

HUMAN NEURAL CREST-DERIVED POSTNATAL CELLS EXHIBIT REMARKABLE EMBRYONIC ATTRIBUTES EITHER *IN VITRO* OR *IN VIVO*[§]

Riccardo d'Aquino^{1#}, Virginia Tirino^{1#}, Vincenzo Desiderio^{1#}, Michèle Studer², Gabriella Cusella De Angelis³, Luigi Laino⁴, Alfredo De Rosa⁴, Diego Di Nucci², Sabata Martino⁵, Francesca Paino¹, Maurilio Sampaolesi^{3,6} and Gianpaolo Papaccio^{1*}

¹Department of Experimental Medicine, Section of Histology, Tissue Engineering and Regenerative Medicine Division, (TERM), Second University of Naples, Naples, Italy

²Telethon Institute of Genetics and Medicine (TIGEM), Developmental Disorders Program, Via P. Castellino 111, Naples, Italy

³Department of Experimental Medicine, Section of Human Anatomy, University of Pavia, Pavia, Italy

⁴Department of Dentistry, Oral Biology and Surgery, Second University of Naples, Naples, Italy

⁵Department of Experimental Medicine and Biochemical Science, University of Perugia, Via del Giochetto, 06126 Perugia, Italy

⁶Interdepartmental Stem Cell Institute, Catholic University of Leuven, O&N I Herestraat 49, Leuven, Belgium

[#]These authors contributed equally to this study.

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Abstract

During human embryonic development, odontogenic tissues, deriving from the neural crest, remain undifferentiated until the adult age. This study was aimed at characterising the cells of the follicle enveloping the dental germ, due to its direct origin from neural crests. Sixty dental follicles were collected from patients aged 18 to 45 years. This research has clarified that dental follicles, if extracted in a very early stage, when dental roots did not start to be formed, contain a lineage of cells, characterised by a high degree of plasticity in comparison with other adult stem cell populations. In particular, we found that these cells share the following features with ES: (i) high levels of embryonic stem cell markers (CD90, TRA1-60, TRA1-81, OCT-4, CD133, and SSEA-4); (ii) mRNA transcripts for Nanog and Rex-1; (iii) broader potency, being able to differentiate in cell types of all three germ layer, including smooth and skeletal muscle, osteoblasts, neurons, glial cells, and adipocytes; (iv) high levels of telomerase activity; (v) ability to form embryoid bodies; (vi) ability, after injection in murine blastocysts, to be localised within the inner cell mass; (vii) no teratoma formation after injection; (viii) *in vivo* tissue formation after transplantation. Our results demonstrate that these cells represent a very easy accessible and extraordinary source of pluripotent cells and point out the fact that they own the cardinal feature of embryonic stem cells.

Keywords: Human adult stem cells, neural crest, embryonic markers, differentiation, pluripotency, embryoid bodies.

The authors declare no potential conflicts of interest.

*Address for correspondence:

Gianpaolo Papaccio
Department of Experimental Medicine
Section of Histology, TERM Laboratory
2nd University of Naples
via L. Armanni 5, I- 80138 Naples, Italy
Telephone Number : +39 081-5666014
FAX Number: +39 081-5666015
E-mail: gianpaolo.papaccio@unina2.it

Introduction

During human embryonic development, neural crest cells migrate from the most dorsal region of the neural tube by day 30 to the head, neck, and other regions of the body. In the craniofacial region, these cells penetrate the mesenchyme, reinforcing it, and then differentiate into cartilage, bone, neurons, glial cells, and connective tissues of the cranium and face. In the trunk region, these cells differentiate into peripheral neurons and muscular-connective tissue of large arteries. In addition, the extracellular matrix exerts an important signalling cue, acting as an epigenetic guide of gene expression. Therefore, neural crest cells are *strictu sensu* pluripotent cells (Betters *et al.*, 2010; O'Rahilly and Muller, 2007). However, to date they have been isolated only from embryonic tissues at an early stage of development (Zhou and Snead, 2008).

Embryologically, teeth are ectodermal organs originating from sequential reciprocal interactions between oral epithelial cells (ectoderm) and cranial neural crest-derived mesenchymal cells (Chai *et al.*, 2000; Imai *et al.*, 1996). The epithelial cells give rise to enamel forming ameloblasts, and the mesenchymal cells form all other differentiated cells (e.g., dentine forming odontoblasts, pulp, periodontal ligament) (Volponi *et al.*, 2010). Among the transient structures of teeth, the dental follicle that is a loose ectomesenchyme-derived connective tissue sac surrounding the enamel organ and the dental papilla of the developing tooth germ before eruption, it is known, up to now, that it contains progenitor cells for cementoblasts, periodontal ligament (PDL) and osteoblasts (Volponi *et al.*, 2010).

This led us to search for an adult source of neural crest cells. On the assumption that the craniofacial region of the adult body largely originates from these cells, we focused on this area. We decided to study the dental sac as a potential source of these cells because of the clinical observation of the persistence during adult life of the dental follicle, which envelops the crown of impacted teeth.

In this study, we provide evidence that the dental follicle comprises cells having all the characteristics of neural crest cells. The follicle of impacted teeth sealed within maxillary and mandible bones can be collected in humans with a simple surgical procedure, and their cells quickly selected and cultured *in vitro*.

We show that Dental Follicle neural crest-derived cells (DFCs) constitute an embryonic-like stem cell population found within the adult, which co-express early neural progenitor markers and vascular endothelial growth factor receptors, including Brn3a and flk-1, and retain embryonic markers and transcription factors, such as Oct-4, Nanog, TRA1-60, and TRA-1-80-1.

All these markers, according to National Institute of Health (NIH) guidelines, are specific for human embryonic stem cells. After challenging them with differentiation cues, DFCs easily and efficiently gave rise to neurons, osteoblasts, adipocytes, and other cytotypes; thus, they have the same pluripotency as neural crest cells (Lin *et al.*, 2006). Due to their embryonic features, which are assessed in this study and include, among others, (i) the ability to form embryonic bodies; (ii) integration into the inner cell mass of blastocysts; (iii) pluripotency both *in vitro* and *in vivo*, and (iv) a very high level of telomerase activity, the clinical use of these cells may advance reconstruction therapy.

Materials and Methods

Subjects, dental follicle extraction, digestion, and culture Sixty early stage dental follicles, extracted from dental germs in which the roots were completely absent, were collected during wisdom tooth extraction from patients aged 18 to 45. Each patient was checked for systemic and oral infection and diseases before extraction. Only disease-free patients were selected for follicle collection. Each follicle was gently removed and dipped in a digestive solution (penicillin 100 U/mL/streptomycin 100 µg/mL, claritromycin 500 µg/mL, 3 mg/mL type I collagenase, 4 mg/mL dispase in PBS (phosphate buffered saline) for 1 h at 37 °C. Once digested, the solution was filtered with 70 µm Falcon strainers (Becton & Dickinson (BD), Franklin Lakes, NJ, USA) and the cells immersed in MEGACELL culture medium, supplemented with 10 % foetal calf serum (FCS), bFGF 2 µM, 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (pen-strep) (all purchased from Invitrogen, San Giuliano Milanese, Milan, Italy) and placed in 75 mL flasks with filtered valves. Flasks were incubated at 37 °C in a 5 % CO₂ and the medium changed twice a week. As soon as cells became confluent, they were subdivided into new flasks.

Stem cells were sorted for SSEA-4 antigen at passage 5, using a FACS Aria II BD (BD Biosciences) when their number was at least 1,000,000 per flask. Human gingival fibroblasts were used as controls.

Colony efficiency assays and proliferation potential

To evaluate colony efficiency and proliferation potential, single cells obtained by limiting dilutions were plated.

Clone and cell numbers were evaluated. After three weeks of culture, cells were stained with 0.1 % (w/v) toluidine blue in 1 % paraformaldehyde. The number of clones (>50 cells) was counted.

Fluorescence-activated cell sorting

At least 1,000,000 cells per sample were detached using 0.02 % EDTA solution in PBS and pelleted (10 min at 1,000rpm), washed in 0.1 % bovine serum albumin (BSA) in PBS at 4 °C, then incubated in a solution of 1 µL antibody/9 µL 0.1 % BSA in PBS. Cells were then washed in the same solution (see above) and were ready for observation. Antibodies were the following: SSEA-4, TRA1-60, TRA1-81, CD90, CD133, OCT-4, flk-1 (all Santa Cruz, Santa Cruz, CA, USA), Tuj1, Brn3A, SMA (BD Biosciences), all mouse anti-human. For Tuj1, Brn3A, SMA and OCT-4 analyses, cells were fixed in 4 % paraformaldehyde in PBS, with 0.2 % Triton X100 for 30 min at 4 °C, washed twice in 0.1 % BSA in PBS and then incubated with antibody.

Reverse transcriptase polymerase chain reaction (PCR) analysis

Total RNA was extracted from 1,000,000 non-sorted cells for each analysis by homogenisation in TRI Reagent (Sigma-Aldrich, Milan, Italy), following the manufacturer's instructions, incubated with DNase (Takara Bio Ink, Shiga, Japan) for 1 h at 37 °C and stored at -80 °C. cDNA synthesis was carried out from total RNA using Superscript II reverse transcriptase (Invitrogen Celbio Italy, San Giuliano Milanese, Milan, Italy) and oligo (dT)₁₂₋₁₈, following the manufacturer's instructions.

PCR analyses were performed using a TC-312 thermal cycler (Techne, Burlington, NJ, USA), under the following conditions: 2 min denaturing step to 94 °C, 35 cycles of 94 °C for 30 s, 54 °C for 45 s, 72 °C for 1 min and a final extension step at 72 °C for 4 min. The PCR mixture contained 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.2 µM of each primer. The primer sequences were: forward *Rex-1* 5'-GGCAAAGACAAGACACCAG-32; and reverse 52-TTTCCCACATTCCGCA CAG-32; *Nanog* primers 5'-GCTGAGATGCCTCACACGGAG-3' (forward) and 5'-TCTGTTTCTTGACTGGGACCTTGTC-3' (reverse); β-actin 5'-TGT GAT GGT GGG AAT GGG TCA G-3'; reverse β-actin 5'-TTT GAT GTC ACG CAC GAT TTC C-3'. The PCR annealing was performed at 54 °C as the β-actin primers are from mouse and contain some mismatch to human. This can be overcome for qualitative analysis of human samples by using a reduced annealing temperature. The amplification products were separated on 2 % agarose gel in Tris acetate EDTA (TAE) buffer. PCRs were performed on RT-negative samples to exclude DNA contamination.

Histochemistry

Fifteen days after isolation, cells were challenged for alkaline phosphatase production (ALP). Cells were washed in PBS 1M and fixed in 4 % paraformaldehyde in PBS, with 0.2 % Triton X100 for 30 min at 4 °C, then washed twice in 0.1 % BSA in PBS at room temperature for 10 min each. Cells were covered using ALP standard

solution, incubated in the dark for 8 h. ALP activity was measured using 100,000 cells/sample, detached by PBS/EDTA 0.02 %, and centrifuged for 10 min at 140 g. The pellet was incubated with 1 mL of BMPurple solution (Roche, Segrate, Milan, Italy) for 8 h in dark. Supernatant was read in a spectrophotometer at 615 nm. As control, gingival fibroblasts were used. The values were expressed as a ratio between sample and BMPurple stock solution. BMPurple solvent was used as blank.

Sphere cluster formation (embryoid bodies)

In order to observe if dental follicle cells were able to form embryoid bodies, cells were plated at a density of 60,000 cells/well in 6-well ultra low attachment plates (Corning Inc., Corning, NY, USA) in Mega cell medium supplemented with 1 % methylcellulose, progesterone (10 nM), putrescine (50 μ M), sodium selenite (15 nM), transferrin (13 μ g/mL), insulin (10 μ g/mL; Sigma-Aldrich), human EGF (10 ng/mL), and human bFGF (10 ng/mL; Sigma). Fresh aliquots of bFGF were added every other day. After culture for 48-72 h, spheres were visible under inverted phase-contrast microscopy (Nikon TS 100, Nikon, Tokyo, Japan).

Telomeric repeat amplification and quantification protocol (TRAPeze ELISA)

Assays were performed using the TRAPeze ELISA Telomerase detection Kit (MP QBiogen, Irvine, CA, USA), according to the manufacturer's instructions. In brief, cell extracts were incubated with biotinylated telomerase substrate oligonucleotide at 30 °C for 30 min. The extended products were amplified by PCR using Taq polymerase (Amersham Biosciences, Orsay, France), biotinylated telomerase substrate oligonucleotide, reverse primers, and a deoxynucleotide mix containing dCTP labelled with dinitrophenyl. The PCR conditions were: 33 cycles of 94 °C for 30 s and 55 °C for 30 s on a GeneAmpPCR System 9700 thermocycler (Applied Biosystems, Courtaboeuf, France). The amplification products were immobilised on streptavidin-coated microtiter plates and then detected by antidinitrophenyl antibody conjugated to horseradish peroxidase. After addition of peroxidase substrate (3,3',5,5'-tetramethylbenzidine), the amount of TRAP products was determined by measuring the absorbance at 450 and 690 nm. To confirm the results of the ELISA, amplified products were resolved by 12.5 % nondenaturing. Gels were stained with SYBRgreen (Invitrogen) (dilution 1/10,000), and PCR amplification products were visualised under UV light. Furthermore, because telomerase has an essential RNA component, aliquots of samples were treated with RNAase to assess the specificity of the reaction. Telomerase activity was measured twice on protein samples obtained from each cell fraction from two independent preparations. To compare the different stem cell populations in the same experiment, assays were performed on 1,800 sorted cells to be in the semiquantitative conditions (OD values between 0.2 and 1.5). Data are means of four different experiments. Data were statistically compared by *t* tests. The protein concentration was determined using the QuantiPro BCA

assay kit (Sigma-Aldrich) and BSA as a standard. The tested samples were the following: i. HeLa, cancer cell line used as positive control; ii. cells positive and negative for SSEA-4 after sorting (day 0); and iii. cells positive and negative for SSEA-4 after 18 and 46 d of adipogenic differentiation.

Injection of DFCs in murine blastocysts

SSEA-4⁺ cells were selected from dental follicles of healthy subjects. The use of human DFC cells for injection into murine blastocysts was approved by the Internal Ethic Committee. Green fluorescent protein (GFP) cells were obtained after infection of SSEA-4⁺ cells with lentivirus. The presence of human-specific DNA within the blood and organs of mouse embryos was evaluated by PCR amplifying an 850-bp fragment of the α -satellite region of the human chromosome 17, using primers corresponding to the primer pair 17a1/17a2 as described by Warburton *et al.* (1991).

Ten murine blastocysts were isolated from donor C57BL/6 on day 3.5 of gestation, and 40 SSEA-4⁺/GFP⁺ cells were injected into each blastocyst. After 24 h, 48 h and 72 h, blastocysts were observed at phase contrast using fluorescent light microscopy.

In vivo teratoma formation

NOD/SCID mice (genotype NOD/NCrCrl-Prkdcscid) were obtained from Charles River Italia (Calco, Lecco, Italy) and housed in the single-barrier animal facility of the Faculty of Medicine, University of Pavia. Mice were fed *ad libitum* with a standard diet and water. In order to assess the capability of DFC to form teratomas *in vitro*, 5,000,000 cells were injected in each of 12 NOD-SCID mice into the skin fold of the inner thigh, using a 27 gauge syringe, of four mice per group. After 2-3 weeks, animals were sacrificed and emerging tissue material was dissected. Tissue was fixed in formalin and embedded in paraffin. Sections were stained in haematoxylin-eosin according to standard procedures. This examination was performed according to our internal ethic committee.

In vitro differentiation

Osteoblasts and bone

To differentiate cells into osteoblasts and then in a bone tissue, we used α -MEM culture medium supplemented of a 20 % foetal bovine serum (FBS), 1 % pen-strep, dexamethasone (0.1 μ M, Sigma-Aldrich), ascorbate-2-phosphate (50 μ M, Sigma-Aldrich), and β -glycerophosphate (100 μ M, Sigma-Aldrich). Flasks were incubated at 37 °C under 5 % CO₂, the medium changed twice a week, and cells were not split for 5 weeks.

As we have previously shown (Laino *et al.*, 2005, 2006), extended culture in high percentage FBS stimulates differentiation. This, together with the long period during which cells were not split, to favour mechanical stability and extracellular matrix deposition, helps osteoblastic differentiation and bone formation. After differentiation, cells were challenged for bone markers including RUNX-2 and OC at FACScanning; and OC, BAP, BSP, collagen type 1, and RUNX-2 at the immunofluorescence.

Skeletal muscle

To stimulate myogenic differentiation, sorted cells were co-cultured with C2C12, a mouse myogenic cell line, and tested for their capacity to fuse with C2C12 and participate in new myofibre formation. Human stem cells were cocultured with C2C12 (1:5) in ATCC medium: Dulbecco's modified Eagle's medium (DMEM, Invitrogen), with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 1.0 mM sodium pyruvate, 90 %, permissive for myogenic differentiation, added of 10 % FCS, for one week and fixed with 4 % paraformaldehyde. Double immunofluorescence was performed with anti-myosin antibody (rabbit anti-mouse, Santa Cruz) and an anti-human nuclear lamins A and C monoclonal antibody (Novocastra, Newcastle, U.K.).

Smooth muscle

To obtain smooth muscle differentiation, cells were placed in MEGACELL medium (see above) with 2 % FBS and 10 ng/mL transforming growth factor (TGF) β . Smooth muscle differentiation was obtained in 4-5 d either spontaneously or with this technique. After differentiation, cells were challenged for SMA antigen.

Adipocytes

To obtain adipocytes, the culture medium was supplemented with 10 % FBS, dexamethasone (1 μ M, Sigma-Aldrich), recombinant human insulin (10 μ M, Sigma-Aldrich), indomethacin (200 μ M, Sigma-Aldrich), and 3-isobutyl-1-methyl-xanthine (IBMX, 0.5 mM, Sigma) twice a week for six weeks. In order to identify lipid-laden fat cells, immunohistochemical analyses were performed with a DAKO CYTOMATION kit (En Vision + System-HRP-AEC, Dako Italia, Milan, Italy) according to the manufacturer's protocol. Antibodies used were anti-adiponectin and anti-PPAR- γ (Peroxisome proliferator-activated receptor- γ) (purchased from AbCam, Cambridge, UK).

Astrocytes, oligodendrocytes and neurons

1,000 DFCs (third passage) were plated onto Lab-Tek Chamber-Slides (Nalge Nunc International, Rochester, NY, USA) and incubated in normal growth medium: MEGACELL (Sigma-Aldrich) with 2 % foetal bovine serum (Euro Clone, Sizzano, PV, Italy), 10 ng/mL bFGF, Ascorbic acid 0.005 g/250 mL (Sigma-Aldrich), 1 % L-Glutamine and 1 % Penicillin-Streptomycin (Euro Clone). Cells were maintained at 37 °C in humidified 95 % air/5 % CO₂. After 24 h we substituted the MEGACELL medium with DMEM (Euro Clone) supplemented with different trophic factors to induce neuronal differentiation. Brain Derived Growth Factor (Sigma-Aldrich) 50 ng/mL, Platelet Derived Growth Factor-AA 10 ng/mL (Sigma-Aldrich), and 135 ng/mL basic Fibroblast Growth Factor (BD Biosciences) were administered singularly to the cells. Control cells were incubated in DMEM alone. Medium was replaced every 3 d for 14 d.

After differentiation, cultures were fixed with 4 % paraformaldehyde in pH 7.4 PBS for 30 min at room temperature. After three washes, fixed cells were incubated with blocking solution containing 10 % FBS and 0.1 %

TritonX-100 (Sigma-Aldrich) in PBS for 1 h at room temperature.

Primary antibodies were added to 1 % FBS and 0.01 % TritonX-100 in PBS at 4 °C overnight and included the followings: mouse monoclonal-anti-Map2; -anti-Map5, -anti beta III tubulin Tuj1; anti sulphatide O4; -anti glial fibrillary acid protein (GFAP) (Dako, Milan, Italy); rabbit monoclonal anti chondroitin sulphate proteoglycan Ng2 (Dako). The secondary antibodies were FITC-conjugated Donkey anti-rabbit IgG 488 nm and rhodamine-conjugated goat anti-mouse IgG 594 nm (Molecular Probes, Eugene, OR, USA).

Slides were fixed using Vectashield with DAPI (Vector Laboratories, DBA Italia, Segrade, Italy) to counter-stain nuclei. As internal control, untreated DSCs were fixed and labelled with the same antibodies as above described.

All the fluorescence signals were analysed with a Nikon TE 2000S microscope at excitation/emission wavelengths of 540/565 nm (TRITC, red), 450/505 nm (FITC, green), and 340/400 nm (DAPI, blue). Images were acquired with the Olympus F View II camera and the Olympus Cell^F software (Olympus Italia, Segrade, Milan, Italy). Experiments were made in quadruplicate.

In vivo transplantation

After *in vitro* differentiation, we performed transplantation experiments *in vivo*, designed to observe if the obtained differentiation *in vitro* was, at least, maintained *in vivo*. To this aim, SSEA-4 sorted DFC pellets were re-suspended in 0.3 mL culture medium and layered onto Gingostat (Gaba Vevas, San Giuliano Milanese, Italy) a commercial type I collagen, made of native lyophilised collagen from animal origin (equine). Collagen sponges were cut in 10x10 mm squared pieces and then sterilised under UV exposure within Petri dishes, and incubated at 37 °C with 5 % CO₂ for 30 min to allow colonisation. Plating efficiency assay was performed counting free-floating cells 30 min after plating.

Cells were placed onto the same scaffolds and used as controls. Scaffolds colonised with DFC cells were transplanted in subcutaneous pockets on the back of 12 nude mice (NU/NU, Charles River). Animals were sacrificed 30 or 60 d after transplantation. Procedures were approved by our internal small animal ethics committee. After each sacrifice the transplant area was dissected and used for normal histology as well as for immunofluorescence in order to demonstrate the human origin of the tissue (HLA antibody, Santa Cruz). Experiments were made in quadruplicates.

Statistical analysis

Student *t*-test (two-tailed) was used for statistical evaluation. Level of significance was set at $p < 0.05$.

Results

Dental follicle culture

We obtained 53 primary cell lines from sixty tissue specimens cultured in MEGACELL at 10 % FBS and frozen in 1 mL FBS/10 % DMSO under liquid nitrogen. These lines were capable of growing up to 23 culture

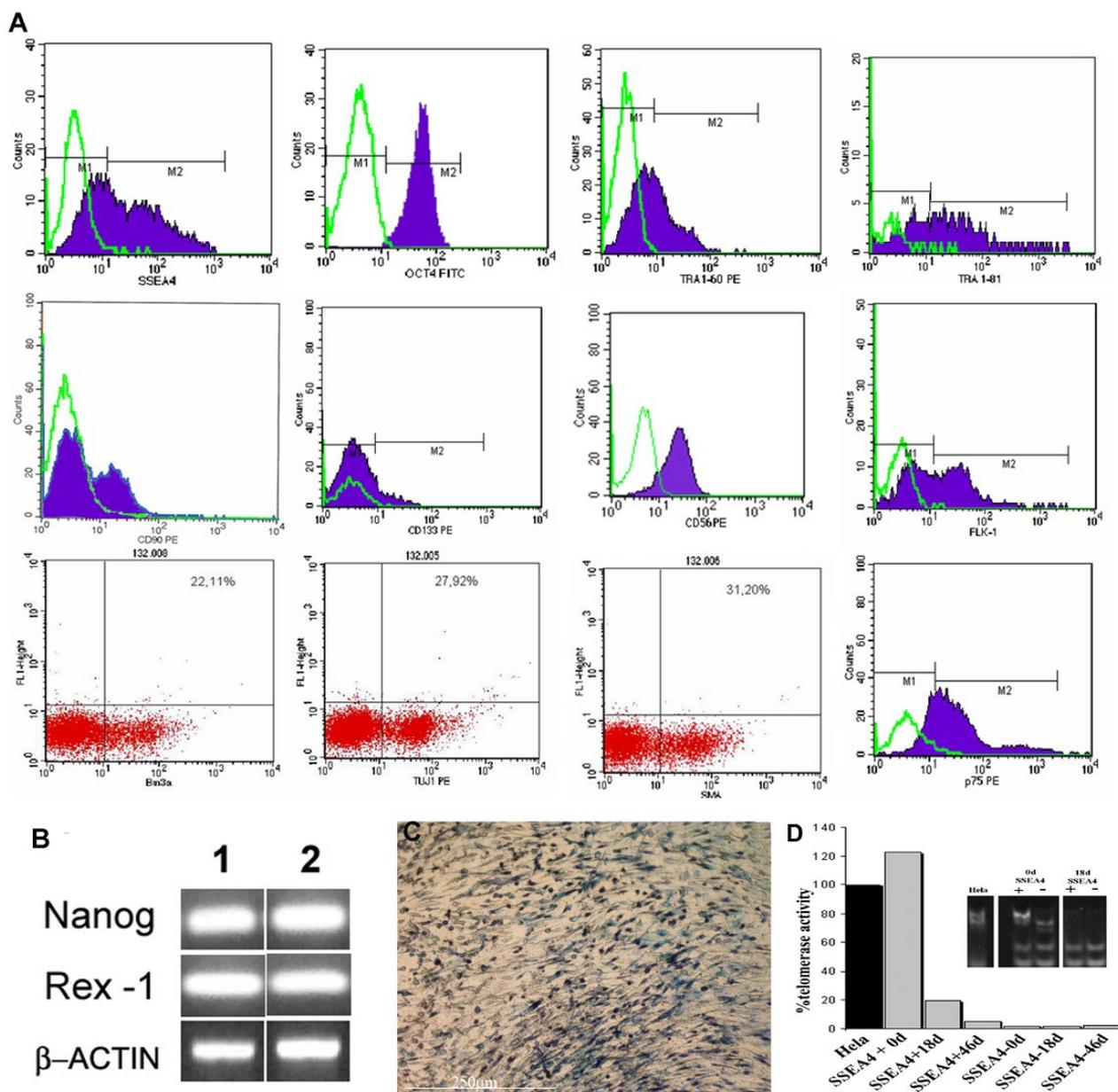


Fig. 1. (A) Fluorescence activated analysis of dental follicle cells (DFCs) shows a strong positivity for SSEA-4, OCT-4, TRA 1-60, TRA 1-81, CD 90, CD133, CD56, flk-1, Brn3a, Tuj1, SMA and p75; (B) PCR analysis for Nanog and REX-1; (C) ALP histochemical assay. The whole population is clearly positive (blue staining). (scale bar = 250 μ m); (D) The telomerase activity of DFCs (SSEA-4⁺sorted cells) shows that SSEA-4⁺ DFCs, sorted at day 0, display high levels of this activity compared to DFCs differentiated in adipogenic medium for 18 d (shown in both gel and histogram) and for 46 d (shown in the histogram).

passages. All the other cell samples were considered “early passages” because the cells become senescent between the 2nd and 4th culture passage. All cell lines showed cells with fibroblast shape. Any differences in terms of growth and morphology were detected to different passages of culture. All experiments were performed at 5th culture passage. At this passage, we did not observe variations on the pluripotent potential in term of gender and age of donors, also because we used only follicles from germs in which dental roots were completely absent.

Antigenic characterisation

Fluorescence activated cell assays, performed at day 15 after cell selection, showed that follicle cells expressed high

levels of positivity for SSEA-4, OCT-4, TRA1-60, TRA1-81, and flk-1 antigens, which are all markers of embryonic stem cells. The percentage of positivity was about 80 % for each embryonic marker (Fig. 1A). In addition, cells were positive for CD90 and CD133 antigens, although at lower percentages. Then we challenged follicle cells for neural crest cell markers, including CD56, Brn3a, Tuj1, and SMA. In this case, the percentage of positivity was about 30 % (Fig. 1A). Conversely, positivity for differentiated neuron antigens, including p75, was very low (Fig. 1A).

Furthermore, with RT-PCR, we found that these cells expressed high levels of mRNA for embryonic stem cell transcription factors, including Nanog and Rex-1 (Fig. 1B).

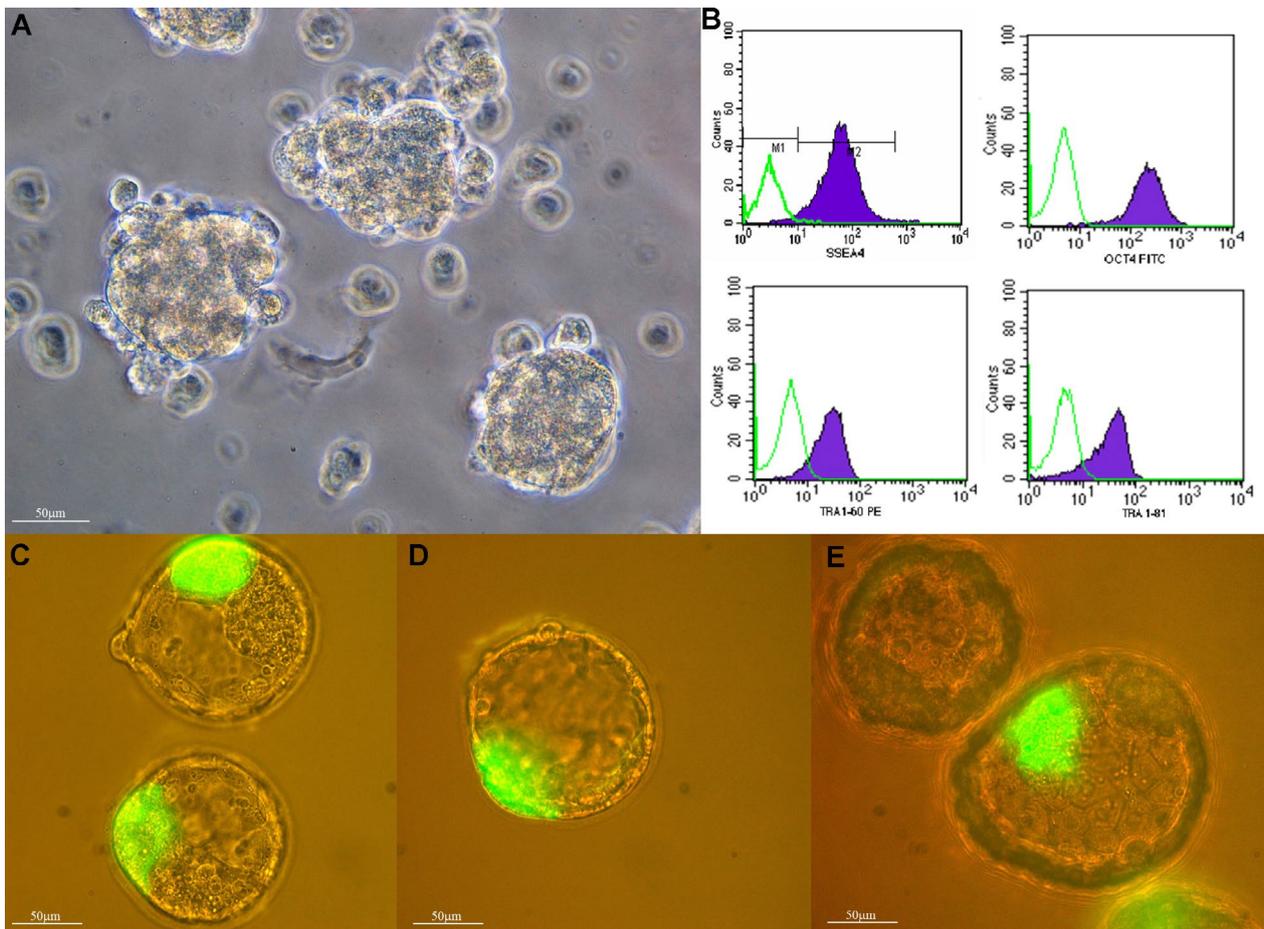


Fig. 2. (A) SSEA-4⁺ cells forming spheres *in vitro* (scale bar=50 μ m); (B) Fluorescence activated cell sorter analysis on DFCs forming spheres showing an average positivity of 99% for SSEA-4, OCT-4, TRA 1-60 and TRA 1-81. (C) Blastocyst injection of GFP⁺/DFCs. Fluorescent plus contrast phase microscopy observation reveals the presence of fluorescent cells within the inner cell mass, 24 h after injection (scale bar = 50 μ m); (D) 48 h after injection (scale bar = 50 μ m); and (E) 72 h after injection (scale bar = 50 μ m).

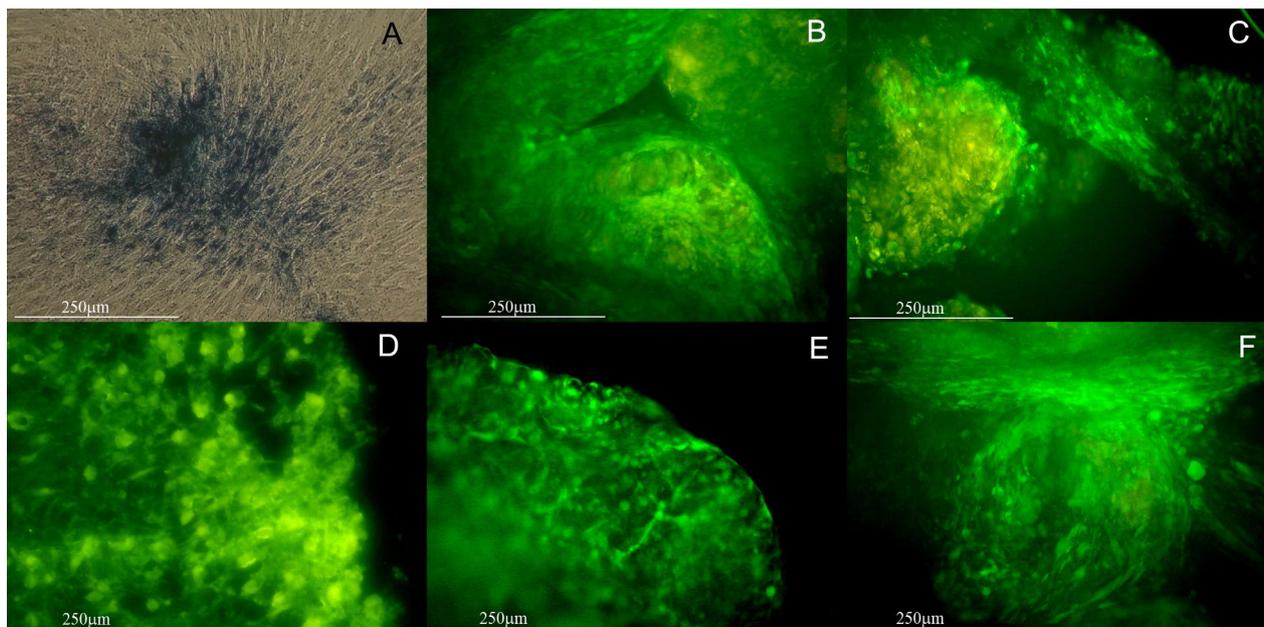


Fig. 3. Bone differentiation of DFCs: (A) Histochemical assay indicates that differentiated cells were positive for ALP (scale bar = 250 μ m); (B) Representative image of Osteocalcin positivity (scale bar = 250 μ m); (C) Representative image of Bone Alkaline Phosphatase (BAP) positivity (scale bar = 250 μ m); (D) Representative image of Bone SialoProtein (BSP) positivity (scale bar = 250 μ m); (E) Representative image of Type I Collagen positivity (scale bar = 250 μ m); (F) Representative image of Runx-2 positivity (scale bar = 250 μ m).

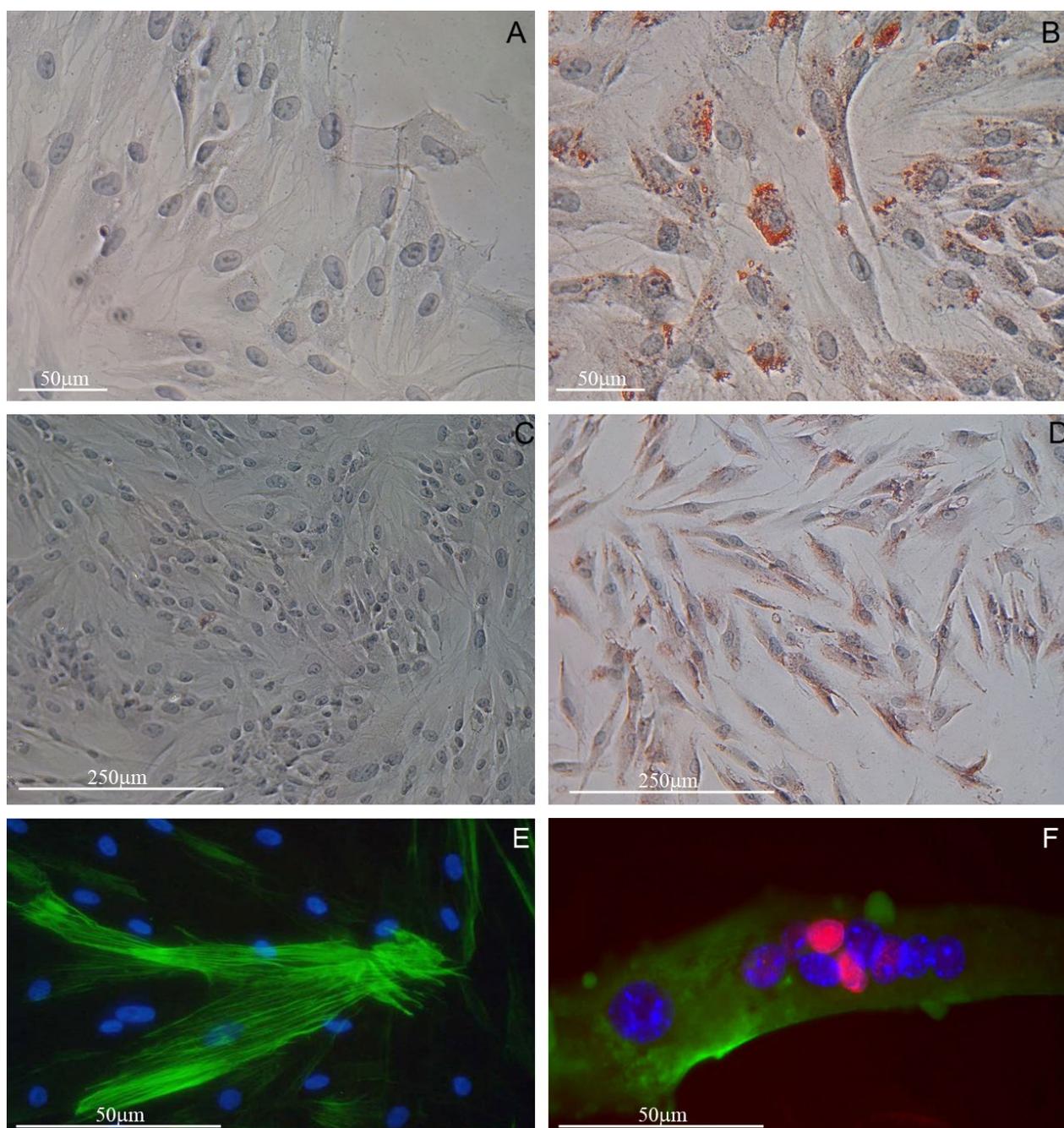


Fig. 4. Image showing adipogenic, myogenic and smooth muscle differentiation of DFCs: **(A)** Control of non-differentiated cells for PPAR γ (scale bar = 50 μ m); **(B)** DFC-derived adipocytes immunostained for PPAR γ in red (scale bar = 50 μ m); **(C)** Control of non-differentiated cells for Adiponectin (scale bar = 250 μ m); **(D)** DFC-derived adipocytes immunostained for Adiponectin in red (scale bar = 250 μ m); **(E)** smooth muscle differentiation of DFCs. Image representative of SMA positivity (scale bar = 50 μ m); **(F)** myogenic differentiation. DFCs were co-cultured with mouse C2C12 myotubes. Myosin-positive fibres are marked with a polyclonal anti-myosin (FITC), human nuclei are marked with a nuclear antilamin A and C (TRITC). Two human nuclei, in red, within a myotube demonstrate cell fusion (scale bar = 50 μ m).

At this stage, cells were also markedly positive for ALP: actually, positivity within the flask was diffuse in the whole cell layer (Fig. 1C).

Telomerase activity

Having found all the previous shown embryonic features, we decided to perform assays in order to observe follicle cell telomerase activity, which is a commonly used parameter showing the lifespan of a cell and which is highly expressed in embryonic stem cells. We measured

telomeres with the TRAPeze ELISA detection Kit, and we found a considerably high level in SSEA-4⁺ sorted follicle cells compared to the control (Fig. 1D). The assay was performed using the same cell population observed at a different time of differentiation (18 and 46 d) induced by adipogenic medium. As shown in this study, the rate of telomerase activity is considerably high in SSEA-4⁺ cells at day 0 and significantly decreases in SSEA-4⁺ cells after 18 and 46 d of adipogenic differentiation. This observation is important because this high level of telomerase activity

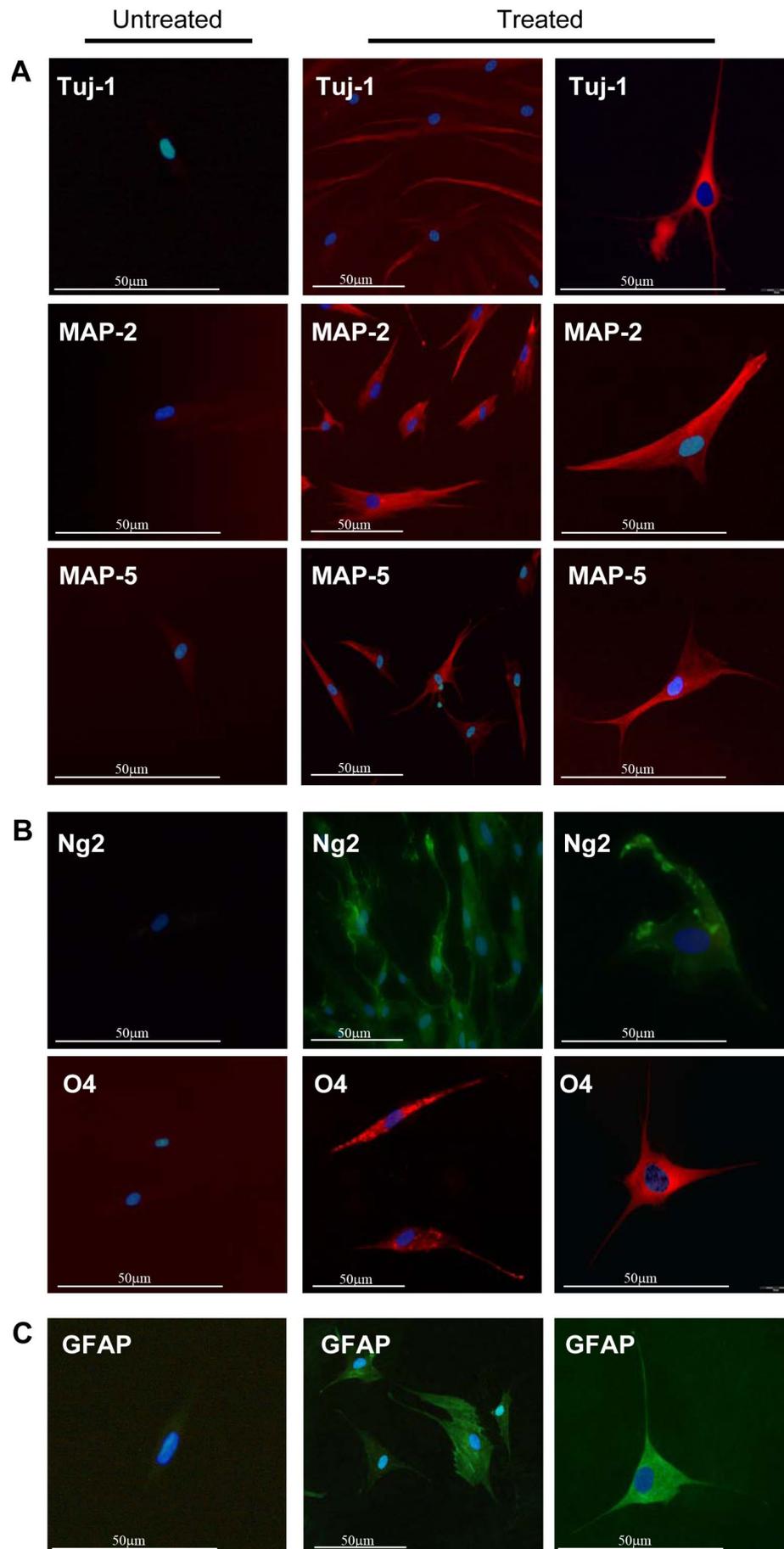


Fig. 5. Image showing neural differentiation of DFCs. **(A)** TUJ1, MAP-2, MAP-5, neuronal differentiation by BDNF (scale bar = 50 μ m); **(B)** NG2, O4, oligodendrocyte differentiation by PDGFAA (scale bar = 50 μ m); **(C)** GFAP, astrocyte differentiation by FGF (scale bar = 50 μ m).

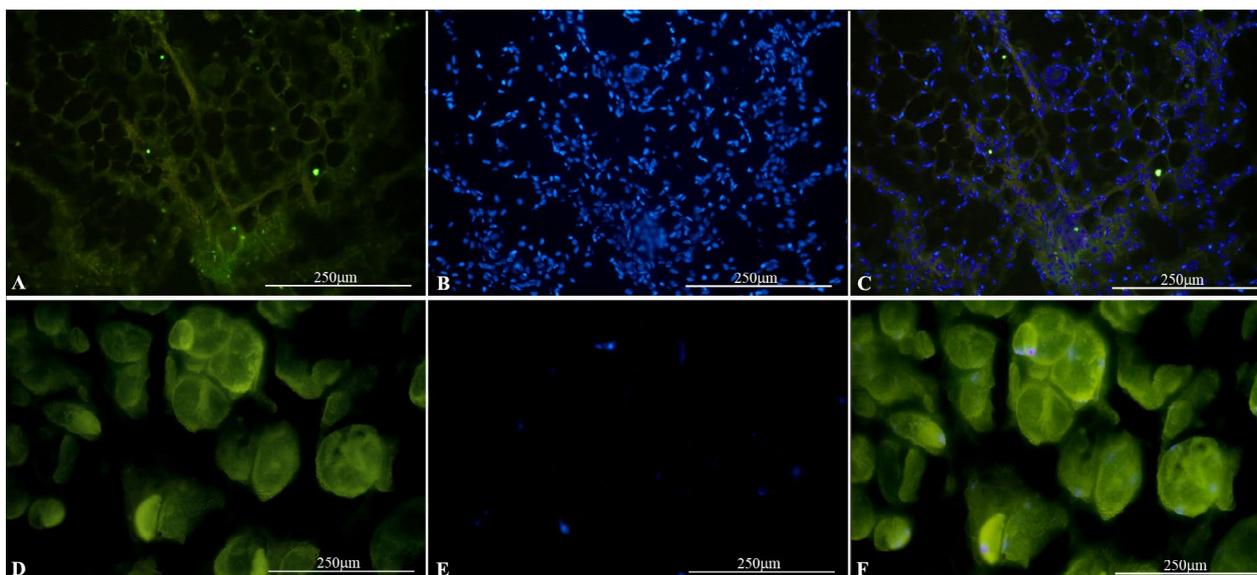


Fig. 6. Image showing the results of SSE4-sorted DFC-transplanted cells in nude mice. After 60 d cells formed either a vascularised adipose tissue (A,B,C) or a skeletal muscle (D,E,F) both positive for HLA-1 (nuclei are stained with DAPI). Merge in C and F. (scale bars = 250 μ m).

was found to be comparable to that usually detected in embryonic cells. The latter further demonstrates the similarity of these cells with embryonic cells.

Sphere cluster formation (embryoid bodies)

Then we organised other experiments in order to ascertain the ability of DFCs to grow in suspension in a serum free medium supplemented with growth factors. This method was described for the first time to select neural stem cell through neurosphere formation and has been largely used as a selection method for embryonic stem cells (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992). In fact, embryonic cells, selected on the basis of their ability to form sphere clusters, commonly called “Embryoid Bodies”, were found to be able to differentiate and build different tissues of the three embryonic germ layers when injected into permissive hosts or stimulated with different media *in vitro*. In our experiments, we found that DFCs were able to form sphere clusters; actually, embryoid bodies were observed already after 24 h in SSEA-4⁺ cultures (Fig. 2A), while SSEA-4⁻ cells, used as controls, did not form spheres. After 7 d of culture, spheres obtained from SSEA-4⁺ cells were seeded in standard plates with 10 % FBS. Cells migrated to from the spheres within a few hours and adhered to the bottom of the flasks, assuming a polygonal shape. These cells resulted to be smaller in size when compared with SSEA-4⁻ cells. After a week, we performed additional tests for SSEA-4, OCT 4, TRA 1-60, and TRA1-81 antigen on adhered cells. We found that almost all the cells obtained from spheres were SSEA-4⁺, OCT 4⁺, TRA 1-60⁺, and TRA1-81⁺ (Fig. 2B), whereas the cells that did not form spheres were completely negative for all antigens. This result further emphasises the “embryonic” nature and behaviour of DFCs.

Blastocyst injections

In order to further observe their embryonic features, we performed a series of key experiments designed to test whether DFCs directly contribute to embryonic

development. To this end, we microinjected these cells into mouse blastocysts. In our first set of experiments, the cells were injected into murine blastocysts isolated from C57BL/6 donors on day 3.5 of gestation.

In total, 30 blastocysts were microinjected with GFP⁺/DFCs. Ten of these injected blastocysts were examined under fluorescence microscope (Nikon Instruments, Florence, Italia). Interestingly, in each blastocyst, GFP⁺/DFCs cells were found within the inner cell mass. Injected cells were viable at 24 h (Fig. 2C), 48 (Fig. 2D) and 72 h after the injection when GFP⁺/DFCs cells were integrated with blastomeres (Fig. 2E). This evidenced that cells were able to induce the formation of chimeras. Only in one case we found that the blastocyst expelled the injected GFP⁺ cells after 24 h.

In order to produce chimeric formation, 20 injected blastocysts were implanted in mouse uterus. After 12.5 d of pregnancy they were explanted and examined for human DNA. Human DNA was not detected in the samples.

Teratoma formation

In order to explore and evidence all the characteristics of these cells, we performed DFC transplantation experiments in nude mice, following the design described in the M&M section. Our aim was to observe the eventual capability of DFCs to give rise to teratomas after transplantation. In all of the 12 cases DFC injections did not lead to teratoma or tumour formation in mice. This seems to be the only main difference between DFCs and embryonic cells.

Differentiation *in vitro*

In addition to all the previous experiments, we performed classical differentiation assays with the aim to demonstrate the pluripotency of DFCs.

Osteoblast and bone differentiation

After 3 weeks of cell culture with basal medium and without passages, cells started to induce the formation of hemispheric ossification centres. The appearance of these

centres of mineralisation was scattered within each flask, with several centres per flask. They then grew to build rounded 3D structures, which became macroscopically observable at day 40. Thereafter, the tissue continued to expand and, by day 50, several calcified nodules of newly synthesised tissue were evident in each flask. These differentiated cells were positive for ALP (Fig. 3A) and osteocalcin at immunofluorescence (Fig. 3B). Using antibodies specific for bone matrix proteins, we found that all the newly produced tissue was positive to protein markers present in mineralised bone (Fig. 3C,D,E), including bone alkaline phosphatase (BAP), bone sialoprotein (BSP), and collagen type I. The transcription factor RUNX-2 was also markedly expressed (Fig. 3F).

Adipogenic differentiation

Adipocyte differentiation was stimulated with dexamethasone. Cells cultured in this adipogenic medium differentiated into adipocytes, as assessed by specific staining for lipids. After 4 weeks from selection, approximately 50 % of cells differentiated into adipocytes containing a lipid content strongly positive for PPAR- γ (Fig. 4A,B) and adiponectin (Fig. 4C,D). The cytoarchitecture was typical of multivacuolar pre-adipocytes.

Skeletal muscle differentiation

In order to stimulate differentiation into skeletal muscular cells, sorted cells were co-cultured with C2C12, and tested for their capacity to fuse with them. Double immunofluorescence staining revealed 32 % of cell fusion (Fig. 4E).

Smooth muscle differentiation

To stimulate smooth muscle differentiation, cells were placed in MEGACELL medium with 2 % FBS and 10 ng/mL TGF β . Smooth muscle differentiation was obtained in 4-5 d. Immunofluorescence performed using SMA antigen showed that the differentiation ratio was of 35-38 % (Fig. 4F).

Astrocytes, oligodendrocytes and neuron differentiation

To assess the pluripotency of DFCs toward cells of neural lineages, we treated these cells with different tropic factors selectively.

To generate neurons, oligodendrocytes and astrocytes were incubated with BDNF (50 ng/mL), PDGF-AA (10 ng/mL), and bFGF (135 ng/mL) respectively for 14 d. At this time point, we performed an indirect immunocytochemistry analysis to evaluate the expression of selective markers of neural differentiation. After 14 d, cells treated with BDNF expressed neuronal markers: Tuj-1, MAP-2, and MAP-5 (Fig. 5A). Interestingly, the expression of Tuj-1 indicates the presence of mature neurones. Yet, when DFCs were treated with PDGF-AA, the oligodendrocytic markers Ng2 and O4 were expressed at high levels (Fig. 5B). Similarly, cells treated with FGF expressed the astrocytic marker GFAP (Fig. 5C).

Accordingly with the expression of neural markers, the morphology of treated cells was changed and appeared neural-like. As expected, expression of neural proteins and

changes in cell morphology were not observed in untreated DFCs.

In summary, DFCs have neuronal differentiation capabilities *in vitro* with the following percentages: neurons 19 %, oligodendrocytes 18 %, and astrocytes 17 %.

In vivo transplants

SSEA-4 DFCs were transplanted using a collagenous scaffold, into nude mice. Results showed that, after *in vivo* transplantation, different human tissues were obtained: an adipose tissue of Human origin (Fig. 6A,B,C) a Human adult bone and a Human skeletal muscle (Fig. 6D,E,F), while the scaffold was completely reabsorbed. In all the cases a blood supply was present and a vascular network established.

Discussion

Stem cells participate in organogenesis, cell turnover, and repair from injury. These processes require cell trafficking, the oriented or directed movement of cells toward a particular anatomic destination (Laird *et al.*, 2008). In the human adult body, other than bone marrow, one of the main sources of mesenchymal stem cells are niches that store these cells that are recruited upon request (Gurtner *et al.*, 2008; Paino *et al.*, 2010; Rim *et al.*, 2005; Shi *et al.*, 2010; Sviderskaya *et al.*, 2009).

Adult stem cells, as well as progenitor cells, are present within the body inside these niches and are unipotent, self-committed cells. Only in some cases, these cells can efficiently generate more cytotypes or a whole tissue. Dental pulp stem cells, which are derived from ectomesenchymal cells with the participation of neural crest-cell migration during organogenesis, can differentiate into osteoblasts, adipose cells, neurons, and smooth muscle and fuse their nuclei with murine myotubes (d'Aquino *et al.*, 2007; Laino *et al.*, 2005; Laino *et al.*, 2006). Moreover, stem cells (SCs) with neural crest characteristics have been isolated from the bulge area of cultured human hair follicles (HFs) exhibiting clonal multipotency that can give rise to myogenic, melanocytic, and neuronal cell lineages (Yu *et al.*, 2006; Yu *et al.*, 2010).

Embryologically, teeth are ectodermal organs originating from sequential reciprocal interactions between oral epithelial cells (ectoderm) and cranial neural crest-derived mesenchymal cells. The epithelial cells give rise to enamel forming ameloblasts, and the mesenchymal cells form all other differentiated cells, e.g., dentine forming odontoblasts, pulp, periodontal ligament (Volponi *et al.*, 2010).

Odontogenic tissues, deriving from the neural crest, remain undifferentiated during the first years of postnatal life, after which they develop the dental and periodontal structures of the secondary dentition. During tooth formation, the dental bud and crown are surrounded by an envelope that can be compared to a "placenta": this is called the "dental follicle" and provides undifferentiated cells for dental tissue building. The dental follicle persists in the adult because of its role in dental organogenesis, which occurs in humans up to the 12th year of life.

Therefore, we undertook this study with the aim to characterise the cells of the early stage dental follicle when it has not yet started to produce dental roots and cementum in particular. We found that the expression of typical ES (Embryonic Stem cell) markers, including the transcription factors Oct-4, Nanog, and REX-1, on cells of the dental follicle is of such a high level as to be of extreme interest. Actually, these cells, called DFCs, not only express Oct-4, Rex-1, and Nanog, but also SSEA-4, TRA1-60, TRA1-81, CD133, and CD90 at very high percentages (from 80 % up to 100 %): all those antigens are markers usually expressed by embryonic pluripotent stem cells. Since SSEA-4, Oct-4, and Nanog are specific markers of embryonic stem cells (Pan and Thomson, 2007; Stojkovic *et al.*, 2004), epiblastic cells, and primordial germ cells (Virant-Klun *et al.*, 2008) the presence of DFCs in an adult tissue reinforces the concept that they are a population of pluripotent or embryonic-like stem cells that directly belong to the neural crest. Actually, the 100 % of positivity for the CD56 antigen reinforces the thought that these cells directly derive from neuroectoderm. In addition, ES properties include not only the expression of Oct-4, Nanog, and Rex-1, as well as pluripotency, but also the capability for sphere formation under the appropriate conditions (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992), blastocyst inner cells mass integration (Durr *et al.*, 2003; Harder *et al.*, 2002), and high telomerase expression. These are all properties of DFCs, as demonstrated in this study. In addition, we did not observe variations on the pluripotent potential in term of gender and age of donors, because we used only follicles from germs in which dental roots were completely absent and at 5th passage of culture. Regarding to chimeras formation, we tried to find human cells in mouse embryo of 12.5 days by sectioning and screening for the presence of any green fluorescent cells, or by total DNA extraction screening for the presence of human related DNA sequences. Unfortunately, we were not able to detect human DNA. Mouse embryonic development is much different from that human, and probably the different pathways involved did not allow human cells to proliferate and contribute to organ formation. Besides this, there is the chance that a low number of human cells integrate in mouse tissue but were not detected by the assays we performed. Although this, the integration with blastomeres, followed up to 72 h is a rare and uncommon event. Therefore, we stress the importance of the presented data, obtained in almost all the cases, and in a considerable number of injected blastocysts, evidencing the tendency of these cells to form chimeras, other than their "embryonic" features and behaviour. DFCs did not form teratomas, after injection into nude mice (12 injected nude mice). Albeit teratoma formation is a general attitude of embryonic stem cells (Nussbaum *et al.*, 2007; Pomper *et al.*, 2009), this result means that cells share many features with embryonic cells, but not all of them, demonstrating that these cells do not have a cancerous potential and, therefore, can be ready for use in therapeutic applications without creating oncological problems.

It is known that culture environments alter the patterning of cells in ways that modify their fates and

even their developmental potential (Joseph and Morrison, 2005). Similar concerns have been addressed in neural crest stem cells, leading to the conclusion that after culture these cells often lose or change their properties, so that in many cases it is much better to select them directly by flow cytometry (Bixby *et al.*, 2002; Morrison and Spradling, 2008; Morrison *et al.*, 1999). Therefore, we have used fluorescence activated cytometry and found high levels of all embryonic markers, demonstrating that in this procedure does not reduce the capabilities of dental follicle cells.

Regarding dental structures, our group has obtained significant results demonstrating that dental pulp stem cells differentiate into several lineages including skeletal muscle (Spath *et al.*, 2010) and mainly into osteoblasts that give rise to bone (Laino *et al.*, 2005; Laino *et al.*, 2006), which after transplantation is remodelled into adult lamellar bone containing vessels (d'Aquino *et al.*, 2007). These cells have been successfully used for human grafts (d'Aquino *et al.*, 2009); moreover, these cells have been used to test their performance on different scaffolds, demonstrating an involvement of cadherin and integrins in their behaviour (Graziano *et al.*, 2007; Mangano *et al.*, 2010). Similar tissue-forming capabilities are shown by DFCs.

To date, the dental follicle has been found to be mainly involved in cementogenesis (Handa *et al.*, 2002). To our knowledge, there are no studies evidencing that dental follicular cells not only express embryonic markers but also have many other features and behaviours of embryonic cells as we have reported here. Some studies have shown that the dental follicle contains committed precursor cells that give rise to cementoblasts, periodontal ligament, and osteoblasts (Morsczech *et al.*, 2005; Yokoi *et al.*, 2007). It has been also encountered as a stem cell niche together with dental pulp and periodontal ligament (Mitsiadis *et al.*, 2007).

Because of its composition, biology, and features the dental follicle may be considered to be composed of cells originating from neuroectodermal structures during development that retain their characteristics and capabilities in the adult. After birth, these cells play a crucial role in steady state conditions during tissue turnover. Furthermore, on tissue damage, these cells could be recruited from their niche and home to damaged areas in order to participate in several cell and tissue repair processes. Our data, summarised in Table 1, have evidenced that these pluripotent cells can be paramount for several therapeutic uses. Actually, DFCs are cells with many features of embryonic stem cells: in particular, their antigen expression and high levels of mRNAs of embryonic transcription factors, and a telomerase activity that is comparable with that of embryonic cells points to an embryonic cell status. Adding to this their ability to form embryoid bodies, their capability integrate in the inner cell mass after being injected in a murine blastocyst, and their efficient differentiation into several cytotypes including osteoblasts, neurons, glial cells, vessels, adipose cells, and many other cell types, highlights the potential interest of these cells.

In vivo transplantation experiments clearly demonstrate that DFCs pluridifferentiation is maintained after grafting

Table 1. Human ES cells vs. DFCs.

Marker name	Human ES Cells	DFCs
SSEA-1	✗	✗
SSEA-4	✓	✓
TRA 1-60	✓	✓
TRA 1-81	✓	✓
ALKALINE PHOSPHATASE	✓	✓
OCT-4	✓	✓
Telomerase activity	+++	+++
Teratoma <i>in vivo</i>	✓	✗
Chimera formation	✓	✓
Pluripotency <i>in vitro</i>	✓	✓

Embryonic features of human ES compared with DFCs. Modified from NIH stem cells report June 2001 "Stem cells: Scientific progress and future research directions".

and that either adipose or skeletal muscle tissues were obtained. Moreover, a vascular supply, which is of paramount importance, was observable in all the cases.

In conclusion, in this study we have shown that DFCs have several capabilities and features including expression of embryonic markers in a high percentage of cells (ranging from 80 % up to 100 %); expression of markers of embryonic neural crest cells; high levels of telomerases and a related long lifespan similar to embryonic stem cells; formation of embryoid bodies in culture; integration within the inner cell mass of murine blastocysts after injection; differentiation toward all lineages (pluripotency), which is maintained after *in vivo* grafting; and complete and vascularised bone, skeletal muscle and adipose tissue generation. Taken together, our findings open up a new scenario in which these cells, which are present in an adult human body, can be used for clinical application, although their particular antigenic profile, being in the adult body. We think that they will help the understanding of tissue regeneration and in solving several clinical problems.

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