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A New Population of Human Adult Dental Pulp Stem Cells: A Useful Source of Living Autologous Fibrous Bone Tissue (LAB)

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ABSTRACT: Stem cells, derived from human adult dental pulp of healthy subjects 30–45 years of age, were cultured, and cells were selected using a FACSorter. A new c-kit$^+$/CD34$^+$/CD45$^-$ cell population of stromal bone producing cells (SBP/DPSCs) was selected, expanded, and cultured. These SBP/DPSCs are highly clonogenic and, in culture, differentiate into osteoblast precursors (CD44$^+$/RUNX-2$^+$), still capable of self-renewing, and then in osteoblasts, producing, in vitro, a living autologous fibrous bone (LAB) tissue, which is markedly positive for several bone antibodies. This tissue constitute an ideal source of osteoblasts and mineralized tissue for bone regeneration. In fact, after in vivo transplantation into immunocompromised rats, LAB formed lamellar bone-containing osteocytes.

Introduction: Recently it has been reported that human dental pulp stem cells (DPSCs) are detectable, in humans, only up to the age of 30 years and that they are able to produce in vitro only sporadic calcified nodules and to form, after transplantation in vivo, a mineralized tissue.
Materials and Methods: Stem cells, derived from human adult dental pulp of healthy subjects 30–45 years of age, were cultured, and cells were selected using a FACSorter. Light microscope, histochemistry, immunofluorescence, and RT-PCR analyses were performed to study both stem and differentiating cells.
Results and Conclusions: A new c-kit$^+$/CD34$^+$/CD45$^-$ cell population of stromal bone producing cells (SBP/DPSCs) has been selected by FACSorting, expanded, and cultured. These SBP/DPSCs are highly clonogenic and, in culture, differentiate into osteoblast precursors (CD44$^+$/RUNX-2$^+$), still capable of self-renewing, and in osteoblasts, producing, in vitro, a living autologous fibrous bone (LAB) tissue. This new-formed tissue is markedly positive for several antibodies for bone, including osteonectin, bone sialoprotein, osteocalcin, fibronectin, collagen III, and bone alkaline phosphatase (BALP). Cells producing LAB can be stored at −80°C for a long period of time and are an extraordinary source of osteoblasts and mineralized fibrous bone tissue. In this study, we also showed that, in aged humans, stem cells can be detected from their pulps. The produced LAB is a fibrous bone tissue resembling the human bone during mineralization, with an external layer formed by osteoblasts markedly positive for osteocalcin. This newly formed tissue constitute an ideal source of osteoblasts and mineralized tissue for bone regeneration. In fact, after in vivo transplantation into immunocompromised rats, LAB formed lamellar bone containing osteocytes.
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Key words: dental pulp, adult stem cells, fibrous bone, osteoblasts, differentiation

INTRODUCTION

Several studies have been carried out to verify if stem cells could become a source of stable differentiate cells capable of inducing tissue formation. Among these, mineralization of hard tissues is of great importance in normal growth and development, and problems in mineralization are common in oral pathologies.[1,2] Recently, the role of stem cells for hard tissue formation has considerably increased attention of researchers as these cells can be a possible, fascinating source of stable differentiated cells, capable of inducing bone formation and control hydroxyapatite crystal growth. Dental pulp stem cells (DPSCs) have already been identified, albeit in a very few number of cases and only from subjects up to 30 years old.[1,3] These stem cells, under specific stimuli, differentiate into several cell types, including neurons, adipocytes, and nestin$^+$ cells. Moreover, it has been shown that they

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TABLE 1. CHARACTERISTICS OF SAMPLES

Sample no.	*Age*	*Tooth*	*Sex*	*Nodules*†
1	36	28	♀	24 ± 2
2	23	18	♀	28 ± 4
3	18	18	♂	26 ± 3
4	20	38	♂	35 ± 6
5	30	28	♂	35 ± 4
6	23	48	♀	27 ± 2
7	33	44	♀	28 ± 2
8	21	18	♂	29 ± 3
9	35	38	♀	28 ± 3
10*	23	18	♂	0
11	29	28	♀	25 ± 3
12*	23	38	♂	0
13	21	38	♂	25 ± 3
14	45	28	♂	29 ± 3
15	37	38	♀	28 ± 2
16	24	18	♂	28 ± 3

* Teeth numbers 10 and 12 belong to the same subject, in which pulps no stem cells were found.

† Number of calcified nodules counted per 25-cm^2 flask in each subject. At least three flasks per subject were obtained. Data are mean ± SD.

form sporadic dense nodules in vitro, but undergo mineralization and bone or dentin-like formation only when grafted in vivo.[2–5]

Using different stem cell markers and a different technique, we have successfully isolated and selected for the first time a distinctive and highly enriched population of stem cells derived from dental pulps in aged adult humans. This stem cell population displays a terrific capability of self-expanding and differentiating in pre-osteoblasts, which are able to self-maintain and renew. They differentiate in osteoblasts, already producing in vitro a living autologous fibrous bone (LAB) tissue. This hard tissue is an useful, extremely interesting source for autotransplants because of its ability to be continuously produced in vitro and to survive after being stored at +4°C for 24 h. In this paper, we stress that stem cells can be obtained from dental pulps of subjects between 30 and 45 years of age using specific antibodies for stromal stem cells; therefore, 30 years should not be considered as a critical age limit. The stem population that we have selected, called stromal bone producing DPSCs (SBP/DPSCs), seems to be quite different from the pulp stem cell population previously described and not selected by flow sorting, mainly for its ability to induce the formation of LAB, which after transplantation into immunocompromised rats, formed a lamellar bone containing osteocytes.

MATERIALS AND METHODS

Subjects, dental pulp extraction, digestion, and culture

Human dental pulp was extracted from molars ($n = 16$) of healthy subjects 30–45 years of age (Table 1) after informed consent. Each subject, before extraction, was checked for systemic and oral diseases and pretreated a week before with professional dental hygiene. Before extraction, the dental crown was covered with a 0.3% chlorexidin gel (Forhans) for 2 minutes. Dental pulp was obtained using a Gracey curette. Pulp was removed and immersed in a digestive solution: 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml claritromycin in 4 ml 0.1 M PBS, with the addition of 3 mg/ml type I collagenase and 4 mg/ml dispase, for 1 h at 37°C. Once digested, the solution was filtered onto 70-μm Falcon strainers (Becton & Dickinson, Sunnyvale, CA, USA). After filtration, cells were immersed in α-MEM culture medium, added with 20% FBS, 100 μM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Milan, Italy). The cell suspension was centrifuged (10 minutes at 140*g*), and the pellet was resuspended in 12 ml of the same culture medium and placed in 25-cm^2 flasks. Flasks were incubated at 37°C in a 5% CO_2, and the medium was changed twice a week.

Monoclonal antibodies, immunofluorescence, and flow-cytometry: FITC, PE, and cychrome-labeled monoclonal antibodies (mAb) against c-kit, CD34, CD45, anti-RUNX-2 transcription factor, and isotype-matched controls were purchased from Santa Cruz. Anti-CD14 and anti-CD44 mAb were purchased from MBL (Woburn, MA, USA). Phenotype analysis of the stem cell population was performed at days 22 and 40 of culture using anti-c-kit, anti-CD34, anti-CD45 mAb or anti-CD14, anti-CD44, and anti-RUNX-2. For indirect immunofluorescence assay, FITC-labeled goat anti-mouse anti-sera (Santa Cruz) were used. Stem cells were always detached using a 10-minute treatment at 37°C with PBS/0.02% EDTA. For cell sorting experiments, stem cell cultures at day 22 were harvested and analyzed for c-kit, CD34, and CD45 expression. A cell population, characterized by low forward scatter and c-kit and CD34 expression, was selected and sorted by using a FACSVantage flow cytometer (Becton Dickinson, Mountain View, CA, USA). Flow cytometry, cell sorting, and data analysis were performed using FACSVantage apparatus equipped with a water-cooled argon ion laser (488 nm, 150 mw) and the CellQuest Software (Becton Dickinson). Both positive cells for c-kit and CD34 were sorted and collected. Nonsorted cells were used as controls.

At day 40, cells were examined for the following monoclonal mouse anti-human antibodies: CD14, CD44 (MBL), and osteocalcin (Santa Cruz), and the transcription factor RUNX-2 (Santa Cruz). The secondary antibodies were goat anti-mouse (FITC; Santa Cruz). For RUNX-2 analysis, cells were fixed in 4% paraformaldehyde in 0.1 M PBS, with 0.2% TritonX100 for 30 minutes at 4°C, washed twice in 0.1% BSA in 0.1 M PBS, and incubated with RUNX-2 antibody. Isotypes and nonprobed cells were used as controls.

Colony efficiency and proliferation potential assays: Low-density sorted cell suspensions were plated in 96-well plates to obtain one or two cells per well. After 3 weeks of culture, cells were stained with 0.1% (wt/vol) toluidine blue in 1% paraformaldehyde. The number of colonies (>50 cells) were counted. A comparison between young (up to 29 years old) and old (30–45 years old) subjects was performed.

Living autologous bone (LAB) number evaluation

Living autologous bone formation was evaluated. The number of calcified nodules per flask was counted, and a comparison between young and old subjects was performed. Data are given as means ± SD.

Induction of adipogenesis in vitro: We observed the adipogenic differentiation of sorted and cultured cells in vitro, in cells placed in separate flasks whose culture medium was added of 10^{-8} M dexamethasone twice a week for 6 weeks, starting at day 15 of culture. Sudan black and Oil red-O staining were used to identify if lipid-laden fat cells were present.

Histology, histochemistry, and immunofluorescence: Differentiated cells and calcified matrix were removed from flasks using a solution of 50/50 1 M trypsin and 0.5% EDTA, and fixed in 4% paraformaldehyde in 0.1 M PBS for 48 h at 4°C, pH 7.4, washed in 0.1 M PBS, pH 7.4, at 4°C, dehydrated, embedded in paraffin, and sectioned (5 μm thick). Slides were stained with H&E, alizarin red, and Schmorl silver nitrate. For histochemistry and immunofluorescence, cells were washed in 0.1 M PBS and fixed in 4% paraformaldehyde in 0.1 M PBS, with 0.2% TritonX100 for 30 minutes at 4°C, and washed twice in 0.1% BSA in 0.1 M PBS at room temperature for 10 minutes. Cells were covered using alkaline phosphatase (ALP) standard solution, incubated in dark for 8 h. ALP activity was performed using 100,000 cell samples, detached by means of PBS/EDTA 0.02% and centrifuged for 10 minutes at 140*g*. The pellet was incubated with 1 ml of BMPurple solution (Roche) for 8 h in dark. The supernatant was read in a spectrophotometer at 615 nm. As a control, c-kit$^-$/CD34$^-$ cells were used. The values were expressed as a ratio between samples and the BMPurple stock solution. BMPurple solvent was used as blank.

For immunofluorescence, cells were washed twice in 0.1 M PBS at room temperature for 10 minutes, fixed in 4% paraformaldehyde in 0.1 M PBS for 48 h at 4°C, pH 7.4, washed in 0.1 M PBS, pH 7.4, at 4°C, and incubated overnight at 4°C with antibodies. LAB samples were embedded in TBS (tissue freezing medium; Triangle Biomedical Sciences, Durham, NC, USA) and cryosectioned (Cryostat 1720 Digital MGW; Lauda, Leika, Germany), fixed in 100% ethanol for 30 minutes at 4°C, washed in 0.1 M PBS, left for 60 minutes in PBS/6% milk, and incubated with antibodies at 4°C overnight.

Antibodies for cells or LAB were the following: osteonectin, fibronectin (Novo Castra, Newcastle, UK), bone sialoprotein (BSP; BIODESIGN International), BALP (US Biological), all mouse anti-human; osteocalcin and collagen III (Santa Cruz) were goat anti-human. The secondary antibodies were goat anti-mouse (FITC) and mouse anti- goat (PE conjugated; Santa Cruz). Cells and LAB were observed under the fluorescence microscope (Fluorescence microscope Axiovert 100; Zeiss).

RT-PCR analysis: Total RNA was extracted from 1,000,000 cells of 18-, 30- and 37-year-old subjects at each time-point (day 22 for nondifferentiated cells and 40 and 60 days for differentiated cells) by homogenization in TRI Reagent (Sigma, Milan, Italy), following the manufacturer's instructions and stored at –70°C until the assays. cDNA synthesis was carried out from total RNA using Superscript II reverse transcriptase (Invitrogen Celbio Italy, San Giuliano Milanese, Milan, Italy), using oligo $(dT)_{12–18}$ and Moloney murine leukemia virus RT (10 U/μl) in 20 μl at 42°C for 50 minutes.

PCR analyses were made in triplicates using a TC-312 thermal cycler (Techne, Burlington, NJ, USA), in which samples underwent a 2-minute denaturing step to 94°C, followed by 35 cycles of 94°C for 30 s, 54°C for 45 s, 72°C for 1 minute, and a final extension step at 72°C for 4 minutes. The PCR mixture contained 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.2 μM of each primer. The primer sequences were as follows: forward RUNX-2, 5′-CACTCACTACCACACCTACC-3′; reverse RUNX-2, 5′-TTCCATCAGCGTCAACACC-3′; forward β-actin, 5′-TGTGATGGTGGGGAATGGGTCAG-3′; reverse β-actin, 5′-TTTGATGTCACGCACGATTTCC-3′. The amplification products were separated on a 2% agarose gel in Tris acetate EDTA (TAE) buffer. PCRs were performed on RT-negative samples to exclude DNA contamination.

Transplantation: LAB samples, each measuring ~1.5 × 1 cm (n = 2/rat), were transplanted into the dorsal surface of 10- to 12-week-old immunocompromised rats. Rats (Wistar rats n = 5; Charles River Laboratories, Calco, Lecco, Italy) were immunocompromised using cyclosporin A (Sandimmun; Novartis, Origgio, Varese, Italy) at a dose of 15 mg/kg body weight, administered 4 h before transplantation and then daily for 2 weeks. During the last 2 weeks, the daily dose was reduced gradually down to 6 mg/kg body weight. LAB, being an already formed hard tissue (fibrous bone), did not need a scaffold support for transplantation. All procedures were approved by our internal small animal ethics committee. Transplants were recovered at 4 weeks after transplantation, fixed with 4% formalin, decalcified with buffered 10% EDTA, pH 7.4, and embedded in paraffin. Sections (5 μm thick) were deparaffinized and stained with H&E.

RESULTS

Adult pulp stem cells were obtained from 16 permanent teeth from subjects 18–45 years of age (Table 1). Stem cell isolation was performed by enzymatic digestion, and cells were placed in culture medium. By day 1 of culture, cells were adherent and often aggregated in groups (Fig. 1A). Spherical-like clusters were present until the fifth day of culture. Stem cells, after being placed separately, quickly expanded, forming adherent, elongated cells, with thin expansions (Fig. 1B). To determinate the proliferation rate and the clonogenic potential of cells, we performed a limiting dilution assay. After 3 weeks of culture, 88% of wells (422/480), which were initially plated with one or two cells, contained colonies (formed of >50 cells), with a doubling time of ≈4 days, showing a very high clonogenic capability.

Immunoreactivity profiles of cultured cells were performed, detecting specific antigens for stem cells. In particular, we selected a stem/progenitor cell population by testing the following markers: c-kit[6] CD34,[7] and CD45.[6] Until now, for dental pulp stem cell detection,

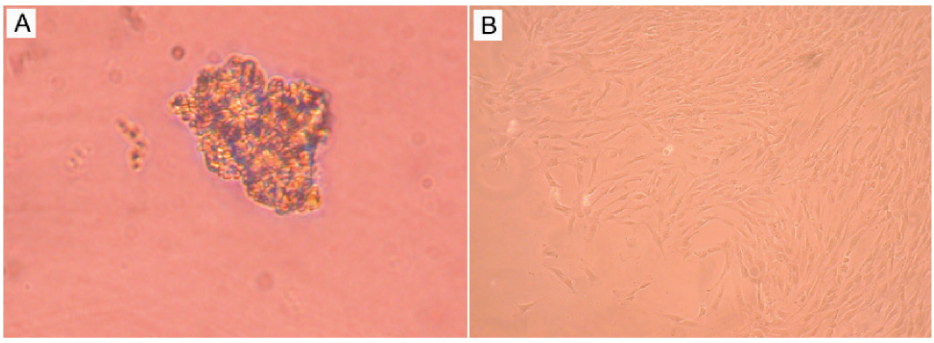

FIG. 1. (A) Aggregated stem cells at day 1 of culture. Cells were adherent and formed aggregates, called embryoid bodies (original magnification, ×400). (B) Expanded stem cells in culture at day 15. Cells are adherent, elongated in shape, with thin expansions (original magnification, ×100).

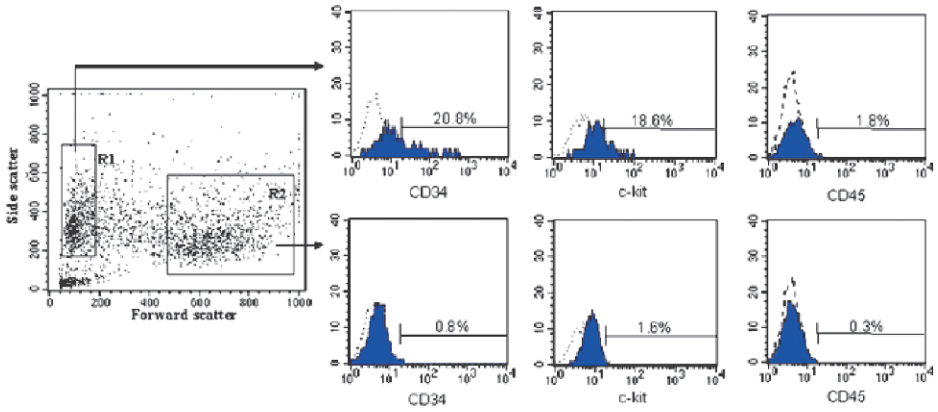

FIG. 2. Representative FACS of cells (belonging to a 23-year-old subject), performed at 22 days of culture. Using FACS analysis, two different cell populations were evidenced and respectively called R1 and R2 based on their size and granularity. A significant number of dental pulp stem cells, belonging to the R1 population, were positive for c-kit and CD34, but negative for CD45. Within the R2 population, only a small and not significant number of cells were found to be positive for those markers. Therefore, only positive cells for c-kit and CD34 belonging to the R1 population were sorted and used for the experiment. Control isotypes PE-conjugated and FITC-conjugated were negative.

c-kit (CD117) has not been used, although it is a useful stem/progenitor cell marker that specifically interacts with the stem cell factor (SCF) and neural crest precursors.[6,8] Previously, for dental stem cells, instead of CD34, STRO-1 has been used,[3,4] which detects only a 5% fraction of $CD34^+$ stem cells.[7] Moreover, CD34 detects an hematopoietic progeny, albeit only up to a 3% of the population,[9] whereas it is a marker for primitive pluripotential stem cells stromal precursors.[9] CD45 is a marker of hematopoietic stem cells and progenitors.[9,10] This explains the rationale of our choice that successfully led to our results.

Cytometric flow analysis, performed at day 22 of culture, showed that, cell population was $c\text{-}kit^+$ and $CD34^+$, but $CD45^-$ (Fig. 2). Isotypes and nonprobed cells, used as controls, were negative. Interestingly, positivity for c-kit and CD34 by expanded and cultured primary pulp cells clearly indicated their stromal origin because cells were $CD45^-$. We selected and sorted these $c\text{-}kit^+/CD34^+/CD45^-$ cells: these SBP/DPSCs stem cells were used to continue the culture by adding a 20% FBS to induce cell differentiation.[11] At day 30, these cells formed aggregates (Fig. 3A), which started to produce an extracellular matrix (Fig. 3B). By day 36, these cellular aggregates further grew, forming several mineralized hemispheric centers (Figs. 3C and 3D). The appearance of these centers was scattered within each flask, and their numbers were dependent on cellular density. The initial number of aggregates was of 600 ± 56 per flask. Then, because of confluence of several aggregates into the same nodule, the final number of calcified nodule per 25 cm^2 ranged between 24 ± 2 and 35 ± 6 (see Table 1). By day 50, mineralized tissue formed a network of newly synthesized LAB (Figs. 3C and 3D). LAB stopped growth only when the medium was not sufficient to cover the whole newly formed structure. The final thickness of this tissue was in relationship to the medium level height; we obtained mineralized tissue up to 15 mm of thickness. When confluent, cells stopped to proliferate, although they continued to form further LAB. After 7 months of culture, no signs of senescence such as stopping of proliferation or significant signs of cell death were observed.

Results have shown that, when comparing stem cells and differentiated cells obtained from young (up to 29 years) and old (30–45 years) subjects, no appreciable differences were found. In particular, neither stem population nor their expansion rate was comparable when subjects were 40–45 years old. Only in one subject, 23 years old, were no stem cells found in two dental pulps, as shown in Table 1. In addition, the number of calcification centers and

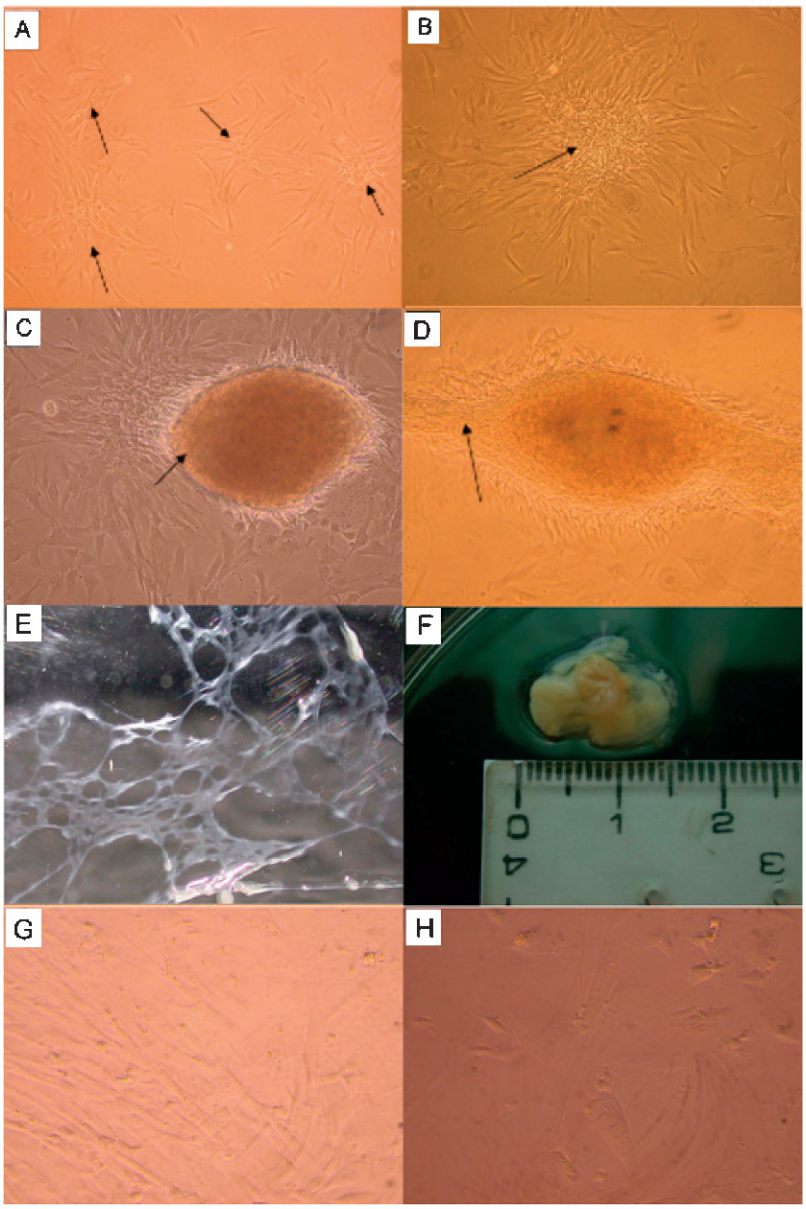

FIG. 3. (A) Differentiating cells, at day 30 of culture, started to deposit, within the flasks, a mineralized matrix forming aggregated centers (arrows; original magnification, ×100) and (B) producing an extracellular matrix (arrow; original magnification, ×100). (C) By day 36, these cellular aggregates grew further, forming several mineralized hemispheric centers (arrow; original magnification, ×200). These centers (D) further grew at day 40, when they started to build a trabecular network (arrow; original magnification, ×200). All pictures belong to a 38-year-old subject. (E) A network of newly synthesized LAB is observable at day 50 (original magnification, ×1). (F) From the flask, a single 12-mm bone nodule of LAB has been taken (original magnification, ×1). Cells and LAB belong to the same 37-year-old subject. Cells that were not selected at the FACSorting and cultured as controls showed a mixed population of differentiated cells, including a great number of (G) fibroblasts and some osteoblasts, often isolated or surrounded by (H) fibroblasts (original magnification, ×200). Cells belong to a 24-year-old subject.

LAB nodules were comparable when counted in young and old subjects, without appreciable differences (Table 1). Cells that were nonselected at the FACSorting and cultured as controls showed a mixed population of differentiated cells, including a great number of fibroblasts and some osteoblasts (Figs. 3E and 3F). Moreover, within these flasks, only occasionally calcified centers were found, but they never become LAB nodules.

SBP/DPSCs are pluripotent stem cells because they can differentiate toward other cytotypes. For instance, we induced adipocyte differentiation by adding 10^{-8} M dexamethasone[12] to cultured (c-kit$^+$/CD34$^+$/CD45$^-$) cells. After 37 days (15 days after stimulation), they presented with a lipid content that was stained with Sudan black (Fig. 4A), whereas cells that were not treated with 10^{-8} M dexamethasone did not undergo adipocyte differentiation and were negative for Sudan black staining (Fig. 4B). In addition, we performed, on the same samples, the Oil red-O staining, which was positive in 10^{-8} M dexamethasone-treated cells (Fig. 4C). By day 60, analysis of LAB tissue showed that it was surrounded by cuboidal cells forming an external cell monolayer, which was completely superimposable to the osteoblasts that in vivo are commonly located along the surface of the trabeculae during the ossification process, whereas the remaining cells were located within the tissue matrix (Fig. 5A). Moreover, LAB was markedly positive for alizarin red (stained in red or orange; Fig. 5B) and Schmorl (stained in black; Fig. 5C) stainings, both detecting calcium deposits. Histochemically, LAB showed a significant positivity for ALP (Fig. 5D). In particular, we showed that ALP activity (Fig. 5E) increased as well as cells that were differentiated, concomitantly to the decrease of the BMPurple salts that did not react; in fact, differentiated cells showed a significant level of activity ($p < 0.001$) toward

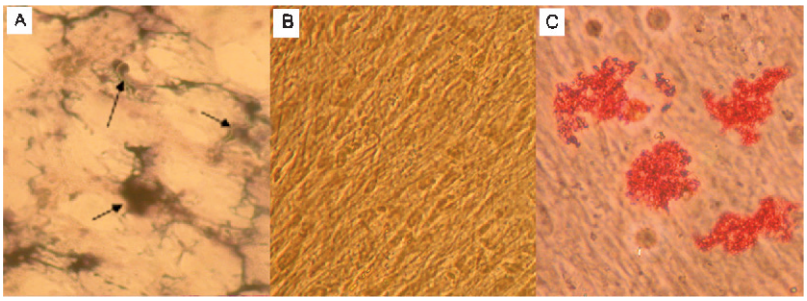

FIG. 4. (A) Adipocyte differentiated cells belonging to sorted cells after 10^{-8} M dexamethasone supplementation to the culture medium (dexamethasone was added twice a week for 6 weeks) are stained with Sudan black, specific for adipocytes. Lipid content is stained in black (arrows; original magnification, ×100). (B) Differentiated cells, nonsupplemented with dexamethasone, used as controls, were negative (original magnification, ×200). (C) Differentiated cells, treated with dexamethasone, were also stained using Oil red-O (original magnification, ×200). All the pictures were obtained from cells of a 23-year-old subject.

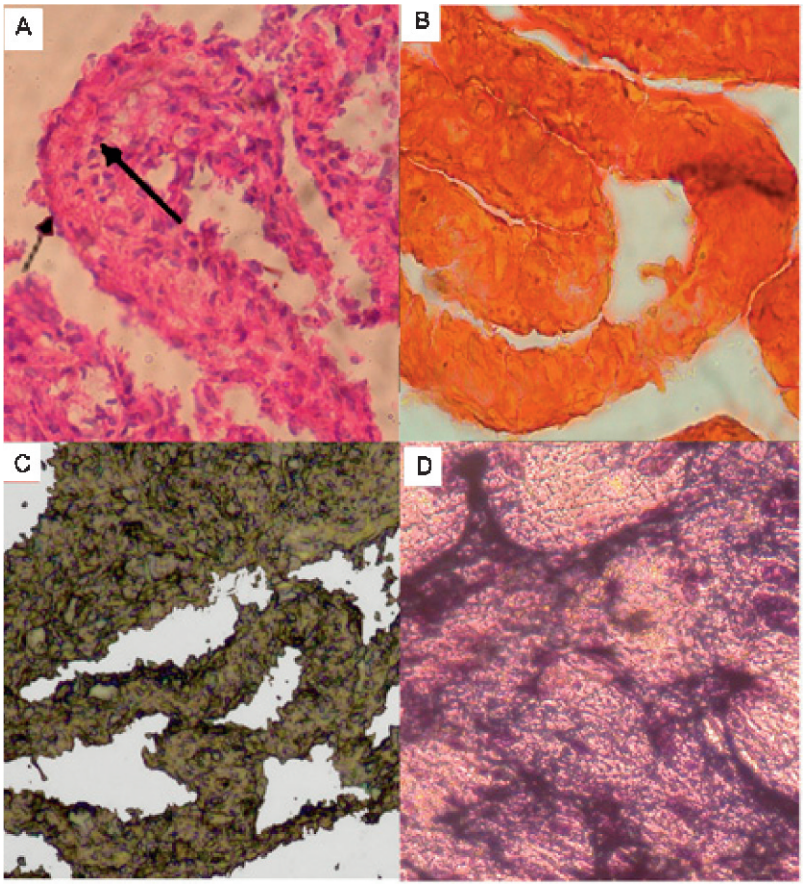

FIG. 5. The newly formed LAB stained with H&E. (A) Cells either form an external cuboidal layer (arrow) or are detected within (thick arrow) a densely stained matrix (original magnification, ×100). (B) LAB is also markedly stained with alizarin red (original magnification, ×100). (C) Schmorl silver nitrate (original magnification, ×100) and (D) ALP (original magnification, ×200). All pictures were obtained from cells of a 20-year-old subject. (E) ALP activity, read in a spectrophotometer at 615 nm, was expressed as the ratio between equal volumes of sample supernatant and BMPurple stock solution (1 ml). BMPurple solvent was used as blank. c-kit$^-$/CD34$^-$ cells were used as controls. *$p <$ 0.001; **$p < 0.01$.

c-kit$^-$/CD34$^-$ control cells. In addition, data showed that age did not significantly affect ALP activity (Fig. 5E).

Cells forming LAB were probed for differentiating markers, including CD44 (which detects adherent mesenchymal cells),[13] osteocalcin (a specific protein produced by osteoblasts, which is detectable both in cell membranes and within the tissue matrix),[14] and RUNX-2 (an early transcription factor for osteoblast precursors).[14–16]

Using immunofluorescence, LAB was largely positive to markers for fibrous bone tissue such as fibronectin, collagen I and III (Fig. 6A), BSP and BALP (Fig. 6B), osteonectin, and osteocalcin; in particular, osteoblasts forming the monolayer surrounding the new formed trabeculae of the LAB were intensely positive for osteocalcin (Fig. 6C), further indicating that these cells were osteoblasts involved in the ossification process. Results showed that sorted and cultured cells, by day 50, albeit not more positive for stem cell markers, were positive for CD44 (100%), RUNX-2 (68.65 ± 2.0%), and osteocalcin (28.45 ± 1.7%). RT-PCR analysis for RUNX-2 has shown that the mRNA transcripts

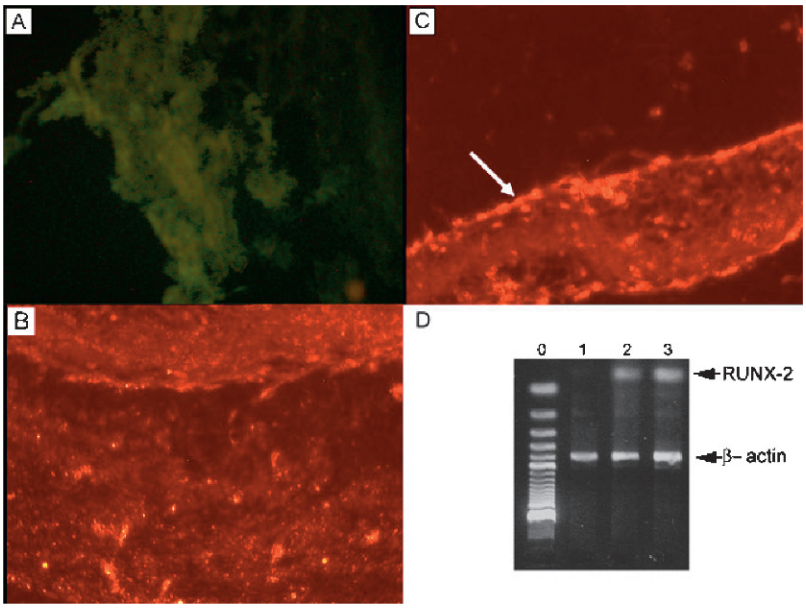

FIG. 6. The newly formed LAB contains several specific bone tissue proteins and is stained with several antibodies. The new formed tissue is diffusely positivity for (A) collagen III and (B) for BALP (original magnification, ×100). Cells were obtained from a 29-year-old subject. (C) Osteoblasts, of cuboidal shape, form an external layer (arrow) along newly formed trabeculae of the LAB and are markedly positive for osteocalcin (original magnification, ×200). Cells were obtained from the same subject (29 years old). (D) Representative RT-PCR analysis performed for RUNX-2. RNA was extracted from 18-, 30-, and 37-year-old subjects, and RT-PCR analyses were performed in triplicate. Agarose gel shows the RUNX-2 PCR products in days 22 (lane 1, predifferentiation time), 40, and 60 (lanes 2 and 3, differentiation times). At each time as positive control a β-actin PCR product was amplified.

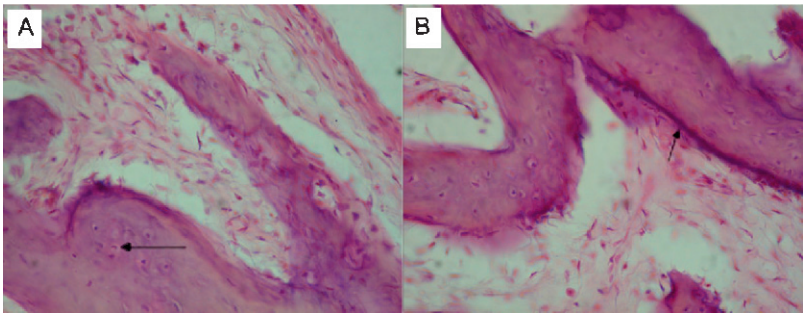

FIG. 7. In vivo lamellar bone formation after LAB transplantation in immunocompromised rats. (A) Osteocytes (arrow) are embedded in lamellar bone, surrounded by loose connective tissue. The transplanted LAB belongs to a 23-year-old subject (original magnification, ×400). (B) Well-developed lamellar bone whose trabeculae show osteoblasts (arrow) surrounding the tissue and osteocytes within it. The transplanted LAB belongs to a 36-year-old subject (original magnification, ×400).

of this transcription factor were present in differentiated cells at days 40 and 60 but not in sorted and not yet differentiated cells at day 22 (Fig. 6D).

To assess their capability to survive and restart differentiation after being stored, cells were frozen and kept for 6 months at –80°C and then thawed and placed in culture medium. After adhering, they started to synthesize new LAB with the same characteristic of the tissue formed by nonfrozen cells. This suggests that they can retain their differentiate potential after long-term storage.

In addition, we showed that LAB transplantation into immunocompromised rats, in all cases, formed a lamellar bone with osteocytes within the bone and osteocytes surrounding the trabeculae (Figs. 7A and 7B).

DISCUSSION

In this study, we describe the remarkable ability of ex vivo expanded human pulp stem cells, taken from adult teeth (DPSCs) and selected by flow cytometry, to differentiate into osteoblasts and produce in vitro a living autologous fibrous bone tissue (LAB). A steady cell population called SBP/DPSCs was established from these selected cells.

Previous studies have shown that stem cells isolated from the pulp of adult human teeth and expanded in vitro showed an ~9% positivity for STRO-1, an antibody that identifies a cell surface antigen expressed by stromal precursors in human bone marrow as well as in erythroid precursors,[17,18] which is considered an early marker of mesenchymal stem cells.[18–20] Actually, STRO-1 recognizes a stromal cell precursor of pericyte cells and ~5% of $CD34^+$ stem cells.[7,19] In humans, CD34 identifies a cell surface antigen expressed by the most primitive stromal other than hematopoietic stem cells, gradually lost after lineage committed progenitors differentiation.[6,9] We have found that adherent dental pulp cells are $CD34^+$ (10.09% of the whole population). Because $STRO\text{-}1^+$ cells are also $CD34^+$, we can infer that previously isolated dental pulp stem cells are likely present in $CD34^+$ population.

For the first time, we have challenged adult dental pulp stem cells with c-kit, a well known and widely used marker for stem cells.[6,9] Our data show that it is highly expressed in dental pulp cells (16.81%). Until now, c-kit, but not CD34, has been reported to be expressed in human mesenchymal stem cells isolated from bone marrow,[21] although CD34 has been found to be variably expressed in

murine mesenchymal stem cells (MSCs).[22] Head and neck hard tissues of the body have, other than a mesodermal origin, a neural crest source, and it is has been shown that c-kit is expressed in neural crest-derived cells, such as melanocyte precursors.[8] The latter is compatible with a presence of c-kit expressing cells in the area of developing teeth. Therefore, we have associated CD34 and c-kit expression to isolate a population of stromal stem cells of neural crest origin.

The absence of positivity for the hemopoietic marker CD45, which recognizes the leukocyte common antigen, monocytes, and T-cell subsets,[9] suggests that, within isolated pulp cells, hematopoietic progenitors are not present. This strengthens the finding that $CD34^+$ and c-kit^+ cells are stromal stem cells.

Once purified, the $CD34^+/cKit^+/CD45^-$ cells were placed in culture, and they further proliferated, starting to secrete an extracellular matrix containing proteins highly expressed in the new formed fibrous bone tissue, such as osteonectin, fibronectin, collagen III, BSP, and BALP (day 50). The appearance of a mineralized matrix was confirmed using a specific calcium deposit staining showing the formation of a LAB, other than by high levels of ALP, whose activity significantly increased in differentiated cells and was not affected by the age of the subject. Moreover, the newly formed trabeculae of the LAB, showing an external layer of cuboidal cells highly $osteocalcin^+$, further showed that these cuboidal cells are osteoblasts involved in the mineralization process. This study showed that no differences were found when comparing stem cells and differentiated cells obtained from young (up to 29 years) and old (30–45 years) subjects, regarding their expansion rate and number of calcification centers and LAB nodules obtained per well.

It is already known that adult pulp stem cells differentiated either after specific stimulation or after unknown stimuli, toward several cell types, including odontoblast-like, neurons, adipocytes, myocytes, and nestin-positive cells. It is also well known that postnatal stem cells exert an extraordinary plasticity and that individual stem cells, when expanded to colonies, retain their multilineage potential.[12,17] Our selected cells are also capable of differentiating into other lineages, as we have shown.

Cytometric flow analysis, made on differentiated cells, at day 40, showed that these cells were negative for all the stem cell antigens and resulted in $CD14^-$, $CD44^+$, $osteocalcin^+$, and $RUNX\text{-}2^+$, showing that these cells undergo an osteoblastic differentiation. In fact, these cells strongly express RUNX-2, a transcription factor essential for osteoblast differentiation[14,23]; this transcriptional factor is closely related to the promotion of ossification[14,24] and targeted disruption of RUNX-2 results in complete lack of fibrous bone formation by osteoblasts.[25] We have found that at days 40 and 60, a noticeable expression of RUNX-2 mRNA transcripts is detected at RT-PCR analysis, confirming the presence of this transcription factor within the cells. In particular, our findings show that cells are 70% $RUNX\text{-}2^+$ and 30% $RUNX\text{-}2^-$ and $osteocalcin^+$, whereas this transcription factor is not present at day 22 in not yet differentiated cells. Therefore, among our cell population, the minority number (~30%, $RUNX\text{-}2^-$ and $osteocalcin^+$) is formed by terminally differentiated osteoblasts, whereas the majority of the cells (~70%) is formed by their precursors or pre-osteoblast cells, still $RUNX\text{-}2^+$, which can easily expand and subculture, suggesting the existence of self-renewal cells. By day 40, all cells are $CD44^+$, an integral membrane protein interacting with components of extracellular matrix, including osteonectin, bone sialoprotein, collagens, and fibronectin, to maintain organ and tissue structure.[13]

We also found that SBP/DPSCs are highly clonogenic, showing a high proliferation potential, and are able to self-maintain for long time, still living in culture after 6 months without showing appreciable signs of senescence. Moreover, both tissue and cells, after storage at +4 or −80°C (for long-term), have shown a high vitality and capability of quickly restarting both proliferation and tissue production. Therefore, these cells are capable of retaining their differentiate potential after long-term storage. Moreover, after in vivo transplantation into immunocompromised rats, LAB samples have been found to be able to form a lamellar bone with osteocytes within the bone and osteoblasts around the lamellae. This further shows that LAB samples, once transplanted in vivo, being an already formed hard tissue, do not need the use of a scaffold and are quickly and easily remodeled into a lamellar bone, most probably to osteoclast recruitment.

In conclusion, this study has shown evidence in several interesting topics: (1) within human pulp, taken from adult teeth (DPSCs) of subjects up to 45 years of age, by means of flow cytometry, a rather consistent number of $c\text{-}kit^+/CD34^+/CD45^-$ stem cells have been selected; (2) these cells, called SBP/DPSCs, can be considered stromal pluripotent stem cells because they express c-kit and CD34 but not CD45 and are able to differentiate into different lineages; (3) SBP/DPSCs, within the culture medium, proliferate and differentiate into preosteoblasts, which still retain a self-renewing capability, and in osteoblasts, form a LAB; (4) LAB is a hard tissue whose trabeculae are surrounded by osteoblasts; (5) this tissue, without the need of scaffold, is able, once transplanted in vivo, to form a lamellar bone with osteocytes; (6) this newly formed tissue may be useful in several pathologies requiring bone tissue growth and repair; and (7) differentiated cells and LAB can be frozen at −80°C and stored for long time, without losing their abilities.

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