Production of Human Dental Pulp Cells with a Medicinal Manufacturing Approach

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Abstract

Introduction: Human dental pulp cells (HDPCs) are generally isolated and cultured with xenogeneic products and in stress conditions that may alter their biological features. However, guidelines from the American Food and Drug Administration and the European Medicines Agency currently recommend the use of protocols compliant with medicinal manufacturing. Our aim was to design an *ex vivo* procedure to produce large amounts of HDPCs for dentin/pulp and bone engineering according to these international recommendations. **Methods:** HDPC isolation was performed from pulp explant cultures. After appropriate serum-free medium selection, cultured HDPCs were immunophenotyped with flow cytometry. Samples were then cryopreserved for 510 days. The post-thaw cell doubling time was determined up to passage 4 (P4). Karyotyping was performed by G-band analysis. Osteo/odontoblastic differentiation capability was determined after culture in a differentiation medium by gene expression analysis of osteo/odontoblast markers and mineralization quantification. **Results:** Immunophenotyping of cultured HDPCs revealed a mesenchymal profile of the cells, some of which also expressed the stem/progenitor cell markers CD271, Stro-1, CD146, or MSCA-1. The post-thaw cell doubling times were stable and similar to fresh HDPCs. Cells displayed no karyotype abnormality. Alkaline phosphatase, osteocalcin, and dentin sialophosphoprotein gene expression and culture mineralization were increased in post-thaw HDPC cultures performed in differentiation medium compared with cultures in control medium. **Conclusions:** We successfully isolated, cryopreserved, and amplified human dental pulp cells with a medicinal manufacturing approach. These findings may constitute a basis on which to investigate how HDPC production can be optimized for human pulp/dentin and bone tissue engineering. (*J Endod* 2015;■:1–8)

Key Words

Cryopreservation, human dental pulp, immunophenotyping, MSCA-1, osteo/odonto-blast differentiation, tissue engineering

Dental research currently explores the potential of cell-based products and tissue engineering protocols to be used as alternatives to usual pulp/dentin and bone therapies. In this context, stem/progenitor cells appear to be particularly appropriate because of their high expansion ability and differentiation potential both *in vitro* and *in vivo* [1]. If bone marrow and adipose tissue are considered potential sources of stem/progenitor cells, painful collection protocols, the decline of the amount of stem/progenitor cells with age, the necessity of general anesthesia, reduced proliferation capacity, and risk of morbidity at the collection site encourage the search for alternative candidates [1, 2]. Human impacted third molars are frequently removed for therapeutic reasons and the loose connective tissue they contain; the dental pulp appears to be a valuable source of stem/progenitor cells for pulp/dentin and bone engineering. Indeed, it contains various cell populations that exhibit osteo/odontoblastic differentiation capabilities and that can be cryopreserved for periods of time greater than 6 months [3–5]. Interestingly, human dental pulp cell (HDPC) populations were recently successfully used for regenerating human pulp/dentin and bone [6, 7].

Cell-based products for tissue engineering are now referred to as human cellular tissue-based products or advanced therapy medicinal products, and guidelines from the American Code of Federal Regulation of the Food and Drug Administration (21 CFR Part 1271) and the European Medicines Agency (European Directive 1394/2007) define requirements for appropriate cell production. These "good manufacturing practices" include recommendations regarding laboratory cell culture procedures to ensure optimal reproducibility, efficacy, and safety of the final medicinal product [8, 9]. In particular, the Food and Drug Administration divides *ex vivo* cultured cells into "minimally" or "more than minimally" manipulated samples according to function of the use or not of procedures "that might alter the biological features of the cells." In this context, most if not all HDPC culture protocols that have been reported so far are unsatisfactory. Indeed, the use of xeno- or allogeneic cell culture media and long-term cell amplification are known to alter the quality of the final cell-based product [10–12]. These findings make the design of new HDPC isolation, characterization, cryopreservation, and amplification *ex vivo* procedures that are compliant with good manufacturing practices regarding medicinal products necessary [5, 7, 13].

The aim of this study was to define a protocol for obtaining a clinical scale number of HDPCs that possess osteo/odontoblastic differentiation potential. Therefore, HDPCs were grown from dental pulp explants on extracellular matrix–coated dishes, and their storage and amplification were performed in serum-free medium (SFM) by using xenogeneic-free products. After appropriate SFM selection, cell immunophenotype, viability, growth kinetics, karyotyping, and differentiation capacity were analyzed to validate our *ex vivo* protocol.

Materials and Methods
Isolation and Amplification of HDPCs

Healthy impacted human third molars were collected from donors aged 13–17 years with informed consent of the patients and their parents in accordance with the World Medical Association's Declaration of Helsinki and following a protocol approved by the local ethics committee. Teeth between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) were used [14]. Dental pulps were aseptically, gently extirpated from pulp cavities with fine tweezers, and the apical part of the radicular pulp was removed with a scalpel to prevent contamination by dental papilla cells. Pulps were then washed twice with phosphate-buffered saline containing 100 IU/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Saint Aubin, France), referred to as P/S from herein, placed onto a sterile glass slide and cut with a scalpel into 0.5- to 2-mm^3 explants. The latter were cultured on dishes precoated with an equal mixture of human placental collagens I and III at a final concentration of 0.5 μg/cm^2 (ABCellBio, Paris, France). HDPCs outgrowing from the explants (referred to as passage 0[P0]-HDPCs) were detached from the culture dish after 2 weeks of culture with xeno-free recombinant protease TrypLe Select 1X (Life Technologies), counted, and either cryopreserved (see below) or plated (5 × 10^3 cells/cm^2) for amplification. The same protease was used for all subsequent passages.

Isolation Success Rate, Cell Outgrowth Surface Area, and Metabolic Assays

Three SFMs called SFM-1, SFM-2, and SFM-3 were tested for assessing HDPC outgrowth formation from the explants. Ninety pulp explants from 5 donors were pooled together to reduce interpatient variability and then separated into 3 equal groups to be cultured in SFM-1, SFM-2, or SFM-3. Explants were then seeded on collagen precoated 12-well plates and cultured in SFM-1 (composition: SPE-IV/EBM [a medium containing clinical grade human albumin, α-minimum essential medium (α-MEM), 25 ng/mL rhIGF-1, and 0.33 ng/mL rhFGF-2; ABCellBio] and P/S), SFM-2 (Dulbecco's Modified Eagle's Medium [DMEM]/F-12/Glutamax [Life Technologies], P/S, 5 ng/mL FGF-2 [R&D Systems, Lille, France] and 5 μg/mL insulin [Umuline, Lily, Neuilly-sur-Seine, France]) or SFM-3 (DMEM/F-12/Glutamax, 1% (v/w) Insulin/Transferrin/Selenium [Life Technologies], and P/S). Cultures were performed for 10 days and then fixed with 10% formalin and stained with a 0.5% crystal violet solution. The isolation success rate was defined as the percentage of explants that give rise to at least 1 HDPC outgrowth. The cell-covered surface area around each explant was calculated by using Image J software (National Institutes of Health, Bethesda, MD). For metabolic activity assessment, first-passage (P1) HDPCs from 5 different donors were pooled and then seeded (5 × 10^3/well) in collagen precoated 96-well plates. Cells were cultured for 68 hours in 200 μL SFM-1, SFM-2, or SFM-3, and then 40 μL of a 5-mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added for 4 hours. Formazan product was solubilized with 99% dimethyl sulfoxide (Sigma-Aldrich) for 15 minutes,

and metabolic activity was measured as absorbance at 590 nm with a Multiskan FC microplate reader (Thermo Fisher Scientific, Courtaboeuf, France).

Multicolor Flow Cytometry

Three million P1-HDPCs cultured in SFM-1 were stained with 17 fluorochrome-conjugated antibodies (Table 1). The nucleic acid dye 7-Amino-actinomycin D (7AAD; BD Biosciences, Le Pont de Claix, France) was used for the exclusion of nonviable cells. Samples were acquired on a BD FACSCanto II Flow cytometer (BD Biosciences) as uncompensated events and recorded as FCS 3.0 files. Analysis and compensation were performed using FlowJo vX software (FlowJo, Ashland, OR). The percentage of cells positively stained corresponded to the percentage of cells present within a gate established so that <1% of the measured positive events represented nonspecific binding by the fluorochrome-conjugated isotype-matched control. Additional fluorescence minus one controls were used for CD271, Stro-1, CD146, and MSCA-1.

Cryopreservation

HDPCs outgrowing from explants (P0-HDPCs) were detached with TrypLe Select 1X and counted. Cells were suspended (10^6/mL) in a solution containing 10% dimethyl sulfoxide and 90% cryogenic SFM (CRYO3; Stem Alpha, Saint-Genis l'Argentière, France). Cells were thoroughly mixed in cryotubes and immediately transferred to an isopropanol-filled Cryobox (Nalgene, Rochester, NY). Samples were then frozen in liquid nitrogen for 510 days.

Cell Viability, Cumulative Doubling Number, and Doubling Time Determination

Post-thaw P1-HDPC viability was compared with that of fresh P1-HDPCs. Cells were stained with trypan blue to discriminate between live and dead cells, and they were counted with a Cellometer auto T4 (Nexcelom Bioscience, Lawrence, MA). For cumulative doubling number (CDN) and doubling time (DT) analysis, HDPCs from P1 to P4 were plated (5 × 10^3 cells/cm^2) on T12.5 flasks (Corning Inc, Corning, NY) until they reached 80%–90% confluence. CDN and DT were calculated from the addition of doubling number counts and the time of culture according to the following formulae:

$$CDN = \ln(nf/ni)/\ln 2 \qquad (1)$$

$$DT = CT/\text{doubling number}(nf = \text{final number of cells} \text{ at } 80\% \text{ confluence, } ni = \text{initial number of cells,} \text{ and } CT = \text{culture time}) \qquad (2)$$

Karyotyping

Post-thaw P4-HDPCs were exposed for 3 hours to 0.7% colcemid (Life Technologies) diluted in the culture medium, and then cells were detached and centrifuged. The pellet was then resuspended in 0.075 mol/L KCl for 2 minutes at room temperature. Cells were centrifuged again, resuspended in methanol acetic acid (3:1) fixative, and stored at $-20°C$ for at least 2 days. G-band staining was performed with the Leishman-Giemsa cocktail.

Osteo/odontoblastic Differentiation and Mineralization Quantification

Post-thaw HDPCs were plated (4.2 × 10^3 cells/cm^2) on plastic dishes and amplified. Once confluence was reached (3–4 days), cells

TABLE 1. Fluorochrome-conjugated Monoclonal Antibodies Used for Immunophenotypic Analysis

Marker	Isotype	Supplier	Reference	Fluorochrome
7AAD	NA	BD Biosciences	559925	Staining solution
CD73	Ms IgG1, κ	BD Biosciences	561258	PE-Cy7
CD10	Ms IgG1, κ	BD Biosciences	555375	PE
CD13	Ms IgG1, κ	BD Biosciences	557454	APC
CD29	Ms IgG1, κ	BD Biosciences	559883	APC
CD44	Ms IgG2b, κ	BD Biosciences	560532	APC-H7
CD49a	Ms IgG1, κ	BD Biosciences	559596	PE
CD90	Ms IgG1, κ	BD Biosciences	555595	FITC
CD105	Ms IgG1, κ	BD Biosciences	560839	PE
CD166	Ms IgG1, κ	BD Biosciences	559263	PE
CD14	Ms IgG2b, κ	BD Biosciences	560180	APC-H7
CD34	Ms IgG1, κ	BD Biosciences	555824	APC
CD45	Ms IgG1, κ	BD Biosciences	560777	V500
HLA-DR	Ms IgG2a, κ	BD Biosciences	561224	V500
CD271	Ms IgG1, κ	BD Biosciences	560834	PerCP-Cy5.5
Stro-1	Ms IgM, λ	Biolegend	340104	AF647
CD146	Ms IgG1, κ	BD Biosciences	560846	FITC
MSCA-1	Ms IgG1, κ	Biolegend	327308	APC

NA, not applicable.

were cultured for 4 weeks in a differentiation medium consisting of DMEM/F-12/Glutamax supplemented with 5% fetal calf serum (Life Technologies), P/S, 10^{-7} dexamethasone, 100 μmol/L ascorbate-2-phosphate, and 10 mmol/L β-glycerol phosphate (all from Sigma-Aldrich). Control cells were cultured in the amplification (control) medium.

For gene expression analysis with real-time polymerase chain reaction, total RNA was extracted by using the RNeasy Mini kit (Qiagen, Courtaboeuf, France). Reverse transcription (RT) was performed from 500 ng total RNA with Prime Script RT reagent kit (Takara; Ozyme, Montigny-le-Bretonneux, France) according to manufacturer's instructions. Real-time polymerase chain reaction amplifications were performed in a 20-μL reaction mix containing 10 μL Fast Start Universal SYBR green master (Roche, Mannheim, Germany), 4 μL RT template diluted 1:3 in sterile water, 300 nmol/L of each primer, and 4 μL sterile water. Amplification was performed in Rotorgene (Qiagen). Thermal cycling conditions consisted of an initial denaturation step at 95°C for 2 minutes and then 40 to 50 cycles of 95°C for 15 seconds and an annealing/extension step at 60°C for 30 seconds. Gene-specific primer sequences were as follows: osteocalcin (OCN), forward: 5'-GAAGCCCAGCGGTGCA-3', reverse: 5'-CACTACCTCGCTGCCCTCC-3'; alkaline phosphatase, forward: 5'-AGCCCTTCACTGCCATCCTGT-3', reverse: 5'-ATTCTCTCGTTCACCGCCCAC-3'; dentin sialophosphoprotein, forward: 5'-ATATTGAGGGCTGGAATGGGGA-3', reverse: 5'-TTTGTGGC TCCAGCATTGTCA-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-ATGGGGAAGGTGAAGGTCG-3', reverse: 5'-TAAAAGCAGCCCTGGTGACC-3'. The GAPDH housekeeping gene was used for sample normalization. Samples were performed in duplicate. The gene expression level was calculated as $2^{-\Delta Ct}$. The GAPDH Ct value was subtracted from the Ct value of the target gene, and results were expressed as relative gene expression to GADPH.

For the quantification of mineralization, HDPCs were fixed for 15 minutes in 10% formalin and then incubated in an alizarin red S (ARS) solution (pH = 4.1–4.3) (Sigma-Aldrich) for 20 minutes. ARS staining was visualized with a DM 750 phase-contrast microscope (Leica, Wetzlar, Germany) coupled to a color camera (ICC 50 HD; Nikon, Champigny-sur-Marne, France). Images were acquired with LAS EZ 2.0 software (Leica). Staining was quantified by removing ARS with a 10% acetic acid solution for 30 minutes, heating at 85°C for 10 minutes, centrifugation at 20,000g for 15 minutes, and neutralization with 10% ammonium hydroxide. Supernatants (150 μL) were dropped on a 96-well plate and read at 405 nm with a Multiskan FC microplate reader.

Statistical Analysis

Values were presented as mean ± standard deviation, and differences were analyzed using the Mann-Whitney U test for nonparametric analysis. The number of independent samples from different donors (n) is indicated in the figure legends. A P value <.05 was considered to be significant.

Results

Efficient isolation and expansion of HDPCs ex vivo require the use of an optimal culture medium. The isolation success rate, cell-covered dish surface area around the explant, and cell metabolic activity were determined for 3 different SFMs. After explant culture for 10 days, SFM-1 showed an outgrowth success rate 19.6% and 10% higher compared with SFM-2 and SFM-3, respectively (Fig. 1A). The cell outgrowth surface area around the explant increased in SFM-1 cultures by 2.78- and 5.64-fold compared with SFM-2 and SFM-3 cultures, respectively (Fig. 1B). HDPC metabolic activity was higher in SFM-1 than SFM-2 and SFM-3 (Fig. 1C). For these reasons, SFM-1 was selected for additional HDPC isolation and expansion experiments. Explant cultures performed with SFM-1 on collagen-coated dishes showed that the first HDPC started to grow from the explants after 3 to 5 days (Fig. 1D). Two weeks later, pooling of cells outgrowing from the explants from 1 dental pulp allowed harvesting of about 10^{6} HDPCs (data not shown).

A multiparametric immunophenotypic analysis of cultured HDPCs was performed with a panel of 17 cell surface markers (Table 1). The gating tree was set so that the initial forward scatter/side scatter (FSC/SSC) gating represented the cell distribution based on size and intracellular composition. Then, the gate was set on 7AAD-negative, live cells (Fig. 2A). All HDPCs expressed the cell surface marker CD73, which was therefore chosen as a basis versus additional markers (Fig. 2B). Almost all CD73-positive HDPCs (≥97%) expressed the mesenchymal markers CD10, CD13, CD29, CD44, CD90, CD105, and CD166, whereas CD49a was expressed by a lower number of cells (85.3%) (Fig. 2C). The hematopoietic markers CD14 (monocyte marker), CD34 (early hematopoietic marker), CD45 (leukocyte marker), and human leukocyte antigen-DR (HLA-DR) (dendritic cell and macrophage marker) were expressed by very few cultured HDPCs
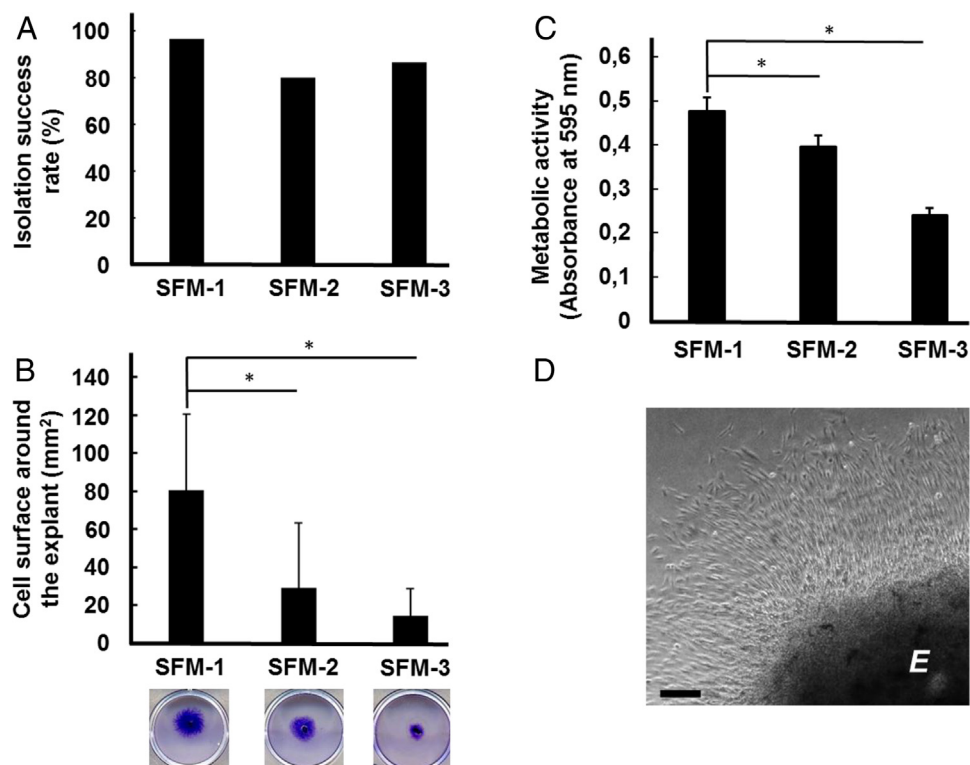
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Figure 1. Selection of SFM for *ex vivo* culture of HDPCs. (*A*) SFM-1 (SPE-IV, insulin-like growth factor–1/fibroblast growth factor–2 [IGF-1/FGF-2]–containing medium) slightly increased the outgrowth success rate by 19.6% and 10% compared with SFM-2 (FGF-2/insulin-containing medium) and SFM-3 (insulin/transferrin/selenium-containing medium). (*B*) The cell surface area around the explant was 2.78- and 5.64-fold larger with SFM-1 compared with SFM-2 and SFM-3, respectively (*n* = 5). (*C*) HDPC metabolic activity was significantly increased in SFM-1 compared with other media (*n* = 7). (*D*) After 3 to 5 days of explant (E) culture in SFM-1, fibroblastlike HDPCs started to grow from the explant. $*P < .05$. Error bars: mean ± standard deviation. Scale bar = 100 μm.

(≤1%) (Fig. 2*D*). We then focused our immunophenotyping on the stem/progenitor cell markers CD271 (LNGFR), Stro-1, CD146 (MCAM), and MSCA-1. We observed that CD271 and Stro-1 were expressed by a very low number of cultured HDPCs (≤1%) but that CD146 and MSCA-1 were expressed by about 40% and 15% of cultured HDPCs, respectively (Fig. 2*E–H*). The expression level of stem/progenitor cell markers was similar from P1 to P4.

The viability of P1-HDPCs after 510 days of cryopreservation was compared with that of fresh P1-HDPCs. Results indicated that the viability of post-thaw HDPCs was significantly decreased compared with freshly isolated cells (Fig. 3*A*). However, this diminution had no significant impact on cell DTs that were stable and similar from P1 to P4 (≈40 hours) for both populations expanded in parallel (Fig. 3*B*). Cumulative doubling numbers after 4 passages were also similar (≈9) (Fig. 3*C*). The morphology of post-thaw and fresh HDPCs at P4 was fibroblastlike (Fig. 3*D*). The genomic stability of expanded post-thaw P4-HDPCs was shown by the observation of a normal karyotype upon G-band analysis (Fig. 3*E*). Mycoplasmas were not detected in random selected cultures of post-thaw cells using the MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME; data not shown).

The osteo/odontoblast differentiation potential of post-thaw HDPCs was confirmed by culturing cells in a specific differentiation medium containing dexamethasone, ascorbic acid, and β-glycerol phosphate. After 4 weeks of culture, the expression of genes coding for the osteo/odontoblastic proteins osteocalcin, alkaline phosphatase, and dentin sialophosphoprotein was significantly increased compared with cells maintained in the amplification (control) medium (Fig. 4*A*).

In addition, ARS staining (Fig. 4*B*) and quantification (Fig. 4*C*) showed a higher mineralization degree in HDPC cultures maintained in the differentiation medium compared with cultures performed in the control medium.

Discussion

Current international guidelines refer to cell-based products derived from *ex vivo* amplified cells as human cellular tissue-based products or advanced therapy medicinal products, and their production thus requires the use of medicinal manufacturing procedures that do not or minimally alter the biological features of the cells. In this study, we designed an original *ex vivo* protocol for the isolation, long-term cryopreservation, and short-term amplification of HDPCs that includes enzyme-free cell selection and the use of xenogeneic-free products and SFM. This protocol is easy, safe, fast, and cost-effective, and it allowed for the production of a clinical scale number of mesenchymal pulp cells maintaining their osteo/odontoblastic differentiation potential.

As was previously shown for the isolation of human adipose or Wharton jelly stem/progenitor cells, minimal manipulation leads to higher safety and efficacy of cell production (15, 16). In this study, we selected exclusively impacted third molars between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) to minimize the risk of pulp tissue contamination with oral microorganisms and to avoid the cell stress that results from crown root mechanical separation during the pulp recovery from older teeth (4, 14). HDPC isolation at around the crown-completed stage
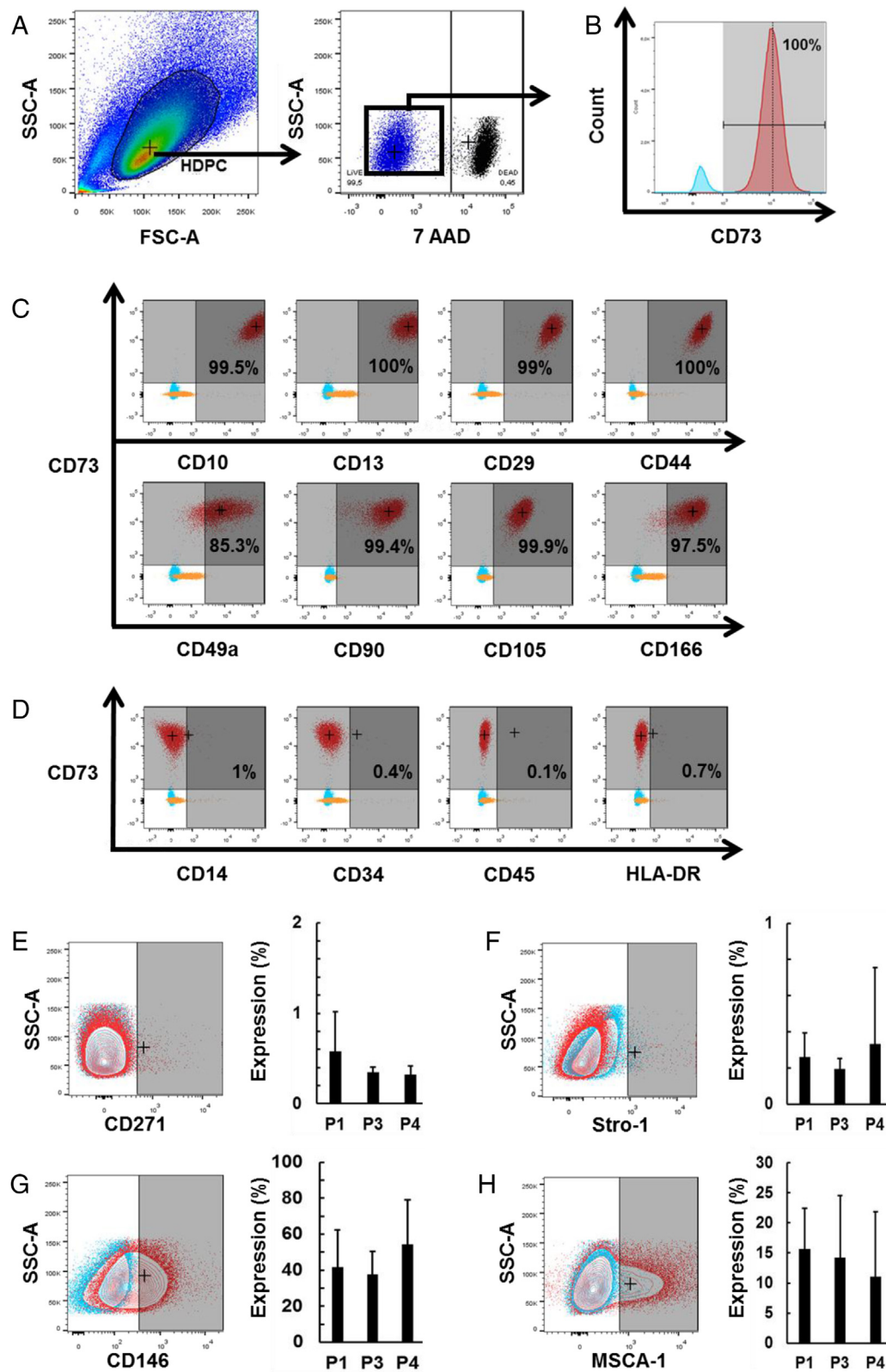
Figure 2. Immunophenotypic analysis of P1-HDPC cultured in SFM-1 with multicolor flow cytometry. The gating tree was set as follows: (*A, left*) Initial gating (black polygon) representing the cell distribution based on size (FSC-A) and intracellular composition (SSC-A); (*A, right*) The gate was then set on 7AAD-negative live cells, colored in blue. (*B*) All HDPC expressed the cell surface marker CD73 (*red peak*) which was therefore chosen as a basis versus additional markers. *Blue peak*: isotype control. (*C*) Almost all CD73-positive HDPC (≥97%) expressed the mesenchymal markers CD10, CD13, CD29, CD44, CD90, CD105 and CD166, whereas CD49a was expressed by a lower number of cells (85.3%). (*D*) The hematopoietic markers CD14, CD34, CD45 and HLA-DR were expressed by very few cells (≤1%). Data from (*A*), (*B*), (*C*), and (*D*) are representative of five independent experiments. Expression levels of the stem/progenitor cell markers CD271 (LNGFR) (*E*), Stro-1 (*F*), CD146 (*G*), and MSCA-1 (*H*) were maintained during cell passaging and did not differ significantly (*n* = 5). Error bars: mean ± SD.
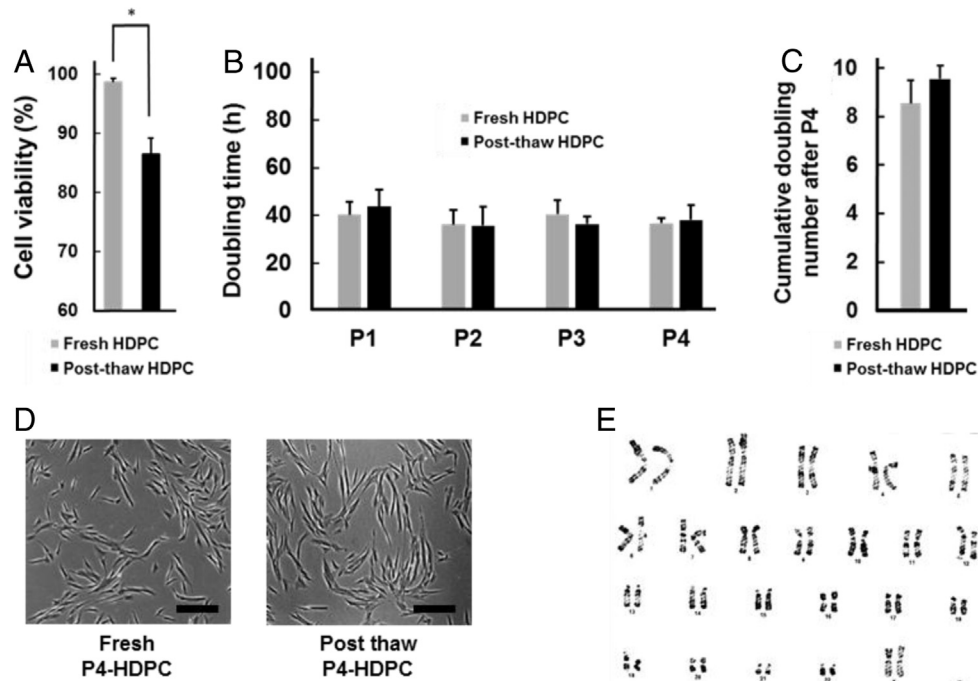
Figure 3. Post-thaw HDPC amplification in SFM-1. (*A*) The viability of post-thaw HDPCs was significantly decreased compared with freshly isolated cells (*n* = 5). (*B*) Cell DTs were stable and similar from P1 to P4 ($\approx$ 40 hours) for both populations (*n* = 5). (*C*) CDNs at P4 were similar ($\approx$ 9) (*n* = 5). (*D*) The morphology of post-thaw was fibroblastlike and similar to that of fresh HDPCs. (*E*) Post-thaw P4-HDPCs showed a normal karyotype on G-band analysis (the karyotype shown is representative from 4 patients). *$P < .05$. Error bars: mean $\pm$ standard deviation. Scale bar = 100 μm.

was previously associated with short cell DTs and high growth rate (17), and we obtained cell DTs and growth rates similar to those reported by these authors.

Two main methods have been described for isolating cells from tissues: explant culture and mechanical/enzymatic disruption associated with either cell adhesion on a plastic dish or cell sorting (3, 18). However, because of the longer handling time compared with explant cutting and the possible consequences of the tissue enzymatic dissociation on cell fate, enzymatic dissociation of tissues appears not to be adapted to medicinal manufacturing (19). In addition, tissues and cells exposed to collagenase, an enzyme frequently used to recover HDPCs from the pulp tissue, are considered to be "more than minimally manipulated" by the Food and Drug Administration. Likewise, cell selection by sorting procedures appears to not be ideal because of the prohibitive cost and complexity of the technique (7, 20). On the contrary, the minimal, easy, and stressless cell handling brought by the explant culture appears to be a beneficial procedure for isolating HDPCs (21).

Cell production under good manufacturing practices procedures implies the use of xeno-free materials and reagents to prevent the risk of viral, bacterial, or prion contamination and the possible induction of immunizing effects in the final recipient (11, 22). For this reason, xeno-free dissociating reagents such as TrypLe or Accutase (Life Technologies, Carlsbad, CA) have been recommended for cell passaging instead of the xenogeneic animal trypsin commonly used (23, 24). Likewise, if cell culture medium supplementation with xeno- or allogeneic serum remains permitted, it should be limited to "cases for which a valid alternative cannot be found" (European directive 1394/2007). Indeed, in addition to the risk of pathogen contamination and induction of immunizing effects, industrial production of serum is associated to product variability,

and the serum itself can promote early cell differentiation (11, 22). For these reasons, we decided to use SFM. HDPC culture in SFM was previously reported, but results are not directly comparable because cells were isolated by enzymatic dissociation of the pulp tissue and by using a serum-containing medium (25). HDPC isolation in SFM was previously associated with a lack of proliferation, and, to the best of our knowledge, we are the first to isolate, cryopreserve, and efficiently amplify HDPCs entirely in serum-free and xenogeneic-free conditions (26).

Several authors have shown that *ex vivo* cultured cells can include several populations such as stem/progenitor cells, fibroblasts, and perivascular cells (1, 18, 21, 27). Our multiparametric immunophenotypic analysis of specific surface markers indicated that more than 97% isolated HDPCs presented a mesenchymal profile. We also observed variable expression of cell surface molecules that were previously described as mesenchymal stem cell/progenitor markers (20, 28). Indeed, we detected the expression of CD146 and MSCA-1 by about 40% and 15% of HDPCs, respectively, whereas CD271 and Stro-1 were expressed by a very low number of cells. These results suggest the presence of distinct cell populations in the cultured HDPCs. The proportion of cells expressing Stro-1 and CD271 was low and similar to that reported by others in explant cultures (21). The perivascular marker CD146 has been related to high clonogenicity and multipotency, and the large number of cells expressing CD146 in our cultures might reveal/characterize an HDPC cell state (28). We report an average expression of MSCA-1 by cultured HDPCs similar from P1 to P4. Because this marker is related to high mineralized tissue cell differentiation potential in human bone marrow stem/progenitor cells, it may be used for selecting and monitoring HDPC populations having this capacity (29, 30). Studies are ongoing in our laboratory to test this possibility.
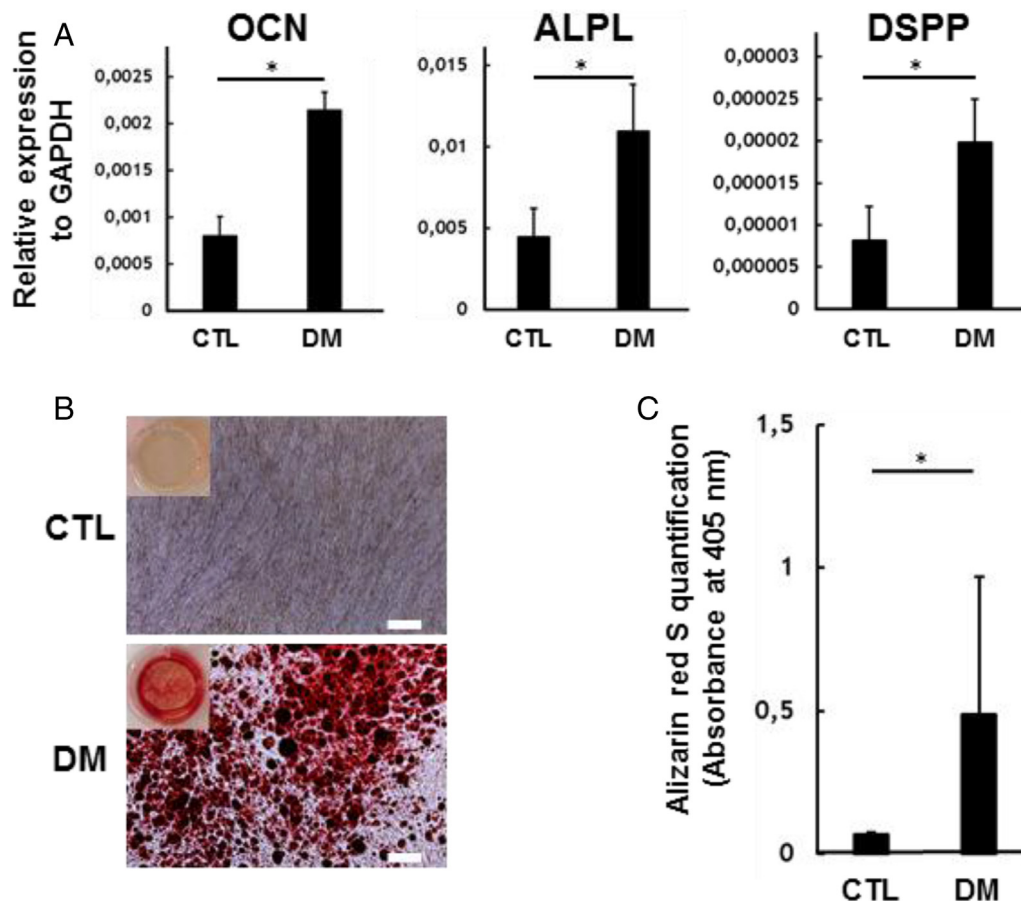
Figure 4. Determination of the osteo/odontoblast differentiation potential of post-thaw HDPCs. (*A*) The expression of osteocalcin (OCN), alkaline phosphatase (ALPL), and dentin sialophosphoprotein (DSPP) genes was significantly increased after 4 weeks of culture in the differentiation medium compared with cells maintained in the amplification (control) medium ($n = 4$). (*B*) ARS staining and (*C*) quantification showed a higher mineralization degree in HDPC cultures maintained in the differentiation medium compared with cultures performed in the control medium ($n = 4$). *$P < .05$. Error bars: mean ± standard deviation. Scale bar = 100 μm. CTL, control medium; DM, differentiation medium.

Prolonged *ex vivo* amplification of human cells is currently not recommended to limit the risk of modifying the phenotype of HDPCs isolated from the explants and preventing their future differentiation into cells capable of forming mineralized tissues (10, 18, 31). In this context, culture conditions that maintain the cell fate with the possibility to rapidly obtain clinical scale numbers of cells are required (9). Our HDPC cultures in SFM-1 gave rise to about 10^6 cells from 1 pulp after 2 weeks of explant culture (not shown). This result is comparable with that obtained for dental pulp stem cells in serum-containing medium (5). Importantly, because the cell DTs remained constant from P1 to P4 (≈ 40 hours), we calculated that more than 25.10^7 cells could be theoretically obtained after 4 passages with 1 pulp, which is likely to be a sufficient number for 1 pulp regeneration, 1 bone socket filling after tooth extraction, or 1 localized periodontal treatment (13, 32). Interestingly, the average number of cells expressing each of the 4 mesenchymal stem cell/progenitor markers remained similar from P1 to P4, suggesting that the cell fate was not profoundly affected by our culture conditions. Our results further indicate that cryopreservation in SFM had no negative impact on cell DTs and cumulative cell numbers. Although the viability of cells cryopreserved in SFM was decreased compared with fresh cells, it remains similar to that previously reported (33). Importantly, rapid amplification of post-thaw HDPCs up to P4 did not lead to genomic instability, which would be potentially harmful for the recipient patient.

Cell culture on rigid plastic dishes can potentially alter cell fate, and the latter is better maintained on extracellular matrix–coated surfaces (12, 34). Accordingly, we precoated the culture dish surface with human placental collagens I and III that were chosen because they are the 2 most abundant collagens in the dental pulp extracellular matrix. Moreover, it was previously reported that HDPC culture was successfully performed on collagen surfaces (35), so we postulated that collagen offered an appropriate surface to maintain the fate of cells outgrowing from the pulp explants and their differentiation potential. Osteo/odontoblast stem/progenitor cells isolated from human dental pulp were recently successfully used for pulp/dentin and bone engineering (6, 7). Interestingly, we found that HDPCs cultured in a specific differentiation medium after short-term amplification clearly expressed an osteo/odontoblast differentiation potential.

In summary, we designed, in this study, specific conditions for HDPC isolation, storage, and amplification with a medicinal manufacturing approach. These conditions include minimal tissue manipulation, enzyme-free isolation, use of xenogeneic-free products, and SFM culture. They allow for the expression of stem/progenitor cell markers and preserve amplification kinetics without inducing karyotype abnormality while maintaining the differentiation potential of HDPCs into osteo/odontoblast cells. These findings may constitute a basis on which to investigate how HDPC production can be optimized for human pulp/dentin and bone tissue engineering.

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