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In Vitro Long-Term Expansion and High Osteogenic Potential of Periodontal Ligament Stem Cells: More Than a Mirage

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Abstract

The periodontal ligament displays a reservoir of mesenchymal stem cells which can account for periodontal regeneration. Despite the numerous studies directed at the definition of optimal culture conditions for long-term expansion of periodontal ligament stem cells (PDLSCs), no consensus has been reached as to what is the ideal protocol. The aim of the present study was to determine the optimal medium formulation for long-term expansion and stemness maintenance of PDLSCs, in order to obtain a sufficient number of cells for therapeutic approaches. For this purpose, the effects of three different culture medium formulations were evaluated on PDLSCs obtained from three periodontal ligament samples of the same patient: minimum essential medium Eagle, alpha modification (α-MEM), Dulbecco's modified Eagle's medium (DMEM), both supplemented with 10% fetal bovine serum (FBS), and a new medium formulation, Ham's F12 medium, supplemented with 10% FBS, heparin 0.5 U/ml, epidermal growth factor (EGF) 50 ng/ml, fibroblast growth factor (FGF) 25 ng/ml, and bovine serum albumin (BSA) 1% (enriched Ham's F12 medium; EHFM). PDLSCs grown in EHFM displayed a higher PE-CD73 mean fluorescence intensity compared with cells maintained in α-MEM and DMEM, even at later passages. Cells maintained in EHFM displayed an increased population doubling and a reduced population doubling time compared with cells grown in DMEM or α-MEM. α-MEM, DMEM and EHFM with added dexamethasone, 2-phospho-L-ascorbic acid, and β-glycerophosphate were all able to promote alkaline phosphatase activity; however, no calcium deposition was detected in PDLSCs cultured in EHFM-differentiation medium. When EHFM-, α-MEM- and DMEM-expanded PDLSCs were transferred to a commercial culture medium for the osteogenesis, mineralization became much more evident in confluent monolayers of EHFM-expanded PDLSCs compared with DMEM and α-MEM. The results suggest EHFM is the optimal medium formulation for growth and stemness maintenance of primary PDLSCs. Moreover, EHFM confers higher osteogenic potential to PDLSCs compared with cells maintained in the other culture media. Overall, the results of the present work confirmed the advantages of using EHFM for long-term expansion of mesenchymal cells in vitro and the preservation of high osteogenic potential.

Keywords

periodontal ligament, osteoblast, stem cells, regeneration

Introduction

In vitro cultured autologous mesenchymal stem cells (MSCs) within passage 5 have been approved for clinical applications in stem cell-based treatments. Many researchers have shown that expansion attenuates the stemness of MSCs, so contributing to a reduced therapeutic potential. However, the number of cells that can be obtained by a single sample is limited, so an intense ex vivo periodontal ligament stem cell (PDLSC) expansion is required before application. We define a new culture medium formulation able to assure

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long-term expansion of PDLSCs without the impairment of osteogenic potential. In this paper, we aim to provide important insights into the optimization of culture methods to promote the clinical-scale expansion of PDLSCs.

The periodontal ligament (PDL) represents a fibrous network connecting the cementum of the tooth root and the alveolar bone. The PDL serves many functions, such as tooth support, tooth nutrition and protection against pathogens from oral cavities[1]. During periodontal disease, the attachment apparatus and tooth-supporting structures are disrupted. Moreover, as the periodontium is a complex structure comprised of hard (cementum and bone) and soft (gingiva and periodontal ligament) tissues, the regenerative capabilities are very limited[2]. To prevent the events associated with periodontal diseases, clinicians and researchers have worked together to investigate novel regenerative approaches, such as guided tissue regeneration, the administration of enamel matrix derivatives and growth factors, and bone grafting[3]; however, most of these approaches have led to unsatisfactory results.

The evidence of MSCs in PDL was first provided by Melcher and McCulloch by in vivo and histological studies[4,5]. Since the first attempt to isolate PDLSCs, numerous studies have demonstrated the role of these cells in periodontal tissue regeneration[6]. Recently, Bartold and Gronthos reported the minimum defining criteria required for cells to be termed PDLSCs[7,8]. Briefly, PDL-derived cells showed a capacity to adhere to a plastic support and express CD29, CD44, STRO-1, STRO-4 and CD146. Moreover, PDLSCs express typical MSCs-associated markers (CD73, CD90, CD105, CD166) and the lack of expression of endothelial (CD31), hematopoietic (CD14, CD34, CD45, CD79a) and immune antigens (HLA-DR, CD40, CD54, CD80 and CD86). Functionally, cultured PDLSCs could be defined by their multipotency in vitro as confirmed by their ability to differentiate into adipocytes, chondrocytes and osteoblasts. Furthermore, PDLSCs can regenerate periodontal tissue when transplanted into animal models[7]. The capacity of these cells to differentiate in alveolar bone, cementum, peripheral nerves and blood vessels has also been shown[9–11].

One of the major complications in PDLSC-based approaches is the limited number of cells that can be obtained by a single sample (on average 1250 cells)[12,13]. As a consequence, an intense ex vivo PDLSC expansion is required before application, and the number of expansions that are sufficient for application should be established in advance. This consideration, in addition to the evidence that culture conditions strongly influence the efficacy of PDLSCs, encourages researchers to establish the most efficacious culture medium to maintain the stemness of PDLSCs.

The aim of the present study was to determine the optimal medium formulation for long-term expansion and stemness maintenance of PDLSCs to obtain a sufficient number of cells for therapeutic approaches. For this purpose, minimum essential medium Eagle, alpha modification (α-MEM) and Dulbecco's modified Eagle's medium (DMEM), largely used to culture PDLSCs, were compared with a new medium formulation previously used to expand MSCs derived from human vessels[14,15]. While previous studies compared the cells obtained from many patients, this study characterized the effects of media formulations on PDLSCs derived from the same patient.

Materials and Methods

Samples and Cell Culture

The study was conducted according to the Declaration of Helsinki on medical protocol and ethics after the approval of the regional Ethical Review Board of Central Calabria, Italy (reference for Magna Græcia University of Catanzaro). After the surgical extraction, nine impacted third molars (six inferior; three superior) were collected from three patients aged 19–25 years (mean age = 22.7 years) recruited at the Unit of Oral Surgery, Academic Hospital of Magna Graecia University of Catanzaro, Italy. PDL was collected by scraping the root surface from the middle third to the apical third, without involving apical papilla, and then minced. Small pieces of tissue were digested using 2 mg/ml collagenase type I solution (no. 17018029; Gibco, Life Technologies, Milan, Italy) for 2 hours at 37°C. PDLSCs obtained from three PDLs of the same patient (in triplicate) were seeded into a six-multiwell plate (Eppendorf, Milan, Italy) with different culture media and incubated at 37°C in a humidified atmosphere of 5% CO_2. In this study, we used and compared three different culture media: α-MEM (no. 22561021; Life Technologies, Milan, Italy) and DMEM (no. D5796; Sigma Aldrich, Milan, Italy), which were supplemented with 10% fetal bovine serum (FBS; no. 10270106; Life Technologies), 100 U/ml penicillin and 100 µmol/ml streptomycin (no. P4333; Sigma Aldrich); and Ham's F12 medium (no. 21765037; Gibco, Life Technologies) supplemented with 10% FBS, 0.5 U/ml heparin, 50 ng/ml epidermal growth factor (EGF), 25 ng/ml fibroblast growth factor (FGF), 1% bovine serum albumin (BSA), 100 U/ml penicillin, and 100 µmol/ml streptomycin (EHFM)[14,15]. When the cell cultures reached 80–90% confluency between 7 and 14 days, the PDLSCs were sub-cultivated as the first cell passage. The cells were seeded at 4000 cell/cm^2. When the cells became 80–90% confluent, they were sub-cultured until passage 10. At each passage, the cells were harvested and processed for flow cytometry analysis. For each experiment, cells derived from at least three different individuals at passages 3–5 were used.

Flow Cytometry Analysis

The immunophenotypic profile of PDLSCs was investigated by flow cytometry as previously reported[16]. PDLSCs cultured in α-MEM, DMEM or EHFM were re-suspended in 100 µl of phosphate-buffered saline (PBS) and stained for 30 min on ice using the following fluorochrome-conjugated

antibodies: PE-CD14 (clone M5E2; Becton Dickinson, Franklin Lakes, NY, USA), PE-CD34 (clone AC136; Miltenyi Biotec, Bergisch Gladbach, Germany), PE-CD45 (clone 5B1; Miltenyi Biotec), PE-CD73 (clone AD2; Miltenyi Biotec), PE-CD90 (clone DG3; Miltenyi Biotec), and FITC-CD105 (clone 43A4E1, Miltenyi Biotec)[17]. The cells were washed twice with PBS and then analysed using the fluorescence-activated cell sorting BD FACScan™ system (Becton Dickinson). The data file was analysed by FlowJo software version 8.8.6 (Becton Dickinson).

Population Doubling Assay

Population doubling and the population doubling time were determined as follows. A total of 3.6×10^3 PDLSCs from each sample were seeded on six-well plates. The cells were passaged upon reaching 80–90% confluence and until passage 9. The PDLSCs were counted at each passage and the population doubling (n) was calculated as follows:

$$3.3 \times \log (\textit{harvested cell number}/\textit{plated cell number}).$$

As a result, the population doubling time at each passage and until passage 9 was calculated as follows:

$$(T_{Px} - T_{Px-1}) \,/\, n_x$$

where T_{Px} = time at passage (P) x, T_{Px-1} = time at passage $x-1$, and n_x = population doubling at passage x.

The population growth curve was determined as follows:

$$Nx!$$

where Nx = number of cells at passage x with x ranging from 1 to 9.

Osteogenic Differentiation

The PDLSCs were cultured in α-MEM, DMEM or EHFM until passage 4. Then, the cells were plated at a density of 4000 cell/cm^2 for histological and molecular analysis. The next day, the media were replaced with osteogenic differentiation media obtained by adding 0.1 μM dexamethasone (no. D4902; Sigma Aldrich), 50 μg/ml 2-phospho-L-ascorbic acid (no. 49752; Sigma Aldrich), and 10 mM β-glycerophosphate (no. G9422; Sigma Aldrich) to α-MEM, DMEM and EHFM. Osteogenic differentiation was also induced in standardized conditions by culturing PDLSCs in a commercial osteogenic differentiation medium (no. A1007201; StemPro™ Osteogenesis Differentiation Kit, Life Technologies).

Colorimetric Detection of ALP Activity

After 3 and 7 days of induction, the cells grown in 24-well plates and maintained under normoxic conditions were washed twice with PBS and then fixed with 10% formalin for 10 minutes. After fixation, the cells were washed twice with PBS and stained with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT; no. B3804; Sigma Aldrich) for 30 minutes and then washed twice with distilled water. All procedures were performed at room temperature.

Alizarin Red S Staining

After 21 days, the cells grown in 24-well plates and maintained under normoxic conditions were washed twice with PBS and then fixed with 10% formalin for 10 minutes. After fixation, the cells were washed twice with PBS and stained with 2% solution of Alizarin Red S (red colour; no. A5533; Sigma Aldrich), pH 7.2, for 30 minutes, and then the cellular matrices were washed with distilled water. All procedures were performed at room temperature.

Quantitative Real-Time Polymerase Chain Reaction

After 7, 14 and 21 days of differentiation, the cells were rinsed with PBS, and total cellular RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The purity and the integrity check of each RNA sample, the reverse transcription of RNA and quantitative real-time polymerase chain reaction (q-RT-PCR) were performed as previously reported[18]. Briefly, cDNA was synthesized from 1 μg of RNA using a reverse transcriptase system kit (no. 4368814; Thermo Fisher Scientific). q-RT-PCR was performed using SYBR Green universal PCR master mix (no. 4368706; Life Technologies). The reactions were performed in triplicate and analysed using the ΔΔCt method with glyceraldehyde 3-phosphate dehydrogenase as a normalization control. Primer sets used in this study are reported in Table 1.

Statistical Analysis

The experiments were performed at least three times. Descriptive data are summarized as the mean ± standard error of the mean (SEM). The statistical analysis was performed by a one-way analysis of variance followed by a Student's t test using Microsoft Excel and GraphPad Prism 5. In each analysis, a p-value <0.05 was considered to be statistically significant.

Results

Flow Cytometry Analysis

The expression of mesenchymal markers was assessed via fluorescent-activated cell sorting analysis. From the results it appeared that the PDLSCs were negative for hematopoietic (CD34 and CD45) and monocyte markers (CD14; positive rate <2%). Conversely, a strong expression of mesenchymal markers CD73, CD90 and CD105 was observed in all experimental conditions (positive rate >90%; Fig. 1). Moreover, the quantitative evaluation of CD73, CD90 and CD105 showed a typical expression pattern in the cells maintained

Table 1. Primers Used for q-RT-PCR Analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ALP	TAAGGACATCGCCTACCAGC	TGGCTTTCTCGTCACTCTCA
OCN	TGAGAGCCCTCACACTCCTC	ACCTTTGCTGGACTCTGCAC
OPN	TGAAACGAGTCAGCTGGATG	TGAAATTCATGGCTGTGGAA
RUNX2	CACCATGTCAGCAAAACTTCTT	TCACGTCGCTCATTTTGC
COLI	GTACTGGATTGACCCCAACC	ACCAGACATGCCTCTTGTCC
COLII	TCCAGATGACCTTCCTACGC	ATCCTTCAGGGCAGTGTACG
IBSP	TGAATACGAGGGGGAGTACG	TAGCCATCGTAGCCTTGTCC
MSX2	ACACAAGACCAATCGGAAGC	CAGCCATTTTCAGCTTTTCC
GAPDH	GGCTCTCCAGAACATCATCC	TTTCTAGACGGCAGGTCAGG

ALP: alkaline phosphatase; Col: collagen; GADPH: glyceraldehyde 3-phosphate dehydrogenase; IBSP: integrin binding sialoprotein; OCN: osteocalcin; OPN: osteopontin; q-RT-PCR: quantitative real-time polymerase chain reaction.

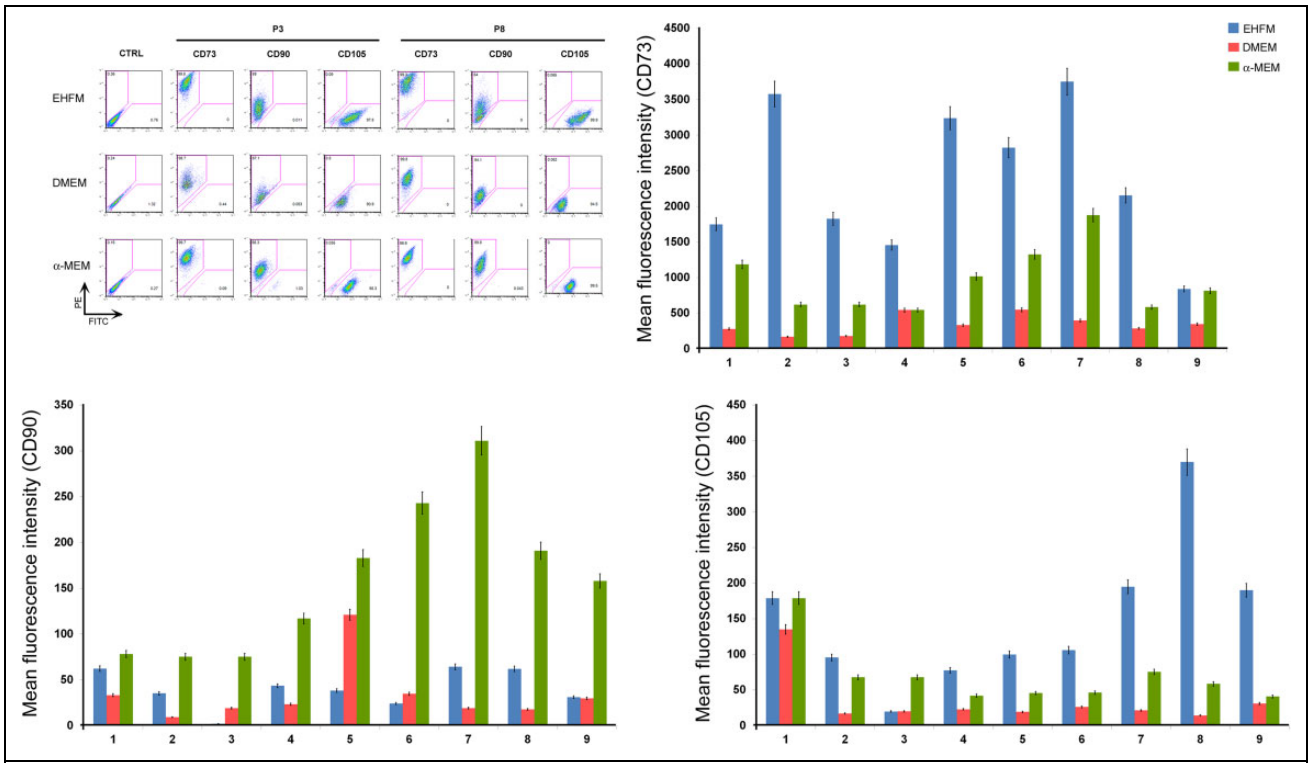

Fig. 1. Characterization of PDLSCs. PDLSCs maintained in EHFM, α-MEM and DMEM were characterized for the expression of hematopoietic and mesenchymal markers. Flow-cytometric analysis of CD14, CD34, CD45, CD73, CD90 and CD105 expression in PDLSCs at passage 3 and passage 8 (A). The percentages of positive cells are indicated. The expression levels of CD73 (B), CD90 (C) and CD105 (D) in PDLSCs maintained in EHFM, α-MEM and DMEM are shown until passage 9 and expressed as the mean fluorescence intensity. Data are presented as the mean ± SEM. For statistical analysis, a one-way analysis of variance followed by a Student's *t* test was applied.
α-MEM: minimum essential medium Eagle, alpha modification; DMEM: Dulbecco's modified Eagle's medium; EHFM: enriched Ham's F12 medium; PDLSC: periodontal ligament stem cell; SEM: standard error of the mean.

in the different culture media. Interestingly, the PDLSCs grown in EHFM displayed a higher PE-CD73 mean fluorescence intensity than the cells maintained in α-MEM and DMEM ($F_{(2,57)} = 48.32$; $\alpha < 0.001$; Fig. 1), even at later passages; on the other hand, the PDLSCs grown in α-MEM showed a significant increase in the PE-CD90 mean fluorescent intensity with respect to the cells cultivated in EHFM or DMEM ($F_{(2,57)} = 19.89$; $\alpha < 0.001$; Fig. 1). Although not statistically significant, the expression level of CD105 in EHFM appeared to be higher than in DMEM and α-MEM at advanced passages (P4–P9).

Effects of Culture Media on Cell Proliferation

The effects of the different media formulations were evaluated on the PDLSC population doubling, population

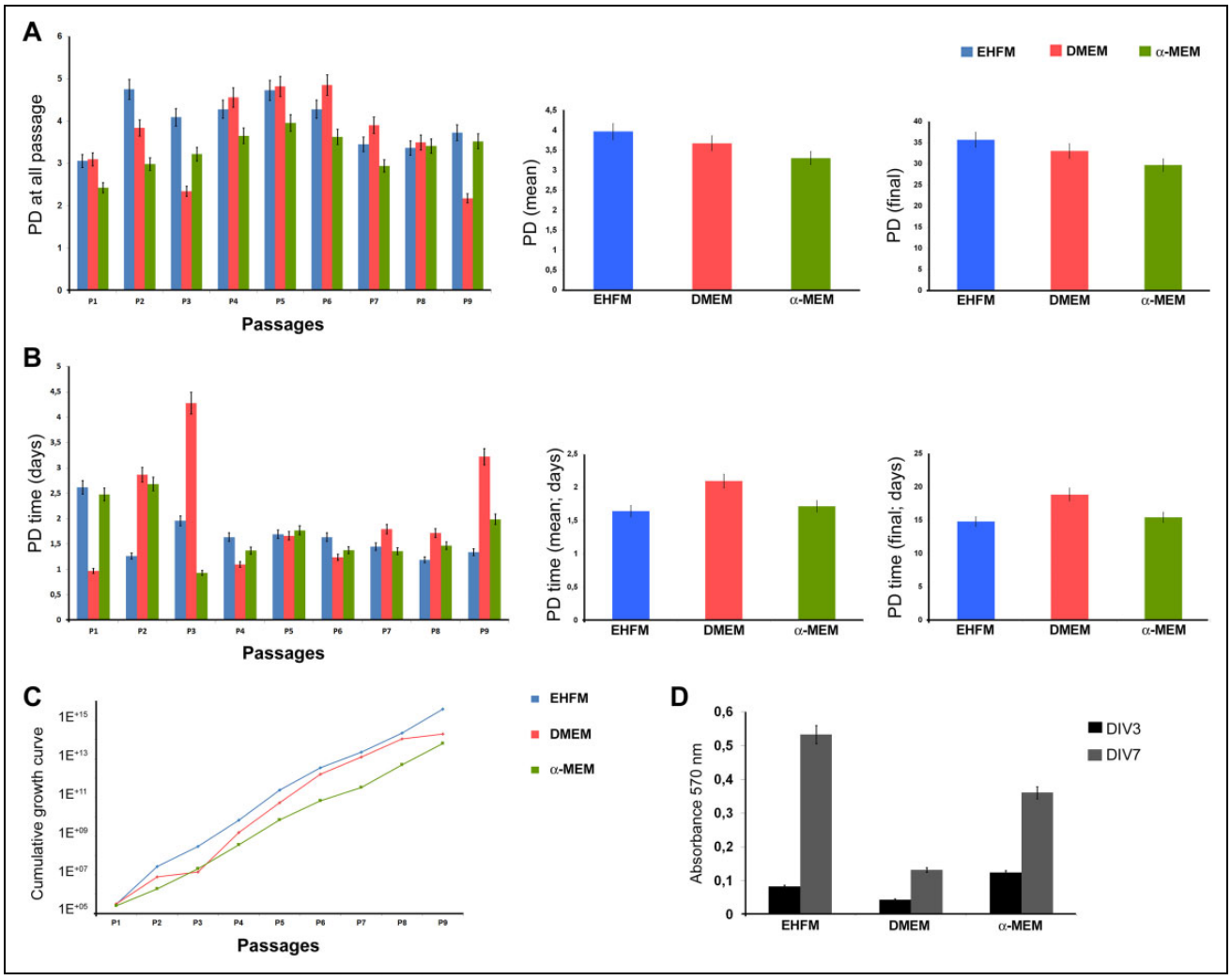

Fig. 2. Cell proliferation assay. The proliferation of PDLSCs was determined by the means of population doubling (A) and population doubling time (B) at all passages, mean score and final score. The growth curve of PDLSCs was determined until passage 9 (C). MTT analysis showed the proliferation of PDLSCs at DIV3 and DIV7 in EHFM, α-MEM and DMEM (D). Data are presented as the mean ± SEM.
α-MEM: minimum essential medium Eagle, alpha modification; DIV: day in vitro; DMEM: Dulbecco's modified Eagle's medium; EHFM: enriched Ham's F12 medium; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PDLSC: periodontal ligament stem cell; SEM: standard error of the mean.

doubling time and growth curve. Independent of the medium used, the population doubling score appeared heterogeneous during the entire period of culture (Fig. 2A). The comparison of the mean and final population doubling among the different media indicates that the PDLSCs grown in EHFM exhibited a significant increase in population doubling compared with the cells grown in DMEM and α-MEM. The population doubling time, similarly, was recorded at every passage (Fig. 2B). The comparison of the mean and final population doubling time among the different cultures shows that the PDLSCs maintained in EHFM exhibited a reduced population doubling time than the cells maintained in DMEM and α-MEM. From the study of the growth curve of PDLSCs, it appeared clear that all media formulations are able to induce cell proliferation even at later passages (Fig. 2C). A higher cell number was observed in the cultures maintained in EHFM than in those maintained in α-MEM and DMEM (+1827% versus DMEM and +5937% versus α-MEM at passage 9). The evaluation of the proliferation rates of the PDLSCs in the different culture media showed an increased cell growth of the cells maintained in EHFM with respect to the cells grown in DMEM and α-MEM (Fig. 2D).

Osteogenic Differentiation via the Addition of Ascorbic Acid, β-Glycerophosphate and Dexamethasone

The extent of PDLSC osteogenic differentiation in EHFM, DMEM and α-MEM was evaluated using a BCIP and NBT substrate, Alizarin Red S staining and q-RT-PCR. All of the osteogenic media promoted ALP activity at day 7, and there

were no differences between EHFM and the other media. Alizarin Red S staining indicated that mineralized nodes were present only in DMEM and to some extent in α-MEM. The maintenance of PDLSCs in the EHFM-differentiation medium resulted in the absence of calcium deposition as evaluated at day 21 (Fig. 3A).

When the PDLSCs were cultured in EHFM, α-MEM or DMEM enriched with osteo-inductive factors, the mRNA expression of all of the selected osteoblastic markers was differentially altered. The EHFM-cultured cells showed a strong upregulation of ALP, collagen II (ColII) and integrin binding sialoprotein (IBSP) on day 7 of induction (Fig. 3B). ALP and IBSP expression continued to increase, peaking on day 21 of differentiation, whereas ColII decreased during differentiation. Conversely, osteocalcin (OCN) appeared upregulated on day 21. In DMEM, the expression profile of the selected genes appeared homogeneous. Indeed, the ALP, OCN, runt-related transcription factor 2 (Runx2), collagen I (ColI), ColII and IBSP genes appeared to be upregulated on day 14 while the mRNA levels were reduced at the third week (Fig. 3C). Interestingly, the α-MEM-cultured cells displayed a very strong upregulation of ALP, osteopontin (OPN), OCN, Runx2, ColII, IBSP and (Msh Homeobox 2) Msx2 during the second week of induction. While the Msx2 mRNA levels were reduced at day 21, the expression of all of the other genes was still upregulated (Fig. 3D). The expression of most of the osteoblast markers (OPN, OCN, ColI, Runx2, IBSP and Msx2) was lower in the EHFM-cultivated cells than in the cells maintained in α-MEM and DMEM, above all during the first 2 weeks of osteogenic induction (Fig. 3E). Conversely, ColII and ALP expression appeared higher in the EHFM-cultured cells than in DMEM- and α-MEM-cultured cells on day 7 and days 7–21, respectively. The OPN, OCN, Runx2, and IBSP mRNA levels were higher in the EHFM-cultured cells than in the other media at the third week of induction.

Osteogenic Differentiation in a Commercial Culture Medium

The differentiation process of EHFM-, DMEM-, and α-MEM-expanded PDLSCs in a commercial culture medium (no. A1007201; StemPro™ Osteogenesis Differentiation Kit) was assessed by BCIP/NBT assay, Alizarin Red S staining and q-RT-PCR. Both the EHFM- and α-MEM-expanded cells showed positivity for ALP activity at day 3; however, the ALP staining reported in the plates of EHFM-expanded cells appeared higher than that of the α-MEM- and DMEM-expanded cells. Alizarin Red S staining indicated the presence of diffuse mineralized nodes in the EHFM-expanded cells, whereas few nodes were reported in DMEM and α-MEM. (Fig. 4A, F).

After 7, 14 and 21 days of culture, the expression levels of the selected osteoblastic genes were determined in triplicate by q-RT-PCR. The results revealed that when the EHFM-, α-MEM- or DMEM-expanded PDLSCs were cultured in a commercial culture medium for the osteogenesis differentiation, the mRNA expression of all of the selected markers was differentially altered. The strong upregulation reported at day in vitro (DIV)7 for the ALP, Runx2 and ColI genes in the EHFM-expanded PDLSCs was reduced during the differentiation process as confirmed by the levels observed at DIV14 and DIV21 (Fig. 4B). Conversely, Msx2 was upregulated later in differentiation, peaking on day 14 of induction. Osteogenic induction in the DMEM-expanded cells markedly enhanced IBSP expression at DIV7; in addition, the ALP gene and, to a lesser extent, the OCN and ColII genes were upregulated during the second week of induction by peaking on day 14, 14, and 21 of induction, respectively. Weak effects have been reported on the transcript expression of other osteogenic differentiation marker genes, such as Runx2, ColI, OPN and Msx2 (Fig. 4C). The α-MEM-expanded cells showed a strong upregulation of OPN during the second and the third week of osteogenic induction. OCN expression appeared upregulated on day 14, whereas no effects have been reported on the expression of the other selected genes (Fig. 4D). In terms of the comparison between expression profiles, the EHFM-expanded cells showed reduced mRNA levels of the OPN, OCN, ColII and IBSP genes during differentiation (Fig. 4E). Of note, the ALP, Runx2 and ColI mRNA levels were higher in the EHFM-expanded cells than in the DMEM- and α-MEM-expanded cells.

Discussion

The optimization of culture systems for the expansion and differentiation of mesenchymal cells constitutes one of the most important targets in the field of regenerative medicine. Indeed, up to now, many culture systems have been tested; however, no consensus has been reached as to the ideal medium formulation. This study compared the effects of three different culture medium formulations: α-MEM, DMEM, and EHFM. The formulation of α-MEM was designed to closely approximate the protein composition of cells, and it contains a higher concentration of amino acids and nucleotides than DMEM[19]. The formulation of EHFM has been previously optimized to expand the MSCs derived from human vessels[14,15].

We hypothesized that the expansion of PDLSCs in EHFM could account for the maintenance of their undifferentiated status; to support this hypothesis, we profiled the expression levels of mesenchymal markers at every passage until passage 9. Our results confirmed a significant higher expression of CD73 in the EHFM-expanded cells than in the α-MEM- and DMEM-expanded cells. CD73, also known as ecto-5'-nucleotidase, is a cell surface enzyme involved in the generation of adenosine from adenosine triphosphate, which in turn activates adenosine receptors[20]. Many authors have demonstrated the involvement of adenosine signaling in osteoblast differentiation and in MSC proliferation[21,22]; accordingly, we speculate that the strong upregulation of

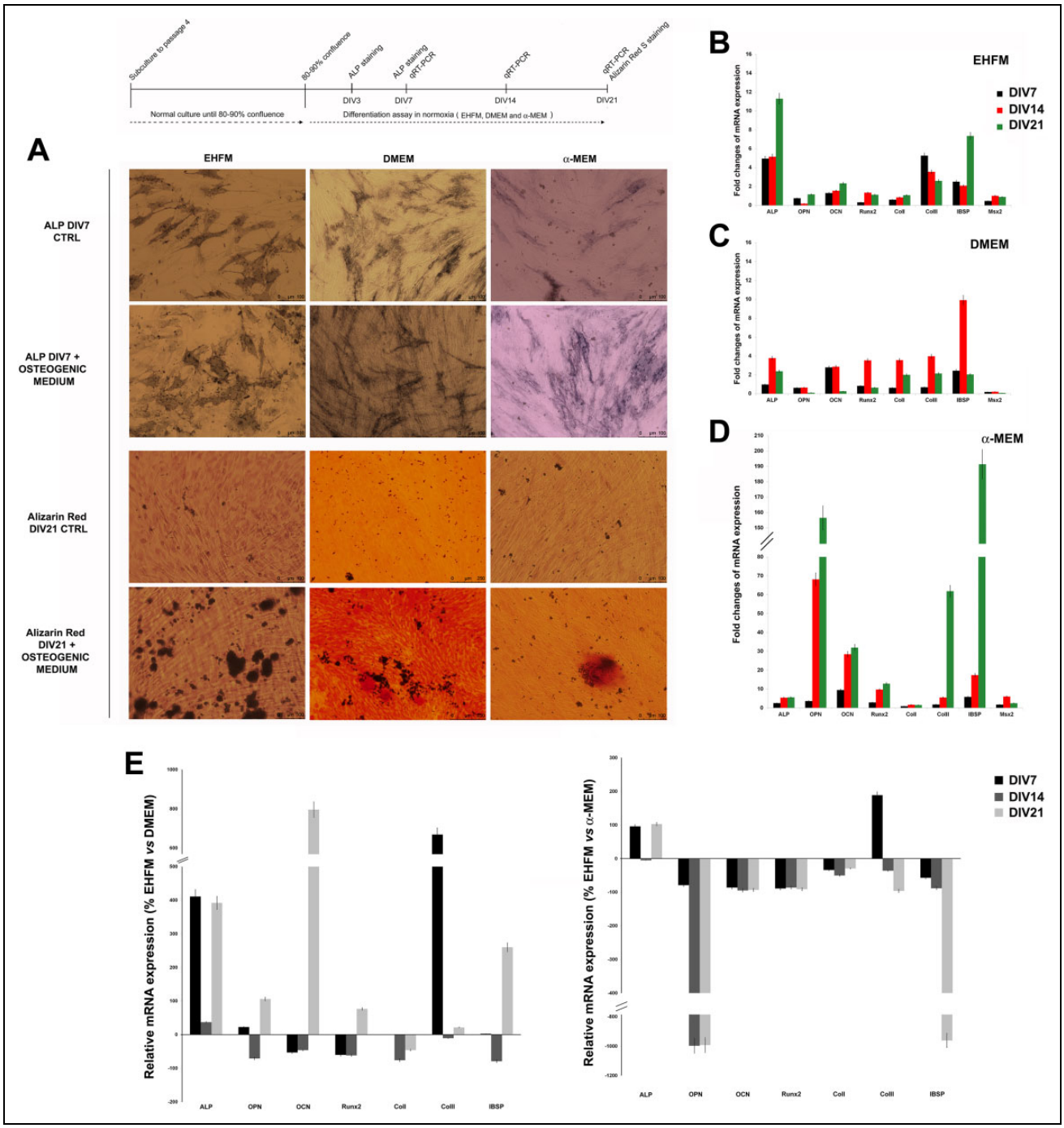

Fig. 3. Osteogenic differentiation of PDLSCs cultured in EHFM, α-MEM and DMEM enriched with dexamethasone, ascorbic acid and β-glycerophosphate. Figure shows the schema of the experimental set up. Detection of the osteogenic differentiation of EHFM-, α-MEM- and DMEM-cultured PDLSCs in basal conditions and in the presence of osteo-inductive factors through ALP activity and Alizarin Red S staining (A). Relative mRNA expression levels of ALP, OPN, OCN, Runx2, Coll, CollI, IBSP and Msx2 in EHFM-, α-MEM- and DMEM-cultured PDLSCs after differentiation culture for 7, 14 and 21 days (B–D). Relative mRNA expression of all osteogenesis markers in EHFM-expanded PDLSCs at 7, 14 and 21 days was expressed as % of DMEM and α-MEM (E). Data are presented as the mean ± SEM.
α-MEM: minimum essential medium Eagle, alpha modification; ALP: alkaline phosphatase; Col: collagen; DMEM: Dulbecco's modified Eagle's medium; EHFM: enriched Ham's F12 medium; IBSP: integrin binding sialoprotein; OCN: osteocalcin; OPN: osteopontin; PDLSC: periodontal ligament stem cell; SEM: standard error of the mean.

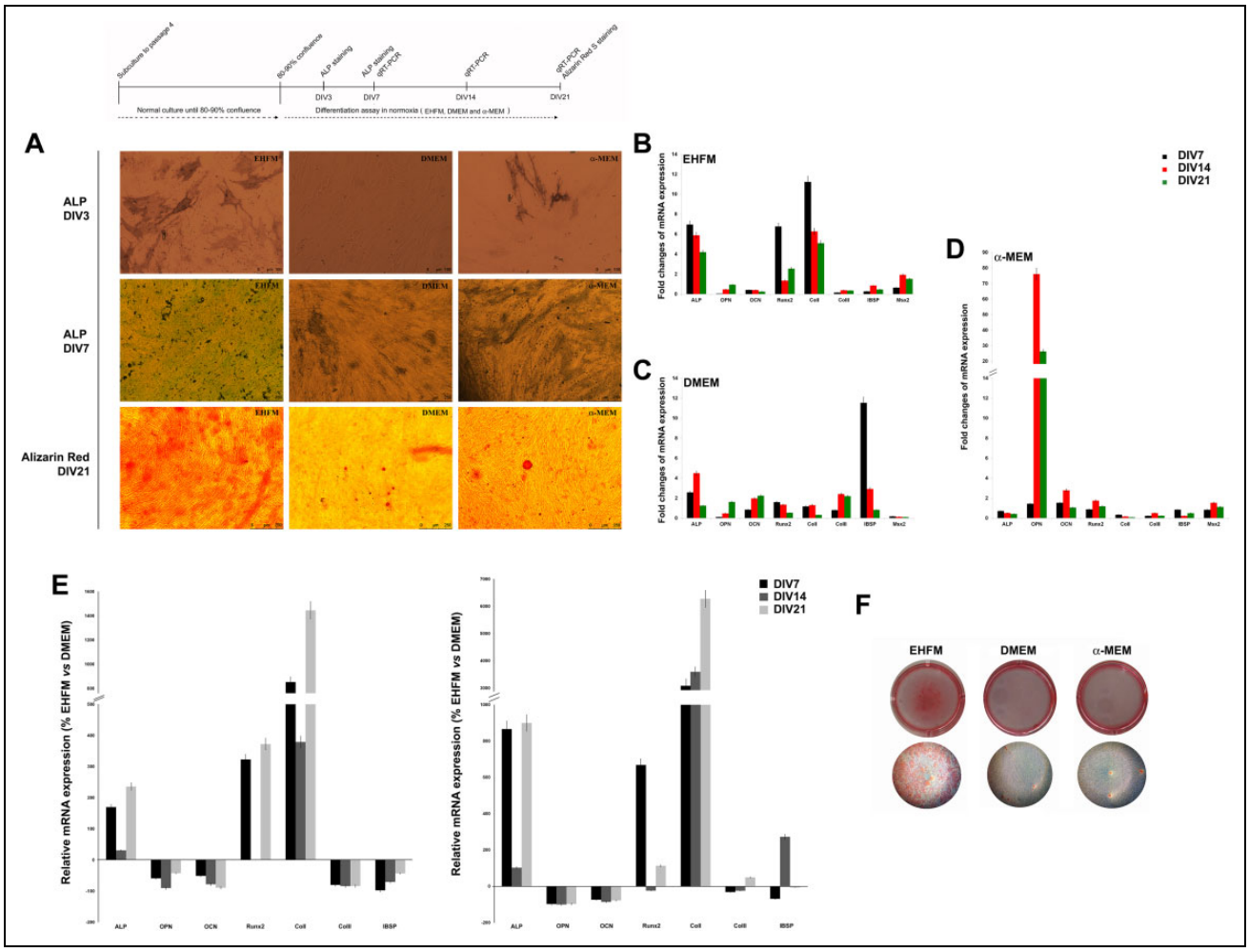

Fig. 4. Osteogenic differentiation of EHFM-, α-MEM- and DMEM-expanded PDLSCs in standardized culture medium for the osteogenesis (Life Technologies). Figure shows the schema of the experimental set up. Detection of the osteogenic differentiation of EHFM-, α-MEM- and DMEM-expanded PDLSCs in the StemPro™ Osteogenesis Differentiation Kit through ALP activity (A) and Alizarin Red S staining (A, F). Relative mRNA expression levels of ALP, OPN, OCN, Runx2, CoI, CoIII, IBSP and Msx2 in EHFM-, α-MEM- and DMEM-cultured PDLSCs after differentiation culture for 7, 14 and 21 days (B–D). Relative mRNA expression of all osteogenesis markers in the EHFM-expanded PDLSCs at 7, 14 and 21 days is expressed as % of DMEM and α-MEM (E). Data are presented as the mean ± SEM.
α-MEM: minimum essential medium Eagle, alpha modification; ALP: alkaline phosphatase; Col: collagen; DMEM: Dulbecco's modified Eagle's medium; EHFM: enriched Ham's F12 medium; IBSP: integrin binding sialoprotein; OCN: osteocalcin; OPN: osteopontin; PDLSC: periodontal ligament stem cell; SEM: standard error of the mean.

CD73 was associated with the better proliferation ability of the EHFM-expanded PDLSCs with respect to the cells expanded in α-MEM and DMEM. However, to elucidate the specific functions, further investigation is required. These results showed that EHFM better supports the growth of primary and expanded PDLSCs.

Regarding the comparison of α-MEM and DMEM, we reported a higher expression of all mesenchymal markers (CD73, CD90 and CD105) in the α-MEM-expanded cells than in DMEM through the entire culture duration, suggesting α-MEM to be advantageous for stemness maintenance. Our findings are in accordance with those of previous studies which showed that α-MEM supplemented with 15% FBS, 2 mmol/l L-glutamine, 100 mmol/l L-ascorbic-acid-2-phosphate and antibiotics provides better support for PDLSCs in both early and late passages[23]. Moreover, the advantages of using α-MEM versus DMEM have also been reported for human dental pulp cell cultures[24–26]. These results confirm α-MEM as a suitable medium for the expansion of PDLSCs.

The eligibility of EHFM, α-MEM and DMEM for osteogenic differentiation in PDLSCs was also assessed via the application of the most used osteogenic differentiation protocol by adding dexamethasone, ascorbic acid and β-glycerophosphate to culture media. After a 3-week period, we were able to detect mineralization in the confluent monolayers of PDLSCs maintained in DMEM and, to a lesser extent, in α-MEM, whereas no signal was evident in the EHFM-

cultured cells. The evaluation of transcript expression of not only the so-called 'master transcriptional regulator' of osteoblast differentiation (i.e. Runx2)[27,28], but also the markers of different stages in osteoblast genesis[29,30], showed that the Runx2, OCN, OPN, Col1, IBSP, and Msx2 levels were lower in the EHFM-cultivated cells than in the cell maintained in α-MEM and DMEM, above all during the first 2 weeks of osteogenic induction. Even more interesting is the fact that ALP expression was downregulated in the α-MEM- and DMEM-cultivated cells after 14 days of induction, whereas it continued to increase in the EHFM-cultivated cells. This result indicated that the PDLSCs in EHFM still displayed an undifferentiated phenotype after 21 days of induction. These data, together with the absence of mineralization evidence after 3 weeks in EHFM-cultured cells, suggested that the PDLSCs were in the first stage of osteoblast differentiation.

Our results seem to suggest a better performance of DMEM with respect to α-MEM in supporting osteoblast differentiation. Indeed, the level of Runx2 expression in immature osteoblasts is high, but it will be downregulated in immature osteoblasts during osteogenesis. We reported that the expression of the Runx2 gene was upregulated on day 21 in the α-MEM-cultivated PDLSCs compared with those cultured in DMEM, thus suggesting a lack of mature osteoblasts with only a few immature ALP-expressing osteoblasts. Unlike our results, Jung et al. reported that α-MEM-cultured PDLSCs produced a significantly greater amount of mineralized tissues on culture plates than DMEM-cultured cells[23]. Despite the upregulation of Runx2, especially in EHFM and α-MEM-expanded cells, we reported a strong upregulation of IBSP on day 21. Because IBSP is recognized as a late-stage marker for osteoblast differentiation involved in calcium and hydroxyapatite binding[31], its overexpression suggested the involvement of different pathways independent of Runx-2. Altogether, these results suggest that EHFM composition is still sufficient to maintain PDLSCs in an undifferentiated state despite the addition of osteoinductive factors. Moreover, we suggest DMEM as a suitable medium for osteoblast differentiation.

Very interestingly, when the EHFM-, α-MEM- and DMEM-expanded PDLSCs were transferred to a standardized culture medium for the osteogenesis (Life Technologies), mineralization became much more evident in the confluent monolayers of the EHFM-expanded PDLSCs and, to a lesser extent, in DMEM and α-MEM. Accordingly, a strong upregulation of ALP, Runx2 and ColI was only reported in the EHFM-cultivated PDLSCs on day 7. Although ALP and Runx2 expression in the EHFM-expanded cells appeared higher than that of α-MEM- and DMEM-expanded cells, the downregulation of mRNA was reported during the different stages of differentiation, thus confirming that a differentiation process occurred. Moreover, the higher OCN gene expression reported in EHFM-expanded cells, with respect to other media, fits well with the extent of mineralization. These results strongly suggest that EHFM confers higher osteogenic potential to PDLSCs compared with cells maintained in the other culture media.

Conclusions

The results of the present work confirmed a key role for the culture system in the maintenance of human PDLSCs, showing the advantages of using EHFM for the long-term expansion of mesenchymal cells in vitro and the preservation of high osteogenic potential. It is noteworthy that the cells expanded in the different media were obtained from three PDLs of the same patient; hence, we can assume that PDLSC features are determined exclusively by medium formulations. To date, we were able to detect just the total effects of EHFM on PDLSCs; meanwhile, the role of each supplement remains poorly understood. FGF is well known to accelerate PDLSC proliferation. Consistent with our results, previous studies showed that adding 20 ng/ml FGF-2 to DMEM facilitates PDLSC proliferation and inhibits mineralization in the presence of osteogenic inducers[32]. Hasegawa et al. showed that treatment with FGF-2 alone and both heparin and FGF-2 for 3 weeks induced the expression of endothelial-cell-specific markers in α-MEM-expanded PDLSCs[33]. Consistent with our results and those of others, Nagayasu-Tanaka et al. showed that FGF-2 accelerated and enhanced the proliferation of fibroblastic cells derived from PDL and enhanced angiogenesis in vivo[34]. However, the role of FGF-2 on both long-term expansion (more than seven passages) and high expression of mesenchymal markers (i.e. CD73) of PDLSCs has not been previously demonstrated. We assumed that FGF-2 activity alone was not sufficient to maintain high proliferative activity and prolonged expression of pluripotency markers. The cross-talk between FGF and EGF/EGFR signaling pathways may account for most of the results observed in EHFM-expanded cells, according to the observation that the gene expression of EGFR was upregulated by FGF[32]. The role of EGF on PDLSCs has also been previously investigated. In the work of Teramatsu et al., EGF was found to function in controlling the expansion and differentiation of undifferentiated MSCs; in particular, it was suggested that EGF might act on osteoblasts at the early stages of differentiation[35]. In terms of the biological effects of heparin, a clear understanding of the molecular pathways involved in heparin function is still lacking; however, many studies have suggested a role in both the promotion of MSC proliferation and osteoblast differentiation[36–39]. Overall, our results indicate that the EHFM formulation was the 'most suitable protocol' for the expansion and the preservation of the osteogenic potential of PDLSCs; nevertheless, more investigation is required to clarify the specific pathways activated in response to heparin, FGF and EGF as well as the effects on the adipogenic and chondrogenic potential.

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Ethical Approval

The study was conducted according to the Declaration of Helsinki on medical protocol and ethics after the approval of the regional Ethical Review Board of Central Calabria, Italy (reference for Magna Græcia University of Catanzaro).

Statement of Human and Animal Rights

The study was conducted according to the Declaration of Helsinki on medical protocol and ethics after the approval of the regional Ethical Review Board of Central Calabria, Italy.

Statement of Informed Consent

Written informed consent was obtained from each patient.

Declaration of Conflicting Interests

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