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# Explant-derived human dental pulp stem cells enhance differentiation and proliferation potentials

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## Abstract

Numerous stem cell niches are present in the different tissues and organs of the adult human body. Among these tissues, dental pulp, entrapped within the 'sealed niche' of the pulp chamber, is an extremely rich site for collecting stem cells. In this study, we demonstrate that the isolation of human dental pulp stem cells by the explants culture method (hD-DPSCs) allows the recovery of a population of dental mesenchymal stem cells that exhibit an elevated proliferation potential. Moreover, we highlight that hD-DPSCs are not only capable of differentiating into osteoblasts and chondrocytes but are also able to switch their genetic programme when co-cultured with murine myoblasts. High levels of *MyoD* expression were detected, indicating that muscle-specific genes in dental pulp cells can be turned on through myogenic fusion, confirming thus their multipotency. A perivascular niche may be the potential source of hD-DPSCs, as suggested by the consistent $Ca^{2+}$ release from these cells in response to endothelin-1 (ET-1) treatment, which is also able to significantly increase cell proliferation. Moreover, response to ET-1 has been found to be superior in hD-DPSCs than in DPSCs, probably due to the isolation method that promotes release of stem/progenitor cells from perivascular structures. The ability to isolate, expand and direct the differentiation of hD-DPSCs into several lineages, mainly towards myogenesis, offers an opportunity for the study of events associated with cell commitment and differentiation. Therefore, hD-DPSCs display enhanced differentiation abilities when compared to DPSCs, and this might be of relevance for their use in therapy.

**Keywords:** DPSCs • hD-DPSCs • cell differentiation • myogenesis • osteoblasts • chondrocytes

## Introduction

Adult stem cells are capable of replacing cells after injury or disease [1–4]. During the last years, new evidence has highlighted that stem cells are capable of differentiating into more cell types than previously imagined. This phenomenon much probably is related to their plasticity or transdifferentiation potential [5–7]. It is well known that stem cell populations in adult mammals are not fixed entities and that after exposure to a new environment they may be able to populate other tissues and possibly differentiate into other cell types [8].

Almost all adult tissues contain stem cells, including bone marrow, peripheral blood, brain, spinal cord, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver and pancreas [9–12]. A population of post-natal stem cells in human dental pulp, called dental pulp stem cells (DPSCs), has also been identified [13]. DPSCs are of special interest for many reasons including the facility of obtaining dental pulp from any adult and the possibility of employing autologous mesenchymal stem cells. Previous studies have demonstrated that, like osteoblasts, DPSCs express bone markers, such as bone sialoprotein, alkaline

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phosphatase (ALP), type I collagen and osteocalcin [14, 15]. Their differentiation is controlled by bone formation regulators, including members of the transforming growth factor-β (TGF-β) superfamily and cytokines [16].

DPSCs are capable of differentiating into odontoblast-like cells, osteoblasts, adipocytes, smooth and skeletal muscle cells [17–19]. For example, DPSCs may form a woven bone complex *in vitro* [19], which can be remodelled into lamellar bone after *in vivo* transplantation [17, 18]. DPSCs express nestin and GFAP, which are molecules related to the neural crest-cell origin of the dental pulp [20].

The multipotent stem cell content of the dental pulp indicates that this tissue has a big potential for clinical applications. It offers the opportunity to elucidate the cellular and molecular mechanisms that operate during developmental and regeneration of dental and other craniofacial structures, and is thus a subject that deserves further studies.

In this study we analysed human adult DPSCs isolated with an explants-culture method in order to evaluate their differentiation potential into different lineages.

# Materials and methods

## Patients and human dental pulp extraction and culture

Human dental pulp was extracted from third molars of eight healthy individuals (aged 22–35 years), following written informed consent, for experimentation that was specifically approved by our Internal Ethic Committee. Each pulp was cultured separately and no differences were observed between cells obtained from different dental pulps. One week before extraction, each individual was checked for systemic diseases and oral infections and was treated with professional dental hygiene. Immediately before extraction, dental crowns were covered with a 0.3% chlorexidin gel (Forhans, NY, USA) for 2 min. Dental pulp was obtained with a dentinal excavator or a Gracey curette. Pulp was gently removed and rinsed in $Ca^{2+}$-, $Mg^{2+}$-free phosphate-buffered solution (PBS) (Invitrogen, San Giuliano Milanese, Milan, Italy) and dissected into 1–2 $mm^3$ pieces with a scalpel. Fragments were digested for 5 min. at 37°C with 0.2% trypsin (Invitrogen). The cells obtained were discarded, and the remaining tissue fragments washed with MegaCell Dulbecco's modified eagle's medium (DMEM) complete medium (supplemented with 10% foetal calf serum [FCS], 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol) were transferred to a Petri dish coated with fibronectin and cultured as explants in MegaCell complete medium in a 5% $CO_2$ atmosphere at 37°C. After a period of 2 weeks a layer of fibroblast-like cells was generated from adherent explants upon which small round phase-bright cells were observed. The small round cells, appearing to be refractile, were separated from the monolayer cells by washing twice with $Ca^{2+}$-, $Mg^{2+}$-free PBS, followed by a first wash for 1–2 min. with 0.53 mmol/l ethylenediaminetetraacetic acid (EDTA) (Versene, Invitrogen) and a second with 0.5 g/l trypsin and 0.53 mmol/l EDTA (Invitrogen) for 2 to 3 min. at room temperature under visual control. The cells thus obtained (approximately $10^5$ cells/explant) were seeded at $5 \times 10^3$ cells per $cm^2$ on collagen-coated dishes or in poly-D-lysine-coated multiwell plates (BD Biosciences, Milan, Italy) in Mega Cell DMEM complete medium. For cryopreservation, 90% FCS and 10% DMSO was used as the freezing medium. To evaluate the growth rate cells were seeded at a density of $3 \times 10^3/cm^2$ and cultivated for 45 days. Cells were counted every 3 days to evaluate the population doublings.

## Cytometric assays

Flow cytometric examinations were performed on the 22th day of culture. Cells were detached by using a 10 min. treatment at 37°C with PBS 0.02% EDTA, pelleted (10 min. at 1000 rpm) and washed in 0.1% bovine serum albumin (BSA) in 0.1 M PBS at 4°C, then incubated in 10 μl of antibody as indicated by the manufacturer. Phenotype analysis was performed with FITC, PE and Cychrome labelled monoclonal antibodies (mAb) against: c-kit, CD34 (Santa Cruz, CA), CD133 (Miltenyi-Biotec, Bergisch Gladbach, Germany), VEGFR, CD31, CD146 (Abcam, Cambridge, MA, USA) as well as isotype matched controls.

## Calcium imaging

Human dental pulp cells were cultured in 35 mm dishes at a density of $1 \times 10^4/cm^2$ in α-MEM complete medium (see above) supplemented with 20% FCS. Cells were incubated in MEM containing 3 mM Fura-2-AM for 1 hr at 37°C, and then rinsed with Krebs–Henseleit–Hepes buffer (140 mM $Na^+$, 5.3 mM $K^+$ 132.4 mM $Cl^-$, 0.98 mM $PO_4^{2-}$, 1.25 mM $Ca^{2+}$ 0.81 mM $Mg^{2+}$, 5.5 mM glucose and 20 mM Hepes) supplemented with 0.2% fatty acid free BSA. The cells were placed in a 37°C culture chamber on the stage of an inverted fluorescence microscope (Nikon, TE2000E, Florence, Italy), with a cooled CCD camera (512B Cascade, Princeton Instruments, AZ, USA). Samples were illuminated alternately at 340 and 380 nm using a random access monochromator (Photon Technology International, NJ, USA) and emission was detected using a 510 nm emission filter. Images were acquired (1 ratio image/sec.) using Metafluor software (Universal Imaging Corporation, Downington, PA, USA). Intracellular $Ca^{2+}$-dependent fura-2 fluorescence was analysed after local addition/application of 10 μl of endothelin-1 (ET-1) (1 μM) and/or ATP (1 mM) (the addition was made using a Gilson pipette directly into 35 mm dish, under the microscope, whereas fluorescence was analysed). Calibration of the signal was obtained at the end of each experiment by maximally increasing intracellular $Ca^{+2}$-dependent fura-2 fluorescence with 5 μM ionomycin, followed by recording minimal fluorescence in $Ca^{+2}$-free medium. $[Ca^{2+}]_i$ was calculated according to previously described formulas.

## Lentiviral vector cell transduction

Lentiviral vectors expressing β-galactosidase with nuclear localization were provided by Dr. L. Berghella (San Raffaele Science Park, Rome, Italy). To test cell infection, β-galactosidase staining was performed as previously described [21]: cultures were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min. at 4°C, washed again twice with PBS and incubated overnight at 37°C with X-Gal staining solution (1 mg/ml 4-Cl-5-Br-3-indolyl-β-galactosidase (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl2 in PBS), then washed with PBS and conserved at 4°C before observation.

## Differentiation assays

### Osteogenic differentiation

In order to induce osteogenic differentiation, cells at a density of 3500/cm$^2$ were placed in DMEM, with 10% FBS, 1% pen-strep, 0.1 μM dexamethasone (Sigma, Milan, Italy), 50 μM ascorbate-2-phoshate (Sigma), 10 mM β glycerophosphate (Sigma). The medium was changed two times per week. Cells in basal medium were used as controls. To demonstrate the osteogenic differentiation, after 21 days, the cells were fixed and stained for ALP.

### Chondrogenic differentiation (micromass culture)

Cells from subconfluent cultures were released by 0.05% trypsin in 0.01% EDTA, counted and used to generate micromass culture. Briefly, $2.5 \times 10^5$ cells were centrifuged at 500 × *g* in 15 ml polypropylene conical tubes and the resulting pellets were cultured for 4 weeks.

Control cultures were grown in a serum-free chemically defined medium consisting of DMEM supplemented with 1 μM bovine insulin, 8 nM human apo-transferrin, 8 nM BSA, 4 μM linoleic acid, 1 mM sodium pyruvate. To induce chondrogenic differentiation, the control medium was supplemented with 10 ng/ml TGF-β, 100 nM dexamethasone and 250 μM ascorbate-2-phosphate.

Cultures were incubated for 4 weeks at 37°C in an atmosphere containing 5% $CO_2$; the medium was changed every 2–3 days. Cell aggregates were harvested at 2 and 4 weeks then fixed and processed for histology.

### Skeletal muscle cultures

nLacZ infected human dental pulp cells were co-cultured with C2C12 and the percentage of fusion was evaluated. Briefly, human stem cells were co-cultured with C2C12 (1:10 or 1:5) in DMEM complete medium (4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mM sodium pyruvate) added to 10% FBS, for 2 days (this step was used only for co-culturing in proliferating conditions), then cultured for four additional days in DMEM complete medium with 2% horse serum added. Percentage of myogenic differentiation was calculated by comparing the number of β-Gal$^+$ nuclei within myosin positive cells to the total β-Gal$^+$ nuclei. Biochemical differentiation was confirmed by RT-PCR using human specific oligonucleotides for MyoD.

### Smooth muscle differentiation

Cells were plated at a concentration of 8000/cm$^2$ and cultured in DMEM medium supplemented with 2% horse serum for 7 days. Smooth muscle differentiation was induced by addition of 5 ng/ml TGF-β, (TGF-β was added every other day). Cultured cells were then fixed and stained with a mouse monoclonal antibody recognizing human smooth-muscle actin (Sigma) and goat antimouse (FITC) (Santa Cruz, DBA, Milan, Italy) as a secondary antibody.

## Histological analyses

### Pellet culture analysis

Cell aggregates were fixed in 4% formaldehyde in PBS for 10–15 min. and embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin, toluidine blue and alcian blue and viewed using transmitted and polarized light microscopy.

### ALP assay

The ALP assay was performed with the Sigma Alkaline kit. Solution A was prepared by melting 1 ml Fast Violet B Salt pill into 1 ml of sodium nitrite solution 0.1 M. Solution B was prepared by dissolving 1 ml of Naphtol AS-MX phosphatase alkaline solution in 48 ml distilled $H_2O$, then A and B solutions were mixed in a single mixture. Cells were fixed with acetone/citrate 1:2.5 for 30 sec., washed with distilled $H_2O$ for 45 sec., added to alkaline-dye mixture for 30 sec. in the dark, washed with distilled $H_2O$ for 2 min. and incubated into Mayer's haematoxylin for 10 min.

### Skeletal muscle analysis

Tibialis anterior (TA) muscles were removed, fixed in 4% paraformaldehyde, embedded in tissue freezing medium (Jung, Leica Instruments, Wetzlar, Germany), frozen in liquid nitrogen-cooled isopentane, and cut on a cryostat into 10 μm serial sections. Muscles were sectioned entirely, and sections scored for the presence of β-Gal$^+$ nuclei by X-Gal staining.

## Immunohistochemistry

Alpha-smooth muscle actin and sarcomeric myosin were analysed using specific antibodies after cell fixation in 4% paraformaldehyde in PBS and permeabilization in 0.2% Triton X-100 in PBS. Cells were washed in 1% BSA in PBS and incubated for 1 hr at room temperature with red or green alexa fluoro-conjugated goat antimouse or anti-rabbit immunoglobulin to a final dilution of 1:500 in 1% BSA in PBS. Immunofluorescence was detected using a Carl Zeiss Axioplan fluoromicroscope (Jena, Germany).

## RNA extraction and RT-PCR analysis

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Milan, Italy): cells were harvested, lysed with the SV RNA lysis buffer and RNA extraction was performed according to Promega instructions. cDNA synthesis was carried out from total RNA using oligo $(dT)_{12–18}$ and Moloney murine leukaemia virus reverse transcriptase (10 U/ml) (GIBCO-Invitrogen, Carlsbad, CA, USA) in 20 ml at 42°C for 50 min. PCR reactions were carried out in PCR buffer (10 mM Tris-HCl pH 8.4; 500 mM KCl; 50 mM MgS04) with 0.2 μmol primers and 2.5 U Taq PCRx DNA Polymerase (GIBCO-Invitrogen) in a total volume of 50 μl for 30 cycles consisting of denaturation at 95°C for 30 sec., annealing at 54°C to 57°C, according to different pair of primers for 30 sec., and extension at 72°C for 40 sec. after the initial denaturation at 95°C for 2 min. The following oligonucleotides were used: human aggrecan *for* 5′-CACTGTTACCGCCACTTCCC-3′, *rev* 5′-ACCAGCGGAAGTCCCCTTCG-3; human type I collagen *for* 5′-AGGGCTCCAACGAGATCGAGATCCG-3′, *rev* 5′- TACAGGAAGCAGACAGGGCCAACGTCG-3′; human bone sialoprotein (BSP) *for* 5′-CTATGGAGAGGACGCCACGCCTGG-3′, *rev* 5′- CATAGCCATCGTAGCCTTGTCCT-3′; human osteocalcin *for* 5′- ATGAGAGCCCTCACACTCCTC-3′, *rev* 5′-CGTAGAAGCGCCGATAGGC-3′; human MyoD *for* 5′-CGATATACCAGGTGCTCTGAGGG-3′, *rev* 5′-GGGTGGGTTACGGTTACACCTGC-3′; β-actin *for* 5′-ACCAACTGGGACGACATGGAG-3′, *rev* 5′-GGTCAGGATCTTCATGAGGTAGTC-3′. Integrity and equal loading of cDNA in the PCR reactions were checked by quantification of β-actin mRNA levels. The amplified products

were resolved by electrophoresis on a 1.5% agarose gel and visualized using ethidium bromide staining.

### *In vivo* transplantation

Eight- to 10-week-old *Scid/bg* mice were anesthetized with Avertin (0.018 ml/g body weight), and muscle regeneration was induced in TA muscles by injection of 25 μl of 1 mM cardiotoxin (Latoxan, Rosans, France). All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies. The appropriate committee (Animal Ethic Committee) approved this work. After 24 hrs, human dental pulp cells were injected ($8 \times 10^5$ cells in 50 μl of phosphate-buffered saline) into the regenerating TA muscle as previously described [22]. Four weeks after transplantation, the mice were killed, the transplanted and the controlateral TA muscles were removed, fixed in 4% paraformaldehyde, embedded in tissue freezing medium (Electron Microscope Sciences, Washington, PA, USA) and frozen in liquid nitrogen-cooled isopentane. TA muscles were cut on a cryostat into 10 μm serial sections and X-Gal staining assayed β-galactosidase activity in cryostat sections. Seven- to 9-week-old *scid/bg* mice were obtained from Charles River (Calco, Italy) and maintained under pathogen-free conditions. The Institutional Animal Care and Use Committee approved the animal experimentation protocols. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies.

### Statistical analysis

Student's t-test was used for statistical evaluation. Level of significance was set at $P < 0.05$.

## Results

### Phenotypic characterization of human dental pulp explants-derived cells

In this study, we utilized a new method for DPSC isolation based on their selection by explants' culture. Human dental pulp was dissected and the fragments plated on a fibronectin-coated dish. After the initial outgrowth of fibroblasts, small, round, phase-bright cells began to migrate over this coat (Fig. 1A). These cells were harvested periodically by treatment with EDTA and mild trypsinization and were grown on collagen-coated culture surfaces, where they exhibited a triangular morphology (Fig. 1B). When, however, cells harvested from the explants were cultured on poly-D-lysine-coated surfaces, most cells became loosely adherent, others remained in suspension and some contaminating fibroblast-like cells attached firmly to the poly-D-lysine coat. After approximately 72 hrs, clusters of small, round, phase-bright cells increased in size and detached from the culture surface (Fig. 1C).

Human dental pulp explant-derived cells (hD-DPSCs) showed a high proliferation rate, with approximately 25 population doublings and a doubling time of about 48 hrs (Fig. 1D). Cytofluorimetric analysis of hD-DPSCs showed a 37% expression for CD146, whereas haematopoietic stem cell markers (CD133, CD34, CD117) and endothelial markers (VEGFR, CD31) were not present or only at very low levels (1.8% expression for CD34) (Fig. 1E).

### Osteogenic and chondrogenic differentiation of human dental pulp explant-derived cells

In order to obtain differentiation into osteoblasts, we cultured hD-DPSCs and compared their differentiation potentiality with human DPSCs [17–19, 23]. Cells were cultured in osteogenic medium for 21 days, then fixed and processed for ALP staining. DPSCs and hD-DPSCs showed no significant differences in ALP activity, and ALP expression was detected also in the absence of BMP-2 (Fig. 2A). This was confirmed by RT-PCR analysis of osteogenic markers, such as osteonectin, type I collagen and bone sialoprotein (Fig. 2B).

To investigate the chondrogenic potential of hD-DPSCs, we utilized the 'micromass culture' approach that provides an assay for chondrogenesis of similar significance to *in vivo* transplantation assays. Recent studies have shown that the 'pellet culture system' can be used not only with human BMSCs [24, 25].

Human explant-derived DPSCs pellets cultured in the presence of TGF-$\beta_1$ generated a solid three-dimensional tissue structure that could be harvested and processed intact for histology or homogenized for RT-PCR analysis. At week 4 the *in vitro* generated micromass 'beads' exhibited a substantial amount of basophilic, metachromatic matrix, corresponding to the histochemical feature of proteoglycan content in cartilage (Fig. 2C). Both bone- and cartilage-specific markers were transcribed, suggesting a commitment of hD-DPSCs to chondro-osteogenic phenotype (Fig. 2D) in a 3D structure.

### Mutipotency of human dental pulp explant-derived cells in skeletal and smooth muscle cells lineages

We investigated the myogenic potential of human dental pulp cells, analysing their conversion into skeletal and smooth muscle cells phenotype.

For skeletal muscle differentiation, we established a xenogenic cell culture model *in vitro* in which different populations of human DPSCs were tested for their ability to fuse with mouse myotubes. In this model, DPSCs and hD-DPSCs that were previously infected with a nuclear-LacZ lentivirus, were co-cultured with C2C12 myoblasts for 2 days under proliferative conditions then switched to differentiation conditions for an additional 4 days. Multinucleated myotubes were identified by the presence of sarcomeric myosin heavy chain and human nuclei that are unequivocally identified by positive X-Gal staining. Contribution of human dental pulp cells to myotubes was determined by the percentage of human nuclei in the myotubes *versus* total number of human

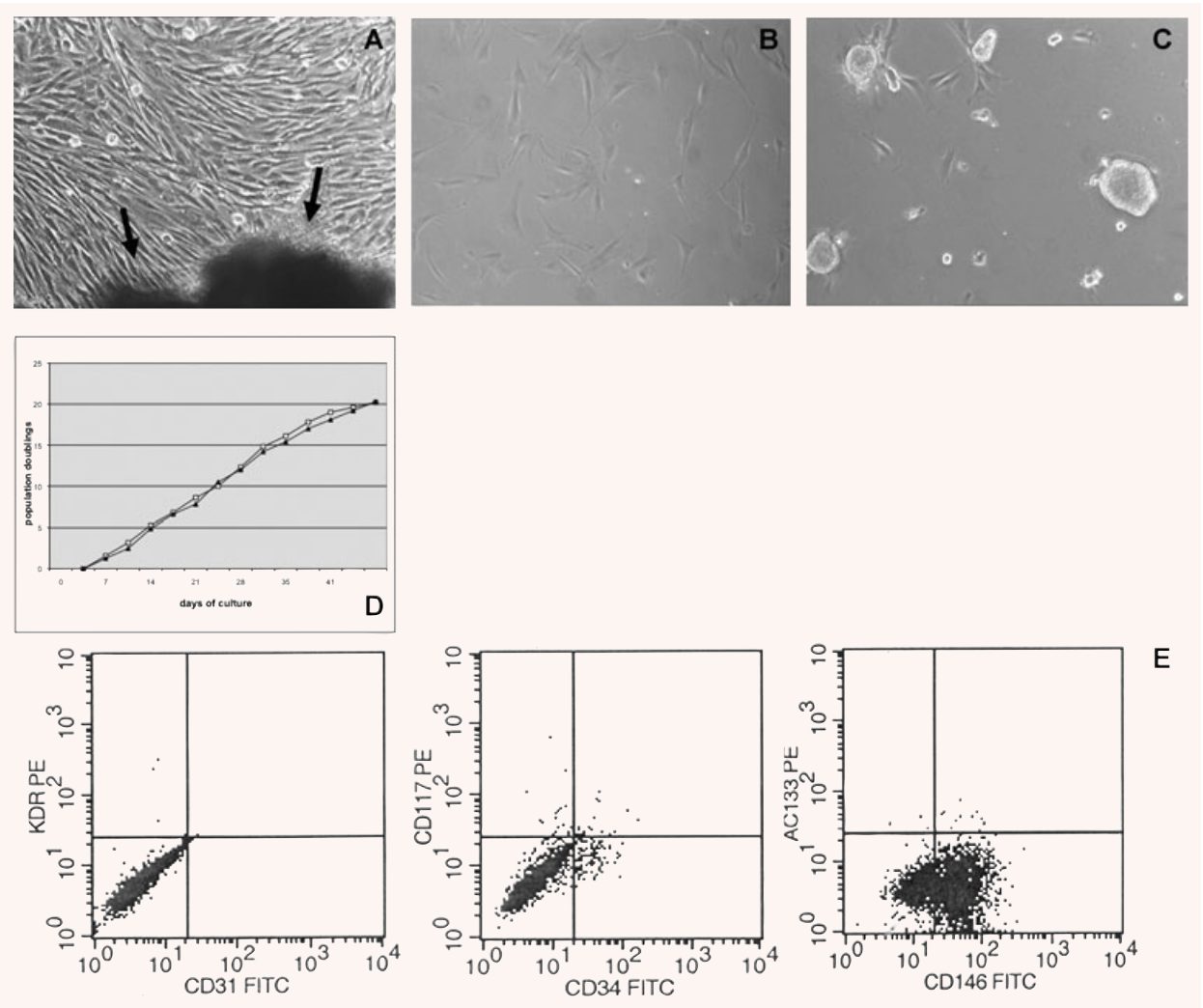


**Fig. 1** Phenotypic characterization of hD-DPSCs. (**A**) Phase-contrast morphology of the cellular outgrowth of a fragment of human dental pulp tissue (dark area, *arrow* highlighted). Round and refractile cells are visible on the top of a layer of fibroblast-like cells (original magnification ×200). (**B**) Phase contrast-morphology of hD-DPSCs plated on collagen coated dish or (**C**) on poly-D-lysine coated dish for 72 hrs (original magnification ×200). (**D**) Population doublings of two different cell populations, cells were counted every 3 days. (**E**) Fluorescence-activated analysis of hD-DPSCs using antibody against endothelial markers (left panel), haematopoietic stem cells markers (middle and right panel), and for mesenchymal stem cell marker CD146 (right panel).

nuclei. The results clearly show that co-culturing of human dental pulp cells with the C2C12 mouse myoblasts resulted in a significant contribution of human nuclei to mouse myotubes (Fig. 3A). In fact, the fusion percentage of hD-DPSCs cells and DPSCs was 23 ± 4% and 15 ± 2%, respectively (Fig. 3B). The presence of human nuclei in mouse myotubes raised the question as to whether any human muscle-specific genes were transcribed. In the *in vitro* myogenesis model, we observed that most 'hybrid myotubes' were positive for multiple human nuclei. However, the staining intensity of these nuclei was not consistent, appearing as a decreasing gradient from one brightly stained nucleus. This observation suggests that the human nuclear proteins from a nucleus were transported to neighbouring mouse nuclei. Therefore, we suggest that mouse muscle-specific transcription factors could translocate in a similar fashion as human nuclei and turn on transcription of human muscle-specific genes. To measure the human muscle gene expression, total RNA was isolated from C2C12 and hD-DPSCs cells co-cultures. RT-PCR was performed with human primers specific for muscle transcription factors. We detected human MyoD gene expression in the co-cultures, indicating

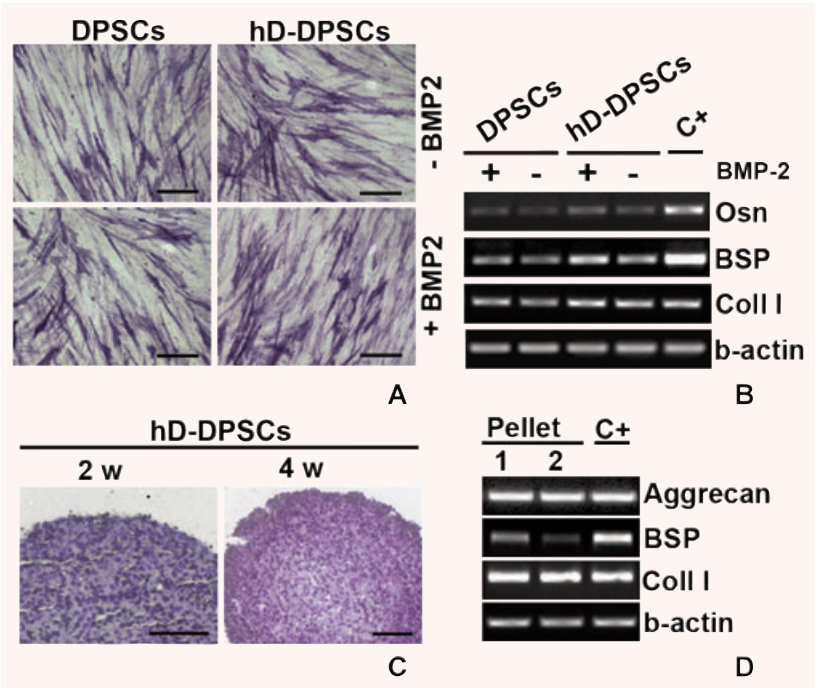


**Fig. 2** Osteogenic and chondrogenic differentiation of hD-DPSCs. (**A**) ALP staining of DPSCs and hD-DPSCs cultured in osteogenic medium. (**B**) RT-PCR analysis for osteogenic markers expression of cultures under the same conditions as reported above; SAOS cell line were used as positive control. (**C**) Toluidine-blue staining of histological sections of pellet beads formed by hD-DPSCs cultured under chondrogenic conditions for 2 weeks (left panel) and 4 weeks (right panel). The metachromatic shift is due to the amount of basophilic matrix contained within the cells. (**D**) RT-PCR expression analysis of specific chondrogenic and osteogenic markers in 4-week pellet cultures; SAOS cell line were used as positive control. The scale bars represent 50 μm in (**A**) and 100 μm in (**B**).

that muscle-specific genes in dental pulp cells can be turned on through myogenic fusion (Fig. 3C).

Smooth muscle differentiation was analysed by treating DPSCs and hD-DPSCs with TGF-β (5 ng/ml) in DMEM medium supplemented with 2% horse serum for 1 week (TGF-β was added every second day). Control cultures were grown in DMEM supplemented only with 2% horse serum (control medium). Then cultures were fixed and stained with a mouse monoclonal antibody recognizing human smooth-muscle actin (SMA) (Fig. 3D). Immunofluorescence analysis revealed that in both cultures undergoing TGF-β treatment (but not in control cultures) a high expression of smooth-muscle actin was found with no significant differences between DPSCs and hD-DPSCs.

## *In vivo* transplantation of human dental pulp cells in regenerating muscle

Our cell culture experiments indicated that human dental pulp-derived cells were capable of fusion with myotubes. However, recruitment to myogenesis of dental mesenchymal stem cells has until now not been investigated *in vivo*. To analyse whether DPSCs and hD-DPSCs can fuse with skeletal muscle fibres in response to physiological stimuli, we chemically induced muscle regeneration in the TA muscle of immunodeficient *scid/bg* mice. It is well known that the number of committed myogenic precursors and the recruitment to myogenesis of resident non-myogenic cells such as fibroblasts or mesenchymal progenitors that populate the muscle tissue increase soon after injury.

Two immunodeficient *scid/bg* mice per human dental pulp cell population were injured with cardiotoxin; 24 hrs after injury human cells, previously infected with a nuclear-LacZ lentivirus, were injected into the damaged muscles ($8 \times 10^5$ cells per muscle). Control animals were injected with phosphate-buffered saline (PBS) into the regenerating TA muscle which was examined 4 weeks after injection for the presence of β-Gal$^+$ nuclei (Fig. 4A).

Transverse cryostat sections showed the presence of β-Gal$^+$ nuclei in regenerating muscle 4 weeks after induction of muscle injury, 95 ± 15 for hD-DPSCs and 30 ± 8 for DPSCs (Fig. 4B). This suggests that both these populations could participate in skeletal muscle regeneration.

The possible function of human dental pulp cells that were recovered after muscle regeneration – whether they directly participate, or just contribute to the recruitment of specific cells in the injured muscle – remains however to be investigated.

## Proliferation and calcium mobilization induced by ET-1 of human dental pulp cells

To investigate the presence of ET-1 receptors in hD-DPSCs cells and DPSCs, we cultured them in 35 mm dishes at a concentration of $1 \times 10^4/cm^2$ in α-MEM 20% FCS medium. Cells were incubated with 3 mM Fura-2-AM for 1 hr at 37°C. Detection of intracellular $Ca^{+2}$-dependent fura-2 fluorescence after local addition of ET-1 (1 μM), revealed a high percentage of ET-1 responsive cells in the hD-DPSCs population as compared to the DPSCs population, 55 ± 9% and 10 ± 2%, respectively (Fig. 5A).

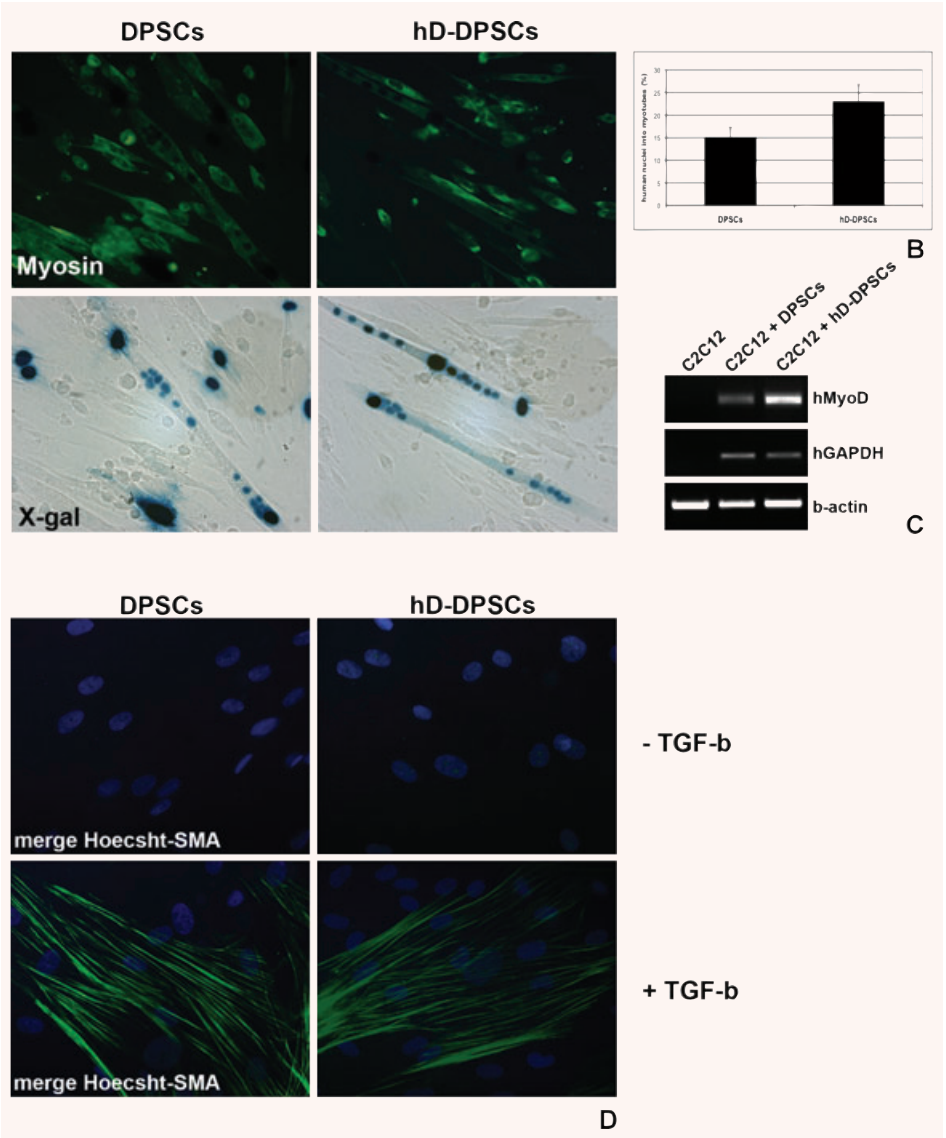


**Fig. 3** Mutipotency of hD-DPSCs in skeletal and smooth muscle cells lineages. (**A**) Human dental pulp explant-derived cells (hD-DPSCs) and human DPSCs were co-cultured with C2C12 for 2 days under proliferative conditions (C2C12) then switched to differentiation conditions (for additional 4 days). Multinucleated myotubes are revealed by the presence of sarcomeric myosin heavy chains. Human nuclei are identified by X-Gal staining positivity (original magnification ×100). (**B**) The percentage of fusion of DPSCs and hD-DPSCs was calculated as ratio between human nuclei in the myotubes and total number of human nuclei. (**C**) RT-PCR analysis of co-cultures shows a consistent expression of human muscle-specific transcription factor MyoD. β-actin was used as loading control. (**D**) Immunofluorescence analysis of human smooth muscle actin in hD-DPSCs and DPSCs, cultured in control medium (DMEM supplemented with 2% horse serum, upper panels), and in differentiating medium (control medium added with growth factor TGF-β, lower panels) . Original magnification ×200.

The proliferative effect of ET-1 was evaluated by BrdU assay (Roche Kit, Milan, Italy). DPSCs and hD-DPSCs cells were treated for 48 hrs with ET-1, then incubated with BrdU 10 μM for 45 min., fixed and stained with monoclonal anti-BrdU FITC-conjugated antibody. The percentage of $BrdU^+$ cells was evaluated by counting at least 250 cells from each experiment (Fig. 5B).

## Discussion

The adult human body contains stem-cell niches that have mainly a perivascular location in different tissues and organs. Among these tissues, dental pulp, entrapped within the 'sealed niche' of the pulp chamber, is an extremely rich site for stem cell isolation. In this study we demonstrated that isolation of human DPSCs by the explant-culture method (hD-DPSCs), allowed the recovery of a population of dental mesenchymal stem cells, which showed a notable proliferation potential, multipotency and a long lifespan.

Interestingly, immunohistochemistry on *in situ* dental pulp samples showed that $STRO\text{-}1^+$ and $CD146^+$ cells were located in close proximity to blood vessels [26], thus confirming the hypothesis that stem cells are present in the wall of blood vessels. hD-DPSCs display a high percentage of $CD146^+$ cells, suggesting a perivascular origin for this population.

We demonstrated that hD-DPSCs, when undergoing differentiation into pre-osteoblasts, deposited an extracellular matrix and

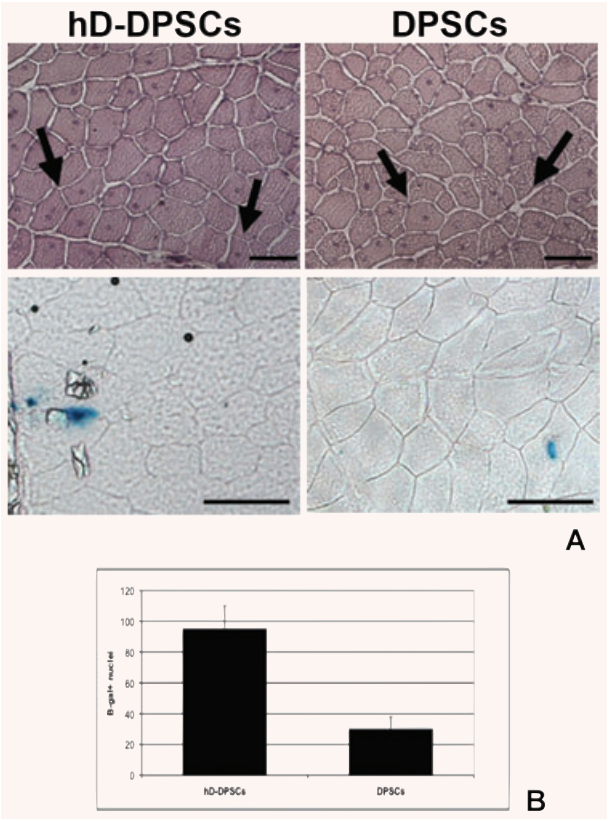


**Fig. 4** Human dental pulp cells transplanted *in vivo* for muscle regeneration. (**A**) Cryostat sections of injured SCID/Bg TA muscle 4 weeks after injection of hD-DPSCs and DPSCs. Haematoxylin and eosin staining shows the presence of regenerating centrally nucleated fibres (*arrows* highlighted), whereas X-Gal staining marks the presence of human dental pulp nuclei previously infected with nuc-LacZ lentivirus TA muscles were sectioned and all sections were scored for the presence of β-Gal$^{+}$ nuclei. (**B**) Transverse cryostat sections showed the presence of β-Gal$^{+}$ nuclei in regenerating muscle 95 ± 15 for hD-DPSCs and 30 ± 8 for DPSCs at 4 weeks after induction of muscle injury. The scale bars represent 50 μm.

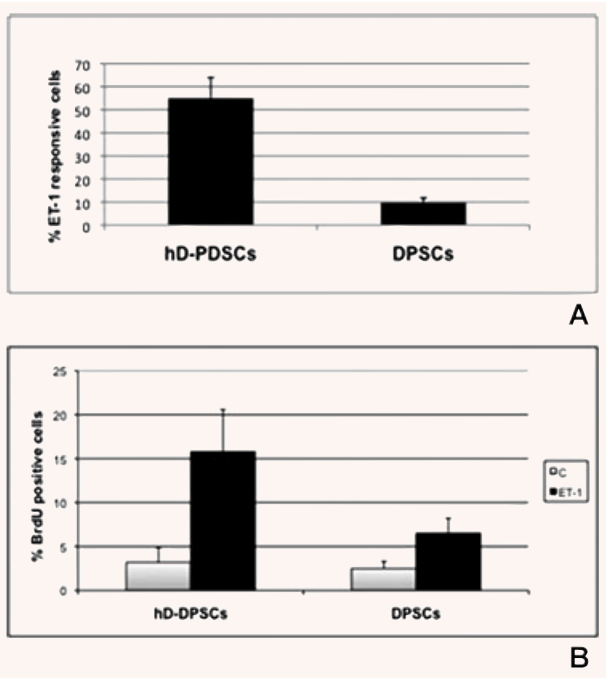


**Fig. 5** Proliferation and calcium mobilization induced by ET-1 of human dental pulp cells. (**A**) hD-DPSCs and DPSCs were incubated in α-MEM containing 3 mM Fura-2-AM. Intracellular $Ca^{+2}$-dependent fura-2 fluorescence is detected after local addition of 10 μl of ET-1 (1 μM). (**B**) Control and ET-1 treated hD-DPSCs and DPSCs were incubated with BrdU 10 μM for 45 min., then fixed and stained with monoclonal anti-BrdU FITC-conjugated antibody. Percentage of BrdU$^{+}$ cells was evaluated by counting for each experiment at least 250 cells.

produced osteonectin, type I collagen and BSP, thus confirming the presence of calcium deposits within the tissue, stressing the effectiveness of the mineralization process.

Chondrogenic differentiation has also been investigated and does not preclude the further development of an osteoblast-like phenotype *in vitro* [27]. We evaluated whether this could be effectively analysed by taking advantage of the histological dimension and the three-dimensional nature of the pellet culture system of hD-DPSCs. This culture method provides a relatively simple *in vitro* assay for testing the osteogenic capacity of putative osteogenic cells strains. Osteogenesis started simultaneously with chondrogenic differentiation and BSP and aggrecan were actively synthesized. The pellet culture of hD-DPSCs is generally taken as a good *in vitro* model of chondrogenesis, and could strike a balance between cell culture and *in vivo* transplantation in cytodifferentiation studies.

The present study highlights that hD-DPSCs are capable of differentiating into several other lineages and to switch their genetic programme to form muscle cells when co-cultured with murine myoblasts. We used differentiating mouse C2C12 myoblasts as an *in vitro* model of myogenesis to test the ability of different human dental pulp cells to fuse with myotubes. The co-culture of human dental pulp cells with C2C12 mouse myoblasts resulted in the contribution of human nuclei to mouse myotubes. This fusion requires cell–cell contact and resulted in the expression of the human MyoD muscle-specific transcription factor. Considering the heterogeneity of hD-DPSCs cultures, further studies are needed to identify the subpopulations of cells that are capable of fusion. In fact, the dental pulp contains fibroblasts, mesenchymal stem cells, neuronal and vascular cells, as well as cells of the immune system. The different degrees of commitment or origin of these cell populations leads to a degree of heterogeneity of the hD-DPSCs. For example, hD-DPSCs samples may contain cells of varying plasticity, i.e. different cell origins or cell populations (*e.g.* mesenchymal and endothelial cells); the latter can be either a problem/limitation or a further possibility of study.

Additionally, our results showed that hD-DPSCs were able to fuse to myoblasts *in vitro* with a higher efficiency than has been previously reported [17]. Given the ability with which hD-DPSCs cells can be expanded and transduced *in vitro*, they appear to be an attractive source of cells that can fuse *in vivo*. To test this possibility, we injected nuclear LacZ-labelled hD-DPSCs and DPSCs into damaged mouse TA muscles. Examination of these mice showed that we were able to detect them in regenerating muscle fibres, indicating the contribution of human cells to regenerating muscle. The frequency of fusion events was low, comparable with that reported for the detection of donor cells after stem cell transplantation. Moreover, the kinetics of differentiation of non-myogenic cells was different to that of the differentiation of committed adult myogenic precursors. This may suggest that hD-DPSCs undergone a longer, possibly multistep process. If this holds true, the minimal presence of hD-DPSCs could be explained with the healthy status of resident satellite cells in *scid/bg* mice. The situation might be substantially different in a dystrophic background characterized by chronic muscle degeneration, in which genetically corrected stem cells could progressively replace the exhausted pool of satellite cells.

Moreover, we challenged DPSCs with ET-1. Endothelins are 21-aminoacid peptides produced in the endothelium, whose main role is in vascular homeostasis as well as in nociception and local inflammation [28–30]. Immunohistochemical detection of the regulatory peptide ET in the endothelium of human dental pulp of developing and mature teeth provides evidence for local ET production and distribution in these tissues. Injection of ET caused vasoconstriction and decreased blood circulation in the pulp of dogs [31], thus demonstrating that receptors for ET also exist in the dental pulp. Although the possible role of ET in developing tissues is far from being clear, the mitogenic effects and the stimulation of proto-oncogenes expression induced by ET in some cells raise the possibility that this peptide might also play a role during tooth development [32, 33].

The perivascular origin of hD-DPSCs was also demonstrated by a consistent $Ca^{2+}$ release in response to ET-1 treatment. Moreover, we found that the response to ET-1 was higher in hD-DPSCs than in DPSCs, probably due to our specific isolation method that promoted perivascular release of stem/progenitor cells. It has been demonstrated that human bone marrow-derived mesenchymal stem cells are genuine perivascular cell precursors based on the response to ET-1 *in vivo* [34]. Our results showed that hD-DPSCs are also genuine perivascular cells exhibiting an increased proliferation rate after treatment with ET-1. The ability to isolate, expand and direct the differentiation of hD-DPSCs *in vitro* to different cell lineages offers an opportunity to study events associated with cell commitment and differentiation. Assay conditions required for efficient differentiation, basal nutrients, cell density, spatial organization, mechanical forces, growth factors and cytokines have a profound influence on hD-DPSCs differentiation. Those cells may produce autocrine and paracrine factors that are essential for lineage progression.

In conclusion, hD-DPSCs have a bigger ability than DPSCs to proliferate and differentiate into multiple cell types *in vitro*. Therefore, the culture and selective differentiation of hD-DPSCs should provide further understanding of dental pulp progenitors and their potential use for new therapeutic approaches in regenerative medicine.

## Conflict of interest statement

Authors declare that they do not have conflict of interest.

## Internal committee approval procedures

This study was approved by our Internal Committee. Patient's informed consent was obtained in a written form and signed. All the procedures were approved by the Internal Ethic Committee.

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee (Animal Ethic Committee).

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