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Impact of increasingly complex cell culture conditions on the proteome of human periodontal ligament stem cells

Asier Fullaondo [a,b], Mar Zalduendo [b,c], Nerea Osinalde [d], Mohammad H. Alkhraisat [b,c,e], Eduardo Anitua [b,c] and Ana M. Zubiaga [a,b]

[a]Department of Genetics, Physical Anthropology and Animal Physiology, University of the Basque Country (UPV/EHU), Leioa, Spain; [b]UIRMI (UPV/EHU-Fundación Eduardo Anitua), University Institute for Regenerative Medicine & Oral Implantology, Vitoria, Spain; [c]Regenerative Medicine Department, BTI Biotechnology Institute, Vitoria, Spain; [d]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Vitoria-Gasteiz, Spain; [e]Oral and Maxillofacial Surgery, Oral Medicine and Periodontics Department, Faculty of Dentistry, University of Jordan, Amman, Jordan

ABSTRACT

Aims: Human periodontal ligament stem cells (hPDLSCs) exhibit an enormous potential to regenerate periodontal tissue. However, their translatability to the clinical setting is constrained by technical difficulties in standardizing culture conditions. The aim was to assess complex culture conditions using a proteomic-based protocol to standardize multi-layer hPDLSC cultivation methodology.

Materials and methods: hPDLSC-derived constructs were created with varying biological complexity. The simplest constructs were monolayer sheets of hPDLSCs cultured with fetal bovine serum (FBS) or Plasma Rich in Growth Factors supernatant (PRGFsn). The most complex constructs were triple-layered cell structures cultured with PRGFsn, with or without PRGF fibrin membrane (mPRGF). Ultrastructure and proteomic analyses were performed on these constructs.

Results: PRGF supernatant improved protein expression related to extracellular matrix, adhesion, proliferation, and migration in hPDLSCs. PRGF fibrin scaffold upregulates proteins for cell activation, respiration, and electron transport. hPDLSCs on fibrin membrane show robust osteogenic potential through differential protein expression (ossification, tissue remodeling, morphogenesis, or cell migration) and overall homeostasis relative to less complex structures.

Conclusion: Our data reveal the far-reaching potential of 3-dimensional constructs in combination with PRGF technology in periodontal regenerative applications.

PLAIN LANGUAGE SUMMARY

What this summary about?: Human periodontal ligament stem cells (hPDLSCs) – cells that can transform into bone-like cells – could be better utilized to regenerate periodontal tissue. Although promising, it's challenging to use these cells in clinical settings due to difficulties in standardizing how they're cultured. To understand the effects of various cell culture methods on the cells' protein makeup and their suitability for regenerative medicine, the researchers created different hPDLSC constructs, from simple cell sheets to more complex, multi-layered structures.

What were the results?: From all culture conditions, the use of Plasma Rich in Growth Factors (PRGF), both as cell culture medium and scaffold, enhances the cells homeostasis and their ability to differentiate into bone-forming cells. Advanced imaging techniques showed good interactions between the cells and the 3D scaffold of PRGF.

What do the results mean?: The findings reveal that the more complex, multi-layered constructs, especially those grown with PRGF, show better differentiation potential and stability compared to simpler structures. This suggests that combining 3D cell constructs with PRGF technology could be a powerful approach for periodontal regeneration in clinical applications.

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CONTACT Eduardo Anitua eduardo@fundacioneduardoanitua.org UIRMI (UPV/EHU-Fundación Eduardo Anitua, University Institute for Regenerative Medicine & Oral Implantology, C/ Jose María Cagigal 19, Vitoria 01007, Spain

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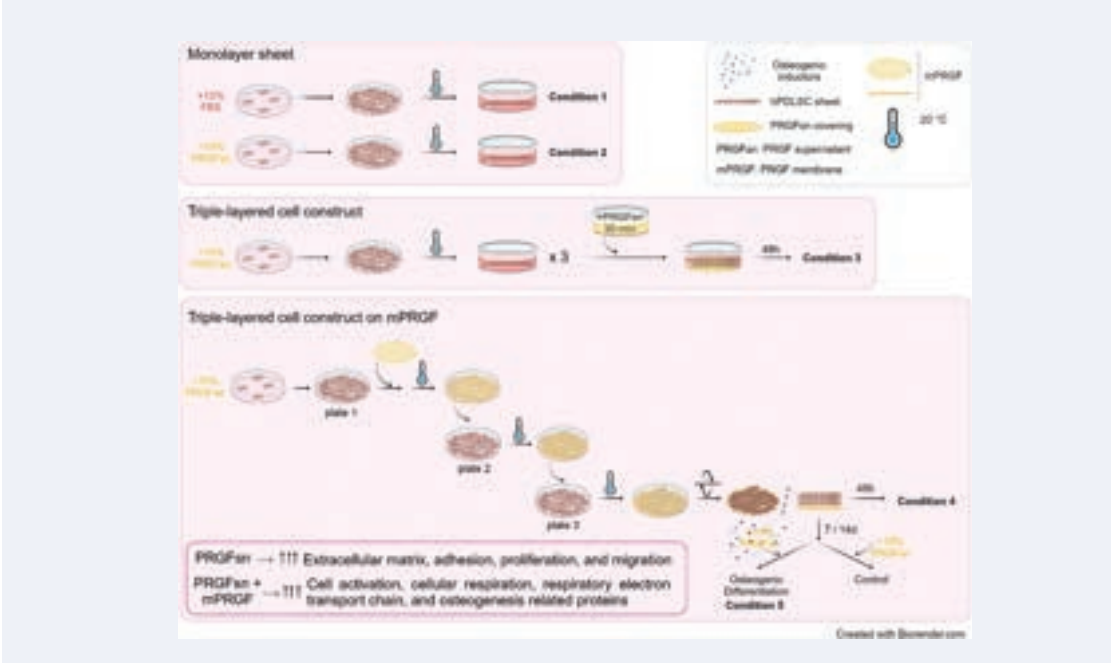

1. Introduction

An efficient periodontal tissue regeneration relies on critical elements that include an appropriate source of growth and differentiation factors necessary for generating an optimal amount of osteogenic tissue. In this regard, the work from our group and others has proposed the use of Plasma Rich in Growth Factors (PRGF) derived from autologous sources for regenerative purposes, given its major composition in growth factors [1–3]. PRGF consists of a plethora of bioactive molecules, from both plasma and platelet origin, involved in relevant processes of tissue regeneration such as cell proliferation, migration and synthesis of growth factors and matrix components, among other elements [4–11]. Due to its versatility, several formulations with different properties can be obtained that confer its applicability in several fields of medicine. In this sense, a liquid formulation, a more viscous preparation with gel-consistence, or indeed, a suturable membrane can be easily obtained [12–14]. PRGF efficacy was originally tested in the Dental area [15,16], but it has been similarly confirmed through numerous clinical studies in the fields of Traumatology [17–21], Ophthalmology [22–24], Dermatology [25,26], Neurology [27], and more recently, Gynecology [28].

The regenerative process requires a suitable environment that supports it. Novel approaches including the use of multilayered cell constructs, alone or in combination with a fibrin membrane scaffold are being considered for regenerative therapies. These stratified structures mimicking physiological tissues might provide an efficient local supply of cells in addition to a high adhesiveness to host tissues due to the preservation of cell–cell junctions and proteins of the cell surface as well as extracellular matrix [29–32]. However, it remains to be determined how these diverse approaches impact the stem cell performance, and to what extent the tissue following these novel procedures is able to engage in periodontal osteogenesis.

A useful way to analyze the physiological or pathological impact of a given cellular process relies on the study of the proteome. Osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs) grown in monolayer cultures has been studied at the proteome level, using gel-based or TMT-based mass spectrometry [33–35]. These studies have revealed a large array of changes that stem cells undergo at the protein level during the process of osteogenic differentiation. They include differential expression of proteins related to cytoskeleton, extracellular matrix organization, metabolic processes or mitochondrial respiration and oxidative phosphorylation, underlying the unique role of PDLSCs in maintaining periodontal tissue homeostasis.

It is presently unknown how tissue engineering technologies impinge on the proteome of hPDLSCs undergoing growth and differentiation. The significance of this research lies in its exploration of the impact of tissue engineering technologies on the proteomic profile of human periodontal ligament stem cells (hPDLSCs) during growth and differentiation. For that, we have carried out a stepwise protocol with cultured hPDLSCs to assess the effect of increasingly complex methodological conditions on their proteomic profile, which could give us an insight on their suitability for regenerative medicine. We report that each tested method provides distinct characteristics to the cells, and that their combination results in an engineered 3-dimensional construct with remarkable osteogenic properties. This research contributes to our understanding of hPDLSCs' potential in regenerative medicine, offering valuable information for optimizing tissue engineering strategies for clinical applications.

2. Materials and methods

This research was approved by the ethical committee of Araba University Hospital (BTI-01-IV/02/20/CST) and was performed following the principles established in the Declaration of Helsinki amended in 2013.

Article highlights

- hPDLSCs-derived constructs of increasing biological complexity have been engineered.
- PRGF supernatant promotes cellular processes such as adhesion, proliferation, and migration.
- PRGF supernatant avoids the possible adverse effects of culturing hPDLSCs in multilayer configurations.
- PRGF fibrin scaffold stimulates the production of energy for biological activities.
- PRGF supernatant activates a partial osteogenic differentiation program in triple-layer constructs on PRGF fibrin scaffolds.

2.1. Preparations of plasma rich in growth factors (PRGF)

Blood from healthy donors was collected into sodium citrated tubes (3.8% wt/v), after written informed consent was signed. Blood was centrifuged for 8 min at 580 g at RT (Endoret System; BTI Biotechnology Institute, S.L., Vitoria, Spain). The plasma rich in growth factors (PRGF) was obtained by collecting the whole plasma column just above the buffy coat.

In order to obtain the PRGF supernatant (PRGFsn) for the enrichment of the culture medium, the whole plasma column from three donors was pooled. Subsequently, calcium chloride was used as plasma activator following the user's guide for PRGF Endoret (BTI Biotechnology Institute). Then, PRGF clot was maintained for 1 h at 37°C in a Plasmaterm oven (BTI Biotechnology Institute) and afterward, centrifuged at 1,000 g for 10 min at RT (5810 Centrifuge, Eppendorf, Hamburg, Germany). PRGF supernatant was finally collected, filtered, aliquoted and stored at −80°C until its use.

Blood from a healthy donor was also collected to prepare the PRGF fibrin membranes (mPRGF) matching the size of a well of 6-well plate [36]. Briefly, for each membrane, 4 mL of PRGF (Figure 1(a)) were activated with calcium chloride in 5 ml rootstock containers (BTI Biotechnology Institute). Following incubation for a few minutes at RT, clot was formed (Figure 1(b)) and then transferred into a fibrin membrane shaper (Figure 1(c)) (BTI Biotechnology Institute). The mPRGF was obtained after pressing for 5 min (Figure 1(d,e)).

2.2. Cell isolation, characterization and culture

A non-impacted wisdom tooth was obtained from one healthy 18-year-old patient during a simple extraction process after written informed consent was provided. Human primary cultures of periodontal ligament stem cells (hPDLSCs) were isolated by the explant method [37–40]. Briefly, periodontal ligament split from the middle third of tooth roots was divided in pieces that were cultured in Dulbecco's modified Eagle's medium (D-MEM/F-12) (Gibco-Invitrogen, Grand Island, NY, USA) with 2 mm glutamine, 50 µg/mL gentamicin, 2.5 µg/mL amphotericin B (all three from Sigma-Aldrich Inc., St. Louis, MO, USA) and 10% fetal bovine serum (FBS) (Biochrom AG, Leonorenstr, Berlin, Germany). Culture medium was changed twice a week. After the first subculture, when the cell culture reached confluence, amphotericin B was removed from the culture medium.

Cell surface antigen expression of hPDLSCs (passage 5) was previously determined by flow cytometry. Following the criteria established by the International Society for Cell and Gene Therapy (ISCT) [41], expression of CD14, CD90 (both from Abcam, Cambridge, UK), CD19, CD34, CD45, CD73, CD105 and HLA-DR (all of them provided by Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was analyzed. Results confirmed the stemness nature of the isolated hPDLSCs. More than 99% of the cells expressed the surface markers CD73, CD90, and CD105. Moreover, the cell population was negative for the rest of the tested antigens (<0.2%). In addition, their ability to differentiate into adipogenic and osteogenic phenotypes was confirmed at cell culture passage number 6 [36].

2.3. Preparation of cell structures

In this longitudinal-like study, cellular structures of increasing complexity were prepared using hPDLSCs at passage 6, derived from the same cryo-preserved cell culture.

Cell Sheet Technology was used to obtain all cell structures for the proteomic analysis. Thus, the use of proteolytic enzymes was avoided preserving the extracellular matrix components and cell–cell junctions. This technology is based on the use of thermoresponsive cell culture dishes that allow the control of cell adhesion and detachment by external temperature changes [42,43].

2.3.1. Monolayer sheets of hPDLSCs

hPDLSCs were seeded on 6-well Nunc UpCell (ThermoFisher Scientific Inc, Waltham, MA, USA) temperature-responsive plates at a density of 10,000 cells/cm^2. Half of the cultures were maintained in FBS-containing culture medium, and the other half were grown with 10% PRGF supernatant instead of FBS (hereafter hPDLSCs' routine culture medium). After reaching a super-confluence state, plates were incubated at 20°C until the cell monolayer was spontaneously detached as a cell sheet floating into the culture medium [30,36].

Figure 1. PRGF fibrin membrane (mPRGF) preparation. (a) PRGF obtained after blood centrifugation (b) is ativated with calcium chloride and afterward the clot is formed. (c) This clot is then transferred into a fibrin membrane shaper and (d) a weigth is placed on top of it. (e) Finally, the mPRGF is obtained by pressing for 5 minutes. Scale bar: 2 cm.

2.3.2. Triple-layered cell constructs of hPDLSCs

hPDLSCs were seeded at 10,000 cells/cm^2 on 6-well Nunc UpCell plates and maintained in the routine culture medium until super-confluence. Cell sheets were obtained as described before. Three cell sheets were then aspirated and sequentially transferred, one on top of the other, to another recipient well. These wells were previously incubated with PRGF supernatant for 30 min to improve the adhesion of the first cell sheet to the plate surface. Triple-layered constructs were incubated with a small volume of routine culture medium under normal CO_2 and temperature conditions for 1 h. After that, 1.5 ml of routine culture medium was added to each well and constructs were cultured for 48 h.

2.3.3. Triple-layered cell constructs of hPDLSCs on mPRGF

To fabricate a more complex structure, hPDLSCs were seeded and cultured similarly to that explained for the preparation of cell monolayer sheets. When the super-confluence state was reached, the culture medium was removed and mPRGF was placed carefully onto the cell sheet. The 6-well plate was then incubated at 20°C. After 45 min, the mPRGF together with the confluent hPDLSCs' monolayer was detached and transferred onto another super-confluent cell culture, allowing the contact between the two cell sheets. After incubating at low temperature, a double-layered construct, along with the mPRGF, was obtained. This process was repeated one more time to complete the triple-layer construct and, finally, the whole construct was turned over and placed into another well so that the fibrin membrane could be in contact with the plastic surface. Triple-layered cell constructs onto the mPRGF were cultured with the routine culture medium for 48 h.

2.4. Ultrastructural analysis of triple-layered cell constructs on mPRGF

The ultrastructure of the most complex construct was analyzed by Scanning Electron Microscopy (SEM) and Confocal Microscopy. Regarding SEM analysis, 3 triple-layered constructs on mPRGF were washed 2 times with PBS after culturing for 48 h. Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate, pH: 7.4, and post-fixed with osmium tetroxide (1% OsO4 in 0.1 M cacodylate) (all reagents provided by Sigma-Aldrich Inc.). After washing again, constructs were dehydrated in increasing concentrations of ethanol, dried at critical point (Autosamdri 814) (Tousimis, Rockville, MD, USA), and gold-sputtered. Finally, constructs were examined in a scanning electron microscope (S-4800) (Hitachi High-Tech Corporation, Tokyo, Japan).

For Confocal Microscopy evaluation, fluorescently labeled fibrinogen (Invitrogen-ThermoFisher Scientific Inc, Waltham, MA, USA) was added to PRGF before clotting with calcium chloride to enable mPRGF to be examined. The subsequent processes for preparing the fibrin membrane and triple-layered constructs were similar to those explained above. After culturing 3 triple-layered constructs for 24 h, they were washed 2 times in PBS, fixed with 4% paraformaldehyde for 1 h at RT and then, hPDLSCs were permeabilised. After blocking nonspecific bindings, an incubation with Phalloidin-TRITC (Sigma-Aldrich Inc.) was carried out for 1 h at RT in order to highlight actin microfilaments. Nuclei were also counterstained with Hoechst. Finally, the constructs were washed and maintained at 4°C until their examination under a LSM880 Airyscan confocal microscope (Zeiss Group, Germany).

2.5. Osteogenic differentiation of triple-layered cell constructs on mPRGF

The aim of this research was the proteomic analysis of hPDLSCs in culture conditions of increasing complexity which in turn promote the regeneration process of the injured tissue after implantation. Regarding periodontal regeneration, obtaining cell structures with specific properties such as improved handling and higher osteogenic potential is an extremely appealing approach. The PRGF membrane confers the necessary firmness to the three-layer constructs on mPRGF while partially activating the osteogenic differentiation programme, therefore it was decided to include these structures for the osteogenesis assays.

hPDLSC layers onto mPRGF were maintained in 6-well plates with 1.5 ml of the routine culture medium. After 24 h, the medium was substituted by a differentiation culture medium consisting of a mixture of dexamethasone, ascorbate-phosphate, and β-glycerolphosphate osteogenic inductors (R&D Systems Inc., Minneapolis, MN, USA) added to the routine culture medium. Triple-layered cell constructs onto mPRGF but maintained without osteogenic inductors were included as negative controls. The culture medium was changed on days 3, 7, and 14. Osteogenic differentiation was not performed for a longer time due to the identification of extracellular matrix deposits before day 14, when the osteogenic potential was tested in the assessment of hPDLSC stemness. The hPDLSCs capacity to differentiate to bone lineage has also been tested at this study time by other authors [44–46].

Two replicates were performed for each condition and study time.

2.6. Sample preparation for proteomic analysis

Two replicates of each cell structure type were assigned for proteomic quantification analysis. After the corresponding culture time for the different structures, hPDLSCs triple-layered composite constructs without mPRGF were washed with phosphate-buffered saline (PBS) 3 times and collected into microtubes to centrifuge at 500 g for 7 min. Then, after discarding the supernatant, pelleted cell structures were stored at −80°C until their analysis. When mPRGF was included in their composition, it was discarded to avoid any interference in the proteomic analysis. Thus, after the washing step, these constructs were incubated in Tryple solution (Gibco-Invitrogen) for 1 h. A cell scraper was used to collect the cells still attached to the fibrin membrane and, finally, cell suspension was filtered through a 70 µm mesh. After centrifugation, cell pellets were washed 3 times in PBS and stored dry at −80°C until their analysis.

2.7. Liquid chromatography-mass spectrometry (LC-MS/MS)

After sample preparation for proteomic analysis, mass spectrometry was performed on an Exploris 480 mass spectrometer, coupled to an Easy-nLC 1200 nanoUPLC System. Peptides were eluted at a flow rate of 300 nl/min with the following percentage of acetonitrile in 0.1% formic acid: 96 min 0–15%, 35 min 15–24%, 22 min 24–32%, 3 min 32–76%, 10 min 76%. Mass spectrometer was operated in DDA (Data Dependent Acquisition) mode in a 3-s cycle time. Full MS scans were acquired from m/z 350 to 1600 with a resolution of 60,000 at m/z 200. Ions were fragmented by higher energy C-trap dissociation with a normalized collision energy of 36%. MS/MS spectra were recorded with a resolution of 45,000 at m/z 200. The maximum ion injection time was set to 50 ms for survey scans and auto for MS/MS scans, whereas normalized AGC target values of 300% were used for both survey and MS/MS scans. A dynamic exclusion time of 40 s was applied and singly charged ions, ions with 6 or more charges, and ions with unassigned charge state were excluded from MS/MS. Data were acquired using Xcalibur software.

2.8. Proteomic data processing and statistical analysis

Raw data files were processed with the MaxQuant software (version 1.6.17.0) [47] using the internal search engine Andromeda. Data originated from the different high pH fractions of the same samples were combined and searched against the UniProtKB-SwissProt database restricted to *Homo sapiens* proteins (version 2020_01). Mass tolerance was set at 8 and 20 ppm at MS and MS/MS levels, respectively. Enzyme specificity was set to trypsin and a maximum of 2 missed cleavages were allowed. Carbamidomethylation of C was selected as fixed modification and oxidation of M, protein N-terminal acetylation, and deamidation of N and Q as variable modifications. Batch-specific TMT correction factors were added based on manufacturer's product data sheet. False discovery rate (FDR) rate was set at 1% at protein and peptide levels.

MS-derived data were analyzed using Perseus software v1.5.6 [48]. Data were filtered out for potential contaminants, proteins identified in the decoy database, and for proteins only identified by site. The log2 transformed intensities were normalized to the median of each sample. The standard two-sided Student's t-test was applied to test for differences in protein abundance. The differential regulation threshold was set to log2-fold changes below −0.5 and above 0.5 with p-values below 0.05. Principal Component Analysis was used for dimensionality reduction and feature extraction as well as visualization of multidimensional data.

2.9. Gene ontology (GO) analysis

To identify the statistically overrepresented GO biological processes, proteomic data were subjected to functional enrichment analyses using the ShinyGO v0.77 tool [49]. The ShinyGO parameters considered were p-value cutoff, FDR, (0.05), species (*Homo sapiens)*, and minimum protein number in the pathway (30). The compilation of all the proteins detected by the proteomics experiment was established as the background proteome of the study. Venn diagrams were made using jvenn [50].

3. Results

3.1. Ultrastructural analysis of triple-layered cell constructs on mPRGF

SEM images of the sample surface and transversal sections of the most complex construct consisting of triple-layered cell structure on mPRGF were captured after culturing with PRGFsn for 48 h. As shown in Figure 2(a–d), one side of the construct consisted of a nude homogeneous fibrin net derived from PRGF while a uniform cell coverage could be observed on the opposite side. The photographs obtained under Confocal Microscopy (Figure 2(e–h)) confirmed the complete integration of hPDLSCs into the PRGF fibrin membrane.

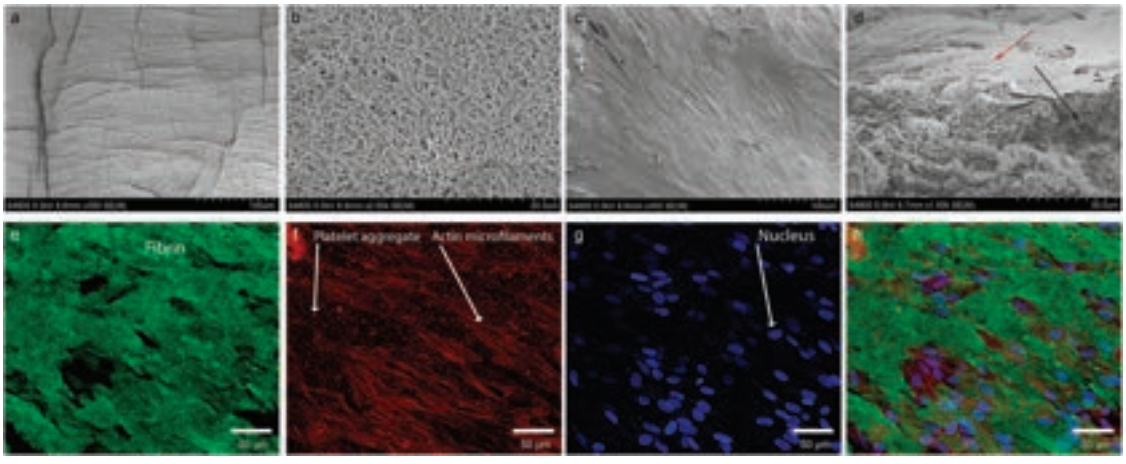

Figure 2. Ultrastructural analysis of the triple-layered cell constructs with mPRGF. SEM microphotographs showing (a, b) The fibrin mesh of mPRGF at the bottom of the construct at different magnifications and (c) A uniform cell coverage on the other side of this structure. (d) The complexity of this composite is shown in a SEM image of a transversal section where the red arrow indicates hPDLSCs and the black one points at the fibrin net of mPRGF. Scale is the distance between the first and last points above the scale value in µm. On the bottom of the figure, a sequence of confocal microscopy captions highlights (e) The fibrin mesh, (f) Actin microfilaments of hPDLSCs and platelet aggregates and (G) hPDLSCs nuclei. (H) Image obtained by overlaying the fluorescence signal from the 3 channels, confirming the cell integration with the mPRGF fibrin membrane. Fibrin mesh, actin microfilaments, and hPDLSC nuclei are fluorescently labeled in green, red, and blue, respectively. Scale bar: 50 µm.

3.2. Osteogenic differentiation of triple-layered cell constructs on mPRGF

Macroscopic photographs of the constructs maintained with or without osteogenic inductors were obtained on days 7 and 14 of cell culture. The size of the cell constructs decreased over the culture time both in the presence (differentiation) and absence of inducers (control), as it is shown in Supplementary Figure S1. This result should be considered for the clinical translation of this tissue engineering methodology keeping in mind the size of the defect in the tissue to be regenerated.

3.3. Proteome profiles of hPDLSC-derived constructs of increasing complexity

We set out to examine the proteome profiles of hPDLSCs cultured for 48 h under conditions of increasing complexity in order to determine the impact of each condition on the shaping of the proteome. The analyzed conditions were as follows (Figure 3(a)): hPDLSCs grown on a single layer and cultured with the routine culture medium (condition 1); hPDLSCs grown on a single layer with the routine culture medium with PRGFsn instead of FBS (condition 2); hPDLSCs grown as triple-layered constructs with the routine culture medium with PRGFsn instead of FBS (condition 3); hPDLSCs grown as condition 3 with the addition of a PRGF fibrin membrane scaffold (condition 4); hPDLSCs grown as condition 4 with the addition of osteogenic differentiation inducers for a total of 7 days (condition 5).

To assess the proteome profiles of periodontal stem cells under each tested condition, a TMT-based quantitative proteomic analysis was carried out, whereby all conditions were analyzed simultaneously, thus reducing any possible bias resulting from separate analyses. Four types of comparisons were carried out: condition 2 *vs*. condition 1, to define the effect of culturing stem cells with PRGF supernatant; condition 3 *vs*. condition 2, to define the effect of culturing stem cells in triple-layered constructions, as opposed to a single layer; condition 4 *vs*. condition 3, to define the effect of a PRGF fibrin

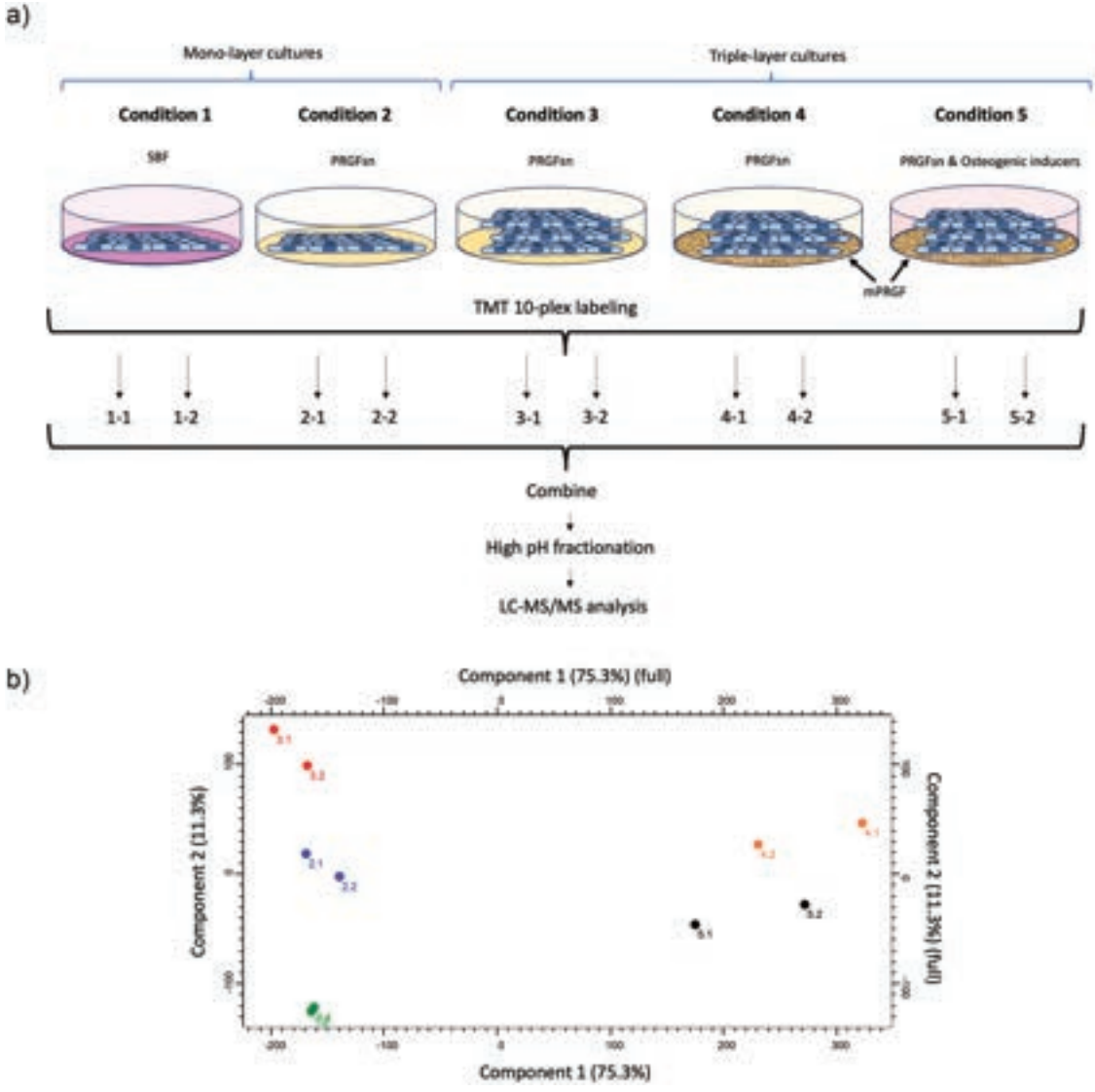

Figure 3. Experimental design and proteomic analysis of hPdlscs. (a) Schematic representation of the experimental setup. hPDLSCs were grown under five different conditions (condition 1–5) and two replicas of each condition were prepared. Upon protein extraction and digestion, peptides were labeled with TMT 10-plex reagent, combined and subjected to high pH fractionation before LC-MS/MS analysis. (b) Principal component analysis (PCA) of proteomics data.

scaffold on the multilayered construct; and condition 5 vs. 4, to define the effect of an osteogenic differentiation process on a triple-layered construct of periodontal stem cells grown over a PRGF fibrin membrane.

Our aim in this research was to select the optimal cell structure for regenerative purposes by comparing different cell culture conditions of increasing complexity. In order to make the assay sufficiently robust, we wanted to avoid as much as possible the inter-assay variability derived from the proteomic analysis technique itself. Thus, we wanted to compare all the structures in a single assay, fixing the donor variable and modifying only the culture conditions. With the same objective, we used cell cultures derived from the same cryopreserved cells and with the same number of cell duplications for all the assays in order to reduce variability between replicates. Giving that in each run 10 samples could be assessed and having 5 conditions, this gave two biological replicates from each condition.

A total of 5,833 proteins were confidently identified and quantified. Principal component analysis confirmed that the proteomes of the tested conditions were clearly different from each other, particularly when the PRGF fibrin membrane was included in the culture (Figure 3(b)). For each comparison, a volcano plot was generated highlighting in red significantly upregulated (log2 fold change > 0.5; p value < 0.05) and in blue significantly downregulated (log2 fold change < −0.5: p value < 0.05) proteins (Supplementary Figure S2). Differentially expressed proteins (Suppl. Table S1) were further considered for Gene Ontology (GO) analyses (Suppl. Table S2).

Regarding the first comparison (2 *vs*. 1), we were interested in determining the impact of administering PRGF supernatant to periodontal ligament stem cell cultures. Interestingly, over 100 proteins appeared upregulated in PRGF-treated cells compared to FBS-treated cells, which could be grouped into GO categories related to extracellular matrix, adhesion, inflammatory response, proliferation, or motility (Figure 4(a)). By contrast, the downregulated fraction included 85 proteins, but no enriched GO categories were detected in this protein subset.

The next comparison (3 *vs*. 2) focused on determining the effect on the proteome of culturing stem cells on several layers. Both single-layered and triple-layered cells were grown with PRGFsn as supplement of the culture medium. The number of differentially expressed proteins increased by about two-fold compared to the previous comparison (259 proteins upregulated and 213 proteins downregulated). Importantly, culturing cells on several layers did not show any significant enrichment on proteins related to cellular stress or apoptosis, suggesting that a multilayer configuration cultured with PRGF supernatant does not negatively impact on stem cell growth. Among the downregulated proteins, the only overrepresented term was vesicular transport, which included several solute carrier proteins (i.e., SLC1A3 and SLC1A5). By contrast, among the upregulated proteins, terms related to proliferation (i.e., FGF2, IGFBP5, PTN), development (i.e., SOD2, SFRP1, LMNA), extracellular matrix (i.e., COL8A1, ITGA2, FBL1) or cell migration (i.e., ICAM1, NTN1, CDH13) appeared enriched (Figure 4(b)).

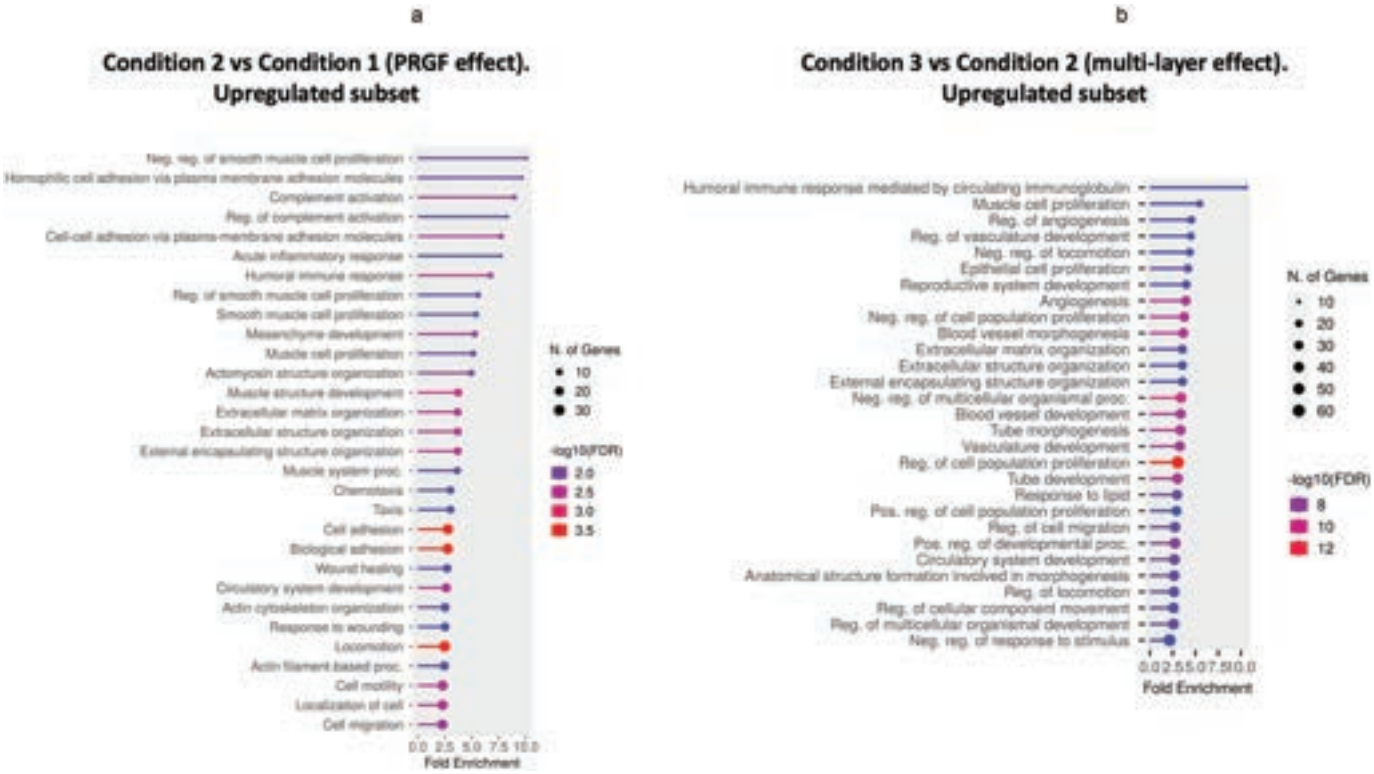

Figure 4. GO analysis of differentially expressed proteins. (a) Enriched GO terms in hPDLSC monolayer cultures grown on PRGFsn or on FBS. (b) Enriched GO terms in hPDLSC triple-layer cultures grown on PRGFsn compared to hPDLSC monolayer cultures grown on PRGFsn. The length of the line indicates the fold enrichment of the GO term, whereas the size of the dot at the end of the line represents the number of genes included in the term. Only GOs of the upregulated subsets are shown, as the downregulated subsets did not exhibit any enriched GO categories. As a background, the total number of proteins identified in each of the comparisons were used.

As a next step on culture complexity, we assessed to what extent the inclusion of a PRGF-derived fibrin membrane affected the features of triple-layered constructs (4 *vs*. 3). Remarkably, the inclusion of a mPRGF led to a significant increase in the number of differentially expressed proteins to about 1,000 (493 upregulated and 487 downregulated), confirming the impact of this component on cell culture. Among the differentially expressed proteins, those related to angiogenesis, extracellular matrix, development, morphogenesis, or migration appeared in the downregulated set (Figure 5(a)), whereas proteins involved in platelet activation, coagulation, respiration, transport, secretion, or cell activation were upregulated in the presence of the fibrin membrane (Figure 5(b)). The term adhesion appeared enriched in both up and downregulated protein set. These results suggest that the PRGF-derived fibrin component modulates the features of a multilayered cell construct by reducing the expression of extracellular matrix, morphogenesis or migration components, and increasing expression of cell activation constituents.

3.4. Proteome profiles of hPDLSC-derived constructs under osteogenic differentiation

The proteome of hPDLSCs differentiated as monolayers in culture has been previously analyzed in detail [33–35]. However, the osteogenic potential of hPDLSCs grown as a multi-layered construct remains to be assessed. In an effort to determine the full potential of a triple-layered construct of stem cells to undergo osteogenic differentiation, cultures were grown on osteogenic differentiation medium over a PRGF fibrin membrane for 7 days. The comparison of condition 5 *vs*. condition 4 showed close to 200 differentially regulated proteins (63 upregulated and 112 downregulated). Among the proteins with an increased expression, we identified many involved in ossification (i.e., ENPP1, CTSK, ALPL), morphogenesis (i.e., GREM2, HEG1) extracellular matrix (i.e., VTN, MMP1), or cell adhesion and migration (i.e., CD63, CEMIP) (Figure 6(b)). By contrast, proteins with a reduced expression were mainly involved in chromatin and chromosome organization (i.e., CBX3, KDM3A, SET) (Figure 6(a)).

We wondered to what extent the difference in incubation time for condition 4 (48 h) and condition 5 (7 days) could account for the observed differential protein expression between these conditions. To get further insight, we repeated the experiment, but this time we kept the same incubation times for the cultures treated with osteogenic inducers and for the control cultures (7 days) and determined by mass spectrometry the proteins that were differentially expressed upon these conditions (Suppl. Table S3). Additionally, we also included a 14-day time-point. Remarkably, triple-layered constructs exhibited many overrepresented GO categories related to osteogenic differentiation within the upregulated protein subset by day 7 of culture, suggesting that the differentiation programme is already in progress at this time-point (Suppl. table S4). By contrast, the downregulated protein subset revealed no enriched GO categories. Moreover, the extent of the differentiation programme was not modified substantially at the proteome level by day 14 of culture compared to day 7.

Next, we compared the GO terms enriched in the two experimental settings at day 7 (Table 1). Some categories that were not common to both experimental settings involved processes such as complement activation, coagulation, extracellular structure, adhesion or angiogenesis (in yellow in Table 1). Furthermore, the enriched categories in the downregulated protein subset of Condition 5 vs Condition 4 related to chromatin structure were absent in the second experiment,

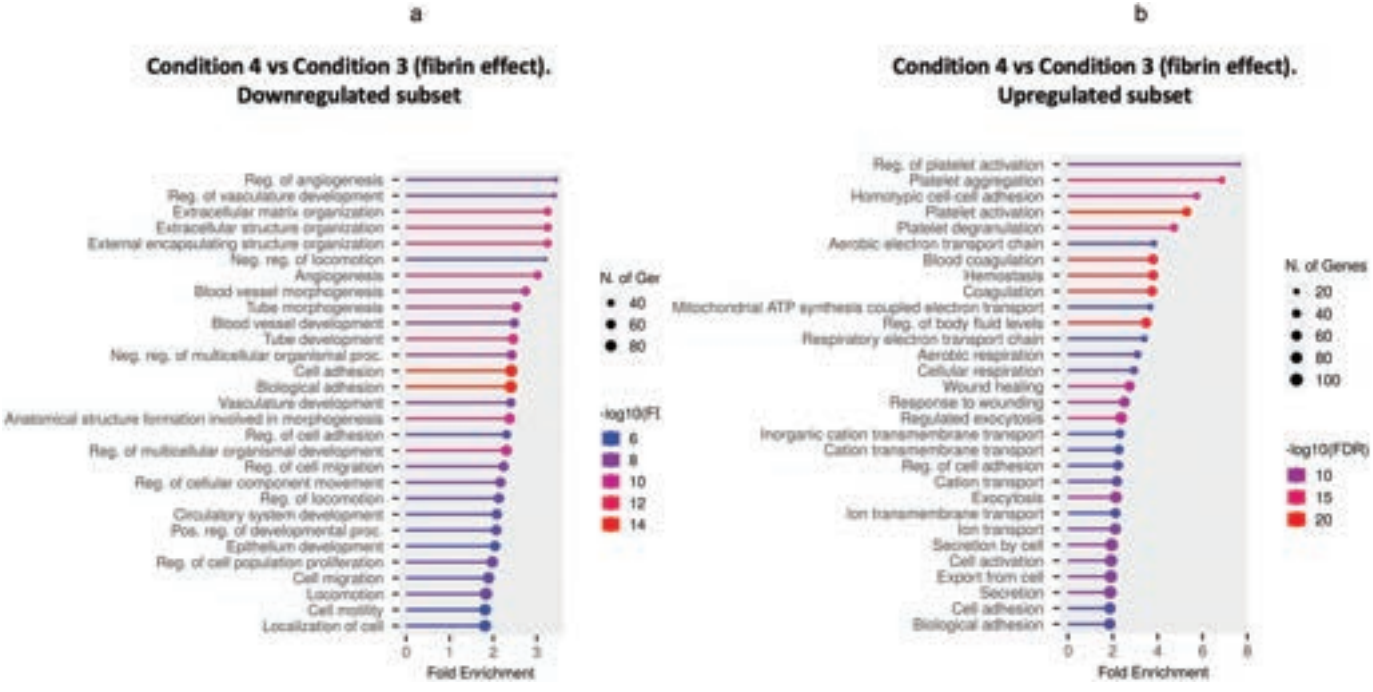

Figure 5. GO analysis of differentially expressed proteins in hPDLSCs grown as triple-layered constructs. GO annotations of significantly enriched terms among (a) Downregulated and (b) Upregulated proteins are shown in the comparison of condition 4 (in the presence of the fibrin membrane) *vs* condition 3 (in the absence of the fibrin membrane). As a background, the total number of proteins identified in each of the comparisons were used.

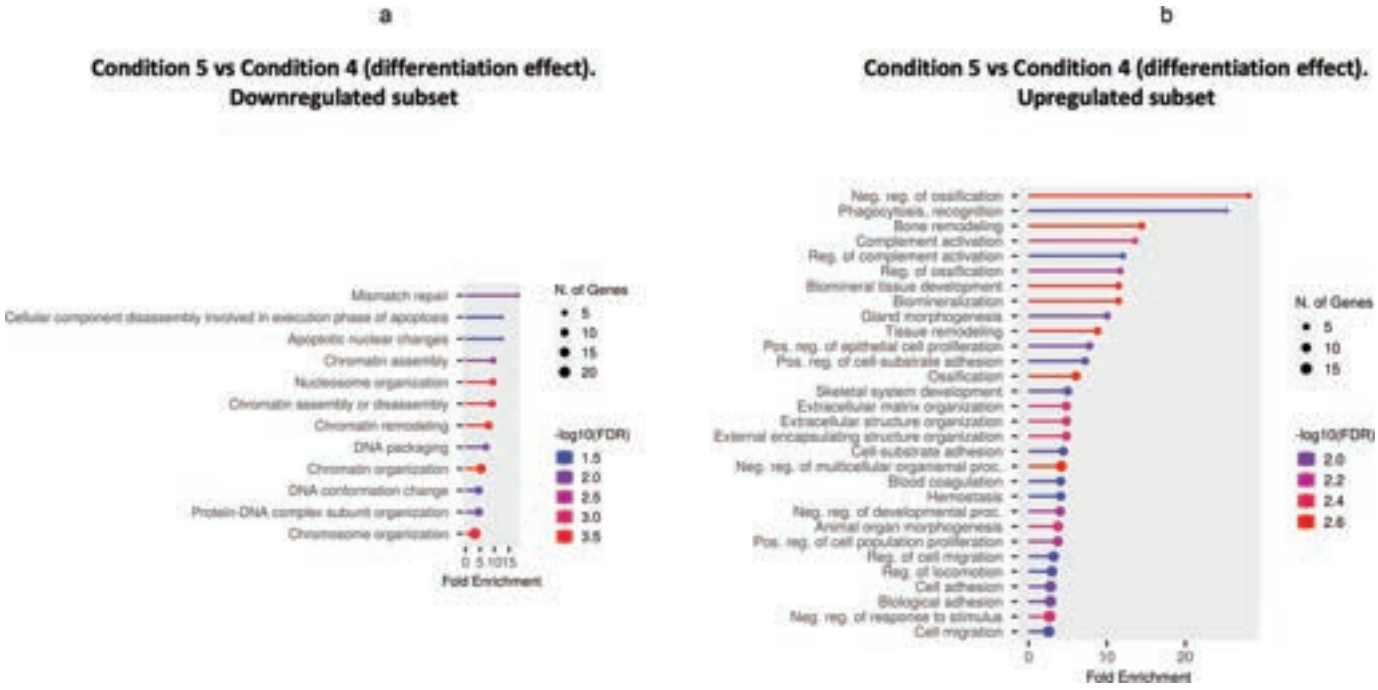

Figure 6. GO analysis of differentially expressed proteins in hPDLSCs grown as triple-layered constructs on differentiation medium. GO annotations of significantly enriched terms among (a) Downregulated and (b) Upregulated proteins are shown in the comparison of condition 5 (in the presence osteogenic inducers) *vs* condition 3 (in the absence of osteogenic inducers). As a background, the total number of proteins identified in each of the comparisons were used.

pointing to differences in the control samples for these effects. However, most categories were shared in both experiments (in green in Table 1), suggesting that the observed impact on the proteome is largely attributable to the osteogenic treatment and not to differences in the incubation times of the control sample in both experiments. Importantly, the common terms were related to ossification, tissue remodeling, morphogenesis, proliferation, or cell migration.

4. Discussion

From a practical perspective, and to accelerate the clinical translation, standardization of the culturing conditions is a milestone. In this study, a stepwise- and proteomic-based protocol has been proposed to examine different culture conditions of increasing complexity, in order to define a standardized methodology for culturing hPDLSCs in a multilayer configuration. Furthermore, this study used PRGF, a specific type of platelet rich plasma with a standardized obtention protocol which confers particular characteristics regarding platelet concentration and cellular composition to it. The growth factors in platelet-rich plasma are mainly coming from three sources: plasma, leukocytes and platelets [3]. For the same patient, the growth factors would vary according to the platelet and leukocyte content in the platelet-rich plasma. In the particular case of PRGF, it excludes leukocytes and has a platelet content 2–3 times higher than the whole blood, reducing the variability in the growth factors and its composition. This has been an echo to the fact that the identity of the raw material is an important factor in cell-base therapy [51]. Following the good manufacturing standard, culture medium supplements like PRGF should not be immunogenic, oncogenic, or carrier of contamination risk [52]. This study confirms that the PRGF does not alter the differentiation capacity of hPDLSCs and thus could be an excellent candidate for a combination with cell-sheet technology in an attempt to improve the properties of the constructs. Furthermore, the fibrin membrane as well as the liquid formulation of PRGF are used to treat different lesions and pathologies in several clinical fields [16,19–21,23,25–27].

Treatment of hPDLSCs with PRGF supernatant had a positive impact on the expression of proteins related to extracellular matrix, adhesion, proliferation or migration, suggesting that this liquid formulation of PRGF used to enrich the cell culture medium could confer an improvement to the culture conditions of periodontal ligament stem cells. This effect was further emphasized in a triple-layer configuration, as we observed an incremental effect on periodontal stem cell function upon implementation of this culture method, arguing for a positive impact of a multi-layered configuration on culture performance. Based on the results of this study, it could be argued that the triple-layer configuration has enhanced the regenerative potential of hPDLSCs by creating a microenvironment that supports cell–cell communication, ECM preservation, and optimal biochemical signaling [30]. The mechanistic advantage of multilayer constructs in improving hPDLSCs lies in their ability to enhance specific biological processes without inducing cellular stress or apoptosis, as demonstrated by the proteomic analysis [36]. Culturing cells as multilayer constructs in the presence of PRGF supernatant led to a substantial increase in differentially expressed proteins compared to single-layered constructs, with about twice as many proteins being upregulated or downregulated. This indicates that the multilayer configuration profoundly impacts the cellular microenvironment and function.

Interestingly, the inclusion of a PRGF fibrin scaffold mediates the upregulated expression of proteins related to cell

Table 1. GO enrichment results of hPDLSCs grown with osteogenic inducers. The GO categories enriched in the two experimental settings (condition 5 (7 days) vs 4 (48 h), column 1; condition 5 (7 days) vs 4 (7 days), column 2) are listed. Shared GO terms between both comparisons are highlighted in green, whereas GO terms exclusively enriched in one of the comparisons are colored in yellow.

A comparison of enriched Gene Ontology terms	
Enriched terms in Condition 5 *vs* Condition 4:	**Enriched terms in Day 7 Differentiation** *vs* Control:
Bone remodeling	Bone remodeling
Reg. of ossification	Reg. of ossification
Biomineral tissue development	Biomineral tissue development
Biomineralization	Biomineralization
Tissue remodeling	Tissue remodeling
Ossification	Ossification
Skeletal system development	Skeletal system development
Neg. reg. of ossification	Reg. of bone mineralization
Neg. reg. of multicellular organismal proc.	Reg. of biomineralization
Animal organ morphogenesis	Reg. of biomineral tissue development
Gland morphogenesis	Bone mineralization
Neg. reg. of developmental proc.	Cartilage development
Pos. reg. of epithelial cell proliferation	Reg. of morphogenesis of a branching structure
Pos. reg. of cell population proliferation	Reg. of morphogenesis of an epithelium
Neg. reg. of response to stimulus	Morphogenesis of a branching structure
External encapsulating structure organization	Reg. of multicellular organismal development
Cell migration	Gland morphogenesis
Reg. of locomotion	Neg. reg. of multicellular organismal proc.
Reg. of cell migration	Animal organ morphogenesis
	Epithelial cell proliferation
	Neg. reg. of response to stimulus
	Reg. of cell migration
Phagocytosis, recognition	Reg. of angiogenesis
Complement activation	Angiogenesis
Reg. of complement activation	Reg. of vasculature development
Blood coagulation	Blood vessel morphogenesis
Hemostasis	Blood vessel development
Extracellular structure organization	Tube morphogenesis
Extracellular matrix organization	Vasculature development
Cell adhesion	Tube development
Biological adhesion	
Pos. reg. of cell-substrate adhesion	
Cell-substrate adhesion	

activation, cellular respiration, and respiratory electron transport chain. It has been reported that mitochondria become activated in a diversity of conditions in response to environmental stimuli [53–56]. In our study, the fibrin membrane also induced mitochondrial activation, consistent with other reports using tissue engineering approaches involving fibrin [57–59], suggesting that fibrin is not solely a scaffold that provides physical support to the cells. Instead, it appears to stimulate the production of energy for biological activities. The material of which membranes for guided tissue regeneration are composed could influence the microenvironment for the proliferation and differentiation of periodontal ligament stem cells. In this sense, a large variety of substrates from different origins has been tested to assess their effect on several cellular processes [60–62]. More physiological scaffolds such as native extracellular matrix composites, have recently attracted much attention in regenerative medicine and tissue engineering [63,64]. However, decellularization techniques involving the use of aggressive treatments must be used to remove cells while preserving the extracellular matrix [65,66].

Our proteomic analysis has revealed that hPDLSCs grown as a multi-layered construct over a fibrin membrane exhibit robust osteogenic potential, as evidenced from the large number of differentially expressed proteins related to ossification, tissue remodeling, morphogenesis, or cell migration that were identified upon induction of osteogenic differentiation. Altogether these results confirm the positive impact of culturing triple-layered hPDLSCs on mPRGF combined with PRGF supernatant on their differentiation potential and overall homeostasis. Interestingly, a number of osteogenesis-related proteins described in the work of Li et al [34] appear already upregulated upon culturing hPDLSCs as multilayer constructs on mPRGF with PRGF supernatant as supplement of the culture medium and without further supply of osteogenesis inducers. This is the case of AKAP13, FST, GJA1, MMP14, SERPINH1, IGFBP3 or HMGA2, among others. From a translational perspective, these findings are highly relevant. The ability to modulate the proteome in a way that supports both homeostasis and differentiation enhances the therapeutic potential of these constructs, offering a promising strategy

for applications in bone regeneration and other complex tissue engineering scenarios. The addition of an osteogenesis-inducing molecules would fully activate this programme, as evidenced by the increased expression of proteins such as AHSG, CTHRC1, GREM1, GREM2, PDFGRA or ALPL that were also described by Li and collaborators [31]. However, considering the possible biological variation between donors, the evaluation of the PRGF impact on hPDLSC from only one donor could be a limitation of this study which warrants further research. Moreover, additional extracellular matrix characterization by transmission electron microscopy is required to enhance our understanding of possible structural variations in it.

Overall, the proteomic alterations observed in this study reflect key mechanisms underlying the regenerative processes facilitated by PRGF-based constructs. The PRGF supernatant would enhance the formation of the cell sheet by enhancing processes of extracellular matrix formation, cell adhesion, cell proliferation or cell migration. Moreover, the use of PRGF fibrin scaffold enhances the cell and mitochondrial activity [53–56]. Furthermore, the fibrin membrane has induced the cell to exhibit robust osteogenic potential. These findings not only validate the use of such constructs for hard tissue engineering but also provide a foundation for optimizing their application in personalized regenerative therapies.

Regarding the clinical translation of the findings of this research, it would be easily guaranteed due to no xenogeneic reagents would be used in the production of triple-layered constructs on mPRGF. The PRGF membrane included in their composition would facilitate their handling as well as provide specific properties that promote the required tissue regeneration. It could be hypothesized that about 600,000 cells would be required for obtaining a triple-layer cell construct (200,000 per well from which one cell layer will be obtained, after reaching the confluence state). Moreover, the versatility of this technology would provide a regenerating structure that could be completely autologous (both the blood for PRGF formulations and the isolated periodontal ligament stem cells would derive from the same patient whose tissue is intended to be regenerated) or allogenic. In addition, the biological properties of PRGF have been tested in healthy patients and also in those with preexisting pathological conditions such as inflammatory or autoimmune diseases, among others [67–70]. With regard to the latter, a simple protocol for inactivating IgE immunoglobulin and reducing the complement activity has recently been described [71], while preserving the physical and mechanical properties of the fibrin membrane after heat inactivation.

Cell Sheet technology offers an interesting alternative to traditional scaffold-based regenerative therapies by addressing key limitations associated with scaffold degradation and biocompatibility [31]. CST has been successfully applied to regenerate various tissues, including the heart, cornea, lung, periodontium, esophagus, and cartilage [31]. This technology enables direct transplantation of cell sheets or the creation of three-dimensional tissue-like structures to repair complex organs. A key advantage of CST is its ability to preserve cell–cell interactions and the extracellular matrix (ECM), critical for maintaining cellular microenvironments and functions [36]. Additionally, CST ensures higher cell survival rates, strong adhesion, better biocompatibility, and enhanced control over tissue structure and immune responses [72–75]. The noninvasive harvesting of cell sheets without proteolytic enzymes further preserves ECM and cell junctions, resulting in high cell densities and improved adhesiveness [29,31,76]. By stacking cell sheets, 3D constructs can be designed to closely mimic physiological tissues, offering a versatile and efficient approach for a wide range of regenerative treatments and biomedical applications [31].

From a future perspective, the application of temperature-responsive polymer-based techniques in the fabrication of cell sheets has been extensively explored in research [73,77,78]; however, significant advancements are still required to enhance their functionality. This study addresses this need by proposing a methodology that closely mimics the physiological conditions of tissue healing, thereby improving cell function, survival, and differentiation toward the regeneration of hard tissues. The plasma-rich growth factor fibrin membrane (mPRGF), prepared from a patient's own blood, simulates the natural process of blood clot formation within a defect. This membrane incorporates all the proteins and biomolecules found in plasma, along with those derived from platelets, embedded within a fibrin scaffold. Such a configuration enables the progressive release of growth factors and biomolecules, effectively creating a "4D construct" that integrates three-dimensional architecture with temporal biomolecule release [2,3,79]. In addition to its biomimetic properties, the PRGF fibrin membrane offers enhanced clinical usability. Its adhesive nature and capacity for suturing provide mechanical stability and facilitate the insertion of cell constructs into defects during surgical procedures [14]. From a functional perspective, the incorporation of mPRGF into cell sheets prevents their shrinkage, stimulates metabolic activity, and promotes extracellular matrix synthesis [36]. At the proteomic level, mPRGF induces the expression of key proteins involved in bone morphogenesis and cellular proliferation while preventing cell death within the construct [36]. Furthermore, PRGF scaffolds exhibit remarkable versatility, as they are compatible with a wide range of cell types, making them suitable for the design of diverse 3D cell constructs. This adaptability positions PRGF scaffolds as a promising tool for regenerating various tissue types. By combining simplicity with efficiency, this methodology has the potential to advance cell sheet technology to a new level, facilitating its clinical application and broadening its therapeutic scope.

5. Conclusions

In view of the results derived from this research, it can be concluded that the use of PRGF formulations offers numerous advantages that improve the cellular behavior in regenerative therapies. Thus, treatment with PRGF supernatant could have a positive impact on the expression of proteins related to extracellular matrix, adhesion, proliferation, or migration. In addition, the inclusion of a PRGF fibrin scaffold could stimulate energy production

for biological activities, as well as provide physical support. In these more complex multilayer structures that include the PRGF fibrin membrane, maintenance in culture with PRGF supernatant would induce the hPDLSC commitment to the osteogenic lineage, and the subsequent addition of osteogenesis inducers would further promote this differentiation program.

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Author contributions

Asier Fullaondo, Mar Zalduendo, Nerea Osinalde, Mohammad H. Alkhraisat, Eduardo Anitua, and Ana M. Zubiaga have made substantial contributions to the conception or design of the work or the acquisition, analysis, or interpretation of data for the work; AND drafting the work or revising it critically for important intellectual content; AND have given final approval of the version to be published; AND agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Asier Fullaondo, Mar Zalduendo, Nerea Osinalde, Mohammad H. Alkhraisat, Eduardo Anitua, and Ana M. Zubiaga authors have read and agreed to the published version of the manuscript.

Disclosure statement

The authors declare the following competing financial interests: Eduardo Anitua is the Scientific Director of and Mar Zalduendo and Mohammad H. Alkhraisat are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology. Asier Fullaondo, Nerea Osinalde and Ana M. Zubiaga declare no conflict of interest. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Ethical conduct of research

This research was approved by the ethical committee of Araba University Hospital (BTI-01-IV/02/20/CST) and was performed following the principles established in the Declaration of Helsinki amended in 2013.

ORCID

Asier Fullaondo http://orcid.org/0000-0001-5387-4762
Mar Zalduendo http://orcid.org/0000-0001-8458-6042
Nerea Osinalde http://orcid.org/0000-0001-8850-7794
Mohammad H. Alkhraisat http://orcid.org/0000-0003-4422-4527
Eduardo Anitua http://orcid.org/0000-0002-8386-5303
Ana M. Zubiaga http://orcid.org/0000-0002-2132-9708

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