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Isolation of pluripotent stem cells from human third molar dental pulp

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Summary. Potent stem/progenitor cells have been isolated from normal human dental pulps, termed dental pulp stem cells (DPSCs). However, no study has described the presence of stem cell populations in human dental pulp from the third molar with embryonic phenotypes. The dental pulp tissue was cultured in media with the presence of LIF, EGF, and PDGF. In the present study, we describe a new population of pluripotent stem cells that were isolated from dental pulp (DPPSC). These cells are SSEA-4$^+$, Oct4$^+$, Nanog$^+$, FLK-1$^+$, HNF3beta$^+$, Nestin$^+$, Sox2$^+$, Lin28$^+$, c-Myc$^+$, CD13$^+$, CD105$^+$, CD34$^-$, CD45$^-$, CD90^{low}, CD29$^+$, CD73^{low}, STRO-1^{low} and CD146$^-$. We have investigated by SEM analysis and q-RT-PCR the capacity of DPPSCs to 3D differentiate *in vitro* using the Cell Carrier 3D glass scaffold into tissues that have similar characteristics to embryonic mesoderm and endoderm layers. These data would support the use of these cells, which are derived from an easily accessible source and can be used in future regeneration protocols for many tissue types that differentiate from the three embryonic layers.

Key words: Dental pulp, Pluripotency, Embryonic markers

Introduction

Embryonic stem (ES) cells can be used as a surrogate for *in vivo* development, enabling the analysis of multilineage differentiation within an *in vitro* environment. Multilineage differentiation of ES cells can be demonstrated by the simple formation of embryoid bodies (EBs), which have cells from all three germ layers (Fuchs and Segre, 2000; Papapetrou et al., 2009). Induced pluripotent stem (iPS) cells can be derived from differentiated somatic cells by inducing a reprogramming process that involves the overexpression of a key set of transcription factors. Comparison of the gene expression profiles of iPS and ES cells has revealed a similar global pattern with significant upregulation of key pluripotency maintenance network genes such as, *OCT3/4*, *NANOG* and *SOX2* (Itskovitz-Eldor et al., 2000; O'Connor et al., 2008). Because pluripotent stem cells have become a current focus in scientific research, many techniques have been developed to demonstrate the actual pluripotency of ES cells or iPS cells. The pluripotency of murine ES (mES) cells (Panopoulos et al., 2011; Rhee et al., 2011) can be tested in three different manners: when injected into the blastocyst cavity, mES cells contribute to all cell types in the chimeric progeny, including the germ layer; when injected subcutaneously, the cells lead to teratoma formation; and when cultured *in vitro*, mESs start to aggregate and generate embryoid bodies (EBs) (Song et al., 2011). The last two techniques are also used to test the pluripotency of human ES/iPS cells.

Information on human dental stem cells is not yet available. However, human dental pulp stem cells (hDPSCs) isolated from the third molars have been used to demonstrate that dental pulp can be used *ex vivo* to obtain multiple cell types. The successful establishment of these cells has increased the possible applications of these stem cells in biological and medical research.

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Abbreviations: DPSC: Dental pulp stem cell. DPPSC: Dental pulp Pluripotent stem cell. DPMSC: Dental pulp Mesenchymal stem cell. SHED: Stem cells from exfoliated deciduous teeth. PDLSC: Periodontal ligament stem cells. DFPC: Dental follicle progenitor cells. SCAP: Stem cells from apical papilla. LIF: leukemia inhibitor factor. EGF: Epidermal growth factor. PDGF: Platelet derived growth factor.

Using a single-colony isolation method, DPSCs have been shown to be capable of self-renewal; multipotent differentiation into a variety of mesenchymal tissues, such as bone, cartilage and muscle; and differentiation into various tissue types from different embryonic layers, including adipose, bone, endothelial, and neural-like cells *in vitro* (Gronthos et al., 2000; Nosrat et al., 2001, 2004; Miura et al., 2003; d'Aquino et al., 2007; Arthur et al., 2008; Honda et al., 2008; Cheng et al., 2008; Huo et al., 2010).

There are still many unanswered questions about stem cells derived from dental pulp. For instance, it is unknown whether there might be a cell population that possesses a greater regenerative potential than the stem cells that are usually isolated from dental pulp. To date, there is not an optimal culture medium that allows adult stem cell amplification without differentiation. Therefore, it is reasonable to hypothesize that naive DPSCs can spontaneously differentiate (self-differentiation) into mature cell lineages via asymmetric cell divisions *in vitro*.

In this study, we reported a new population of pluripotent stem cells isolated from dental pulp. We established a protocol that allows the reproducible isolation and identification of stem cells that we refer to as "dental pulp pluripotent stem cells" (DPPSCs). The cells were isolated by culturing them in media containing LIF (leukemia inhibitor factor), EGF (epidermal growth factor) and PDGF (platelet-derived growth factor). DPPSCs were identified as cells with the phenotypes $CD13^+$, $SSEA4^+$, $OCT3/4^+$, $NANOG^+$, $SOX2^+$, $LIN28^+$, $CD14^+$, $CD29^+$, $CD105^+$, $CD34^-$, $CD45^-$, $CD90^-$, $STRO1^-$ and $CD146^-$. This information could be used in future regeneration protocols for many tissue types of the three embryonic layers. The aim of this study was to confirm the results that we obtained from DPPSCs in our previous studies and to demonstrate the pluripotent capacity of these cells using multiple methods that are widely accepted to prove such capacity. Therefore, we demonstrated the pluripotency of DPPSCs by 3D differentiation into bone and liver-like tissues.

Materials and methods

Patient selection

Healthy human third molars extracted for orthodontic and prophylactic reasons were selected from 20 different patients of different sexes and ages (14-60 years old). The extraction procedure was kept simple to prevent tooth damage.

Primary cells obtained from human molar samples

Immediately after extraction, the third molars were washed using gauze soaked in 70% ethanol, followed by a wash with sterile distilled water. Holding the tooth with upper incisor forceps, an incision was made between the enamel and the cement using a cylindrical turbine bur. A fracture was made on the same line and fragments of the tooth were placed in a Falcon flask containing sterile PBS 1X. The samples were rapidly transported to the laboratory and placed in Petri dishes in a laminar flow hood. Tissues were isolated from the dental pulp using a sterile nerve-puller file 15 and forceps. Cellular separation was completed by digesting the divided pulp tissue with collagenase type I (3 mg/ml) (Sigma) for 60 minutes at 37°C. Cells were then separated using an insulin syringe and centrifuged for 10 minutes at 1,800 rpm. The cell fraction was washed twice with sterile PBS 1X and centrifuged again for 10 minutes at 1,800 rpm at room temperature. Once collected, the cells were counted and seeded in DPPSC medium. In order to establish the primary culture, the cells were grown in 96-, 24- and 6-well culture dishes and in 150 ml flasks coated with 100 ng/ml hFN inside a 5% CO_2 humidified chamber for 3 weeks. The medium was changed every 4 days. During the splitting/passages of DPPSCs, cell density was maintained at 80-100 cells/cm^2 by detaching cells with 0.25% trypsin (Cellgro) and replating every 36-48 hours after the cells were 60% confluent.

Culture medium of DPPSC

The cell expansion medium consisted of 60% DMEM-low glucose (Sigma) and 40% MCDB-201 (Sigma) supplemented with 1X Insulin-Transferrin-Selenium (ITS) (Sigma), 1X linoleic acid-bovine serum albumin (LA-BSA) (Sigma), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 100 units of penicillin, 1000 units of streptomycin (PAA), 2% fetal bovine serum (Sigma), 10 ng/ml hPDGFBB (R&D Systems), 10 ng/ml EGF (R&D Systems), 1000 units/ml hLIF (Chemicon), Chemically Defined Lipid Concentrate (Gibco), 0.8 mg/ml BSA (Sigma) and 55 μM ß-mercaptoethanol (ß-ME, Sigma).

Base medium

The base medium consisted of 60% DMEM-low glucose (Gibco), 40% MCDB-201 (Sigma) with 1X Insulin-Transferrin-Selenium, 1x linoleic acid BSA, 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 100 units of penicillin, and 1000 units of streptomycin (Gibco).

Isolation and culture of human dental pulp mesenchymal stem cells (DPMSC)

Human adult DPMSCs were isolated from the dental pulp of third molars and were suspended in Dulbecco's Modified Eagle Medium (DMEM, Biochrom) containing 2 ng/ml basic fibroblast growth factor (bFGF) and 10% fetal bovine serum (FBS, Hyclone). Cells were plated at a density of 300,000 cells/cm^2. The medium was changed after 72 h and every 2 days thereafter. To

propagate the DPMSCs, the cells were detached at 90% confluency by the addition of phosphate buffered saline (PBS, Biochrom) containing 0.05% trypsin-ethylene-diaminetetraacetic acid (EDTA; Biochrom) and replated at a density of 4,000 cells/cm^2.

Culture of human NTERA-2

NTERA-2 cells were obtained from the ATCC®. Cells were maintained and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37ºC in a humidified atmosphere at 5% CO_2.

3D Culture of DPPSC

For 3D cultures of DPPSC P15, the AggreWell™ and hanging drop culture methods were used for 5 days. For 3D cultures using the Cell Carrier 3D glass scaffold, cells were seeded in 6-well and 24-well plates at a density of 1x10^3 cells per cm^2. The scaffolds were treated with 100 ng/ml hFN in a 5% CO_2 atmosphere 24 hours prior to seeding. The medium was changed every 4 days over a period of 15 days.

Flow cytometry

FACS analysis was carried out the same day of the extraction and again after 2 and 3 weeks of culture initiation. The following fluorochrome-labeled monoclonal antibodies were used: CD13 FITC (eBioscience), SSEA-4 PE (eBioscience), OCT3/4 FITC (RD SYSTEMS), CD45 PE-Cy5 (BD Pharmingen), CD105 FITC (BD Pharmingen), CD34 PE-Cy5 (BD Pharmingen), CD73 PE (BD Pharmingen), CD146 FITC (BD Pharmingen), CD29 PE (BD Pharmingen), STRO-1 FITC (BD Pharmingen), and Nanog FITC (Abcam). For the analysis of control samples, different IgG isotypes coupled to FITC, PE, and PE-Cy5 fluorochromes (BD Pharmingen) were used. The cell suspension (in PBS plus 2% FBS) was incubated for 45 minutes at 4°C in the dark. Later, cells were washed twice with PBS containing 2% FBS and centrifuged for 6 minutes at 1,800 rpm. Depending on the number of cells, the cells were resuspended in 300 to 600 μl of PBS and 2% FBS. All flow cytometry measurements were made using a FACScan cytometer (FACSCalibur) and analyzed using the winMDI 2.8 program. More than 400,000 cells were used from each sample to detect nonspecific unions or autofluorescence.

Cell sorter

SSEA-4$^+$ cells were isolated using a flow cytometer with a cell separator device. These cells were collected on 24-well culture plates. The separation by FACS was carried out in a Coulter EPICS ELITE-ESP®. In total, 250 cells that were positive for SSEA-4 were isolated and cultivated in a 96-well plate that was previously coated with human fibronectin (10 ng/mL) (BD Bioscience) by incubating the plates for one hour at 37°C. SSEA-4$^+$ cells were seeded at a density of 10 cells/well.

Immunophenotypic analysis

Cells were fixed with 4% paraformaldehyde (Sigma) for 4 minutes at room temperature, followed by a methanol (Sigma) treatment for 2 minutes at -20°C. For the nuclear ligand analysis, cells were permeabilized with 0.1 M Triton X-100 (Sigma) for 10 minutes. Slides were incubated sequentially for 30 minutes each with primary antibody FITC coupled anti-mouse IgG antibody. Between each step, the slides were washed with PBS plus 1% BSA (Sigma). Cells were examined using confocal fluorescence microscopy (Confocal 1024 microscope, Olympus AX70, Olympus Optical, Tokyo).

RNA isolation and qRT-PCR

Total cellular RNA samples were extracted using Trizol (Invitrogen). RNA was extracted weekly from the

Table 1. Primers used for amplification.

	FORWARD	REVERSE
Oct3/4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
Klf1	TGGCATCGCGAAAGTGTATC	AAAGGGAGGCGAGCATCTC
c-Myc	GCGTCCTGGGAAGGGAGATCCGGAGC	TTGAGGGGCATCGTCGCGGGAGGCTG
Nanog	AAAGAATCTTCACCTATGCC	GAAGGAAGAGGAGAGACAGT
HNF 3 beta	CTTAGCAGCATGCAAAAGGA	TGCGTTCATGAAGAAGTTGC
Lin 28	TCCCTCTTCCCTCCTCAAAT	TTCCCCTAACCAGATTGTCG
HNF4 alfa	ATTGCTGGTCGTTTGTTGTG	TACGTGTTCATGCCGTTCAT
Nestin	CAGGAGAAACAGGGCCTACA	TGGGAGCAAAGATCCAAGAC
ALB	GCTGTTGGGATGGTCAAAGAAG	GGTTTCGAGGGCAAACGA
AFP	TCCAGTCGAAGATTGGGTCC	GCTTGTGGGTTTCAATCTTTTTATTT
Osteocalcin	GGTGCAGAGTCCAGCAAAGG	AGCGCCTGGGTCTCTTCCTA
Osteopontin	GAAGGTGAAGGTCGGAGTCA	TGGACTCCACGACGTACTCA
Osteonectin	AGGTATCTGTGGGAGCTAATC	ATTGCTGCACACCTTCTC

differentiated cells. Two μg of RNA was treated with DNase I (Invitrogen) and reverse-transcribed using M-MLV Reverse transcriptase (Invitrogen). We analyzed the efficacy of the cDNA (1, 0.1, 0.01, 0.001, 0.0001 dilutions) at different concentrations for all primers using NTERA cells as positive controls. Additionally, we tested the following samples as positive controls: hepatocyte markers from human liver cDNA samples, osteoblast markers from human bone cDNA from (Ambion). Quantitative RT-PCR was performed using CFX96 (Bio-Rad). RT-PCR was performed using 50 ng of cDNA and SYBR Green Supermix (Bio-Rad Laboratories, Inc.). cDNA samples were amplified using specific primers with the following conditions for 40 cycles. The expression levels of genes of interest (Table 1) were normalized against the housekeeping gene GAPDH. The relative expression levels were normalized to Human cDNAs (Positive controls), which were normalized to 1. The result was analyzed using the 2ΔΔCt method.

Multilineage differentiation

Mesoderm differentiation

For 3D bone differentiation. DPPSC were seeded in 6-well plates and in *Cell Carrier 3D glass scaffold* in 24-well plates at a density of $1x10^3$ cells per cm^2. After 24 hours, the differentiation protocol was initiated by using an osteogenic medium: α-MEM containing 10% heat inactivated FBS, 10 mM ß-glycerol phosphate (Sigma), 50 μM of L-ascorbic acid (Sigma), 0.01 μM dexamethasone and 1% penicillin and streptomycin. The medium was changed every 3 days during 21 days.

Endoderm differentiation

For 3D differentiation, $5x10^4$ cells were seeded in a Cell Carrier 3D glass scaffold pre-coated with 2% Matrigel and placed in 24-well plates with RPMI medium (Mediatech) supplemented with GlutaMAX and penicillin/streptomycin and containing 0.5% defined fetal bovine serum (FBS; HyClone) and 100 ng/ml Activin A (R&D Systems). Three days post-induction, the medium was refreshed using the same RPMI-based medium with 100 ng/ml Activin A but replacing FBS by KOSR 2%. After 2 days, definitive endoderm cultures were refreshed with RPMI medium supplemented with GlutaMAX and penicillin/streptomycin and containing 2% KOSR, 10 ng/ml FGF-4 (R&D Systems), and 10 ng/ml HGF (R&D Systems). Three days later the cells were switched to minimal MDBK-MM medium (Sigma-Aldrich) supplemented with GlutaMAX and penicillin/streptomycin and containing 0.5 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich), 10 ng/ml FGF-4, and 10 ng/ml HGF. After another 3 days, the cells were switched to complete hepatocyte culture medium (HCM) supplemented with SingleQuots (Lonza) and containing 10 ng/ml FGF-4, 10 ng/ml HGF, 10 ng/ml oncostatin M (R&D Systems), and 10^{-7} M dexamethasone (Sigma-Aldrich). Differentiation was continued for another 9 days. At each stage, the medium was refreshed every 2-3 days.

Scanning electron microscopy (SEM)

For Scanning Electron Microscopy (SEM), scaffolds were fixed in 2.5% glutaraldehyde (Ted Pella Inc., Redding, CA) in 0.1 M Na-cacodylate buffer (EMS, Electron Microscopy Sciences, Hatfield, PA) (pH 7.2) for 1 hour on ice. After post-fixation for 30 min with 1% OsO_4 (Osmium Tetroxide) for 1 h, the samples were dehydrated in a series of acetone (30%–100%) with the scaffolds mounted on aluminum stubs. The samples were examined with a Zeiss 940 DSM scanning electron microscope.

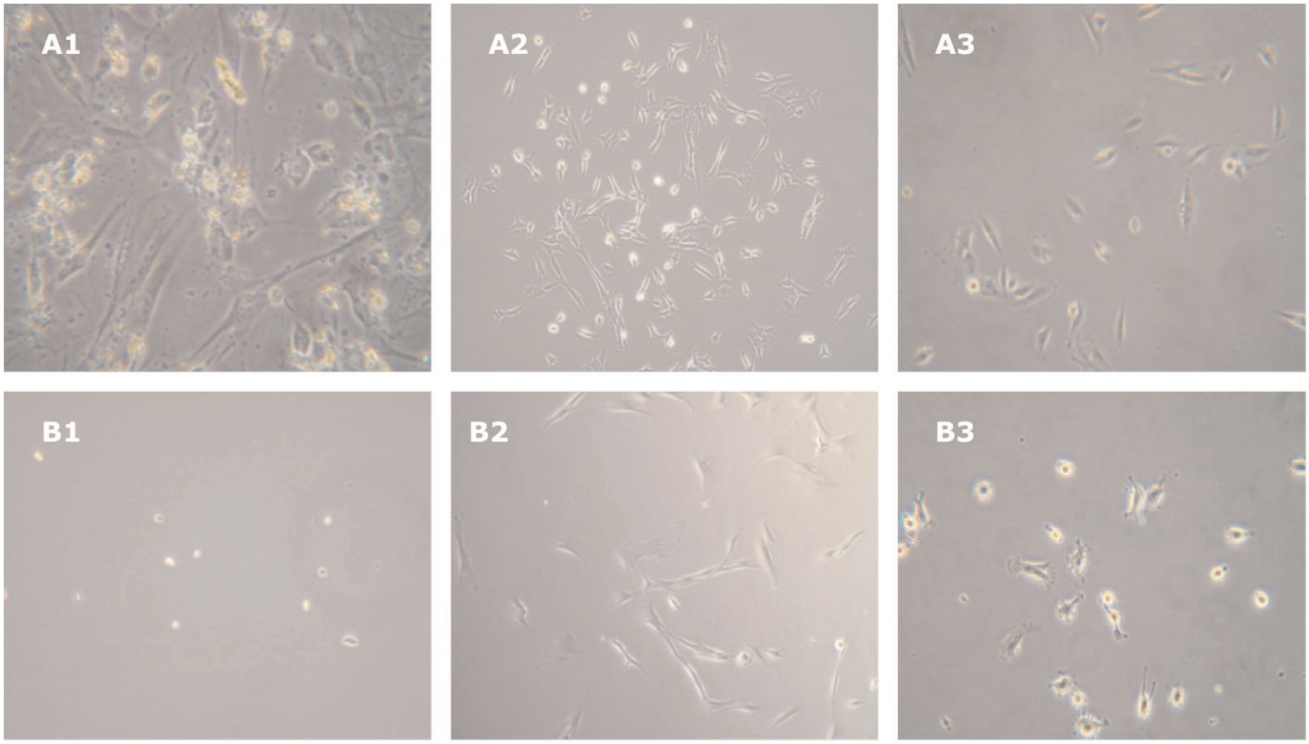
Statistical analysis

For all the data, the statistical test applied was the paired-samples t-test, with statistical significance set at $p<0.05$. Data were analyzed with SPSS Version 16.0 software. The values are expressed as the mean ± standard deviation.

Results

Isolation of dental pulp pluripotent stem cells from third molars of healthy individuals

The studies carried out in this article were directed at isolating and purifying a population of pluripotent stem cells derived from dental pulp (DPPSC) that display a characteristic embryonic marker expression pattern ($SSEA\text{-}4^+$, $OCT4^+$, $NANOG^+$, $hFLK\text{-}1^+$, $hHNF3beta^+$, $Nestin^+$, $Sox2^+$, $Lin28^+$, Myc^+, $CD13^+$, $CD105^+$, $CD34^-$, $CD45^-$, $CD90^{low}$, $CD29^+$, $CD73^{low}$, $STRO\text{-}1^{low}$ and $CD146^-$). We performed cell isolation by using third molar samples obtained from patients (n=25) of both sexes and of different ages (14-60 years old). The samples used were extracted for orthodontic and prophylactic reasons. Cells were seeded at a density of 80-100 cells per cm^2 using a modified medium similar to human multipotent adult progenitor cell medium (Verfaillie et al., 2005), but with the addition of LIF. We were able to purify DPPSCs using a cell sorter by isolating $SSEA\text{-}4^+$ cells from the separated dental pulp cells. The expression patterns of the DPPSC characteristic markers by FACS analysis on the cells isolated by the cell sorter, as well as the cultivated cells with DPPSC media (n=5), were similar to the cells cultivated without isolation, which were also cultured under the same medium conditions for three weeks (Figs. 1A,B, 6). This suggests that DPPSC isolation using our media is more advantageous than using a cell sorter to obtain DPPSC.



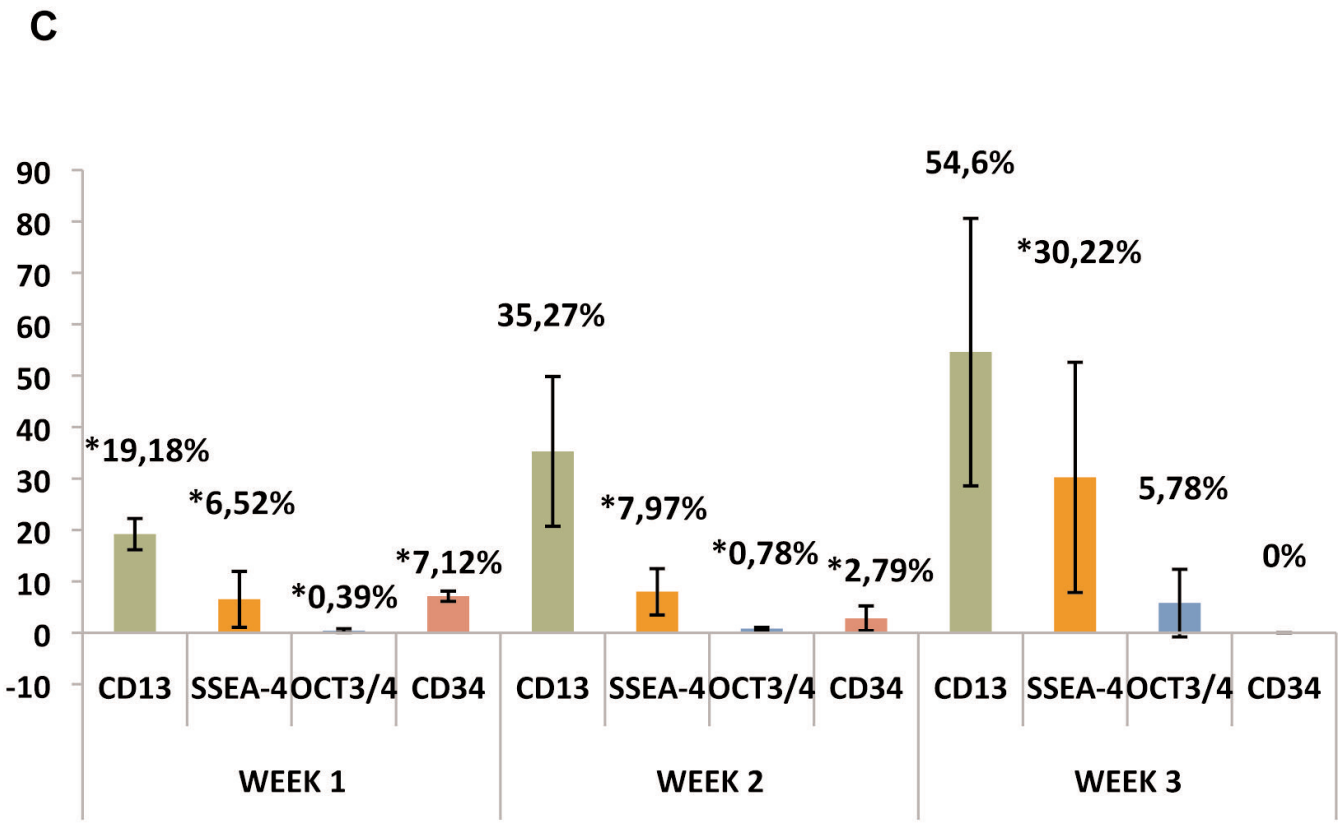


Fig. 1. Cellular morphology of DPPSCs from dental pulp. **A.** Morphology of cells obtained from human dental pulp in primary culture without cell sorter. **B.** Morphology of cells positive to SSEA4 obtained from human dental pulp in primary culture with cell sorter. **C.** Immuno-phenotype by FACS analysis of cultured dental pulp at different time points after culture initiation. Expression levels of SSEA4, CD13, Oct3/4 and CD34 are shown (n=5 *p<0.05).

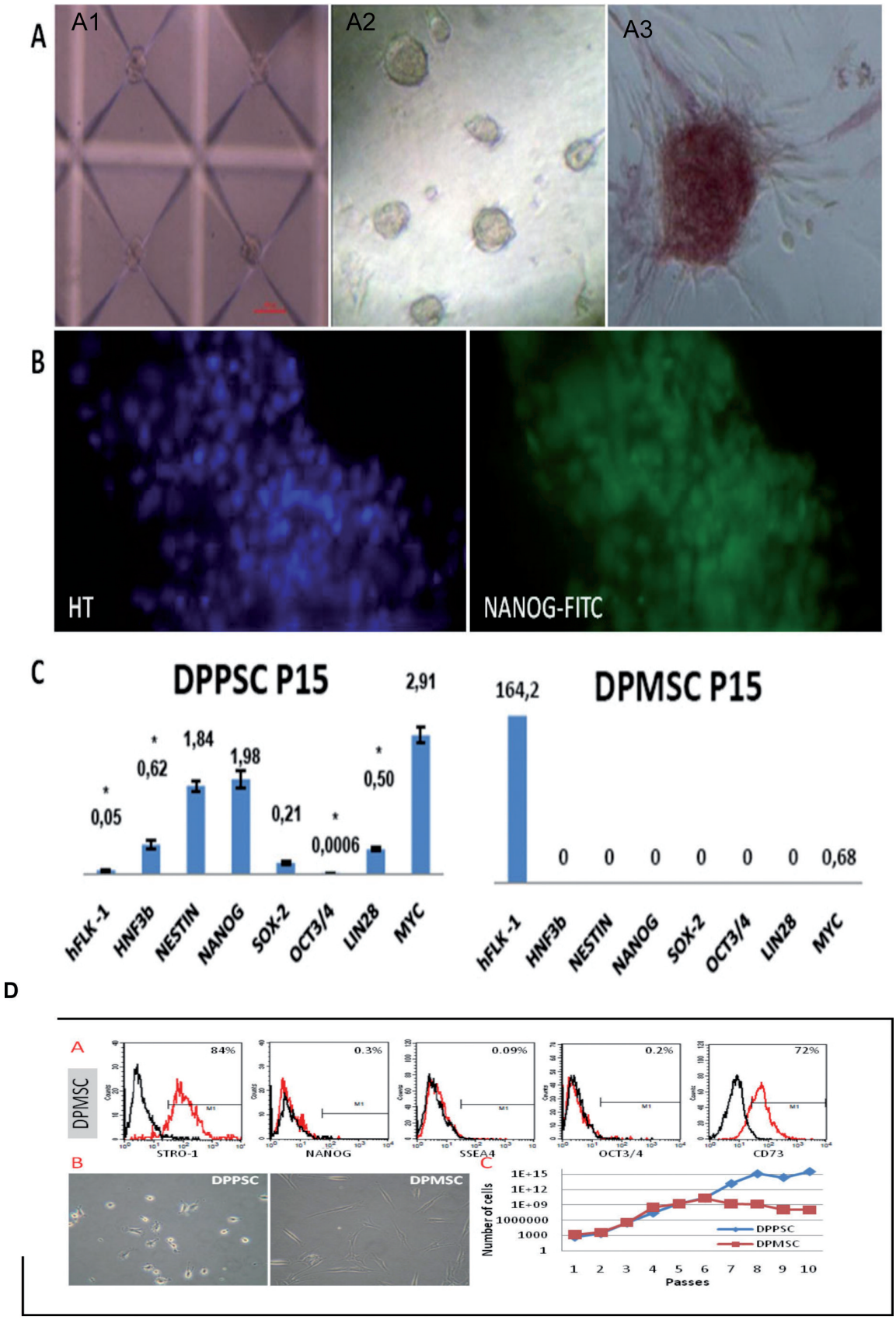


Fig. 2. Characterization of pluripotent stem cells obtained from dental pulp (DPPSCs). **A1:** AggreWell system (Stem Cell Technologies), which utilizes a micropatterned culture surface and centrifugal forced aggregation to direct the formation of EBs by using DPPSCs for 5 days. **A2:** Morphology of DPPSC embryoid bodies by hanging drop. **A3:** Alkaline phosphatase staining of DPPSC embryoid bodies. **B.** Immunophenotype analysis by confocal microscopy shows the expression of NANOG FITC. **C.** qRT-PCR analysis of hFLK-1, HNF3b, NESTIN, NANOG, SOX2, OCT3/4, LIN28, and MYC in the DPPSC and DPMSC at P15. The expression of genes of interest was normalized against the housekeeping gene GAPDH. The relative expression was normalized to cDNAs of NTERA cells as a positive control, which is normalized to 100. The results were analyzed by the 2ΔΔCt method. **D.** Analysis by FACS for DPMSC P15. E: Cell morphology of DPPSCs and DPMSC at P15. F: Cell proliferation curves for both populations.

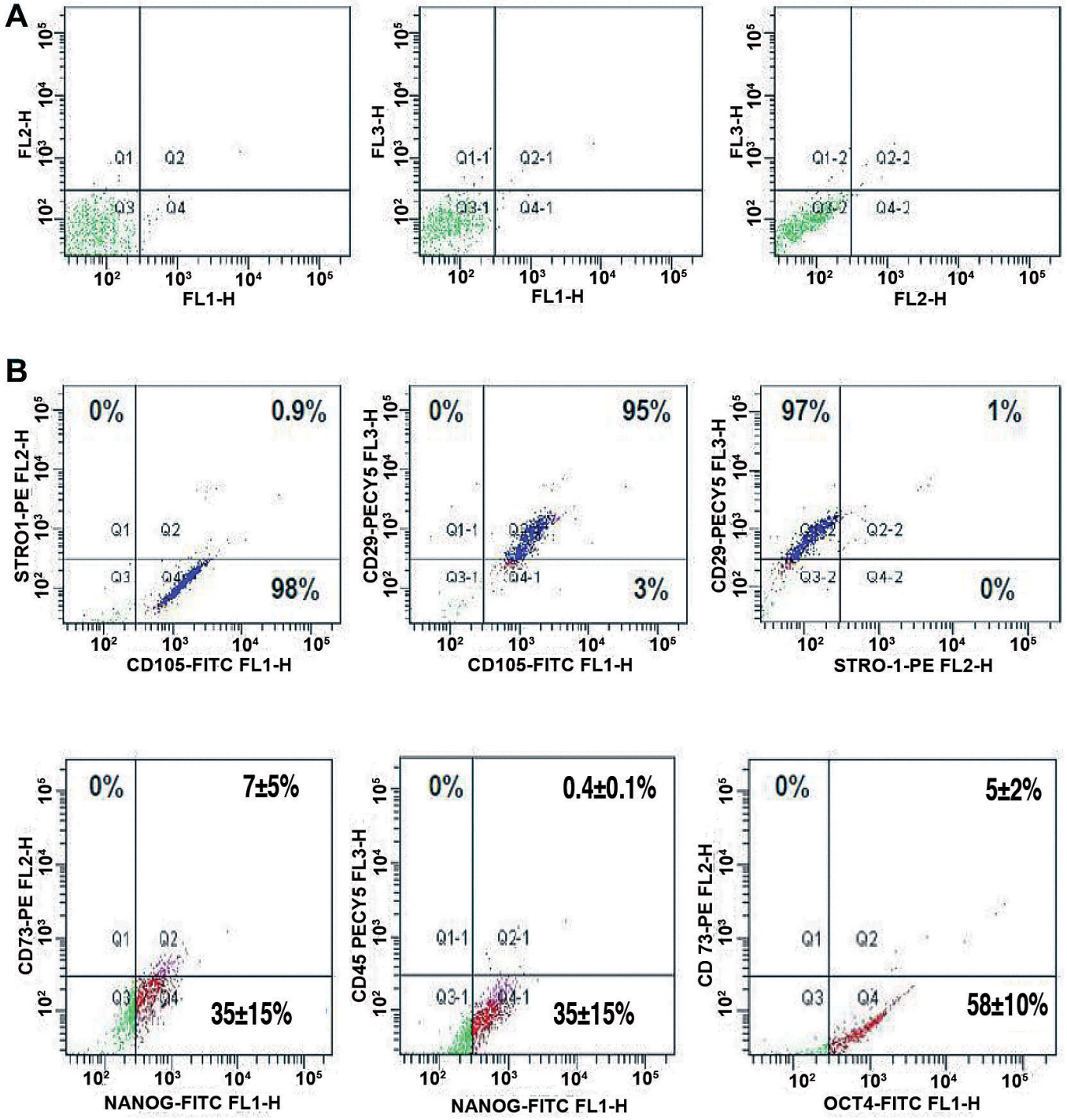


Fig. 3. FACS analysis to observe the phenotype of DPPSCs. **A.** Representation of the analysis of the isotope control IgG1 FITC, IgG1 PE, IgG1 PE-Cy5. **B.** In this graph, cells positive for CD105, CD29, and negative for STRO1 can be observed **C.** In this graph, cells positive for NANOG, OCT4, and negative for CD73 and CD45 can be observed.

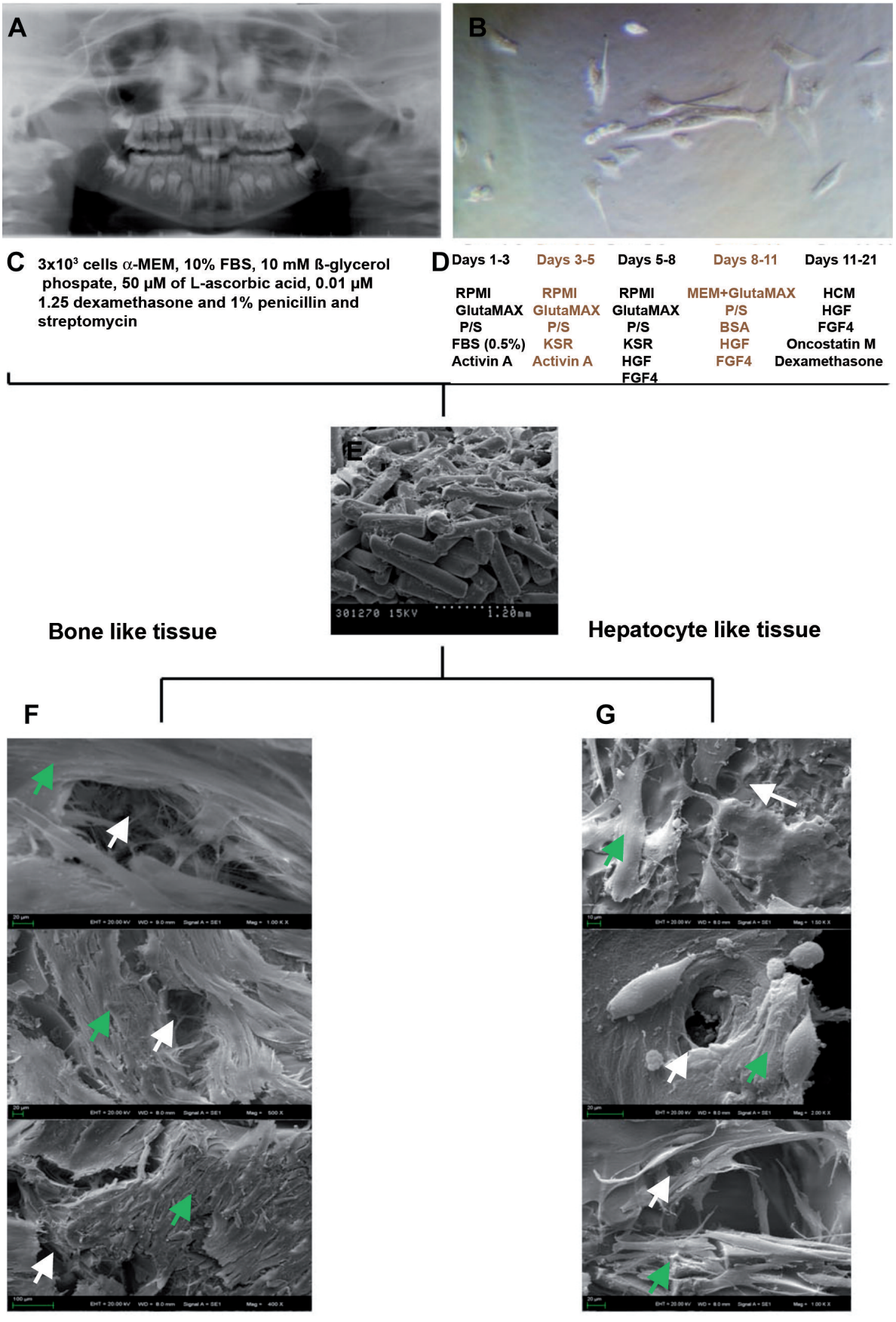


Fig. 4. Differentiation of DPPSCs to endoderm and mesoderm. **A.** Image by RX of the third molar. **B.** Cellular morphology of DPPSCs from dental pulp. **C.** Protocol used for bone differentiation. **D.** Protocol used for Hepatocyte differentiation. **E.** Scanning electron microscope image of Cell Carrier 3D glass scaffold used for both differentiation. **F.** Scanning electron microscopy image of 3D differentiation for 21 days into mesoderm tissues, including bone-like tissue, collagen (white arrow), and cortical (green arrow) structures. **G.** Scanning electron microscope image of 3D endoderm differentiation after 21 days on a Cell Carrier 3D glass scaffold. Hepatic-like cells with large and small open pores that have a fenestra-like appearance (white arrow), ultrastructure of sinusoidal endothelial cells (green arrow) the surface of sinusoidal endothelial cells.

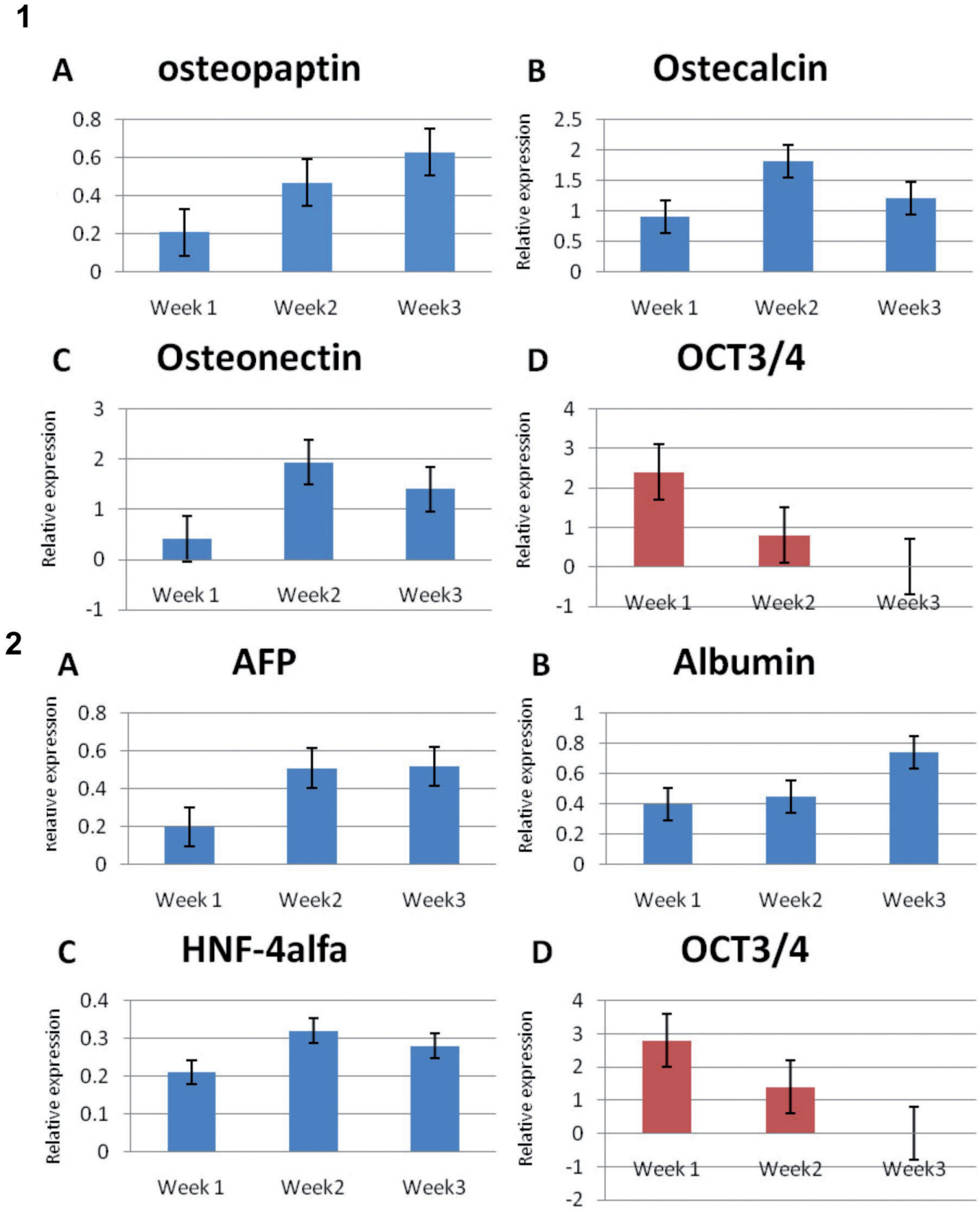


Fig. 5. A-D. Analysis of the expression of Osteopantin, Osteonectin, Osteocalcin and OCT3/4 by qRT-PCR during three weeks of bone-like tissue differentiation. mRNA levels were normalized to GAPDH (a housekeeping gene). The relative expression was normalized to human cDNA from bone cells (n=3), which was normalized to 1. 2: A, B, C, D: qRT-PCR detection of AFP, ALB, HNF 4alfa and OCT3/4 in DPPSCs differentiated to a 3D bone lineage (n=3). mRNA levels were normalized to GAPDH (a housekeeping gene). The relative expression was normalized to human cDNAs from liver cells, which were normalized to 1.

Characterization of DPPSCs

In this study, we analyzed the cell phenotypes of all clones obtained from a sample of dental pulp, cultivated at a density of 80-100 cells per cm^2 and expanded at different passages when the culture reached a confluence of 60%. Figure 1A shows representative images of the morphology of DPPSCs. DPPSCs are characterized by small-sized cells with large nuclei and low cytoplasm content (resembling MAPCs) (Verfaillie et al., 2005) without the typical flat and elongated MSC appearance. FACS analysis was carried out each week during the three weeks of primary culture formation on cell cultures derived from patients who were 14, 17, 18, 28, and 38 years of age in order to observe changes in marker expression (Fig. 1C). An increase in the percentage of specific markers of DPPSC cells (CD13, SSEA-4, OCT3/4, and CD34) was observed with respect to the culture time. The CD13, SSEA-4 and OCT3/4 expression levels increased from 19% to 54%, 6% to 30%, and 0.4% to 6% during weeks 1 to 3, respectively. However, the hematopoietic marker CD34 diminished from 7% to 0% over the same time period. To characterize these cells and learn about their capacity to form embryonic bodies, we performed 3D culture formation with the AggreWell™ and hanging drop culture methods, to determine the possibility of embryoid body (EB) formation by DPPSC (Fig. 2A1,A2). We observed a strongly positive relation for the alkaline phosphatase staining of embryoid body (EB) formation by DPPSC (Fig. 2A3), and we performed immunocytology with NANOG- FITC (Fig. 2B), where we could see the expression of NANOG in the nuclei of embryonic bodies formed by DPPSCs.

One of the main objectives of our study was to compare proliferation and genetic marker expression between DPPSCs and DPMSCs from the same donors. We analyzed genes that are commonly expressed in the three embryonic layers by qRT-PCR of DPPSCs and DPMSC at P15 and found the DPPS cells to be $OCT4^+$, $NANOG^+$, $FLK\text{-}1^+$, $HNF3beta^+$, $Nestin^+$, $Lin28^+$, $Sox2^+$, and Myc^+ (Fig. 2C). We also compared the morphology and proliferation rates, and we found that with an increase in the number of passages, P5 DPMSCs decrease in proliferation, whereas DPPSCs increase in proliferation (Fig. 2B,C).FACS analysis was also carried out for DPMSC and DPPSCs (Figs. 2, 3). FACS analysis showed that the following markers were expressed in DPPSCs at P15: CD105-FITC (87%), CD90-FITC (28%), CD45-PE-Cy5 (0.2%), CD29-PE (97%), and CD146-FITC (0.17%).

Multipotent capacity of the DPPSCs: 3D differentiation of DPPSC into Mesoderm and Ectoderm Tissues

Osteoblast differentiation

Using a 3D culture system, DPPSCs were able to differentiate into both endoderm and mesoderm tissues. After 21 days of culture in bone differentiation medium, DPPSCs gave rise to bone-like tissue that was able to synthesize typical structures, such as collagen and cortical structures, which were detectable by SEM analysis (Fig. 4F). Using qRT-PCR, we analyzed the expression of specific bone tissue genes like Osteonectin, Osteocalcin and Osteopontin (Fig. 5, 1A-D). We also observed a decrease in OCT3/4 expression during the 3 weeks of differentiation. Human bone cDNA (dvbiologics) was used as a positive control, and GAPDH was used as a housekeeping control (HK). Our results indicate that DPPS cells can efficiently differentiate into bone tissue and express specific bone

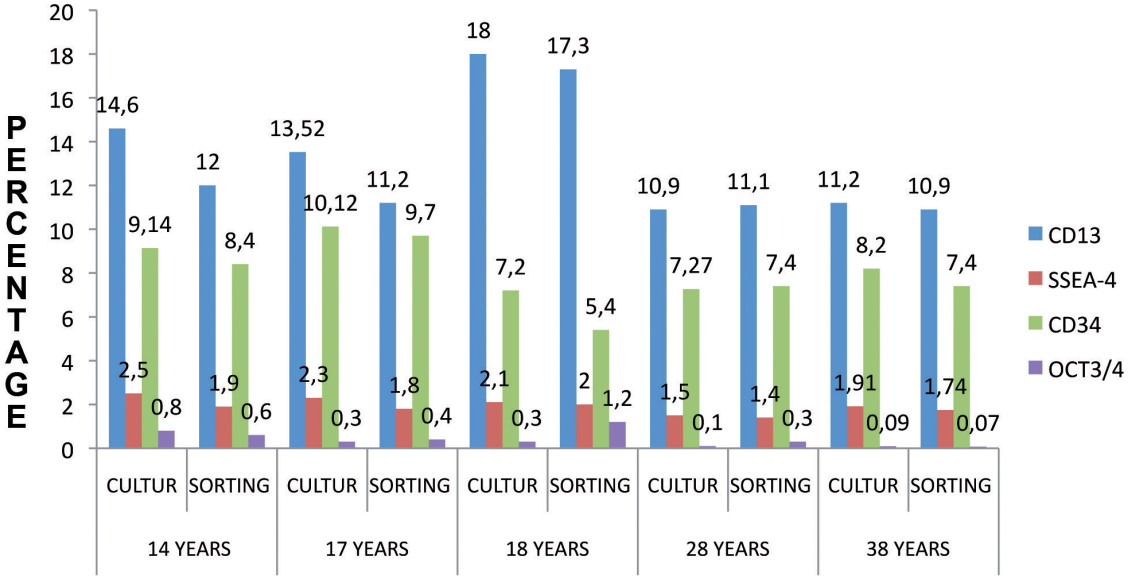


Fig. 6. Analysis by FACS (n=5) to compare the phenotype of DPPSC by using two isolation techniques. 1) Cell sorter (Cells positive to SSEA 4) and 2) by cells cultured with DPPSC media. This experiment was performed using the two DPPSC isolation techniques with the same culture conditions, and FACS analysis was performed after two weeks of culture.

tissue genes.

Endoderm differentiation: differentiation to hepatocytes

In a similar manner to osteoblast differentiation, after 21 days of 3D culture to induce hepatic-like cells differentiation, using SEM and qRT-PCR, we demonstrated that DPPSCs had differentiated into hepatocyte-like cells and were expressing hepatic tissue genes. By SEM examination, large and small pores had a fenestra-like appearance, the surface of sinusoidal endothelial cells and ultra-structures of sinusoidal endothelial cells (Fig. 4G). Using qRT-PCR, we analyzed the the expression of specific hepatic genes such as Albumin, AFP and hepatic nuclear factors HNF4 alfa (Figs. 5, 2A-D). We also observed a decrease in OCT3/4 during the differentiation. Human liver cDNA (Ambion) was used as the positive control, and GAPDH was used as the housekeeping control (HK). Our results indicate that DPPSCs can efficiently differentiate into endoderm tissue and express specific hepatic tissue genes.

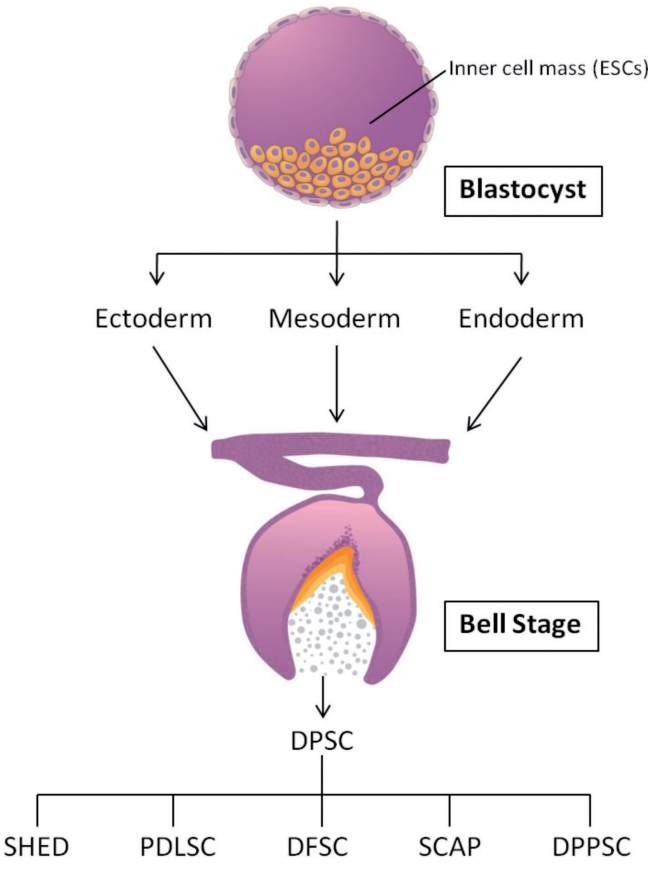


Fig. 7. Schematic classification of the different dental pulp stem cells and their origin.

Discussion

The main goal of our research project was to characterize a novel population of adult pluripotent stem cells obtained from the dental pulp of third molars. Our group is particularly interested in establishing a protocol for the isolation and identification of subpopulations of pluripotential stem cells such as DPPSCs with a phenotype similar to embryonic stem cells, characterized by the expression profile SSEA-4$^+$, OCT4$^+$, NANOG$^+$, FLK-1$^+$, HNF3beta$^+$, Nestin$^+$, Sox2$^+$, Lin28$^+$, Myc$^+$, CD13$^+$, CD105$^+$, CD34$^-$, CD45$^-$, CD90^{low}, CD29$^+$, CD14$^+$, CD73^{low}, STRO-1^{low}, and CD146$^-$. These cells can be applied in future protocols for the regeneration of many types of tissue from the three embryonic layers. While culturing of dental pulp cells, we noticed that when using a medium that contains growth factors such as EGF and PDGF in addition to a STAT3 pathway activator such as LIF (Zeng et al., 2006; Aranda et al., 2009; Sohni and Verfaille, 2011), an increase in the percentage of specific proteins that are representative of pluripotent stem cells was observed. This result indicated to us that, similar to bone marrow, dental pulp could be a very important source for the isolation of pluripotent cells. This evidence opens up pathways for future comparative studies of the regenerative potency of the two cell sources. In addition, because of recent experiments preserving umbilical cord cells, the possibility of freezing these cells seems feasible.

Because wisdom teeth extraction is widely performed in dental clinics, the third molars are the most common source for the isolation of dental stem cells. Given that the third molar is the last tooth to develop, it is normally in an early stage of development and is capable of producing an optimum quantity of dental pulp tissue for the isolation of DPSCs (Mina and Braun., 2004; Iohara et al., 2004; Tecles et al., 2005; Zhang et al., 2005; Liu et al., 2006; Stevens et al., 2008).

We recognize that dental pulp contains (Fig. 7), *ex vivo*, multipliable cells called dental pulp stem cells: exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs) and stem cells from apical papilla (SCAPs). These cells express markers that are associated with cells of the endothelium and/or smooth muscle, such as factor 1 derived from the stroma (STRO-1), vascular cell adhesion molecule-1(VCAM-1), antigen/mucin18 associated with melanoma (MUC-18), and actin of the smooth muscle (Gandia et al., 2008). Other authors have described the presence of lateral stem cell populations in dental pulp (Zhang et al., 2006; Otaki et al., 2007), but there are no reports of the presence of stem cells that are CD13$^+$, SSEA-4$^+$, OCT3/4$^+$, NANOG$^+$ and Sox-2$^+$.

In this work, during cell morphology analysis, the formation of colonies and embryonic bodies with characteristics similar to embryonic stem cells and MAPCs were observed (Verfaillie et al., 2005). It was also observed in various publications that, because

seeding with a higher density and a confluence superior to 80% causes changes in cell morphology and phenotype, the density of cells being seeded should be very low (from 80-100 cells per cm^2), and the cultures should be 60 to 70% confluent before passaging. Contrary to earlier publications, MAPC cultures are heterogeneous, and two cell populations with different phenotypes (large and small) co-habitate. We believe that the small cells are MAPCs (as described by Dr. Verfaillie, 2005); therefore, we collected only the smaller-sized cells for FACS analysis, and those cells gave us the correct phenotype.

For tissue engineering applications, the culture environment should not only support cell survival, but should also provide optimal conditions for matrix synthesis. During this process of tissue formation, the cell shifts from essentially a 2D configuration on the scaffold surface to a 3D configuration embedded in the matrix material. It is well known that there are marked differences in cell expression in a 2D culture compared to a 3D culture (Shin et al., 2004; Braccini et al., 2005; Scaglione et al., 2006). We analyzed the ability of DPPSC to adhere to and migrate in on the scaffold surface, and as shown by SEM these results confirmed that DPPSC had a greater adhesion and migration capacity and, in this way, a higher proliferation and differentiation ability.

The markers associated with pluripotency in stem cells are the transcription factors Oct4, Nanog, and Sox2, which are indispensable for indefinite stem cell division without an effect on differentiation potential (or, put another way, without affecting its capacity for self-renewal). The functional importance of Sox2 and Nanog for altering the cell status has been clearly demonstrated (Chambers et al., 2003; Mitsui et al., 2003; Pan and Pei, 2003, 2005). Nanog has been reported to be a key gene for maintaining pluripotency (Oh et al., 2005), as shown by the capacity for multi-lineage differentiation and perpetual self-renewal of cells expressing this gene. To determine the differentiation potential of the DPPSCs, we analyzed the *in vitro* potential of the cells to differentiate into tissues of each embryonic layer (mesoderm and endoderm) (Takahashi and Yamanaka, 2006; Yu et al., 2007; Zuba-Surma et al., 2009; Ratajczak et al., 2008).

To demonstrate that the DPPSC have the capacity to differentiate into endoderm and ectoderm tissue, we cultured DPPSCs in Cell Carrier 3D glass scaffold with liver differentiation medium. Using SEM and qRT-PCR, we showed that differentiated cells expressed liver cell proteins, such as Albumin, HNF4 alfa, and AFP. In a similar way we demonstrated the potential of these cells to differentiate into tissue of mesodermic origin, namely, bone tissue. Cultivating these cells in an osteogenic culture medium at a density of $3x10^3$ cells per cm^2, we demonstrated that these cells can differentiate into bone tissue expressing specific bone tissue genes, such as osteonectin, osteopaptin, and osteocalcin.

Taken together, these data and the previous evidence of multipotent cells found in adult tissues, which had a certain degree of pluripotency because they were derived from early embryonic cells and maintained in the adult stage, such as very small embryonic-like (VSEL) stem cells in the bone marrow (Zuba-Surma et al., 2009), suggested that dental pulp might be an important source for the isolation of cells with a pluripotent character. It could be speculated that those DPPSCs might be derived from residual undifferentiated cells in the dental pulp.

The characteristics unique to these cells are under investigation, but the current evidence opens pathways to future comparative studies of the regenerative potency between the two sources. In addition, the possibility of freezing them seems feasible, a process that is currently being performed with cells from the umbilical cord.

The relationship between DPPSC and iPS cells should also be investigated. It was demonstrated that the induction of iPSs from stem cells seems to be easier than that from differentiated cells(Yan et al., 2010). One possibility is that the reprogramming process might occur in DPPSCs rather than in the differentiated dermal fibroblasts. Thus, only DPPSCs could selectively expand in culture. Moreover, the number of reprogramming factors could be reduced when using DPPSCs as a target. Further studies are needed to answer these questions.

From the results obtained, we plan to explore new avenues for future studies in regenerative medicine using easily accessible cells (such as DPPSCs) that have an embryonic character and a pluripotential regenerative capacity.

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