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Research Article

Characterization of Dental Pulp Stem Cells Using MALDI-TOF Mass Spectrometry

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Abstract

Dental pulp stem cells (DPSCs) are mesenchymal stem cells that possess multipotent properties, implicating them in tissue regeneration. DPSC culture and identification are mandatory for any experimentation or therapeutic application. Isolation and culture were performed by the explant method, and identification was made by analyzing the peptide profile obtained by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS), which was confirmed by reference flow cytometry. As a proof-of-concept, DPSCs were cultured and identified from 4 of 8 tested teeth, with 1/3 obtained in culture within 170 h after extirpation. After storage at -80°C in 10% dimethyl sulfoxide in complete medium, these four DPSC cell lines were made publicly available by depositing them in the Collection de Souches de l'Unité des Rickettsies. In this study, we propose a rapid and reproducible protocol for the identification of stem cells isolated from dental pulp by MALDI-TOF MS.

Keywords: Dental pulp, Stem cells, Culture, Flow cytometry, Mass spectrometry, MALDI-TOF MS

Introduction

Stem cells are self-renewing cells capable of differentiation into one or more types of specialized cells [1]. Due to their main role in wound healing and the renewal of a tissue's cell pool, they are considered valuable cells for regenerative medicine, resolving some ethical issues related to human embryonic stem cells [2,3]. Accordingly, stem cells can be isolated from adult organs and tissues, including bone marrow [4], the heart [5], the skin [6], the brain [7], the eye [8], skeletal muscle [9] and teeth [10]. Dental tissues are particularly accessible sources for the recovery of stem cells,

and wisdom teeth are generally used for that purpose. In teeth, dental stem cells are found in several areas of the dental organ and are named dental pulp stem cells (DPSCs) [10], stem cells from human exfoliated deciduous teeth (SHEDs) [11], periodontal ligament stem cells (PDLSCs) [12], dental follicle progenitor cells (DFPCs) [13] and stem cells from apical papilla (SCAPs) [14]. These pulpal stem cells represent an important stock of postnatal stem cells and can be cultured and multiplied ex vivo [15].

Two methods are currently used for the isolation and culture of DPSCs: one method is based on the enzymatic digestion of the dental pulp by collagenase/dispase, which can be harmful to cell viability [16]; the other method is based on a primary cell culture from pulp explant that takes longer than obtaining enzyme-digested pulps but allows the isolation of more homogeneous cell populations [17,18].

Matrix-associated laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry (MS) is a tool routinely used in microbiology laboratories for the rapid identification and classification of bacterial species using databases including reference spectra of isolated species [19-22]. The application of mass spectrometry for bacterial identification based on peptide spectra was proposed as early as 1975 [23]. MALDI-TOF-MS also plays an important role in cell proteomics identification [24,25]. It has been previously used as a fast, easy and accurate method to identify signatures of immune cells [26].

The aim of this study was to propose a reproducible protocol for the identification of stem cells isolated from dental pulp by MALDI-TOF MS.

Materials and Methods

Sample collection and cell culture

The teeth used for this study were intact third molars, extracted for lack of space on the dental arch. These third molars were included and free from pathologies, belonging to healthy young adults (18-25 years), without gender differentiation. The fact that these teeth are included, allows them to be preserved from any contamination. These teeth were collected on the same day were collected in an odontology surgical clinic (Marseille, France) in accordance with the guidelines approved by the ethics committee of the Institut Hospitalo Universitaire (IHU) Méditerranée Infection (project approved 11/2017). Extracted teeth were extemporarily immersed into a sterile solution composed of a vital medium (Dulbecco's Modified Eagle Medium F-12: DMEM/F12, Invitrogen, Villebon-sur-Yvette, France) supplemented with 1% streptomycin-penicillin (Thermo Fisher, Grand Island, USA) and stored for 1-7 days at 4°C. The teeth were then decontaminated with 70% alcohol, and the pulp from each tooth wasrecovered as previously described [27] and cultured by using the explant method with modifications [28]. Briefly, each pulp was cutinto fragments the size of which did not exceed 2 mm^3 and put into a culture in T25 flasks (SARSTEDT, Nümbrecht, Germany) containing DMEM/F12 medium supplemented with 1% streptomycin-penicillin, 1% amphotericin B (Bristol-Myers-Squibb, Rueil-Malmaison, France) and 10% heat-inactivated fetal bovine serum (qualified FBS, Gibco, Paisley, Germany). Flasks were incubated at 37°C under a 5% CO_2 atmosphere for 11 days. Primary cells were then observed at 40x and 20x using an inverted microscope (Nikon ECLISE TS 100, France S.A.S). When 90% confluence was observed, the cells were trypsinized (0.05% Trypsin-EDTA; Thermo Fisher) before being duplicated. The reference dental pulp stem cell line (CLS, Eppelheim, Germany) was included in the study as a reference positive control.

Identification of dental pulp stem cells via flow cytometry

Flow cytometry was used as a reference method for DPSC identification [29]. Flow cytometry was performed after 2-4 passages of the cultured cells at 90% confluence. The antibodies used were as follows: negative markers CD45-PC5 (clone 2D1, BD Bioscience, San Jose, California) and CD34-FITC (Beckman Coulter) and positive markers CD90-AF-647 (clone 5E10, BioLegend, San Diego, California), CD105-PE (clone 43A3, BioLegend), and CD73-FITC (clone AD2, BioLegend), including 5 µL of each fluorochrome (isotype control: IgG1 FITC, IgG1 APC, IgG1 PE, and IgG1 PC5 (BeckmanCoulter Company, Marseille, France) in the cell suspension. Analyzing the expression of these markers was recommended by the International Society of Cell Therapy to confirm the mesenchymal phenotype of cells [30]. The reference DPSC line (CLS) was used as a positive control in these experiments.

Identification of dental pulp stem cells using MALDI-TOF MS

DPSCs were identified by MALDI-TOF MS using a previously published protocol to characterize intact immune cells, including

monocytes, T lymphocytes and polymorphonuclear leukocytes [26]. Briefly, 1 µL of primary dental pulp cells was deposited directly on the MALDI-TOF-MS target plate, to which 1 µL of α-cyano-4-hydroxy-cynnamic acid matrix (HCCA; Sigma-Aldrich, St. Louis, USA) was added. The analysis of the MALDI-TOF-MS spectra was performed using a Microflex LT mass spectrometer and FlexControl and FlexAnalysis software (Bruker Daltonics, Massachusetts, USA). DPSCs stored for at least 4 months at -80°C in 10% dimethyl sulfoxide in complete medium were recultivated and identified again by MALDI-TOF MS by comparing the corresponding spectra with the same cells harvested in 4-6 passages before freezing.

Results

Isolation and expansion of cells

This identification method was done on a total of 8 teeth, we harvested DPSCs from the pulp of 3/5 of the teeth that were cultured 24 h after extirpation and from the pulp of 1/3 of the teeth that were cultured one week after extirpation, preserved as described above. One of these 8 cultures became contaminated with yeast despite the presence of antifungals in the culture medium. In the 7 uncontaminated cultures, primary cells were observed between the 11th and 20th day for 4 cultures, whereas no growth was observed in three cultures. These 4 primary cells were duplicated after 90% confluence (Figure 1) and deposited in the strain collection of the Unité des Rickettsies under numbers (CSUR): Q1346, Q1347, Q1349 and Q1610.

Flow cytometry identification of DPSCs

Flow cytometry analyses confirmed the identification of the cells cultured from the dental pulp as DPSCs. Indeed, cells in passage 2 yielded high expression of the mesenchymal stem cell markers CD73 (≥ 75% expression), CD90 (≥ 86% expression), and CD105 (≥ 36% expression) and no significant expression of the hematopoietic stem cell markers CD34 (≤ 5% expression) and CD45 (≤ 11% expression) (Figure 5). The reference cell line used as a positive control yielded the expression of CD73 (95% expression), CD90 (66.4% expression), CD105 (61% expression), CD34 (1.19% expression) and CD45 (1.05% expression).

MALDI-TOF MS identification of DPSCs

The MALDI-TOF MS signature of DPSCs was analyzed. DPSCs were deposited at least twice per spot before and after freezing. Before freezing, several peaks of different intensities were detected, whereas no peak was observed in the negative control matrix (Figure 2). After at least 4 months of freezing at -80°C in 10% DMSO complete medium, the same peaks were observed (Figure 2C).

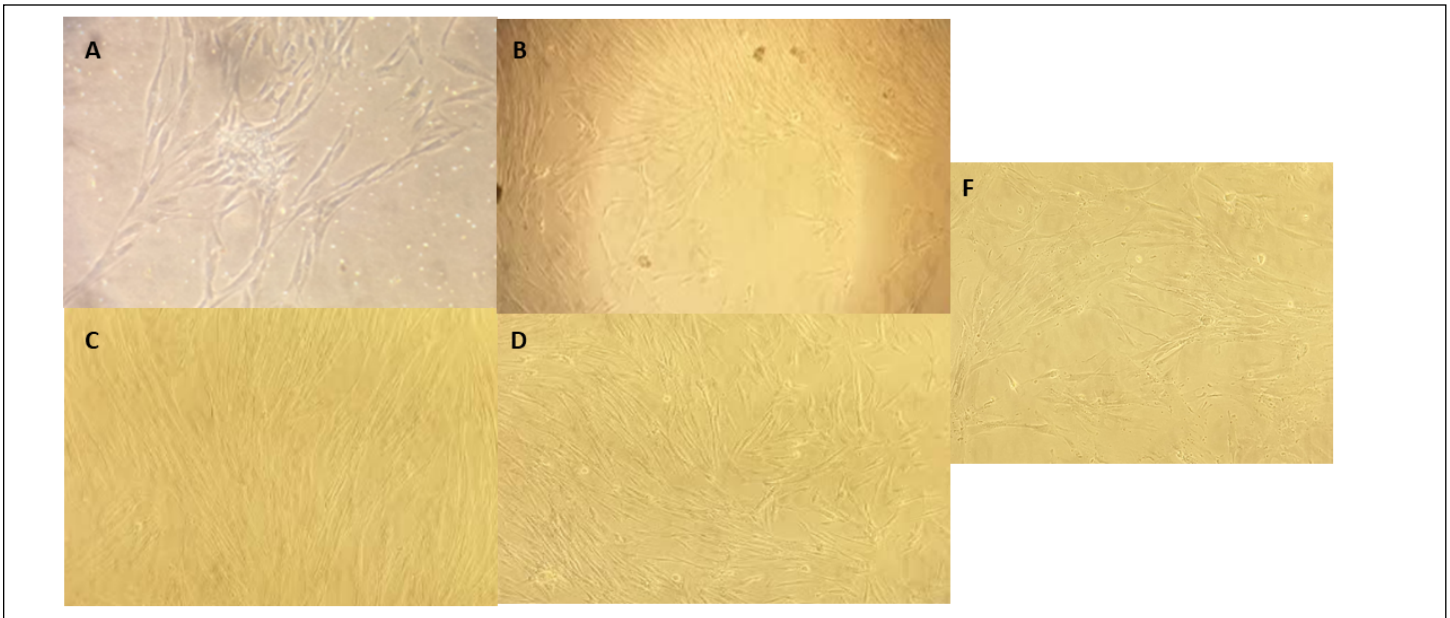

Figure 1: Primary cells of dental pulp in culture. (A) Pulp put in culture 24 h after the extirpation of the tooth (day 13, objective x40). (B) Pulp put in culture 170 h after the extirpation of the tooth (day 22, objective x20). (C) Second passage of DPSCs put in culture 24 h after extirpation (day 9, objective x20). (D) Second passage of DPSCs put in culture 170 h after extirpation (day 8, objective x20). (F) Fifteenth passage of the DPSC line (day 8, objective x20).

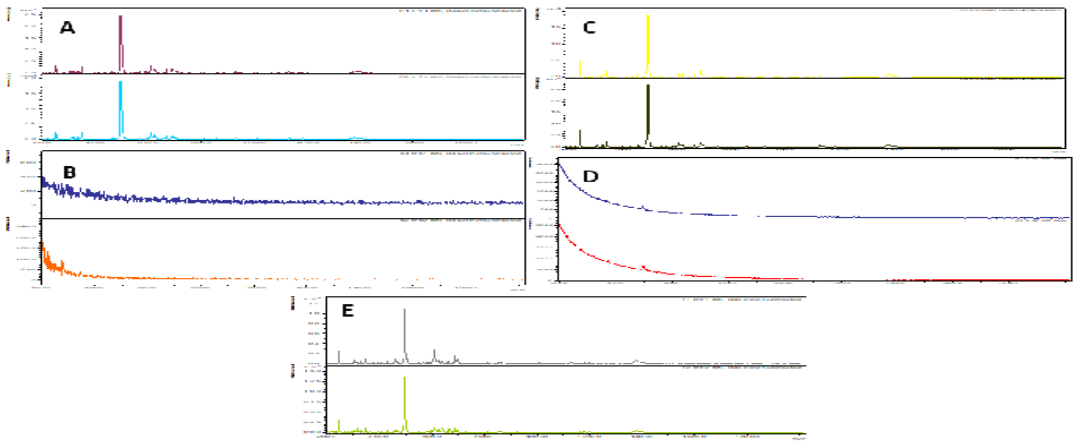

Figure 2: MALDI-TOF-MS spectra of dental pulp stem cells. (A) DPSCs before freezing. (B) Matrix control before freezing. (C) DPSCs after freezing. (D) Matrix control after freezing. (E) DPSC line for reference control.

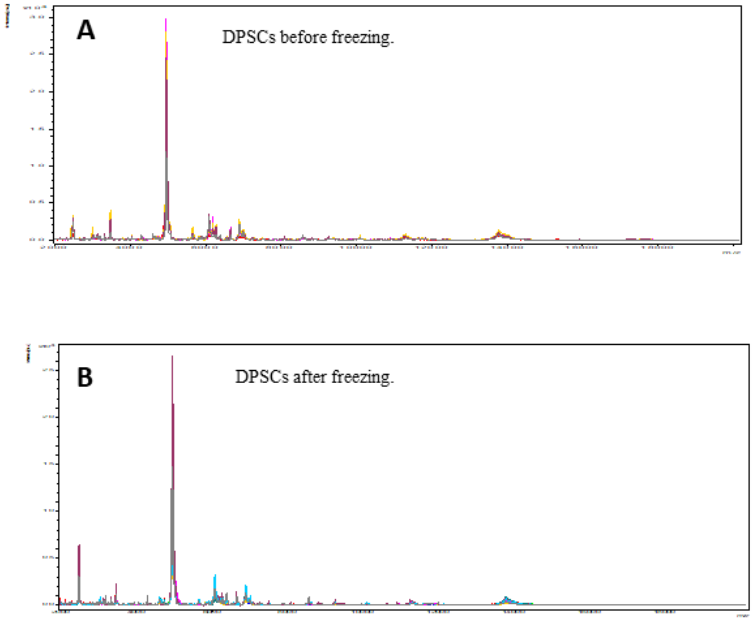

Figure 3: Fusion of MALDI-TOF-MS spectra of dental pulp stem cells. (A) DPSCs before freezing, including the cell line. (B) DPSCs after freezing, including the cell line.

The fusion of all spectra, including that of the cell line, presented the same aspect but differed in the peak intensity (Figure 3). In addition, we tested the spectra of DPSCs obtained after freezing relative to the database created with the spectra before freezing (DPSCs J0). The scores ≥ 2.17 were highly significant (Table 1). Finally, comparing the average DPSC spectra with those of 9 different cell types available in the database showed that DPSC profiles were unique, differing from those of the other cells of human origin (Figure 4).

Discussion

In the present work, DPSCs were recovered from half of manipulated teeth, allowing for the establishment of four DPSC cell lines, which were deposited in a public collection. This result increases the number of publicly available DPSC lines by 5-fold, as only one such cell line had been previously reported (CLS, Eppelheim, Germany). The availability of several DPSC lines is of interest for experimental works to account for the possible individual variations in the properties of these cells and the possible variations linked to

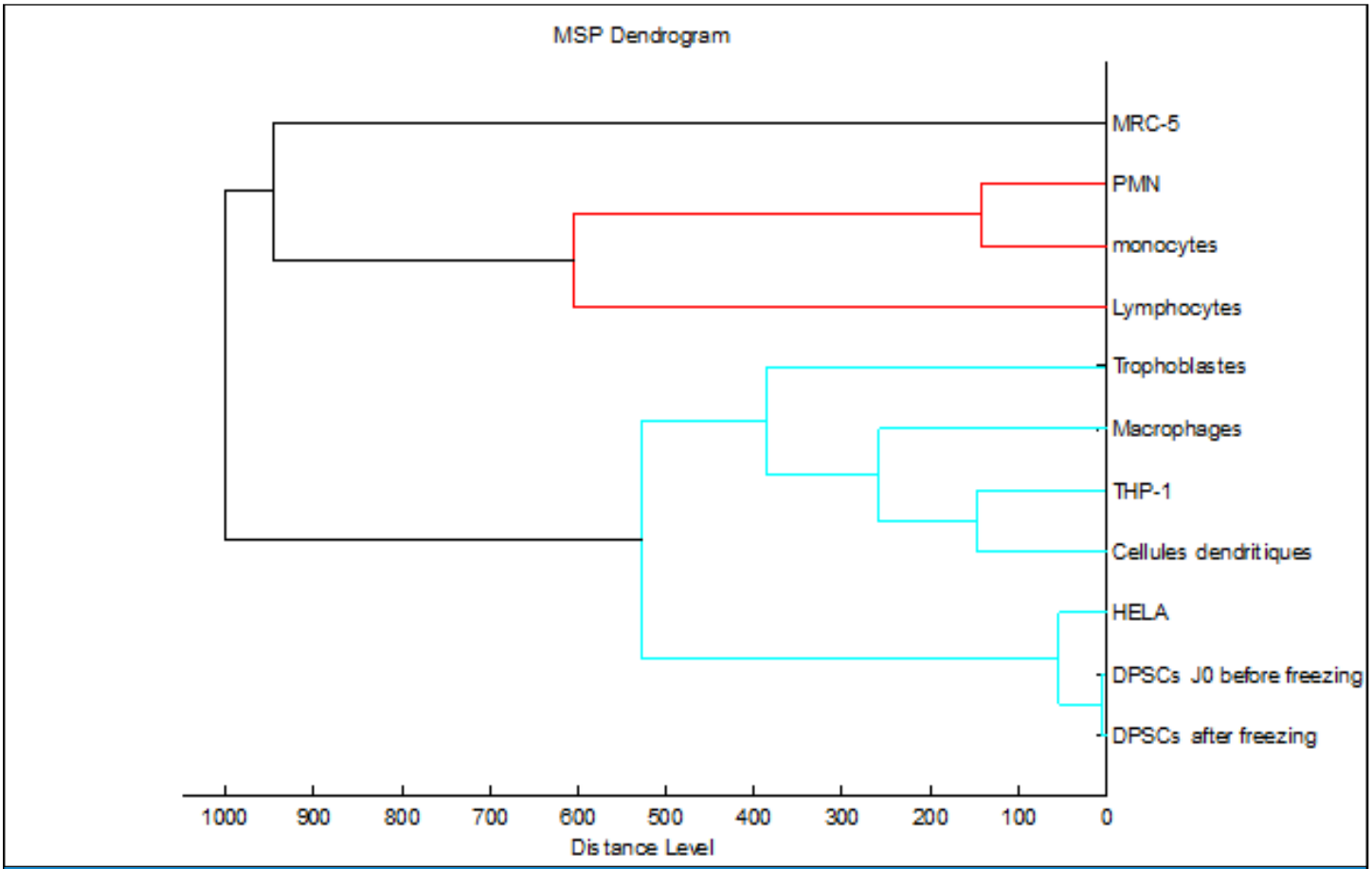

Figure 4: Dendrogram of 9 human cell types and DPSCs.

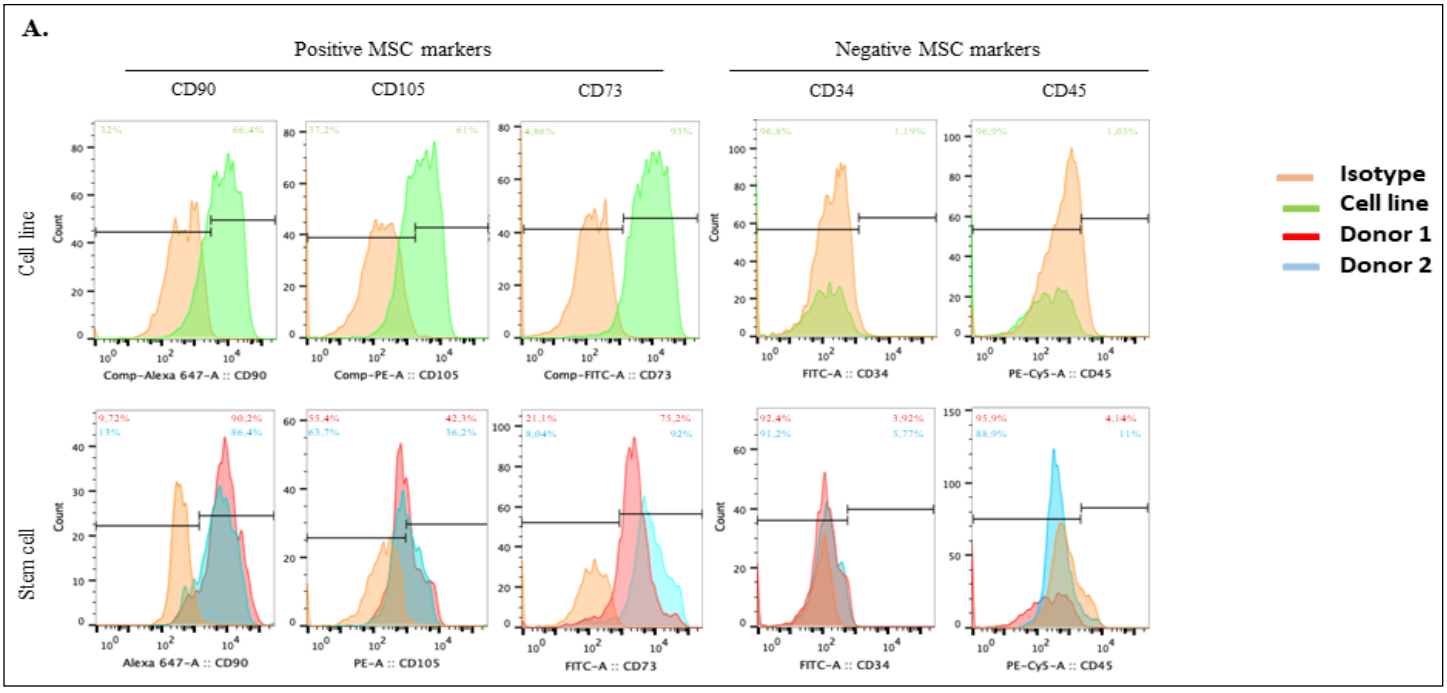

Figure 5: Immunophenotyping of dental pulp stem cells of two patients by flow cytometry compared with dental pulp stem cells by expression of specific antibodies for the mesenchymal markers CD73, CD90, and CD105 and the hematopoietic markers CD34 and CD45.

Table 1: MALDI TOF database tested with the same DPSCs but after freezing

Analyte name after freezing	Best match in the selected databases	Score value
DPSCS 1	**DPSCs J0** (Name of DPSCs database before freezing)	2.303
DPSCS 1		2.303
DPSCS 2		2.231
DPSCS 2		2.178
DPSCS 3		2.453
DPSCS 3		2.519
DPSCS 4		2.596
DPSCS 4		2.592
Cell line		2.407
Cell line		2.203

multiple passages of only one DPSC cell line in different laboratories. This performance illustrates the effectiveness of the isolation and culture protocol reported here. In addition, for the stability of the culture and its maintenance over a long period of time, certain parameters must be taken into account when selecting teeth to not affect the properties of stem cells, namely, the type of teeth, the stage of development of each tooth, and the age of the donor and his genetic background (donor health data) [31].

Here, failure in the recovery of DPSCs in 4/8 teeth was probably due to contamination of the medium by yeast despite the decontamination of the teeth with 70% alcohol before the recovery of the pulp and the presence of antifungal in the culture medium and to the reduced viability of DPSCs when the teeth have been cultured for more than 24 h after their extirpation [32]. In addition, we determined that the culture of DPSCs can be initiated at least one week (170 h) after extirpation, which is new information because the previous record was 120 h [32]. The difference of 2 days could matter as teeth are a somewhat rare clinical material in the laboratory. In our study, the recovery of DPSCs took 11 to 20 days, comparable to the previously published two weeks [10,28].

Furthermore, we used MALDI-TOF MS, which can separate proteins and peptides in complex samples, making it possible to easily and quickly characterize the different signatures of intact immune cells, including monocytes and T lymphocytes in peripheral mononuclear cells [26]. This method was simple and fast and did not require any specific antibodies, such as flow cytometry. Analysis of the fusion spectra of isolated DPSCs showed no significant difference from the stem cell line of the dental pulp. These results were validated by phenotyping. Flow cytometric analysis revealed the expression of CD34, CD90, and CD105 markers and excluded CD34 and CD45 hematopoietic markers, which are features of mesenchymal stem cells [30]. Thus, our results suggest that our cells are indeed stem cells and are consistent with previously reported data [29,33].

In conclusion, we developed a new integrated protocol for the culture, preservation and MALDI-TOF-MS identification of DPSCs. This protocol has the advantage of easy and fast identification of DPSCs without the need for any specific, rare and expensive antibodies contrary to flow cytometry. As a proof-of-concept, DPSCs were harvested from a tooth collected for at least one week (170 h after tooth extraction), and four DPSC lines have been deposited in a public repository.

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Author Contributions

H.O.H. and E.G. conceived and designed the experiments. H.O.H. and M.D. performed the analyses. J-C.G. provided samples. H.O.H., M.D., G.A. and E.T. wrote the main manuscript text and prepared figures. All authors reviewed the manuscript.

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