TOMAZ ALVES DA SILVA NETO

Insights from *in vitro* and *in vivo* studies evaluating the Protease Activated Receptor 1 (PAR_1) potential pathways and associated osteogenic outcomes

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Corrected Version

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ABSTRACT

da Silva Neto TA. Insights from in vitro and in vivo studies evaluating the Protease Activated Receptor 1 (PAR1) potential pathways and associated osteogenic outcomes [dissertation]. São Paulo: Universidade de São Paulo, Faculdade de Odontologia; 2020. Corrected Version.

Periodontal disease has a high prevalence in the worldwide population and can evolve to a destruction process of periodontal support structures: cementum, alveolar bone and periodontal ligament. Regeneration of damaged tissues and organs is considered to be the ultimate treatment objective and has been sought through biological solutions since the current surgical regenerative treatments present some limitations. Thus, recent efforts in the treatment of periodontal disease have focused on cell-based regenerative approaches and cell sheets derived from human periodontal ligament stem cells (hPDLSCs) have attracted wide attention for its regeneration potential as they are able to allow osteoblastic proliferation and to support mineralized matrix formation. The protease-activated receptor type 1 (PAR1) is a widely known G-protein coupled receptor that plays a crucial role in the initiation and maintenance of inflammation and fracture repair, regulating various aspects of osteogenesis and our group has observed that the activation of PAR1 is able to increase the osteogenic activity in hPDLSCs. However, whether PAR1 shows the same effect on hPDLSCs cell sheets is still unknown. The purpose of this study was to evaluate the effect of PAR1 activation on the osteogenic activity in cellsheets obtained from hPDLSCs in an in vivo ectopic bone formation model and to evaluate the cementogenic gene expression outcome related to PAR1 activation under the blockade of PI3K and MAPK/ERK intracellular pathways in hPDLSCs.

Keywords: Periodontal regeneration. Cell sheets. Protease-activated receptor. PAR1. Periodontal ligament cells. Bone tissue engineering.

RESUMO

Silva Neto TA. Perspectivas sobre estudos *in vitro* e *in vivo* avaliando as vias potenciais e desfechos osteogênicos associados ao receptor ativado por protease do tipo 1 (PAR_1) [dissertação]. São Paulo: Universidade de São Paulo, Faculdade de Odontologia; 2020. Versão Corrigida.

A doença periodontal possui uma alta prevalência na população mundial e pode evoluir para um processo de destruição dos tecidos de suporte do periodonto como cemento, osso alveolar e ligamento periodontal. Sabe-se que o objetivo principal de terapias que buscam a regeneração e o reparo de danos teciduais é promover a formação de um novo tecido/órgão com propriedades anatomofisiológicas semelhantes às do tecido original. Na periodontia, os métodos tradicionais têm apresentado algumas limitações no que se refere à regeneração dos tecidos de suporte periodontais e, desta forma, terapias biológicas têm sido avaliadas como uma possível alternativa. Deste modo, o uso de células mesenquimais do ligamento periodontal (CMLPs) tem apresentado potencial terapêutico regenerativo. Além disso, o uso de membranas celulares (MCs) tem despertado atenção devido as suas propriedades macroestruturais e por permitirem a diferenciação em células cementogênicas e osteogênicas. Entre os receptores que possuem papel crucial em processos regenerativos, sabe-se que o receptor ativado por protease do tipo 1 (PAR1) exerce função regulatória em processos de reparo ósseo, regulando principalmente a atividade osteoblástica. Estudos do nosso grupo tem observado que a atividade osteogênica das CMLPs é aumentada a partir da ativação do PAR1. Entretanto, os mecanismos de ação pelos quais este processo ocorre ainda não foram completamente elucidados e tampouco se sabe se esta atividade osteogênica devido a ativação do PAR1 ocorre em MCs. Desta forma, o objetivo deste estudo foi avaliar o efeito da ativação do PAR_1 na atividade osteogênica em membranas celulares de CMLPs em um modelo de formação óssea ectópica *in vivo* bem como avaliar a expressão de genes relacionado à cementogenese na ativação do PAR1 sob o bloqueio das vias intracelulares PI3K e MAPK/ERK em CMLPs.

Palavras-chave: Regeneração periodontal. Cellsheets. Receptor ativado por protease. PAR1. Células do ligamento periodontal. Engenharia de tecido ósseo.

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LIST OF ABBREVIATIONS, ACRONYMS, INITIALS, AND SYMBOLS

ABC	Avidin-biotin complex
ARS	Alizarin Red Staining
BSA	Bovine Serum Albumin
BV/TV	Bone Volume/Tissue Volume
CCD	Charge-coupled Device
CEMP1	Cementum Protein 1
DAB	Diaminobenzidine
DAPI	4′,6-Diamidino-2-Phenylindole
ERK	Extracellular Signal-regulated Kinases
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GBR	Guided Bone Regeneration
GTR	Guided Tissue Regeneration
H&E	Hematoxylin & Eosin
hPDLSCs	human Periodontal Ligament Stem Cells
IgG	Immunoglobulin G
kV	Kilovolts
LPS	Lipopolysaccharide
MAPK	Mitogen-activated Protein Kinases
MicroCT	Micro-computed Tomography
Oct-4	Octamer-binding transcription factor 4
OPG	Osteoprotegerin

PAR1	Protease-activated receptor 1
PBS	Phosphate Saline Buffer
PE	Phycoerythrin
PI3K	Phosphatidylinositol-3-kinase
RT	Room Temperature
RT-Qpcr	Quantitative Real-Time Reverse Transcription PCR
Runx2	Runt-Related Transcription Factor 2
Sox-2	Sex Determining Region Y-Box2
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor-α
WB	Western Blotting
α-MEM	α-Minimum Essential Medium
µA	Micro Ampere
µm	Micrometer

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1 INTRODUCTION

Periodontal disease is a chronic inflammatory biofilm-induced condition highly mediated by host response patterns, which in advanced stages may result in a substantial breakdown of the tooth-supporting tissues, ultimately leading to tooth loss. Periodontal tissue is a specialized distinctive tissue structure composed by alveolar bone, cementum and periodontal ligament. Damage to these structures may severely compromise important biological functions, including bone remodeling, occlusal forces absorption and ultimately the masticatory function. (1)

Current both non-surgical and surgical treatments such as guided tissue regeneration (GTR) and guided bone regeneration (GBR) have been widely utilized in clinical practice in the aim to eliminate the infection's primary cause and provide regeneration, respectively. However, these treatment strategies have been shown to be ineffective in providing complete regeneration of hard and soft periodontal structures and the most common outcome is the formation of long junctional epithelium or the incomplete regeneration of the periodontal structures, and a long-term stable clinical regenerative outcome has not been achieved. (2–4)

Recently, several studies in the cell-based regenerative medicine field have been reported combining tissue engineering and stem cells. (5,6) The use of cell sheets is a promising therapeutic strategy that allows the use of mesenchymal stem cells in a construct that maintains interconnected cells in contact with their extracellular matrix, providing more stability and allowing delivery to possible therapies. (7) Cellsheets utilizing hPDLSCs are well known to mimetize the natural periodontal ligament environment in a regenerative state and to enhance cellular signal communications that potentially stimulate regeneration of periodontal tissues. (3)

The protease-activated receptor type 1 (PAR1) is a G protein-coupled cell membrane receptor that regulates several intracellular signaling pathways related to capable of enhancing osteogenic differentiation and an increase in mineralized matrix deposition. PAR1 activation plays a key role in osteogenesis and bone regeneration (5,8) by the enhance of osteoblast proliferation and differentiation, increase the

synthesis of osteoprotegerin (OPG) in periodontal ligament cells and upregulate and reducing the inflammatory osteoclastogenesis induced by LPS. (9) Furthermore, PAR1 has shown to be associated with bone formation and osteoclasts differentiation impairment at early bone healing early stages in an animal model. (10,11)

Our previous studies showed that PAR1 activation increased osteogenic activity of hPDLSCs demonstrated by an increase in mineralized matrix deposition and osteogenic differentiation. (12,13) However, whether PAR1 shows the same effect on hPDLSCs in a cellsheet in vivo model is still unknown. Here, our study focused on investigating if the hPDLSCs cell sheet osteogenic outcomes are related to PAR1 in an in vivo model.

2 PROPOSITION

i. To evaluate the osteogenic outcomes related to the protease-activated receptor type 1 activation on human periodontal ligament stem cell sheets in an *in vivo* model.

ii. To evaluate whether the cementum protein 1 expression related to the protease-activated receptor type 1 activation on human periodontal ligament stem cells is influenced by the MAPK/ERK and PI3K pathways blockade.

3 CHAPTER I: Protease-activated receptor 1 (PAR1) enhances osteogenesis in periodontal ligament stem cellsheets.

ABSTRACT

Background: Recent studies have revealed the potential use of human periodontal ligament stem cell sheets as a novel therapeutic strategy to possibly achieve tissue regeneration. Protease-activated receptor (PAR_1) is a G-protein-coupled receptor that plays a key role in osteogenesis and although some recent studies have shown that its activation increases bone formation in human periodontal ligament stem cells (hPDLSCs), little is known about its effects in a cell sheet model with hPDLSCs.

Objective: To evaluate the osteogenic effects of PAR_1 activation in hPDLSCs cell sheets using an ectopic bone formation model in Balb c/nude mice.

Methods: hPDLSCs were obtained from 3 different patients using the explant technique. Groups were divided into control medium (clonogenic), osteogenic medium and osteogenic medium plus PAR_1 activation by agonist peptide. Cell phenotype was determined by flow cytometry and immunofluorescence. Calcium deposits formation was quantified by Alizarin Red Staining. Cell sheet microstructure was analyzed through light microscopy, scanning electron microscopy and histology. Immunohistochemistry (bone sialoprotein, integrin β1 and collagen type 1) and histological stains (H&E, Van Giesson and Masson's Trichrome) were performed after 2-months implantation *in vivo.* Ectopic bone formation was evaluated using computed microtomography.

Results: PAR_1 activation increased calcium nodules deposition and enhanced pro-osteogenic bone sialoprotein, collagen type 1 and integrin β1 antibodies expression. Furthermore, PAR_1 was found to be associated with increased bone formation.

Conclusion: PAR_1 activation enhanced ectopic bone formation in a cell sheet of hPDLSCs, indicating the potential of this receptor in cell engineering to be applied in periodontal tissue regeneration.

Keywords: Stem cells; PAR1; Innate immunity.

**Article to be submitted to the Journal of Dental Research in co-authorship with Marinella Holzhausen (Advisor).*

3.1 Introduction

Periodontal disease is a chronic inflammatory biofilm-induced condition highly mediated by host response patterns, which in advanced stages may result in a substantial breakdown of the tooth-supporting tissues, ultimately leading to tooth loss. Periodontal tissue is a specialized distinctive tissue structure composed by alveolar bone, cementum and periodontal ligament. Damage to these structures may severely compromise important biological functions, including bone remodeling, occlusal forces absorption and ultimately the masticatory function. (1)

Current both non-surgical and surgical treatments such as guided tissue regeneration (GTR) have been widely utilized in clinical practice in the aim to firstly eliminate the infection's primary cause and secondly provide regeneration. However, these treatment strategies have been shown to be ineffective in providing complete regeneration of hard and soft periodontal structures and the most common outcome is the formation of long junctional epithelium or the incomplete regeneration of the peri odontal structures, and a long-term stable clinical regenerative outcome has not been achieved. (2–4)

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associated with bone formation and osteoclasts differentiation impairment at early bone healing early stages in an animal model. (10)

Our previous studies showed that PAR_1 activation increased osteogenic activity of hPDLSCs demonstrated by an increase in mineralized matrix deposition and osteogenic differentiation. (11) However, whether PAR_1 shows the same effect on hPDLSCs in a cellsheet in vivo model is still unknown. Here, our study focused on investigating if the hPDLSCs cell sheet osteogenic outcomes are related to PAR_1 in an in vivo model.

3.2 Material and Methods

3.2.1 Ethics Statement

Informed consent and ethical committee approval number 98/2018 were obtained prior to the patient's teeth collection at the clinic of the School of Dentistry of the University of São Paulo (FO-USP). The utilization of balb/c nude mice in this research was also approved by the Ethics Committee (CEUA) of the Chemistry Institute (IQ-USP) under the protocol # 98/2018.

3.2.2 Cells isolation

Periodontal ligament stem cells were harvested from third molars from systemically healthy individuals (n=3, aged from 18 to 30 years). The inclusion criteria were: partially or totally erupted third molars and absence of periodontal disease.

Periodontal ligament tissue specimens were obtained through the scaling of the middle third of the root and hPDLSCs were isolated using the explant technique (12) in alpha-modified Eagle’s medium (α-MEM) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 0.5 mg/mL amphotericin B (Gibco, Invitrogen, Carlsbad, CA, USA) at 37 °C in an atmosphere of 5% CO2 and 95% umidity. (15) After 14

days, cells from the explants achieved a 70% confluence degree and hPDLSCs populations were used in passage 4 for all experiments.

3.2.3 Pluripotency characterization

In order to identify the mesenchymal stem cell phenotype, approximately $5x10^5$ hPDLSCs were incubated in 5% BSA/PBS 1x at 4 °C in dark for 1 hour with the following monoclonal antibodies: PAR1-FITC, OCT4-FITC, SOX2-FITC, STRO-1-FITC (Abcam, Cambridge, UK) CD14-FITC, CD90-FITC (eBioscience, SanDiego, CA), CD34-FITC (Biolegend, USA), CD31-PE, CD-44-PE, CD45-PE (eBioscience, SanDiego, CA) and CD146-PE (Biolegend, USA) for 30 minutes at 4ºC. Unstained control was used to set gates. A total of 10-50,000 events were recorded and data analyzed by FlowJo (Becton Dickinson, Brazil).

3.2.4 Cell sheet culture and experimental design

hPDLSCs at 1×10^6 cells/cm2 were seeded in 100mm plates for 24hrs with α-MEM supplemented with 10% of fetal bovine serum and 50 μg/mL vitamin C to induce cell sheet formation, as previously described. Subsequently, cell monolayers were assigned for one of the following experimental groups: (1) control medium (CTRL) composed of α-MEM supplemented with 10% of fetal bovine serum and 50 μg/mL vitamin C; (2) osteogenic medium (OST) (CTRL + 100nM dexamethasone, 5mM b-glycerophosphate and 50mg/ml ascorbic acid; Sigma, St Louis, MO, USA); and (3) osteogenic medium with the addition the PAR1 agonist peptide (100 nM TFLLR-NH2; Tocris Bioscience Inc., Bristol, UK) (OST+PAR1). 22 The culture medium was changed every 3 days for 14 days.

3.2.5 Alizarin red staining

The hPDLSCs from P2 were seeded in 6-well plates (5 x 10^4 cells/cm^2), in triplicate. After treatment with CTRL, OST and OST+PAR1 groups for 2, 7 and 14 days, cell sheets were washed with 1x PBS, fixed with 4% paraformaldehyde for 15 min at room temperature (RT), washed with 1x PBS again and incubated with a stirring solution of 2% Red Alizarin (pH 4.2) (A5533, Sigma-Aldrich) for 30 min at RT. For qualitative and macroscopic analysis, images were acquired using a microscope CDD camera (D7000, Nikon, Minato, Japan). For the quantitative analysis, we used a 10% ammonium hydroxide solution to dilute calcium deposits and a spectrophotometer was used to measure the absorbance at 405 nm. ARS quantification was calculated using a standard-curve and data were analyzed by using GraphPad Prism software.

3.2.6 Cell sheet characterization

3.2.6.1 Scanning electron microscopy

Cell sheets at 14-days treatment were also fixed with Karnovsky's solution (Thermo Fisher Scientific, Massachusetts, USA) for 24 h. After this period, post-fixation was performed with osmium tetroxide for 2 hours and dehydration, passing the material through an increasing series of alcohol (from 70 to 100%). The samples were further dried under critical point conditions and sputter coated with gold. Observations were then performed by using a scanning electron microscope (SEM; S-4800; Hitachi, Ltd., Tokyo, Japan).

3.2.6.2 Histological profile

Hematoxylin and eosin (H&E) staining of cell sheets was also performed. Briefly, after detachment and fixation in 4% paraformaldehyde for 24 hours, samples were paraffin embedded and sectioned in 4 µm thickness. After the sectioning process, the histological samples were placed in a water bath at 50 ° C and captured to a histological slide and stored in an oven at 65 °C for 12 hours to allow the paraffin remnants to be removed. Sequentially, paraffin removal dehydration protocol was completed using a sequence of xylol and alcohol solutions and the specimens proceeded a routine H&E staining following the manufacturer's guidelines.

3.2.7 Ectopic bone formation assays

In vivo bone formation potential was evaluated using cell sheets pre-incubated with the experimental groups described above for 14-days. Then, cells were detached using a cell scraper (Corning, New York, USA), folded 4 times to acquire a cylinder shape and transplanted to the dorsal region of 6-weeks old mice. After 60 days, mice were euthanized, subcutaneous samples were removed and processed for the analyzes described below.

3.2.8 Computadorized microtomography

After fixation, subcutaneous samples were scanned using a Skyscan 1176 MicroCT System (Bruker Biospin, Billerica, Massachusetts, EUA) with the following acquisition parameters: 45 kV, 8.71 µm resolution and 550 µA. Data obtained were processed, reconstructed into three-dimensional images using NRecon Reconstruction Software (Micro Photonics, Allentown, Pennsylvania, USA) and analyzed using CTAn (Bruker Biospin, Billerica, Massachusetts, EUA) system software. For all reconstructed images, a region of

interest was delimited, a threshold was established individually for all samples in order to avoid artifacts in the final processing.

3.2.9 Immunohistochemistry and histological analysis

Histological procedures were performed as described previously for HE. Masson Trichrome and Van Gieson's stains were carried out following the manufacturer protocol. For immunohistochemistry analysis, after fixation in a 4% paraformaldehyde solution for 30min, the samples were embedded in paraffin and sectioned at 20μm. The cuts were subjected to dewaxing, rehydration, exposure to the specific antibodies and then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, MO, USA) for 10 min. Then, the sections were blocked in 3% hydrogen peroxide solution for 10 min followed by antigen retrieval with 10% citrate buffer for 15 minutes and blockage in 5% BSA (Sigma-Aldrich, MO, USA) for another 30 min. The primary antibodies used for staining included: β1 integrin (Abcam, AB52971), type I collagen (Abcam, AB34710), and bone sialoprotein (Abcam, AB8448). The slides were then incubated with a secondary goat anti-rabbit antibody (IgG, HRP-ABcam, AB205718) followed by a avidin-biotin complex (ABC) incubation for 30min. The diaminobenzidine (DAB) solution was applied every 2 min and the reaction was observed in a microscope. Specimens were dehydrated, cleared and mounted. Images from the specimens were obtained with a light microscope (E600, Nikon, Shinagawa, Tokyo, Japan) and three images of each sample were acquired and quantified using Image J.

3.2.10 Statistical analysis

Data were expressed as mean ± standard deviation (SD) from comparing three different groups. Experiments were performed in triplicate and One-way ANOVA with post-

hoc Tukey's test was used. Data were analyzed based on the comparison between the experimental group (OST+PAR1) with the osteogenic control group (OST) and the negative control (CTRL). A significance *P value* of 0.05 was established for all tests and the data analysis was performed using the GraphPad Software (GraphPad Prism™ Version 6.0c, La Jolla, CA, USA).

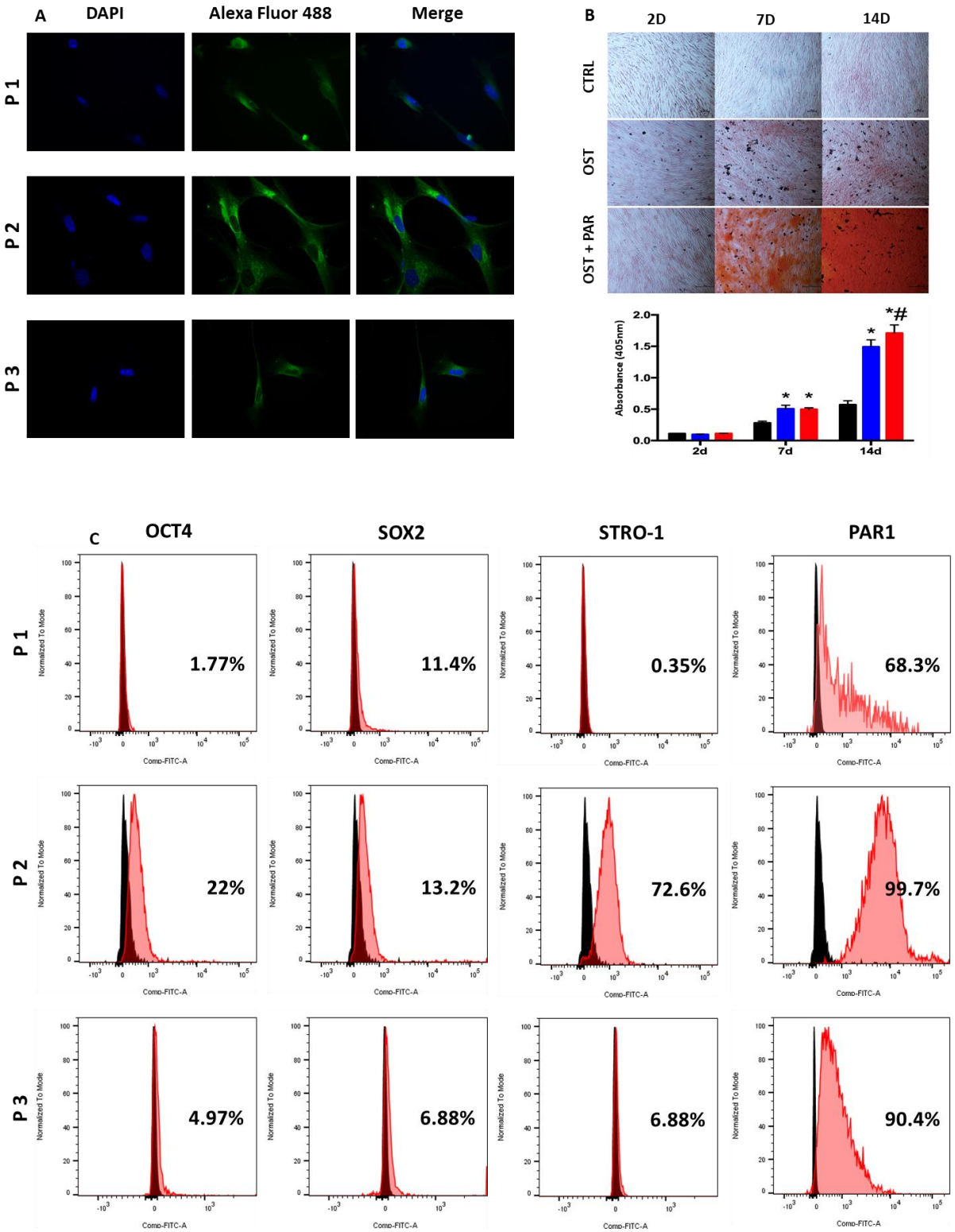
3.3 Results

3.3.1 hPDLSCs characterization and osteogenic potential of PAR_1

We isolated hPDLSCs from three different individuals (P1, P2 and P3) and characterized the cell lines by flow cytometry and immunofluorescence for PAR_1. Although all cell lines were positive for pluripotency surface markers (Figure 3.1C), only P2 presented a unique positive phenotypic profile for OCT-4 (22%), SOX2 (15.2%), and STRO-1 (72.6%) in agreement with the literature to be considered an hPDLSC lineage. (16) Still, only P2 presented a high percentage of $PAR1^+$ hPDLSCs (~100%), as observed by Flow Cytometry and Immunofluorescence (Figure 3.1C and 3.1A).

Further,ARS was carried out using P2 isolated cell line only to evaluate whether the PAR_1 activation played a role in their osteogenic differentiation. In agreement with our findings, PAR_1 significantly promoted an increase in calcium deposits through ARS assay only at P2 hPDLSCs (Figure 3.1B). This way, only P2 hPDLSCs were used in further experiments.

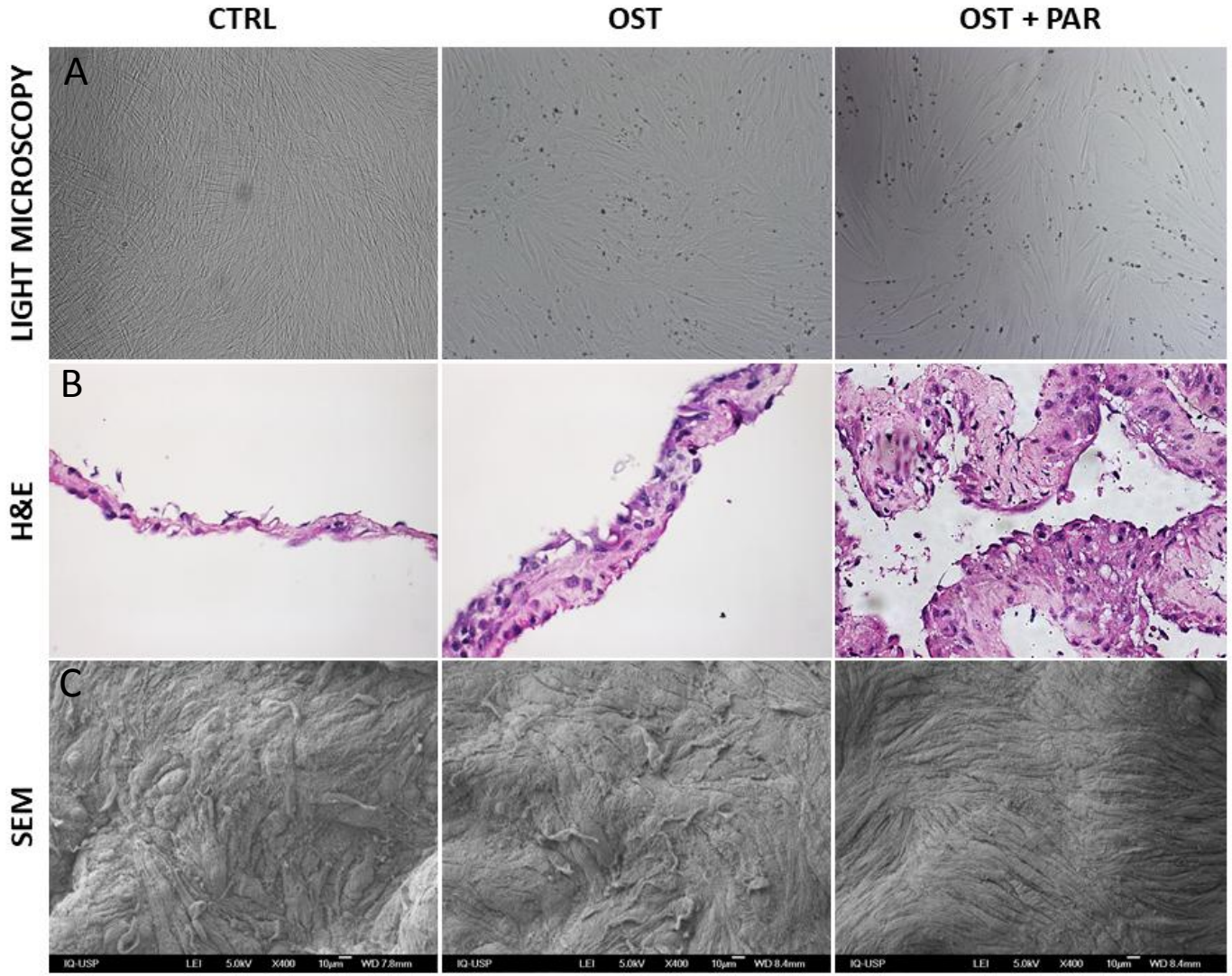
Figure 3.1 – Immunofluorescence, alizarin red staining and flow cytometry. PAR_1 immunofluorescence representative expression in hPDLSCs in relation to DAPI. Images in 60X magnification (**A**), Alizarin red staining for hPDLSCs sheets (P2) in 2, 7 and 14 days (**B**), Flow cytometry analysis showing the percentage of hPDLSCs for each surface stemness and pluripotent embryonic biomarkers (SOX2, OCT4, STRO-1) after 24h of stimuli with mediators. Unstained cells were used to set positive cell populations, $p<0.05$. Results are given as the mean ± SEM (**C**)



3.3.2 Cell sheets characterization

We characterized hPDLSCs sheets by SEM, H&E and direct light microscopy images. After 14 days of culture with the three different groups, hPDLSC sheets were fully formed and able to complete detachment from the dishes. Light microscopy images showed a fully confluent fibroblastic-like profile as expected (Figure 3.2). In addition, images from OST and OST+PAR1 groups, but no CRTL showed the formation of calcium nodules, as also expected. Furthermore, a qualitative analysis of the H&E sections detected thickness degree differences in the OST and OST+PAR1 groups when compared to the CTRL group (Figure 3.2). Finally, OST+PAR1 group showed a more organized and dense fibers network as well as tightness junctions between cells at 400x augmentation when compared to the OST and CTRL groups as observed in SEM images (Figure 3.2).

Figure 3.2 - Morphological cellsheets characterization. (A) Light microscope cellsheets representative images for the 3 groups (40x). (B) Cellsheet histological profile by H&E staining. (C) Microstructural analysis through scanning electron microscopy (400x)



3.3.3 PAR_1 activation in hPDLSCs cell sheets enhanced mineralization after *in vivo* transplantation

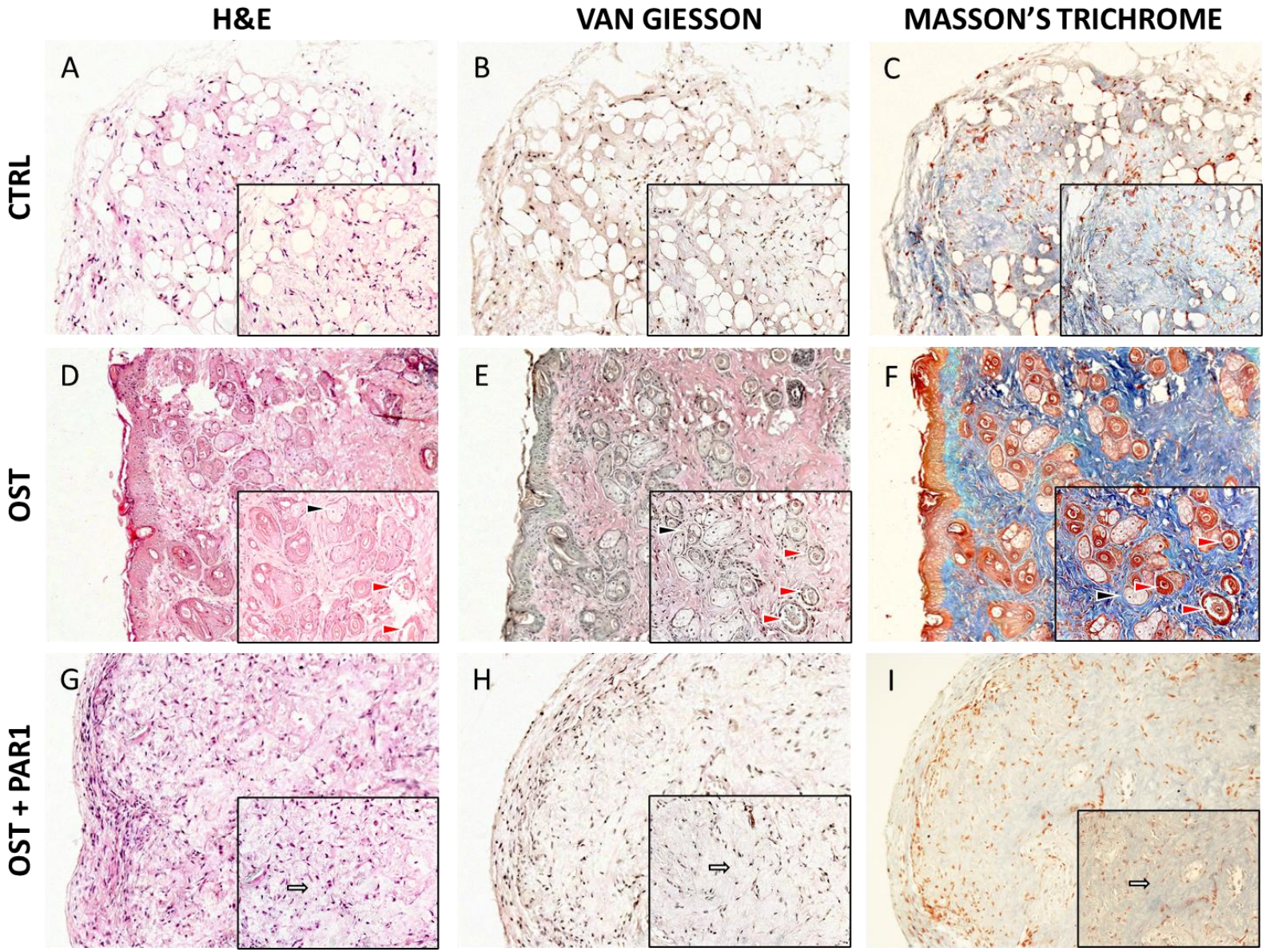
In order to evaluate the osteogenic translational potential of PAR_1 activation in hPDLSCs sheets, we performed *in vivo* experiments with cellsheets subcutaneous transplantation and evaluated the ectopic bone formation through microCT after 2-months. MicroCT results were displayed in bone volume/tissue volume percentage (BV/TV %). The OST+PAR1 group demonstrated a higher ectopic bone formation outcome in comparison with the other groups ($p < 0.05$) (Figure 3.3 C).

Figure 3.3 - Microcomputed tomography (microCT) analysis of ectopic bone formation in vivo after 60 days of implantation (Figure 3A and 3B) and the statistical analysis comparing the BV/TV% between the 3 groups CTRL, OST and OST+PAR1 (Figure 3C). Results showed a higher ectopic bone formation outcome for the group OST+PAR1 in comparison with the other groups. (*), p<0.05 versus control. Results are given as the mean ± SD

3.3.4 Histological findings evidenced an accelerated mineralization process mediated by PAR_1 activation

Histological qualitative analysis from H&E, Masson Trichrome and Van Gieson's stains from the 3 groups after a 2 month transplantation period in nude mice evidenced an acceleration in the osteogenesis process displayed by a greater deposition of neoformed bone matrix, higher prevalence of osteoblast clusters and lower abundance of undifferentiated fibroblast-like cells for the OST+PAR1 group when compared to the OST and CTRL groups (Figure 3.4). Furthermore, a greater neovascularization degree was found in the OST group when compared to the other groups, suggesting that this group was in an intermediate osteogenesis stage. (Figure 3.4)

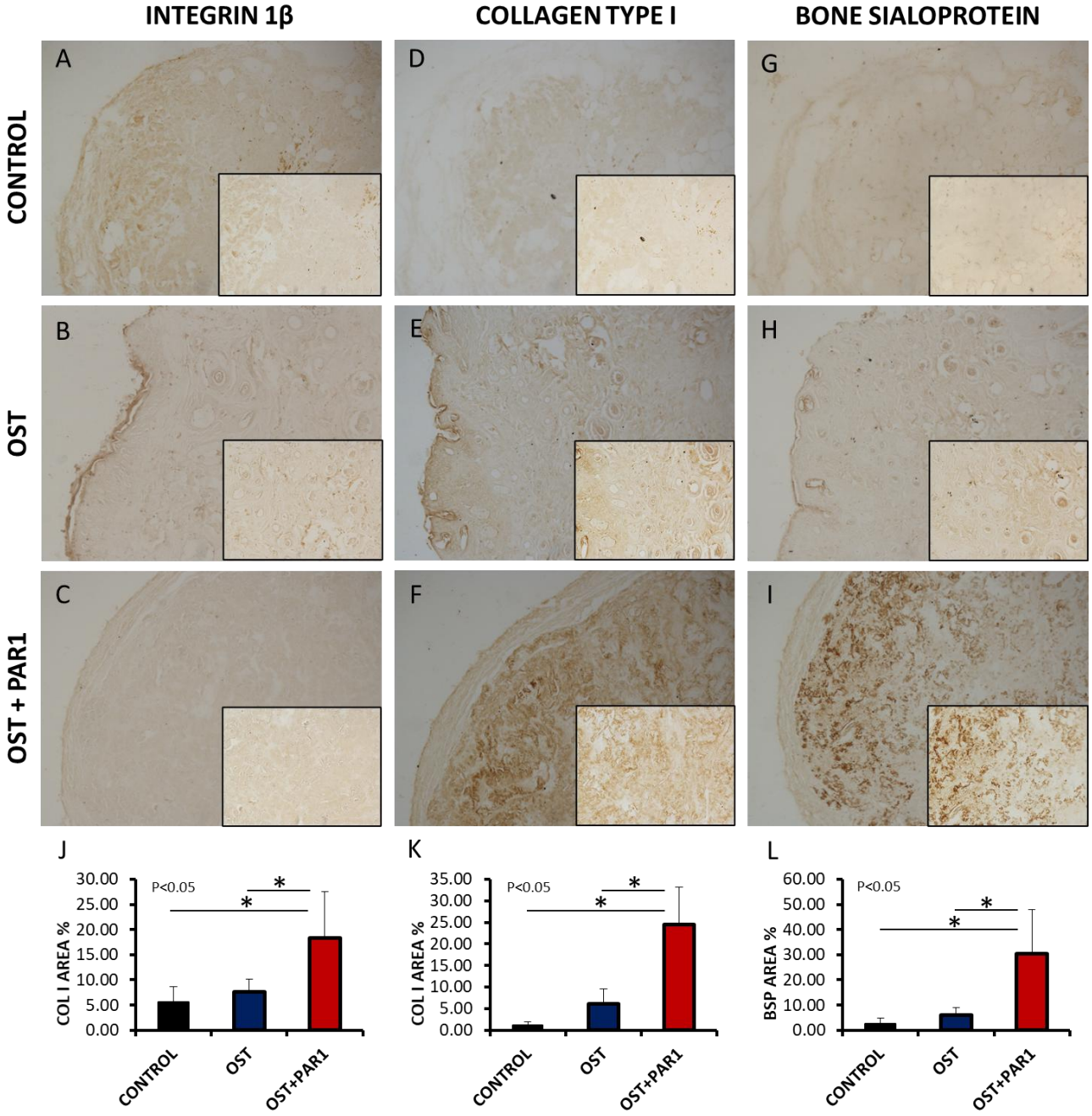
Figure 3.4 - Histological qualitative analysis from H&E, Masson Trichrome and Van Gieson's stains from the 3 groups after a 2 month transplantation period in nude mice. Images at 4x and 20x (small squares). CTRL group displayed connective tissue characteristics with no bone formation detected for the 3 stains **(A, B and C)**. Osteoblast clusters (black solid arrows) and blood vessels (red solid arrows) were detected in higher abundance in the group OST (**D, E and F)** when compared to the other groups. The OST + PAR group showed osteogenesis in a later stage compared to the other groups with bone neoformed matrix with osteocytes (white arrows) presence



3.3.5 PAR_1-induced mineralization in vivo was followed by an upregulation of osteogenic markers

Immunohistochemistry results presented a higher positive expression of bone sialoprotein in the OST+PAR1 group compared to the other groups,. Furthermore, the expression of integrin 1β and collagen type 1 was detected in higher levels at the OST+PAR1 group when compared to the other groups. (Figure 4) MicroCT results were displayed in bone volume/tissue volume percentage (BV/TV %). The OST+PAR1 group demonstrated a higher ectopic bone formation outcome in comparison with the other groups (Figure 3.5).

Figure 3.5 - Histological Immunohistochemistry quantification analysis for the collagen type I, integrin 1β and bone sialoprotein from the 3 groups after a 2 month transplantation period in nude mice. Images at 4x and 20x (small squares). (*) p<0.05. Results are given as the mean ± SD (J, K and L)



3.3 Discussion

In this study we investigated the osteogenic potential of the PAR_1 activation by using a cell sheet engineering strategy in an *in vivo* model. Previous studies have reported the role of PAR1 in the osteogenic processes demonstrating that its absence is associated with derived bone marrow cells migration impairment, an increase in osteoclast colonization, enhanced osteoclastogenesis and a decrease in mineralized bone deposition *in vivo*. (Song et al. 2005; Jastrzebski et al. 2019)

On the other hand, PAR_1 activation has been largely reported to upregulate bone formation by mediating proliferative responses in osteoblasts. (Song et al. 2005; Mackie et al. 2008; Sato et al. 2016) Our group has previously demonstrated that PAR1 activation in hPDLSCs is specifically associated with an increase in mineralized nodule deposition, higher calcium concentration levels and increased hPDLSC proliferation. In addition, PAR1 blockage decreased the calcium deposition, suggesting that PAR1 plays a major role in mineralization and differentiation of hPDLSCs. (Rovai et al. 2019)

In the present study, we isolated hPDLSCs from three patients, characterized the surface marker phenotypes by flow cytometry and purposefully proceeded to evaluate the osteogenic differentiation potential and calcium deposition between the cell lines. The non-specialized mesenchymal stem cells found in the periodontal ligament tissue such as hPLSCs present pluripotency properties that allow differentiation into several cell types, including osteoblasts and cementoblasts. These cells exhibit a phenotype that is compatible with the positive expression of CD-146, OCT4, STRO-1, CD-44 and CD-146 cell markers. (Chen et al. 2006; Nagatomo et al. 2006; Gay et al. 2007; Fujii et al. 2008; Xu et al. 2009) Further, cells with positive expression of STRO-1 were found to be related with periodontal tissue formation and expressed in dental follicle cells in mice. (P. Kémoun et al. 2007; Philippe Kémoun et al. 2007)

Our results demonstrated a strong correlation between the presence of stemness markers (OCT4, CD146, SOX2, CD-90, STRO-1, CD-44) and PAR1 prevalence with a higher calcium deposition found in P2 in the ARS essay. At the same time, P2 was negative for CD14, CD31, and CD34, corroborating with hPDLSCs phenotype present in the literature. (Choi et al. 2015)

Vascularization is a key mechanism in the earliest stages of osteogenesis during ectopic bone formation (Gerber and Ferrara 2000; Bragdon et al. 2017) and angiogenesis is pivotal to enable osteogenesis *in vivo*. (Amini et al. 2012) The absence of vascularization leads to tissue necrosis through hypoxia and nutrients dearth (Phelps and García 2010; Novosel et al. 2011; L et al. 2014). Cellsheets present improved vessel formation capability because the space between the cell layers allows for the formation of capillaries and therefore vascularization in new transplanted grafts. (Sakaguchi et al. 2015)

In this study, we could identify qualitative histological differences between the groups regarding angiogenesis. The CTRL group exhibited necrosis spots and a lack of vessel formation when compared with the OST and OST + PAR1. These findings could be explained through the absence of ascorbic acid in the CTRL group, in which depletion is related to angiogenesis impairment by the inhibition of mature type IV collagen formation. (Telang et al. 2007) Further, the higher histological vascularization found in the OST group can be explained due to the fact that the group was at an earlier stage of osteogenesis when compared to the OST + PAR1 group, suggesting that the osteogenesis acceleration mechanism mediated by PAR1 activation also occurs through an increase in angiogenesis. These findings corroborate with several studies that demonstrated the pro-angiogenic role of PAR1 activation mainly through VEGF increased expression. (Yin et al. 2003; Ma et al. 2005; Hu et al. 2019)

In fact, the enhanced osteogenic profile found in the OST + PAR1 group on the histological findings were consistent with the immunohistochemistry and microCT results in our study. Evaluation of bone volume percentage (BV/TV %) through microCT displayed significantly higher values in the OST + PAR1 group when compared to the OST and CTRL groups.

In the bone neoformation processes, several extracellular molecules produced by differentiating osteogenic cells can be used to identify the stages of bone maturation *in vivo.* (Lekic et al. 1996) Integrin 1β functions as a modulator, facilitating osteoblast attachment, spreading, adhesion, migration, and differentiation in osteogenesis via specific alpha(v)beta(3) signaling pathway (Bellahcène et al. 2000; Campbell and Humphries 2011) and is capable of recognizing ligands with RGD-motifs, like BSP. BSP is an osteoblast differentiation marker expressed during initial stages of bone formation (Harris et al. 2000; Marom et al. 2005) and is also capable of promoting angiogenesis by mediating human endothelial cell attachment and migration. (Bellahcène et al. 2000) In this study, the

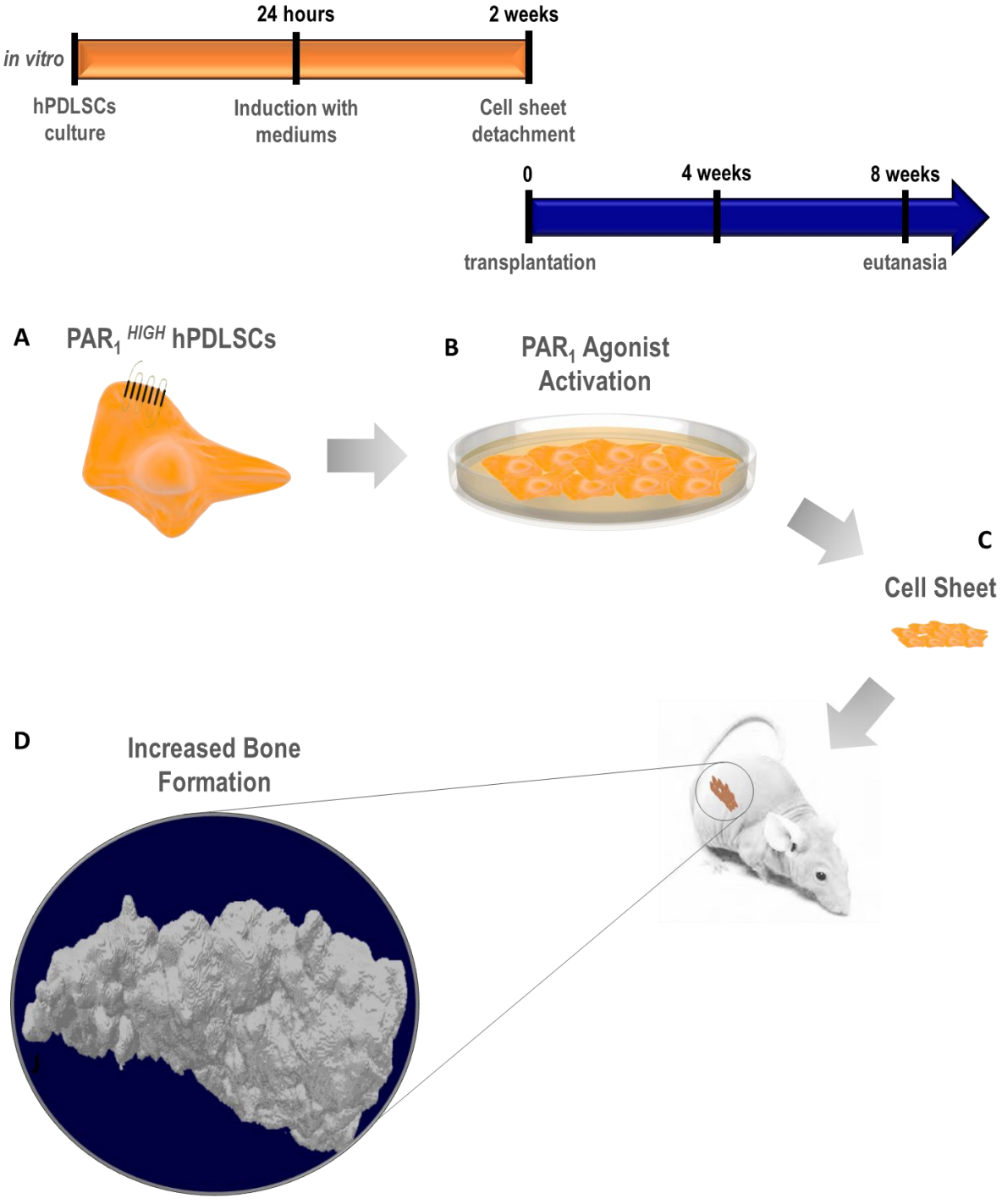
immunohistochemistry assay results for the OST + PAR1 group showed a higher expression for collagen type I, integrin 1β and BSP comparing with the other groups, evidencing an upregulated expression of important osteogenic immunological markers mediated by the PAR1 activation.

Some limitations are present in this *in vitro* and *in vivo* study. First, the research included only one donor cell line for the main experiments, which substantially reduces the external validity of the *in vitro* findings. Secondly, we found great difficulty in isolating mesenchymal strains from patients and a great difference in the surface markers characterization was found among the isolated cells. Moreover, the PAR1 expression was found to be widely variable, limiting the receptor activation associated outcomes for further PAR1 targeting therapies. On the other hand, the use of cellsheets obtained through the removal of third molars seems to be a feasible option for further clinical therapies, since cellsheets present no associated adverse reactions because they can be obtained and implanted in the same donor. Moreover, cellsheets are feasible to be used in regeneration procedures because the preservation of the extracellular matrix provides good structural stability and manageability, allowing this tissue engineering strategy to have a promising future as an option in regenerative treatments.

3.4 Conclusion

The main finding of this study is that the PAR_1 activation in hPDLSCs cellsheets enhanced osteogenesis *in vivo* (Figure 3.6). In addition, *in vitro* results from ARS reinforced that the presence of cell phenotype for stemness and PAR_1 surface biomarkers are associated with an upregulation of osteogenesis in hPDLSCs.

Figure 3.6 - Graphical abstract evidencing the process of obtaining and screening mesenchymal strains with a high prevalence of PAR1 on their cell surface **(A)**, cellsheets harvesting using osteogenic induction and PAR1 activation **(B)**, in vivo transplantation process **(C)** and increased ectopic bone formation in vivo **(D)**



Acknowledgment

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4 CHAPTER II: Protease-activated receptor increases CEMP1 gene expression through MAPK/ERK pathway.

ABSTRACT

Background: PAR_1 is a G-coupled protein receptor that regulates several cellular metabolism processes, including differentiation and proliferation of osteogenic and cementogenic related cells. Our group has demonstrated in previous studies the regenerative potential of human periodontal ligament stem cells (hPDLSCs) *in vitro*. The better understanding of the relationship between intracellular signaling pathways underlying PAR_1 activation and the genes expression related to these processes is crucial.

Objective: The present study aimed to verify the PAR_1 activation affected the Cementum Protein 1 gene expression and to investigate the potential correlation between the MAPK/ERK and PI3K pathways blockage with this expression in hPDLSCs.

Materials and Methods: hPDLSCs were obtained from 3 different donors using the explant technique. The treatments were divided in osteogenic control (OST) (α-MEM, 15% fetal bovine serum, L-glutamine, penicillin, streptomycin, amphotericin B, dexamethasone and beta-glycerophosphate) and osteogenic medium (OST) (CTRL + dexamethasone and beta-glycerophosphate). In addition, the hPDLSCs were treated according to the specific activation of PAR_1 (PAR_1 agonist) and blockage of the MAPK/ERK pathway at 2 and 7 days. The gene expression of CEMP1 was assessed by qPCR.

Results: Overall, the activation of PAR_1 by the agonist peptide after blockage of the MAPK/ERK pathway resulted in a downregulation of CEMP1 gene expression at 2 and 7 days, whereas hPDLSCs without the blockade of this pathway presented an increased gene expression for CEMP1 when compared to the OST group ($P<0.05$). For the PI3K experiment, blockade of this pathway prior to PAR_1 activation did not alter the gene expression of CEMP1 in any of the experimental times when compared to the osteogenic control ($P<0.05$).

Conclusion: The CEMP1 gene expression through the PAR_1 activation seems to be highly mediated by the MAPK/ERK pathway in hPDLSCs.

Key words: Stem cell; PAR1; CEMP1; ERK; MAPK; PI3K.

**Article to be submitted to the Journal of Periodontal Research in co-authorship with Marinella Holzhausen (Advisor), Emanuel da Silva Rovai and Alpdogan Kantarci (The Forsyth Institute).*

4.1 Introduction

The ultimate goal for periodontal therapy is to achieve complete regeneration of lost periodontal tissues. However, despite the effectiveness of periodontal treatment through scaling and root planning in controlling the disease progression, it still fails to provide complete regeneration of lost periodontal tissues (1). In these cases, the formation of a long junctional epithelium takes place as a tissue repair response, reducing the periodontal pocket depth. (2)

Periodontal regeneration is an organized and dynamic process initiated by the migration, adhesion, proliferation, and ultimately differentiation of progenitor cells into osteoblasts and periodontal ligament cells that actively synthesize the tissue components of the functional periodontal insertion apparatus. (3,4) The success of all these steps relies on the availability of growth factors present in the extracellular matrix, which controls the gene expression in these cells. (3,5) In the absence of these molecular and cellular components, regeneration can be compromised, leading to a healing process rather than the formation of the specialized periodontal tissues. (6)

Cementogenesis is considered to be one of the most important processes in periodontal regeneration where several molecules in the extracellular matrix play a major role in cementoblastic proliferation and differentiation, resulting in the formation of a new cementum matrix. (4,7) CEMP1 is a key regulatory protein expressed in periodontal ligament cells involved in the local metabolism in cementogenesis, mediating cementoblastic differentiation and induction of cement deposition. (8) Human periodontal ligament stem cells (hPDLSCs) are well known for their pluripotency properties that enables them to differentiate into osteoblasts, fibroblasts and cementoblasts. (9) A recent study found that hPDLSCs were able to regenerate periodontal ligament and trabecular bone tissues in a periodontal defect *in vivo* model, suggesting a potential role of hPDLSCs in cementogenesis. (10)

PAR_1 is a G protein-coupled receptor that when activated triggers a series of intracellular signaling cascades that mediates cellular responses to a subset of extracellular proteins. It is present in many cell types, including human gingival fibroblasts, gingival epithelial cells, periodontal ligament cells, hPDLSCs, and in the musculoskeletal system on the surface of the cell membrane of osteoblasts, myoblasts and chondroblasts. (5,11-13) PAR_1

intracellular stimuli are usually mediated by the MAPK/ERK, p38 and PI3K/Akt pathways (14) and the persistent stimulation and phosphorylation of the MAPK/ERK is considered to play a major role in cellular responses. (15)

In this research, we evaluated how MAPK/ERK and PI3K/Akt signaling pathways blockage influenced the gene-related expression of CEMP1 after PAR_1 selective activation. Understanding how the signaling pathways associated with the downstream cascade of PAR1 is related to extracellular responses will help us better understand how cellular cementogenic responses are regulated in hPDLSCs.

4.2 Material and Methods

4.2.1 Ethics Statement

Informed consent from donors was acquired prior to the teeth extraction and was revised and approved by the Ethics Committee of the School of Dentistry of the University of São Paulo (FO-USP) under the protocol # 803.811 and procedures were performed according to the Helsinki Declaration.

4.2.2 Cells Isolation, Culture and Phenotyping

Three partially or totally erupted human third molars removed from systemically healthy patients (18 to 30 years old) without periodontal disease or predisponent associated factors were used. hPDLSCs were isolated using the explant technique as described. (14) Briefly, teeth were washed with a phosphate-saline buffer (PBS) right after the extraction procedure and transported in alpha-modified Eagle's medium (α-MEM) (Gibco, Carlsbad, CA, USA) to the laboratory. The removal of the periodontal tissue attached to the middle third of the root was carried out with a curette as described. (14).

To establish the cultures, hPDLSCs were harvested in a 25cm^2 culture flask (Corning, New York, USA) with clonogenic medium (CM) (α-MEM + 100 μg/mL penicillin, 15% fetal bovine serum, 100 μg/mL streptomycin, 0.5 mg/mL amphotericin B - Gibco, Invitrogen, Carlsbad, CA, USA) in an aerobic chamber (37° C, 5% CO2 and 95% humidity). Then, culture medium was changed every 48 hours until the culture reached 80% of confluence, when cells were trypsinized (Gibco, Life Technologies, NY, USA) and subcultures were established. All the experiments were carried out with cells at passage 4.

The characterization of the stemness biomarkers was performed through flow cytometry assay for the following surface antibodies: CD34-FITC CD14-FITC, CD31-PE, CD90-FITC, CD45-PE, CD146-PE and CD44-PE (all from eBioscience, CA, US). Briefly, 5 × 10^5 cells were washed in PBS and incubated with the conjugated antibodies for 30 minutes at 4°C. The cytometry was performed using compensation beads for each specific antibody and negative control with unstained cells was used (FACSort, Becton Dickinson, Brazil). CellQuest software was used to further analysis (Becton Dickinson, Brazil).

4.2.3 Experimental design

(Invitrogen, Carlsbad, CA, USA) per well. hPDLSCs were seeded using a density of 2x10^5 per well in 24-well plates. Experiments were divided in 3 groups, as follows: (1) osteogenic medium (OM) containing 0.1 mM dexamethasone, 2 mM β-glycerophosphate, and 50 μg/mL ascorbic acid; Sigma-Aldrich, St. Louis, MO, USA); (2) osteogenic medium containing the PAR_1 selective agonist peptide (OST + PAR) TFLLR-NH2 (100 nM) (Tocris Bioscience Inc., Bristol, UK) (14); (3) osteogenic medium containing with the blockage of the MAPK/ERK pathway followed by the PAR_1 activation by the selective agonist peptide (ERK + PAR); (4) osteogenic medium containing with the blockage of the PI3K pathway followed by the PAR_1 activation by the selective agonist peptide (PI3K + PAR). To ensure the full pharmacological effect, MAPK/ERK and PI3k pathway blocking drugs were administered into the cultures 30min before the PAR1-selective activation by the agonist peptide. Culture medium was changed every two days.

4.2.4 CEMP1 Quantitative Gene Expression

For the CEMP1 gene expression in hPDLSCs, samples were collected at 2 and 7 days for all groups: OST, OST + PAR, ERK + PAR and PI3K + PAR and the RNA extraction was performed using Trizol (1mL/well) (Invitrogen, CA, USA). Then, a reverse transcription reaction under controlled conditions (25°C for 10 min, 42°C for 60min and 85°C for 5min) using a complementary cDNA strand obtained through a High Capacity cDNA kit (Applied Biosystems, CA, USA) was carried out. The extracted RNA quality and concentration were evaluated using a NanoDrop™ One Spectrophotometer (Thermo Scientific, MA, USA) with an absorbance wavelength of 260 and 280 nm.

The quantitative real-time PCR (RT-qPCR) was performed using a TaqMan Universal Master Mix II kit (Applied Biosystems, CA, USA), TaqMan primers and probes (ThermoFisher, IL, USA) for the following oligonucleotide sequences according to the GeneBank: GAPDH (NM_002046) and CEMP1 (*Hs04185363_s1*). The entire qPCR procedure was performed using StepOne Plus™ System (Applied Biosciences, CA, USA) and divided in the following cycles: 95°C for 10 min, 40 (15 sec) cycles of 95° C, 60°C for 1 min and final cycle starting on 60°C and finishing in 95° C with 20 min duration. Quantification of the relative expression calculated by the ΔΔCT method using GAPDH as the endogenous control. (15)

4.2.4 Statistical Analysis

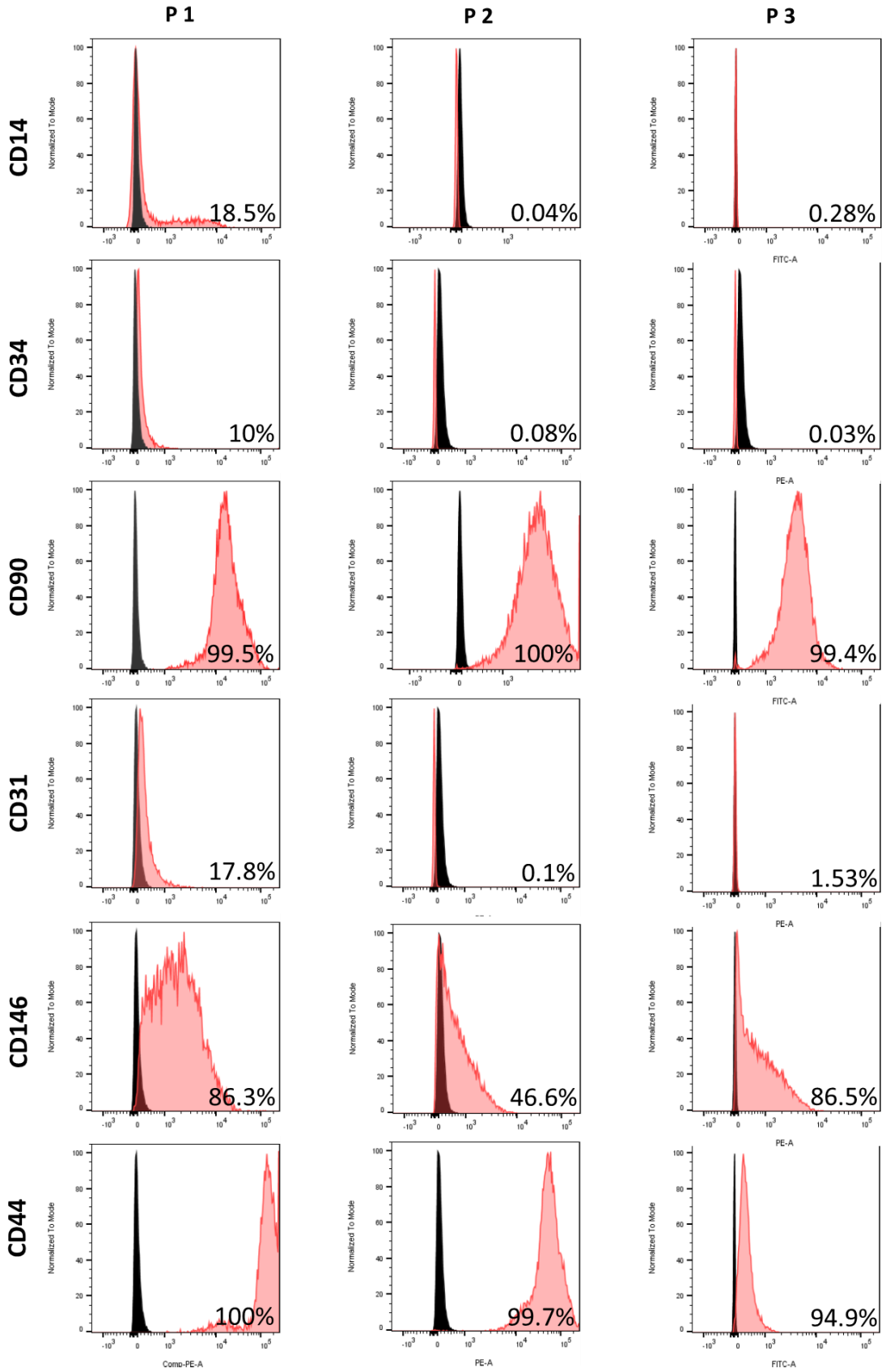
All the experiments were performed in triplicate. Statistical analysis was carried out using the GraphPad Prism 5.01 program (GraphPad Software, La Jolla, CA, USA). The data were expressed as mean ±SD of the four independent experiments performed with hPDLSCs derived from three different patients. ANOVA with pos-hoc Tukey test was used to analyze statistical differences between groups considering a significance level of 5%.

4.3 Results

4.3.1 Cells Phenotype Characterization

Flow cytometry was used in order to examine cell surface markers. Overall, cells presented a low expression of CD14, CD34, CD31 and a high expression of the surface markers CD90, CD146 and CD44, in agreement with the literature to be considered an hPDLSC lineage. (16) (Figure 4.1).

Figure 4.1 - Flow cytometry analysis showing the percentage of hPDLSCs for each surface stemness and pluripotent embryonic biomarkers (CD14, CD34, CD90, CD31, CD146, CD44) after 24h of stimuli with mediators. Unstained cells were used to set positive cell populations



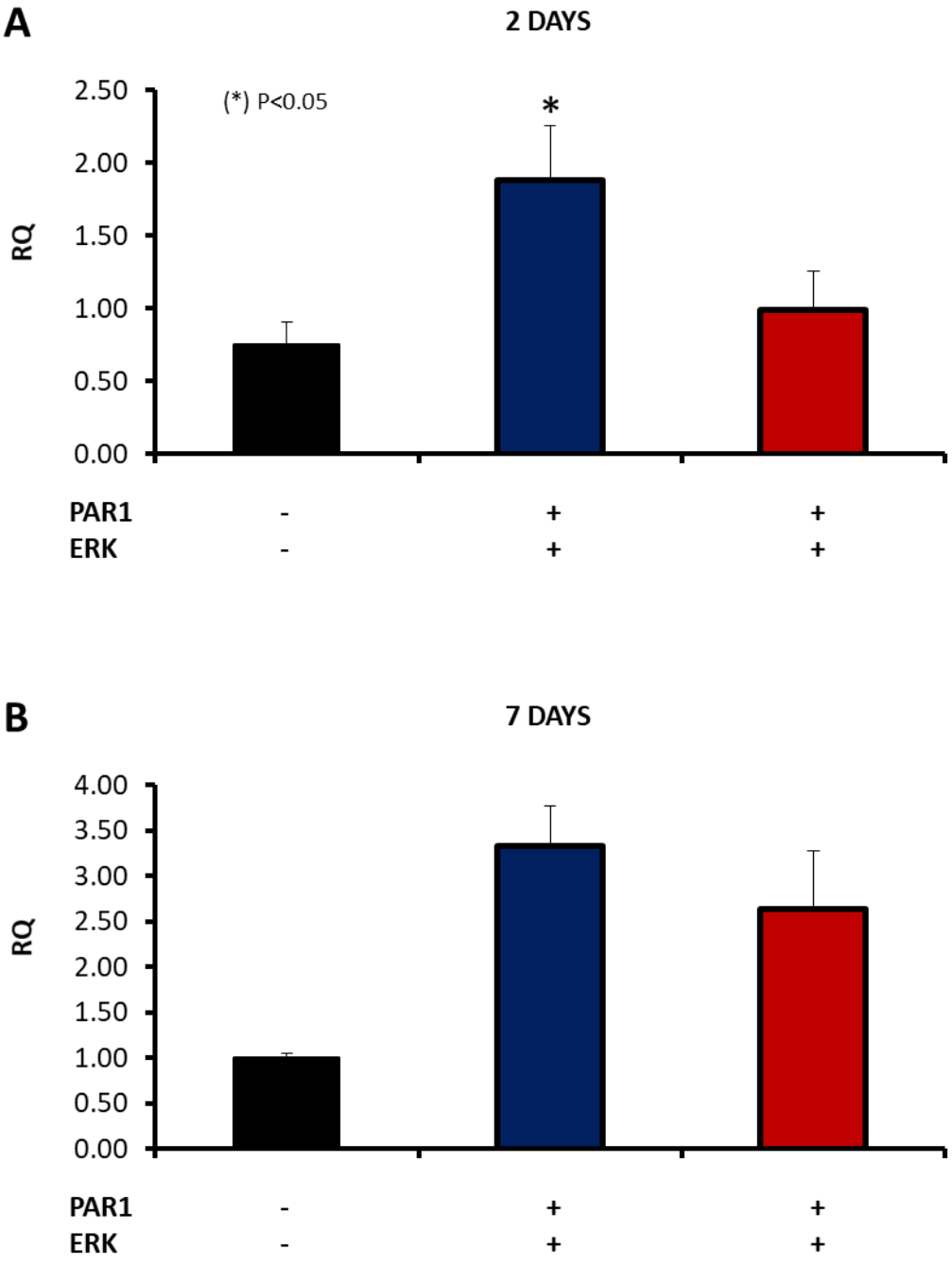
4.3.2 PAR_1 Activation Increased CEMP1 Gene Expression in hPDLSCs

At 2 days, treatment with PAR_1 agonist peptide enhanced CEMP1 gene expression in the OST + PAR group when compared to the osteogenic control (OST) for the two blocking experiments ($p < 0.05$) (Figure 2A and 3A). The same result was found at 7 days of experiment, where CEMP1 gene expression levels were statistically different when compared to OST ($p < 0.05$) (Figure 2B and 3B).

4.3.3 MAPK/ERK Pathway Blockage Downregulated CEMP1 Gene Expression in hPDLSCs under PAR_1 Activation

The blocking effect of the MAPK/ERK phosphorylation pathway resulted in a decreased CEMP1 gene expression found at 2 days of experiment when comparing the OST + PAR group with ERK + PAR group ($p < 0.05$) (Figure 4.2 A). In fact, CEMP1 gene expression levels were at low point where no significant statistical difference was detected between the ERK + PAR and the osteogenic control (OST) ($p > 0.05$) (Figure 2A). On the other hand, this gene expression impairment effect was found to be upregulated after 7 days of treatment and no statistical difference was detected between the OST + PAR and ERK + PAR groups regarding CEMP1 gene expression (OST) ($p > 0.05$) (Figure 4.2 A).

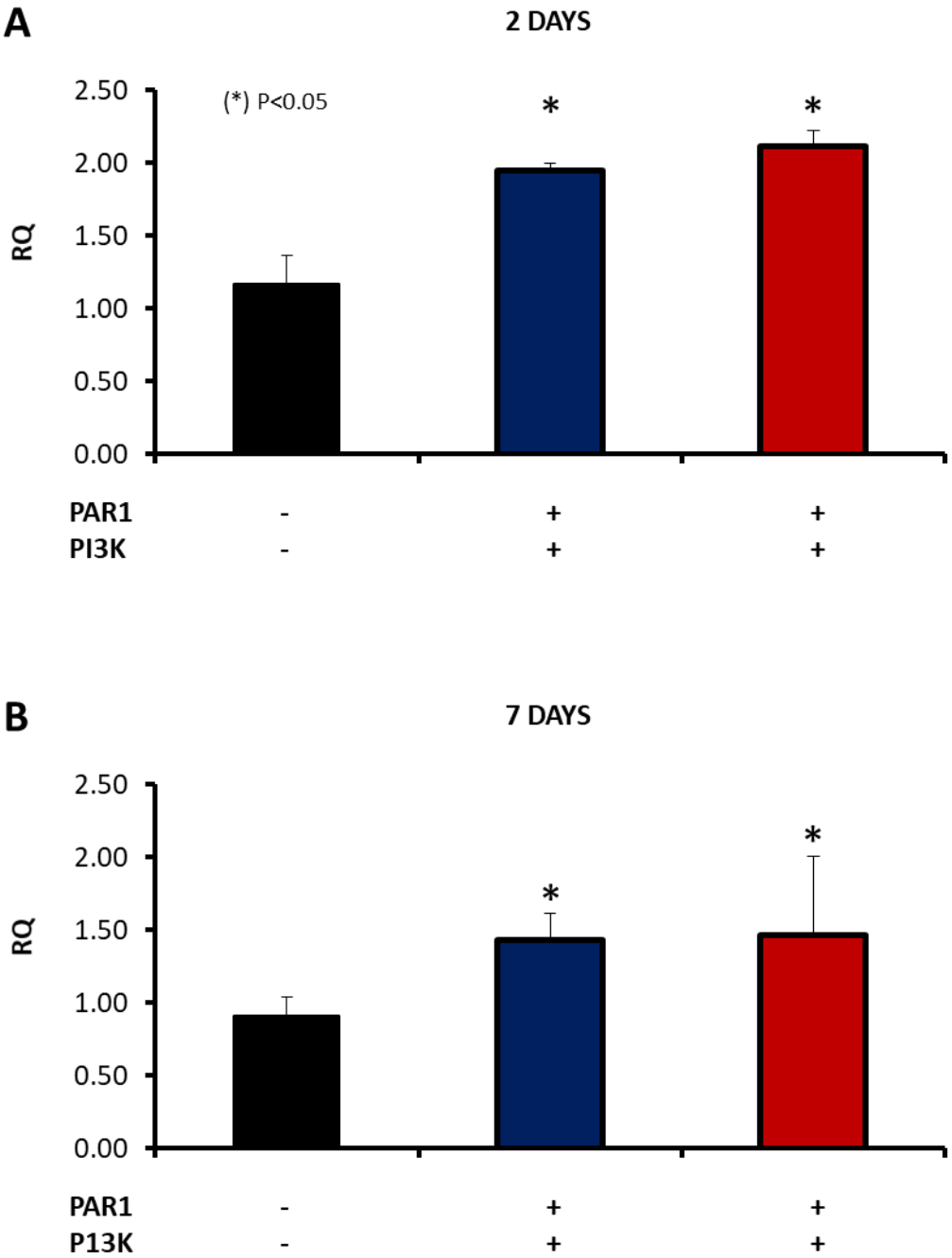
Figure 4.2 - Cementogenic biomarker (CEMP1) relative gene expression in hPDLSCs evaluated by RT-qPCR using GAPDH as endogenous control after 2-days **(A)** and 7-days **(B)** of treatment with the following groups: osteogenic medium (OST), osteogenic + PAR_1 activation by selective agonist peptide (OST + PAR) and osteogenic medium containing with the blockage of the MAPK/ERK pathway (ERK + PAR). (*) = $p<0.05$ versus control (non-stimulated). Results are given as the mean ± SD



4.3.4 PI3K Pathway Blockage Did Not Alter CEMP1 Gene Expression Patterns in hPDLSCs under PAR_1 Activation

There was no difference found in CEMP1 gene expression between the experimental groups (OST + PAR and PI3K + PAR) when PAR_1 was activated after the blockage of the PI3K pathway at 2 and 7 days of treatment ($p > 0.05$) (Figure 4.3 A and 4.3 B).

Figure 4.3 - Cementogenic biomarker (CEMP1) relative gene expression in hPDLSCs evaluated by RT-qPCR using GAPDH as endogenous control after 2-days **(A)** and 7-days **(B)** of treatment with the following groups: osteogenic medium (OST), osteogenic + PAR_1 activation by selective agonist peptide (OST + PAR) and osteogenic medium containing with the blockage of the PI3K pathway (PI3K + PAR). (*) = $p<0.05$ versus control (non-stimulated). Results are given as the mean ± SD



4.4 Discussion

Despite the great advance in knowledge about cellular and molecular events involved in the regeneration of periodontal tissues, achieving complete bone, periodontal ligament and cementum neoformation is still considered a challenge. (1,16-18) Cementogenesis represents one of the main events in periodontal regeneration, once through cementum the new fibers of the periodontal ligament are inserted. (1,19) In this context, hPDLSCs have shown a great potential for regeneration of the periodontal tissues, including cementum. (10,20) A previous study conducted by our group showed that PAR1 activation in hPDLSCs leads to an increase in osteogenic activity. (11) In the present study, we provided evidence for the first time that PAR1 activation by its agonist peptide led to an ERK dependent increase in CEMP1 gene expression, suggesting that PAR1 may have a potential role in cementogenesis.

CEMP1 is the main regulatory protein for cementogenesis. It plays an important role in local metabolism, regulating cementoblastic differentiation and induction of cement deposition. (8) Through the regulation of specific genes, CEMP1 has the ability to change the phenotype from non-mineralizing cells to minarelizing cells (cementoblasts/osteoblasts), resulting in the formation of an extracellular mineralized matrix similar to cementum. (1) In this study, activation of PAR1 resulted in increased expression of CEMP1 in hPDLCs treated with osteogenic medium, suggesting that PAR1 may increase cementogenic activity.

Literature suggests an important role of PAR1 in periodontal regeneration and bone metabolism. (5,11,13,12,20,21) However, despite the fact that PAR1 activation by its selective agonist or thrombin has already been shown to result in increased expression of Runx2 and osteoprotegerin in hPDLCs, (9,11) transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF) by fibroblasts (22) and increased expression of TGF-β, fibroblast growth factor type 1 (FGF-1) and type 2 (FGF-2) in osteoblasts (13,11), the present study was the first to demonstrate a PAR1 relevant role in another process associated with periodontal regeneration.

ERK1/2 and PI3K/Akt are considered the two major PAR1 downstream signaling pathways (9,23,24). In the PI3K/Akt pathway investigation, we found that the PI3K/Akt inhibitor had no impact on the increased CEMP1 gene expression mediated thought PAR1 activation, suggesting that this pathway has little or no role in the results observed.

Conversely, the above-mentioned pathway has already been implicated in increased OPG expression via PAR1 activation in periodontal ligament cells.(9)

When the ERK1/2 pathway inhibitor was used, we observed that the PAR1 effect on CEMP1 gene expression was suppressed, suggesting that PAR1 activation can led to an increase in CEMP1 gene expression in an ERK1/2 dependent manner. The ERK1/2 plays a pivotal role in cell differentiation and proliferation. (25) In fact, in an ERK dependent manner, PAR1 activation is associated with an increase proliferation of dental pulp fibroblasts (26), astrocytes (27) and vascular smooth muscle cells. (28,29) In addition, PAR1 may increases CCL2 expression in human osteoblasts (30), and prostaglandin E2 in mouse osteoblastic cells (31) via ERK pathway. Regarding the relation of CEMP1 and the ERK pathway, a study found that treatment with calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of mesenchymal periodontal ligament cells in a CEMP1 and ERK-dependent manner (32), which corroborates with the findings of present study which shows a role of the ERK1/2 pathway on CEMP1 expression.

Understanding the critical regulators associated with cementogenesis is of great interest for developing molecular therapies for cementum regeneration. Therefore, the present study provides evidence that PAR1 and ERK1/2 may serve as potential targets for the application of therapies related to periodontal regeneration through PDLSCs.

4.5 Conclusion

The main finding of this study is that the PAR_1 activation in hPDLSCs enhanced CEMP1 gene expression *in vitro* (Figure 4.2 A). In addition, CEMP1 increased gene expression in hPDLSCs seems to be related with the MAPK/ERK pathway in a dependent manner.

Acknowledgment

This work was supported by the São Paulo Research Foundation (FAPESP) Grants 17/23158-0 and 2018/13818-5 and CAPES Higher Education Improvement Coordination.

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CONCLUSIONS

i. PAR1 activation enhanced ectopic bone formation using hPDLSCs cellsheets in an ectopic bone formation *in vivo* model, indicating the potential of this receptor in cell engineering to be applied in periodontal tissue regeneration.

ii. Since PAR1 appear to play a major role also in the gene expression of cementogenic factor (CEMP1), acting through a ERK pathway, we can strongly correlate that PAR1 and ERK1/2 may serve as potential targets for the application of therapies related to periodontal regeneration through hPDLSCs.

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[1] According to Vancouver style.

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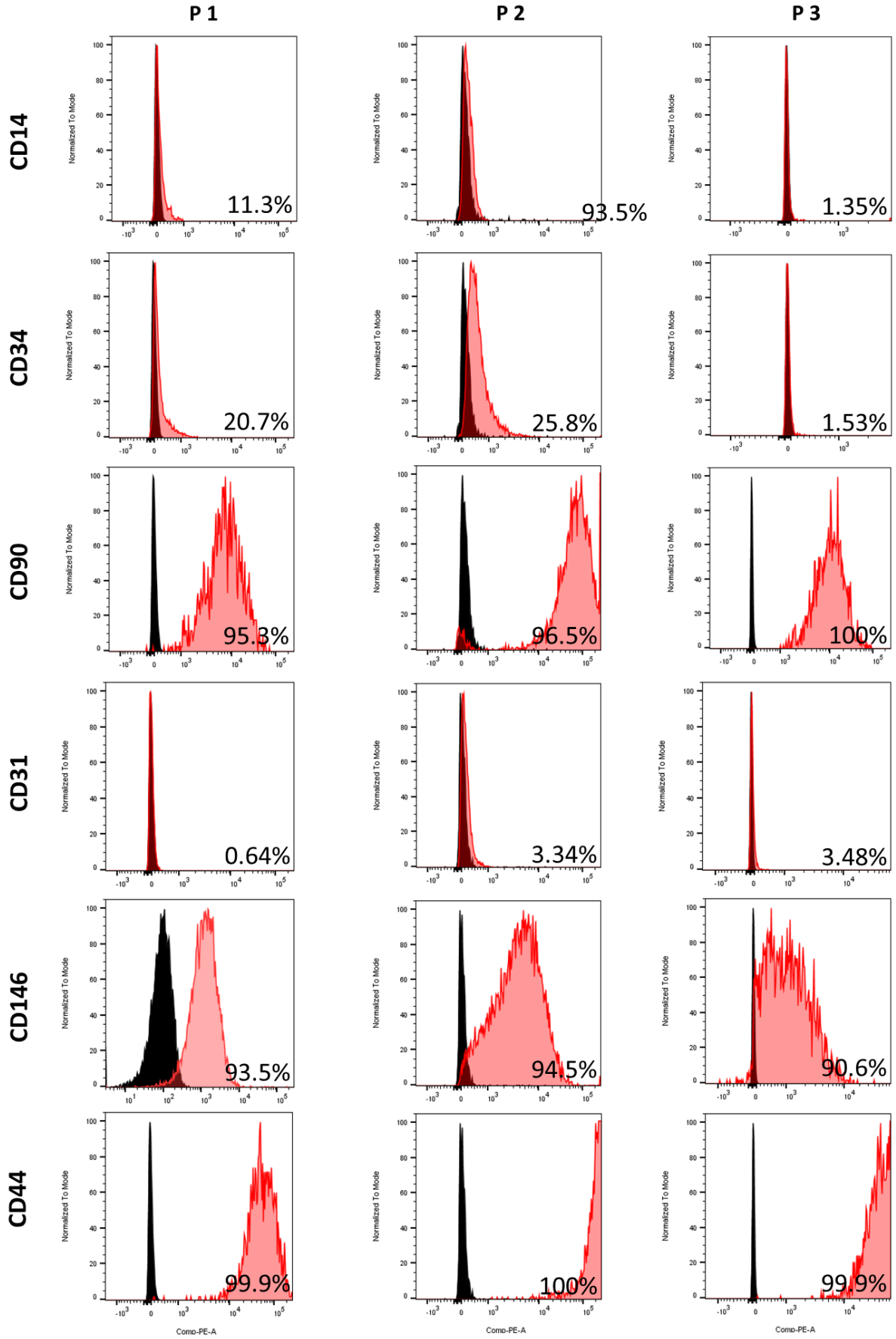
APPENDIX A – Flow cytometry analysis showing the percentage of hPDLSCs for each surface stemness and pluripotent embryonic biomarkers (CD14, CD34, CD90, CD31, CD146, CD44) after 24h of stimuli with mediators. Unstained cells were used to set positive cell populations



ATTACHMENT A – Ethical Committee approval from the Chemistry Institute (IQ-USP)

Universidade de São Paulo
Instituto de Química

DECLARAÇÃO

Declaro que o Projeto "Efeito da ativação do receptor ativado por protease do tipo 1 (PAR1) sobre a atividade osteogênica em membranas de células-tronco do ligamento periodontal humano", certificado sob o número 92/2018, desenvolvido sob responsabilidade do **Prof. Dr. Luiz Henrique Catalani**, com os colaboradores Marinella Holzhausen Caldeira, Letícia Miquelitto Gasparoni, Tomaz Alves da Silva Neto e Tayná Brunheroto Contessoto, foi aprovado pela Comissão de Ética em Uso de Animais (CEUA), do IQ-USP, em 28/03/2018.

O período aprovado por esta Comissão para execução deste projeto é de 28/03/2018 até 29/07/2020, sendo que o número de animais solicitados foram: 45 Camundongos isogênicos machos – linhagem Balb/c Nude.

São Paulo, 30 de outubro de 2019.

Profa. Dra. Daniela Sanchez Bassères
Coordenadora CEUA

ATTACHMENT B – Ethical Committee approval from the School of Dentistry (FOUSP).

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA
Comissão de Ética no Uso de Animais
Tel. (11) 3091 7842
ceuafo@usp.br

Fundada em 1900
"Em constante busca da excelência"

CERTIFICADO

Certificamos que a proposta intitulada : "Efeito da ativação do receptor ativado por protease do tipo 1 (PAR1) sobre a atividade osteogênica em membranas de células-tronco do ligamento periodontal humano, em camundongos BALB/c nude" registrada com o nº 029/2018, sob a responsabilidade da Profa. Dra Marinella Hozhausen Caldeira, Prof. Dr. Luiz Henrique Catalani, Letícia Miquelitto Gasparoni, Tomaz Alves da Silva Neto, Tayná Brunheroto Contessoto, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Faculdade de Odontologia da USP, em reunião de 12/11/2018.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	15/01/2019 a 31/12/2019
Espécie/linhagem/raça	Camundongo isogênico BALB/c Nude
Nº de animais	32
Peso/Idade	20g
Sexo	masculino
Origem	Biotério FCF/IQ da USP

São Paulo, 14 de novembro de 2018.

Profa. Associada Dra. Silvia Vanessa Lourenço
Coordenadora do CEUA-FOUSP

ATTACHMENT C – Ethical Committee approval from the School of Dentistry (FOUSP).

FACULDADE DE ODONTOLOGIA DA UNIVERSIDADE DE SÃO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Titulo da Pesquisa: Efeito da ativação do receptor ativado por protease do tipo 1 (PAR1) sobre a atividade osteogênica de células do ligamento periodontal

Pesquisador: Marinella Holzhausen Caldeira

Área Temática:

Versão: 2

CAAE: 34651214.8.0000.0075

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 803.811

Data da Relatoria: 24/09/2014

Apresentação do Projeto:

Desenho Observacional. Estudo in vitro sobre o efeito da ativação do receptor ativado por protease do tipo 1(PAR1) sobre a atividade osteogênica de células do ligamento periodontal.O estudo será realizado com células do ligamento periodontal de 10 terceiros molares higidos extraidos com indicação ortodôntica.

Objetivo da Pesquisa:

No presente estudo é estabelecida a hipótese de que a ativação do PAR1 em células do ligamento periodontal pode levar ao aumento da atividade osteogênica. Desta forma, o objetivo principal do presente estudo será avaliar o efeito da ativação do PAR1 através de trombina sobre a diferenciação osteogênica das células do ligamento. Além disso, serão avaliados os efeitos da ativação do PAR1 sobre a expressão gênica e proteica de fosfatase alcalina (ALP), osteopontina, osteocalcina, RANKL, OPG e runt-related transcription factor-2 (RUNX2) e expressão proteica de IL-10 e TGF-ß1.

Avaliação dos Riscos e Beneficios:

O risco envolvido ao paciente que aceite participar desta pesquisa é mínimo, haja visto que todo o procedimento de raspagem será feito após o dente ser extraído.

Endereço: Av Prof Lineu Prestes 2227
Bairro: Cidade Universitária **CEP:** 05.508-900
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-7960 **Fax:** (11)3091-7814 **E-mail:** cepfo@usp.br

FACULDADE DE ODONTOLOGIA DA UNIVERSIDADE DE SÃO

Continuação do Parecer: 883.811

O benefício será colaborar no desenvolvimento científico na área da periodontia através de se melhor conhecer o mecanismo de ativação do PAR1 nas células dos tecidos periodontais.

Comentários e Considerações sobre a Pesquisa:

A pesquisa apresenta-se bem delineada, com objetivos e metodologia bem descritos. Atende aos termos de apresentação obrigatória e o cronograma é compatível com a apreciação ética.

Considerações sobre os Termos de apresentação obrigatória:

São adequadamente apresentados o TCLE e a Carta de Autorização para uso de Clínica Odontológica da FOUSP.

Recomendações:

Tendo em vista a legislação vigente, devem ser encaminhados ao CEP-FOUSP relatórios parciais semestrais referentes ao andamento da pesquisa e relatório final ao término do trabalho. Qualquer modificação do projeto original deve ser apresentada a este CEP, de forma objetiva e com justificativas, para nova apreciação

Conclusões ou Pendências e Lista de Inadequações:

Nada consta

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

SAO PAULO, 24 de Setembro de 2014

Assinado por:
Maria Gabriela Haye Biazevic
(Coordenador)

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