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

L. Spath, V. Rotilio, M. Alessandrini, G. Gambara, L. De Angelis, M. Mancini, T. A. Mitsiadis, E. Vivarelli, F. Naro, A. Filippini, G. Papaccio

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

doi:10.1111/j.1582-4934.2009.00848.x
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 with a dentinal excavator or a Gracey curette. Pulp was gently removed and rinsed in Ca²⁺-, Mg²⁺-free phosphate-buffered solution (PBS) (Invitrogen, San Giuliano Milanese, Milan, Italy) and dissected into 1–2 mm³ 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% CO₂ 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 Ca²⁺-, Mg²⁺-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 10⁵ cells/explant) were seeded at a density of 5 × 10³ cells per cm² 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 × 10⁵/cm² 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 22nd 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 Cyochrome 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 × 10⁴/cm² in α-MEM complete medium (see above) supplemented with 20% FCS. Cells were incubated in αMEM 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 PO₄³⁻, 1.25 mM Ca²⁺ 0.81 mM Mg²⁺, 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 Metalfuor software (Universal Imaging Corporation, Downingtown, PA, USA). Intracellular Ca²⁺-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 Ca²⁺-dependent fura-2 fluorescence with 5 µM ionomycin, followed by recording minimal fluorescence in Ca²⁺-free medium. [Ca²⁺i] was calculated according to previously described formulas.

Lentiviral vector cell transduction

Lentivectors 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-CI-5-Br-3-indolyl-β-galactosidase (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgO2 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/cm² were placed in DMEM, with 10% FBS, 1% pen-strep, 0.1 μM dexamethasone (Sigma, Milan, Italy), 50 μM ascorbate-2-phosphate (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 × 10⁵ 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 lino 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% CO₂; 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/cm² 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 nitrate solution 0.1 M. Solution B was prepared by dissolving 1 ml of Naphth AS-MX phosphate alkaline solution in 48 ml distilled H₂O, 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 H₂O for 45 sec., added to alkaline dye mixture for 30 sec. in the dark, washed with distilled H₂O 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 MgSO₄) 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'-CAGTGTATCCGG-CACCTCCC-3', rev 5'-ACCAGGGAAGATCCCTTGCG-3'; human type I collagen for 5'-AGGGTCGCTCAAGAGATGASGAAGG-3'; rev 5'-TACAAGGAAG-GAACGGGCCAAGCTCG-3'; human bone sialoprotein (BSP) for 5'-CATTG-GAGAGGACGCGCAGCCTG-3'; rev 5'-CATACGCTCGTACGCTTGC-3'; human osteocalcin for 5'-ATGAGACCCCTACACTTCTC-3'; rev 5'-GCTAG-AAGGCGGCAGTATGGC-3'; human MyoD for 5'-GGATATACAGGGGCTCT-3'; rev 5'-GGGTTGGTACGCTGACCC-3'; human MyoD for 5'-ACCAACTGGAGACAGTGAG-3'; rev 5'-GGTCAAGATCTTCAGAG-3'; integrity and equal loading of cDNA in the PCR reactions were checked by quantification of β-actin mRNA levels. The amplified products were checked by quantitative PCR using human specific oligonucleotides for MyoD.
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 × 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 contralateral 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 (hDPSCs) showed a high proliferation rate, with approximately 25 population doublings and a doubling time of about 48 hrs (Fig. 1D). Cytofluorimetric analysis of hDPSCs 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 hDPSCs 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 hDPSCs 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 hDPSCs, 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-β1 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 hDPSCs to chondro-osteogenic phenotype (Fig. 2D) in a 3D structure.

**Multipotency 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 hDPSCs 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 nuclei.
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. 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).
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 x 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 x 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).
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-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...
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).

Acknowledgements

This study was funded by the Italian Project of relevant interest 2005 (to E.V.) and 2006 (to F.N.). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

References

1. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell. 2000; 100:157–68.

2. Graf T, Stadtfeld M. Heterogeneity of embryonic and adult stem cells. Cell Stem Cell. 2008; 3: 480–3.

3. Alison MR, Islam S. Attributes of adult stem cells. J Pathol. 2009; 217: 144–60.

4. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med. 2004; 8: 301–16.

5. Krause DS, Theise ND, Collector MI et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell. 2001; 105: 369–77.

6. Anderson DJ, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? Nat Med. 2001; 7: 393–5.

7. Zhou Q, Brown J, Kanarek A et al. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature. 2008; 455: 627–32.

8. Colnot C. Skeletal cell fate decisions within periosteum and bone marrow during bone

© 2009 The Authors
Journal compilation © 2010 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd
regeneration. J Bone Miner Res. 2009; 24: 274–82.
9. Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? Cell. 2001; 105: 829–41.
10. Bluteau J, Coquillart S, Payan Y et al. Haptic guidance improves the visuo-manual tracking of trajectories. PLoS ONE. 2008; 3:e1775.
11. Fuchs E, Segre JA. Stem cells: a new lease on life. Cell. 2000; 100: 143–55.
12. Mitsiadis TA, Barrandon O, Rochat A et al. Stem cell niches in mammals. Exp Cell Res. 2007; 313: 3377–85.
13. Gronthos S, Mankani M, Brahim J et al. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA. 2000; 97: 13625–30.
14. About I, Bottero MJ, de Denato P et al. Human dentin production in vitro. Exp Cell Res. 2000; 258: 33–41.
15. Buchaille R, Couble ML, Magloire H et al. Expression of the small leucine-rich proteoglycan osteoadherin/osteomodulin in human dental pulp and developing rat teeth. Bone. 2000; 27: 265–70.
16. Kettunen P, Karavanova I, Thesleff I. Responsiveness of developing dental tissues to fibroblast growth factors: expression of splicing alternatives of FGFR1, -2, -3, and of FGFR4; and stimulation of cell proliferation by FGF-2, -4, -6, and -9. Dev Genet. 1998; 22: 374–85.
17. d’Aquino R, Graziano A, Sampaolo M et al. Human postnatal dental pulp cells co-differentiate into osteoblasts and endothelialicytes: a pivotal synergy leading to adult bone tissue formation. Cell Death Differ. 2007; 14: 1162–71.
18. Graziano A, d’Aquino R, Laino G et al. Human CD34+ stem cells produce bone nodules in vivo. Cell Prolif. 2008; 41: 1–11.
19. Laino G, d’Aquino R, Graziano A et al. A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res. 2005; 20: 1394–402.
20. Lovschall H, Mitsiadis TA, Poulsen K et al. Coexpression of Notch3 and Rgs5 in the pericyte-vascular smooth muscle cell axis in response to pulp injury. Int J Dev Biol. 2007; 51: 715–21.
21. Sanes JR, Rubenstein JL, Nicolas JF. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. 1986; 5: 3133–42.
22. Ferrari G, Cusella-De Angelis G et al. Muscle regeneration by bone marrow–derived myogenic progenitors. Science. 1998; 279: 1528–30.
23. Laino G, Graziano A, d’Aquino R et al. An approachable human adult stem cell source for hard-tissue engineering. J Cell Physiol. 2006; 206: 693–701.
24. Mastrogiaco M, Cancetta E, Quarto R. Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells. Osteoarthris Cartil. 2001; 9: S36–40.
25. Sekiya I, Colter DC, Prokop DJ. BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. Biochem Biophys Res Commun. 2001; 284: 411–8.
26. Miura M, Gronthos S, Zhao M et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA. 2003; 100: 5807–12.
27. Galotto M, Campanile G, Robino G et al. Hypertrophic chondrocytes undergo further differentiation to osteoblast-like cells and participate in the initial bone formation in developing chick embryo. J Bone Miner Res. 1994; 9: 1239–49.
28. Griswold DE, Douglas SA, Martin LD et al. Targeted disruption of the endothelin-B-receptor gene attenuates inflammatory nociception and cutaneous inflammation in mice. J Cardiovasc Pharmacol. 2000; 36: S78–81.
29. Pomonis JD, Rogers SD, Peters CM et al. Expression and localization of endothelin receptors: implications for the involvement of peripheral glia in nociception. J Neurosci. 2001; 21: 999–1006.
30. Hirata Y, Ishimaru S. Effects of endothelin receptor antagonists on endothelin-1 and inducible nitric oxide synthase genes in a rat endotoxic shock model. Clin Sci. 2002; 103: 332S–5S.
31. Gilbert TM, Pashley DH, Anderson RW. Response of pulpal blood flow to intra-arterial infusion of endothelin. J Endod. 1992; 18: 228–31.
32. Casasco A, Casasco M, Ciuffreda M et al. Immunohistochemical evidence for the occurrence of endothelin in the vascular endothelium of normal and inflamed human dental pulp. J Dent Res. 1992; 71: 475–7.
33. Neuhaus SJ, Byers MR. Endothelin receptors and endothelin-1 in developing rat teeth. Arch Oral Biol. 2007; 52: 655–62.
34. Au P, Tam J, Fukumura D et al. Bone marrow–derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. Blood. 2008; 111: 4551–8.