% FBS was used to induce chondrogenic differentiation. The cultures were incubated for 21 days and at the end of the incubation, chondrogenic

differentiation of the cells was assessed via staining with Safranin O (Merck, Darmstadt, Germany).

For adipogenic differentiation, subconfluent hPDL-MSCs were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml isobutyl-methylxanthine (IBMX; Sigma-Aldrich), 1 µM dexamethasone, and 10 µg/ml insulin (Actrapid; Novonordisc, Bagsvaerd, Denmark) for three weeks. The presence of intracellular lipid droplets, as an indicator of adipogenic differentiation, was confirmed by Oil Red O (Merck Chemicals, Darmstadt, Germany) staining.

To induce myogenic differentiation, hPDL-MSCs were cultured in DMEM supplemented with 5% horse serum (PAA Laboratories), 2% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM hydrocortisone (Galenika, Beograd, Serbia) and 0.1 µM dexamethasone. After 3 weeks, the cells were fixed with ice-cold methanol, stained with 0.3% crystal violet and examined for the presence of myotubes containing three or more aligned nuclei.

RESULTS

Growth characteristics of hPDL-MSCs

MSCs were successfully isolated from the alveolar crestal and horizontal fibers of human periodontal ligament and characterized following the minimal criteria for defining MSCs (Dominici et al. 2006). First, the ability of hPDL-MSCs to grow as spindle-shape fibroblast-like, plastic-adherent cells that give rise to CFU-F colonies was demonstrated (Fig. 1). Namely, after 4 days in culture, flattened cells attached to the culture flasks displaying a fibroblast-like, spindle-shaped morphology (Fig. 1A). These cells gradually grew to form small colonies (Fig. 1B), while the confluence was reached within 7-10 days. No spontaneous differentiation was observed throughout the passages.

Results of CFU-F assay evidenced the clonogenic potential of hPDL-MSCs expanded in culture. However, the clonogenic efficiency of hPDL-MSCs was low since 5.75 and 29.25 colonies were generated from 100 or 1000 plated cells, respectively (Table 1). In addition, a decrease in CFU-F frequencies was observed as the initial plating density was increased.

Analysis of hPDL-MSC proliferation in the cell growth assay showed that the cell numbers of hPDL-MSCs increased to about 6-fold at day 8 after plating ($5x10^3$ cells on day 0 up to $2.95±1.8 x10^4$ cells on day 8), while calculated PDT values were approximately 48 h for $5x10^3$ cells plated.

Phenotypic properties of hPDL-MSCs

Studies performed to characterize the phenotype of the hPDL-MSC comprise usage of a large panel of antibodies specific to known antigens associated with human mesenchymal and hematopoietic lineages. The flow cytometry analyses revealed a high expression of surface markers related to MSCs, such as CD44 (77.90-94.47% positive cells), CD90 (70.35-77.14% positive cells), CD105 (74.98-94.00% positive cells), CD73 (92.71-97.45% positive cells) and CD29 (78.73-98.06% positive cells), as shown in Fig. 2A. As expected, the hPDL-MSCs were negative for hematopoietic stem cell markers, since less than 2% cells expressed CD34, CD11b, CD45 or CD235a antigen (Fig. 2B). At the same time, immunocytochemical staining of the cells demonstrated that the hPDL-MSCs were also positive for the additional mesenchymal cell markers, including Stro-1, fibronectin, vimentin and α-SMA (Fig. 3). Using flow cytometry, we further investigated whether hPDL-MSCs display embryonic stem cell characteristics. Representative histograms shown in Fig. 4 demonstrate that a large fraction of these cells express embryonic stem cell-associated antigens, such as Nanog, Sox2 and SSEA4. The higher percentage of positive cells was revealed for the Nanog molecule (85.49-93.99%) then for the Sox2 marker (73.55-80.17% positive cells), while the number of positive cells ranged from 29.85 to 56.09% for SSEA4 antigen.

Differentiation potential of hPDL-MSCs

As a functional assay to confirm the MSCs iden-

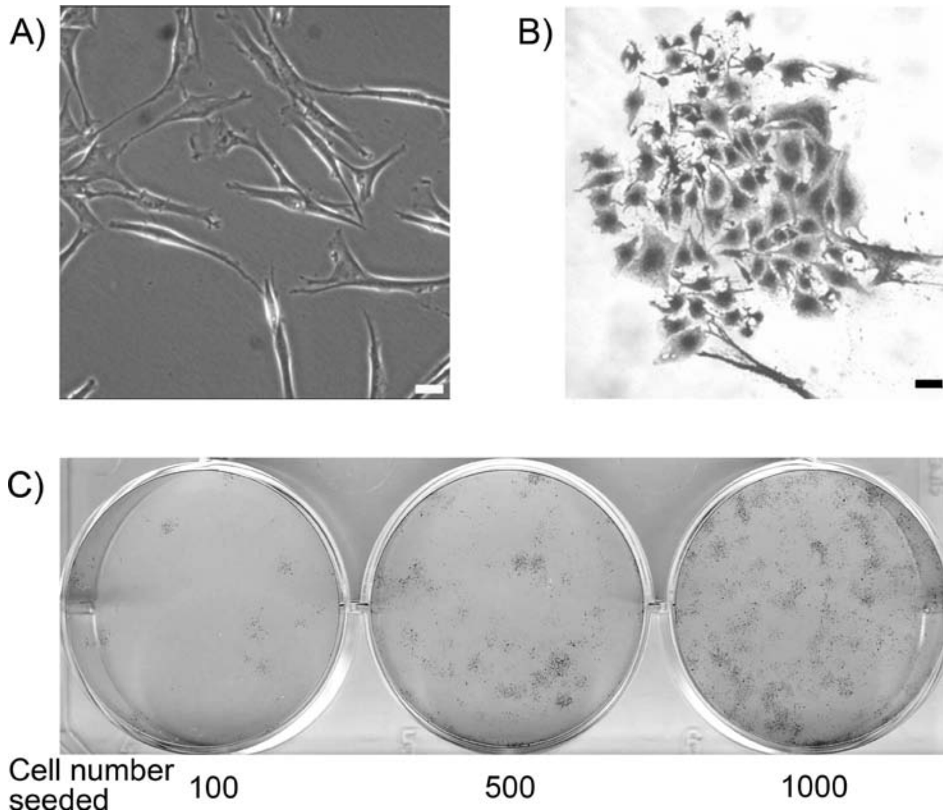

Fig. 1. Morphological and growth characteristics of hPDL-MSCs. (A) Phase-contrast microscopy showing flattened, fibroblast-like morphology of hPDL-MSCs after 4 days in culture. Scale bar: 25 μm. (**B**) Single-cell-derived colony stained with crystal violet after 10 days in culture. Scale bar: 100 μm. (**C**) CFU-F assay. Clonogenic colonies originating from a single cell after 10 days cultivation in GM stained with crystal violet.

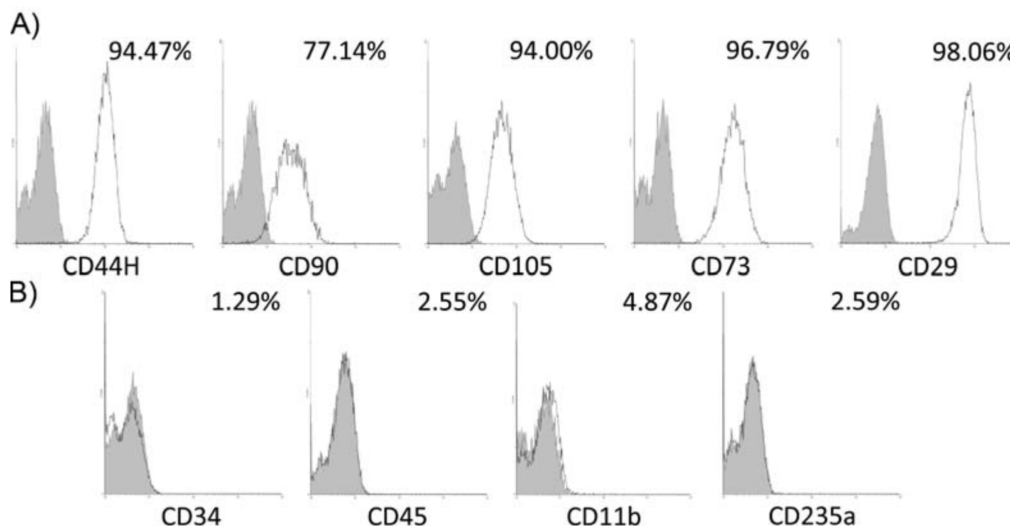

Fig. 2. Immunophenotypic characteristics of hPDL-MSCs. Representative flow cytometry histograms showing the expression (empty histograms) of: (A) selected mesenchymal markers (CD44H, CD90, CD105, CD73, CD29) and (B) hematopoietic markers (CD34, CD45, CD11b, CD235a) by hPDL-MSCs, with percentages of positive cells based on the isotype controls (shaded histograms).

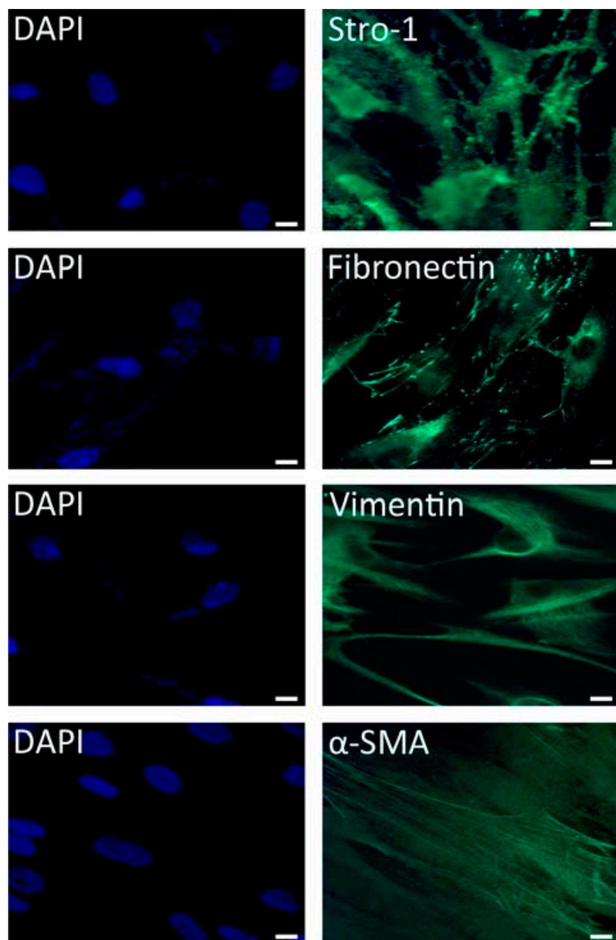

Fig. 3. Representative panels of immunocytochemical detection of mesenchymal markers, STRO-1, fibronectin, vimentin, and α–SMA on hPDL-MSCs. Cells were immunostained with indicated primary antibodies and corresponding FITC-labeled secondary antibodies (green). Cell nuclei were counterstained with DAPI (blue). The samples were analyzed and photographed using an epifluorescence microscope. Scale bars: 25 μm.

tity, we next examined the differentiation potential of hPDL-MSCs and demonstrated their osteogenic, adipogenic and myogenic differentiation capacity.

The osteoblast differentiation capacity was determined by staining the cells for ALP activity after 7-day incubation in osteogenic differentiation medium along with standard culture medium as control. Obtained results confirmed the osteogenic differentiation potential of hPDL-MSCs since a notably increased ALP activity was observed in hPDL-MSC cultures incubated in osteogenic differentiation medium compared with cells exposed to regular growth medium (Fig. 5A). In addition, 3-week-old cultures of hPDL-MSCs incubated in an osteoinductive medium demonstrated the capacity to form Alizarin red positive condensed nodules, confirming that these deposits as calcium mineralized matrix, which is indicative of osteoblasts (Fig. 5B). In the control cultures, these were not observed.

Chondrogenic differentiation was determined by staining of the secreted cartilage-specific proteoglycans with Safranin O. In hPDL-MSCs grown under chondrogenic conditions for 21 days, the presence of chondrogenic proteoglycans was observed by positive Safranin O staining, confirming chondrogenic differentiation (Fig. 5C). As expected, control cultures grown in regular medium were negative for Safranin O staining.

Adipogenic induction was evidenced by the accumulation of lipid-rich vacuoles within the hPDL-MSCs cultured 21 days in adipogenic differentiation medium. The presence of intracellular lipid droplets was confirmed with Oil Red O staining, while in control culture conditions Oil Red O staining was all negative (Fig. 5D).

The myogenic differentiation capacity of hPDL-MSCs was demonstrated by the presence of multinucleated myotubes observed after 16 days of hPDL-MSC incubation in myogenic differentiation medium, while in control cultures incubated in regular growth medium these were not seen (Fig. 5E).

DISCUSSION

The PDL is a specialized, highly vascularized, connective tissue encompassing the root of the tooth. Beside the role in maintaining tooth attachment to the surrounding alveolar bone, PDL possess a regenerative capacity. Potential use of these cells in cell-based therapy for the treatment of damaged periodontia has encouraged researchers to identify PDL-MSCs and explore their characteristics. However, several stud-

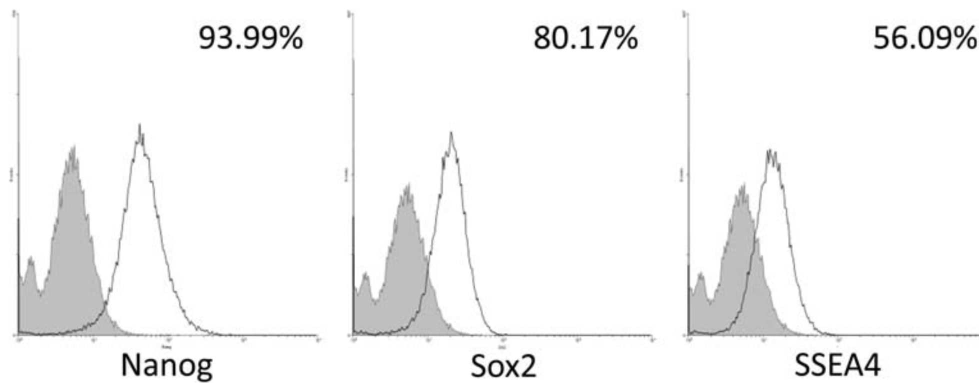

Fig. 4. Embryonic stem cell marker expression in hPDL-MSCs. Representative flow cytometry histograms show the expression of embryonic antigens (NANOG, SOX2 and SSEA4) by hPDL-MSCs compared to isotype controls (solid histograms). Open histograms show specific staining for the indicated marker. The percentages represent the fraction of cells expressing each antigen.

Table 1. The frequency of colony-forming cells from periodontal tissue at different plating densities.

PDL MSCs seeded	CFU-F number	Colony forming efficiency (%)
100	5,75 ± 3,86	5,75 ± 3,89
500	16,25 ± 5,56	3,25 ± 1,34
1000	29,25 ± 9,22	2,29 ± 0,46

Human PDL-MSCs were cultivated at density of 100, 500 or 1000 cells/well in GM for 10 days and stained for CFU-F. The results are presented as mean ± SD for three experiments; each performed in duplicate.

ies indicated that appropriate manipulation of PDL-MSCs is indispensable for their use in tissue engineering (Akizuki et al., 2005; Hasegawa et al., 2005). In the present study, we give evidence that cells isolated from human periodontal ligament tissue in our laboratory satisfy the criteria used to define multipotent mesenchymal stem cells comparable to the features of other postnatal MSCs isolated from various sources (Dominici et al., 2006), as well as of the hPDL-MSCs reported in previous studies (Nagatomo et al., 2006; Iwata et al., 2010). Namely, hPDL-MSCs isolated in this study showed the property to adhere to plastic as spindle-shape fibroblast-like cells when maintained in standard culture conditions. In addition, obtained hPDL-MSCs exhibited the ability to proliferate and to produce colonies from a single cell. By using a colony-forming efficiency assay that determines the CFU-F number, we demonstrated that a minor population within the human periodontal ligament is clonogenic, being in agreement with previous observations made by other authors (Seo et al., 2004). Furthermore, for hPDL-MSCs obtained in this study we demonstrated a high proliferation rate that could be ascribed to the developmental stage of the tissue used. In terms of potential application of hPDL-MSCs in cell-mediated therapies, it is a very important finding that no changes in their proliferation rate were observed throughout the passages.

To confirm stem cell properties of isolated and culture-expanded hPDL-MSCs, immunophenotype analyses of specific markers expression were performed by flow cytometry. As expected, for the obtained hPDL-MSCs a high rate of positivity (70%-90%) was demonstrated for the typical mesenchymal stem cell marker proteins, such CD44H, CD90, CD105, CD73 and CD92. Additionally, the results confirmed the absence of hematopoietic markers,

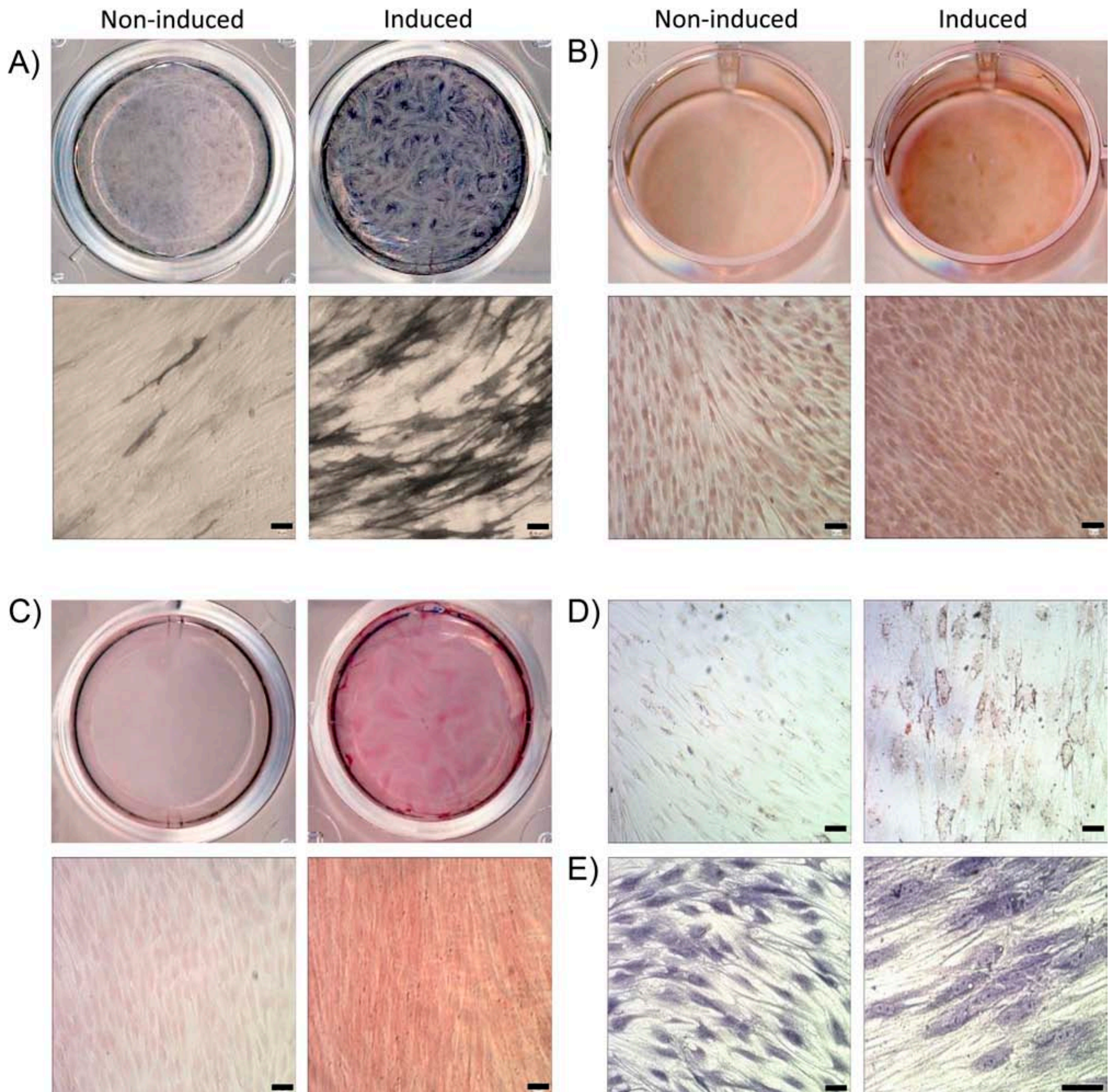

Fig. 5. Multilineage differentiation potential of hPDL-MSCs. (A) Osteogenic differentiation was confirmed by positive staining for ALP activity; (B) Osteogenesis was evaluated by positive staining of extracellular matrix mineralization with Alizarin red; (C) Positive staining of proteoglycans by Safranin O confirmed chondrogenic differentiation of MSCs; (D) Oil Red O staining of intracytoplasmatic lipid droplets demonstrated adipogenic differentiation; (E) Myogenic diferentiation characterised by the formation of myotubes stained with crystal violet. Scale bars: 50 μm.

such as CD34, CD11b, CD45 and Glycophorin-CD235a, on tested cells. Moreover, hPDL-MSCs expressed the early mesenchymal stem cell surface marker, Stro-1, as well as fibronectin, vimentin and α-SMA, intracellular markers that are additional indicators of the mesenchymal origin of the cells,

which is in agreement with previous findings (Gay et al., 2007). Furthermore, we extended the characterization of hPDL-MSCs by analyzing their expression profile for Nanog, Sox2 and SSEA4, embryonic stem cell markers, well known as key regulators of ESC self-renewal and pluripotency, The flow cytometry results showing that hPDL-MSCs were strongly positive (more then 70%) for Nanog and Sox2, with a considerable proportion (30-50%) of hPDL-MSCs positive for the SSEA4 antigen, suggesting that the obtained hPDL-MSCs are primitive and highly multipotent cells. The expression of Nanog, Sox2 and SSEA4 in hPDL-MSCs analyzed in our study is in agreement with reports that human adult MSCs derived from different sources may express ESC markers (Riekstina et al., 2009), as well as with previous findings of positive ESC marker expression in PDL-MSCs (Huang et al., 2009; Trubiani et al., 2010).

However, as the final, most relevant criterion to confirm the stem cell nature of isolated hPDL-MSCs, we analyzed their multilineage mesenchymal differentiation capacity. By the use of specific differentiation media, we successfully demonstrated their multipotent nature, as these cells were able to differentiate into distinct mesenchymal cell lineages, including osteogenic, chondrogenic, adipogenic and myogenic lineages. In this way, we provided evidence that PDL contains cells that are clonogenic, highly proliferative and multipotent, pointing to their capability to regenerate the PDL tissue.

Stem cell therapy is a novel and promising option for periodontal tissue regeneration. Periodontitis is one of the most prevalent oral diseases in adults that leads to the destruction of periodontal tissues caused by pathogenic microflora in the dental plaque (Savage et al., 2009). This microbial community, as a highly organized bacterial biofilm, is resistant to both the applied antimicrobial agents and the host defense mechanisms (Armitage and Robertson, 2009). Bacterial infection induces an inflammatory response of the alveolar bone and soft periodontal tissues and the inflammation cascade often leads to irreversible destruction of tooth-supporting tissue and finally causes tooth loss (Pihlstrom et al., 2005). Regeneration of lost periodontal tissues due to periodontitis has always been the ultimate goal of periodontal therapy. Current regenerative procedures, such as root surface conditioning, guided tissue regeneration, use of bone replacement grafts, application of exogenous growth factors or proteins, can only partially regenerate periodontal tissues (Chen et al., 2010). On the other hand, several studies demonstrated that transplanted autologous PDL-MSCs enhanced the regeneration of periodontal tissue in experimental defect models in animals, as well as in human patients with intrabony defects, suggesting that PDL-MSCs transplantation may be an efficient alternative for the treatment of periodontitis (Kim et al., 2009; Ding et al., 2010; Feng et al., 2010).

In summary, we isolated, expanded and characterized hPDL-MSCs, a population of stem cells that express specific mesenchymal cell markers and possess the ability to self-renew and differentiate into osteogenic, chondrogenic, adipogenic and myogenic cells. The data presented suggest that the obtained hPDL-MSCs can be easily accessible as a potential source for tissue engineering. However, further extensive studies need to be performed in order to better define PDL-MSC function in the process of regeneration *in vivo*, as well as to create suitable scaffolds for periodontium regeneration, with the ultimate goal to develop and optimize new therapeutic strategies in periodontal defects.

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