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con la parathormona (PTHrP) regulan la proliferación de células madre mesenquimales humanas
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Vascular endothelial growth factor (VEGF) and the N-terminal portion of parathyroid hormone-related protein (PTHrP) regulate the proliferation of human mesenchymal stem cells

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Summary

Adipose tissue contains a large number of mesenchymal stem cells (ASCs) residing in their vascular stroma. Although there is controversy regarding the ability to generate bone tissue from these cells *in vivo*, the *in vitro* cells offer a good model of osteogenic differentiation due to its phenotypic similarity with the bone marrow stromal cells (BMSCs) in culture. The differentiation of osteo-progenitor populations of bone marrow is intensely regulated by local factors, such as vascular endothelial growth factor (VEGF) and parathyroid hormone-related protein (PTHrP), which modulate these populations' proliferation in different stages of differentiation. Both the VEGF and the N-terminal fragment of the PTHrP exert osteogenic effects. In this study, we posited that its effects on proliferation of osteo-progenitors are stage dependent of osteoblastic differentiation. After confirming its capacity to *in vitro* differentiation by Runx2 gene expression and accumulation of calcium, the proliferative response to stimuli was analyzed with VEGF or PTHrP (1-36) of ASCs submitted or not to osteogenic induction. VEGF, but not PTHrP (1-36), stimulated the proliferative capacity of uninduced ASCs, whereas BMSCs, but not VEGF, stimulated the proliferation of induced ASCs, corroborating the differential role of this growth in different stages of differentiation.

Key words: *adipose mesenchymal stem cells (ASCs), PTHrP, VEGF, osteogenic differentiation.*

Introduction

In the late 1960s, Friedenstein first described Bone Marrow Stromal Cells (BMSCs) as fibroblastic cells with adhesion to the plastic and tri-linear differentiation capacity, generating osteoblasts, chondroblasts and adipocytes. A fraction of these cells also demonstrated clonogenic capacity when they were cultured in very low density (Colony Forming Units-Fibroblast, CFU-F)¹. It was later noted that this multipotentiality is only inherent in a small part of this heterogeneous cell population, corresponding to Skeletal Stem Cells (SSCs, less than 0.1% of BMSCs and about 12% of CFU-F), which are also the only ones able to produce the stromal necessary for generating hematopoietic niches in bone marrow^{2,3}.

Adipose tissue contains a large number of adherent cells, capable of forming CFU-F and many other features that liken them to BMSCs. Compared to BMSCs, this tissue is easily accessible, such as liposuction discarded in liposuction operations, and isolation of stromal cells is relatively simple^{4,5}. Similar to the SSCs among BMSCs, the stromal vascular fraction (SVF) of adipose tissue is a heterogeneous population of cells, that include the ASCs, which presumably are similarly associated with SSCs to the microvasculature of fat⁶, in consonance with other tissues in which the adult stem cells are associated with the microvasculature in the form of pericytes⁷. Although several authors claim that ASCs have the inherent ability to differentiate into bone-derived cells, to date no research group has demonstrated that this is possible except after trans-differentiating these cells after intensive treatment with BMPs, which has been shown to be a potent osteo-inductor of various cell types⁸. Although the clinical utility of ASCs for bone regeneration has not been demonstrated, these cells may be a convenient model for studying osteoblastic differentiation *in vitro*, because of its easy access and response similar to that of BMSCs to *in vitro* tri-linear differentiation factors⁹.

In the presence of ascorbic acid, dexamethasone and β -glycerol-phosphate the ASCs express markers of osteoblastic differentiation *in vitro*. This is the case of the system consisting of osteoprotegerin (OPG) and activator receptor ligand for the nuclear factor κ B (RANKL), proteins involved in bone remodeling. RANKL is a protein of the tumor necrosis factor α (TNF- α) family that is expressed on the membrane of osteoblasts, which in turn can be secreted by them¹⁰. It binds to the activator receptor for the nuclear factor κ B (RANK) that is present in osteoclast precursors activating its differentiation and maturation to osteoclasts¹¹. OPG is a decoy receptor secreted by osteoblasts that binds to RANKL, preventing it from binding to RANK, thereby blocking the activation of osteoclasts. On the other hand, Runx2 is a key transcription factor in the differentiation of osteoblastic cells¹².

Vascular endothelial growth factor (VEGF) is a key molecule in the regulation of endothelial cell proliferation. It promotes the proliferation, migra-

tion and survival of these cells, as well as their vascular permeability¹³. The expression of VEGF and its receptors in BMSCs has been demonstrated by various studies in cell cultures^{13,14}. In addition, the role of the VEGFR2 receptor as key in osteoblastic differentiation and survival has been shown *in vitro*¹⁵. VEGF induces differentiation in cell cultures of preosteoblasts¹⁴ and stimulates their migration and proliferation^{13,14}. Thus, VEGF seems to be involved in the early stages of bone differentiation, both in skeletogenesis –an important factor in endochondral and intramembranous bone formation– and in adult homeostasis, promoting osteoblastic differentiation and reducing adipogenic differentiation of BMSCs^{16,17}.

Parathormone-related protein (PTH), PTHrP, is a pleiotropic cytokine with important functions in bone tissue¹⁵. It is considered a modulator of bone remodeling and a stimulator of bone formation that promotes osteoblastic differentiation and its survival¹⁵. It is, therefore, an important maturation factor. The post-translational processing of the PTHrP gene generates different fragments, including an N-terminal fragment, PTHrP (1-36), which shows great homology with PTH and acts through the common 1 receptor for PTH/PTHrP, PTH1R¹². The expression of PTHrP in the osteoblastic lineage decreases as it differentiates²⁰, but PTH1R plays a fundamental role in mature osteoblasts and osteocytes, decreasing its apoptosis and increasing its number in periosteum osteoblasts *in vivo*^{18,19}. Previous studies indicate that PTHrP could increase the proliferation of immature osteoblasts through the regulation of Cyclin D1 (promoter) and p27 (inhibitor), both regulators of the cell cycle¹².

In this paper, we hypothesize that the effects of VEGF and PTHrP on cell proliferation of osteoprogenitors are dependent on the osteoblastic differentiation stage. Thus, more undifferentiated populations would be more sensitive to VEGF while the progenitors already committed to osteoblastic differentiation would respond better to PTHrP (1-36). For this purpose, human ASCs from liposuction were used as an *in vitro* model of osteogenic differentiation. After confirming the model's *in vitro* differentiation ability by Runx2 gene expression and calcium accumulation, the proliferative response to stimuli with VEGF or PTHrP (1-36) of ASCs subjected to or not undergoing osteogenic induction was analyzed. VEGF, but not PTHrP (1-36), induced proliferative capacity of undifferentiated ASCs, whereas PTHrP (1-36), but not VEGF, induced proliferation of ASCs previously treated with osteogenic differentiation medium, confirming the differential role of these growth factors in different stages of differentiation.

Materials and methods

Isolation, primary culture and functional study of ASCs

Abdominal subcutaneous adipose tissue was obtained during the surgery of healthy patients using the liposuction technique. Six women were

included in this study with an average age of 40 years (range 25-60 years). All donors gave their informed consent, according to the appropriate clinical protocol. Patients were operated in the Department of Plastic Surgery of HM Hospitals (Madrid, Spain), and tissue sample collection was approved by the Institutional Review Board/Clinical Research Ethics Committee of HM Hospitals (Madrid, Spain). Adipose tissue was digested in 0.075% collagenase solution type I (Invitrogen, Life Technologies, New York, USA) for 30 minutes at 37°C, following the protocol described previously⁴. The cells were then plated on plastic surface (Corning, New York, USA) for 24 hours in DMAX Growth Medium (Invitrogen, Life Technologies, New York, USA) with 10% FBS (Sigma St. Louis, Missouri, USA) and supplemented with antibiotics: penicillin (100 IU/ml) and streptomycin (100 mg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA). Non-adherent cells were removed, and fresh medium was added for the primary culture of the adherent cell fraction for 7 days. The culture medium was replaced every 3 days. At that time, ASCs were functionally phenotyped by flow cytometry and their potential for osteogenic differentiation was analyzed.

Flow cytometry

For flow cytometry analysis, ASCs were re-suspended in PBS (saline phosphate buffer) at a density of 1×10^6 cells/ml, fixed with 2% paraformaldehyde (PFA) and incubated with conjugated mouse monoclonal antibodies (FITC or PE) with anti-CD90, CD29, CD34, CD45, CD106 CD44, CD144, CD31 and KDR (BioLegend, San Diego, CA, USA) for 30 min at room temperature. Cells were washed 3 times with PBS and analyzed by Accuri's C6 Flow System cytometer at 488 nm and 15 mW. Frontal dispersion (FSC), lateral dispersion (SSC) and specific fluorescent marker (LGFL) at 488 nm and 540 nm were automatically obtained for each cell. Data were digitally collected over a dynamic range of 16 million digital data channels. Amplification and logarithmic analysis of fluorescence was performed using BD Accuri™ c6 Analysis Software.

Assays of osteogenic differentiation

Cell lineage osteogenic differentiation medium was used to evaluate the potential for osteogenic differentiation of ASC. For this differentiation, ASCs were cultured for 14 days in the presence of DMEM (Dulbecco's Modified Eagle medium) with 10% FBS, 10^{-8} M dexamethasone, 0.5 mg/ml ascorbic acid and 0.1 M β -glycerolphosphate Sigma-Aldrich, St. Louis, Missouri, USA). The medium was replaced every 3 days, and at the end of the 14-day period, the histochemical red alizarin staining was performed to reveal and quantify the number of cells per microscopic field surrounded by mineralized extracellular matrix stained with alizarin red. A check of osteoblastic differentiation was also performed by means of alkaline phosphatase (ALP) assay, which consisted of the iden-

tification of the red stained deposits indicating alkaline phosphatase activity by the Sigma Aldrich 86R kit. ASCs from six different patients were used in each test.

Cellular proliferation studies

The response related to the proliferation of ASCs to factors such as VEGF (160 pM) and PTHrP (1-36) (100 nM) in the cultures were studied for 24 hours in both growth medium and osteogenic differentiation medium. Measuring the proliferation of these assays, the xCelligent System (Roche Diagnostics, Basel, Switzerland) was used to measure cell proliferation in real time. The xCelligent system provided real-time and end-point proliferation measurements based on readings of cultured plates with electrodes that detected changes in cell morphology, providing a parameter called Cell Index extrapolable to cell proliferation.

Extraction of total RNA and quantitative PCR in real time

ASCs, both at baseline and at differentiated conditions to osteoblasts, were subjected to extraction of total RNA from a cell homogenate with a standard method with guanidyl-phenol-chloroform thiocyanate (Tri-Reagent®, MCR; Cincinnati, Ohio, USA). The purity and quantification of total RNA extracted was determined by spectrophotometry A_{260}/A_{280} (Nanodrop 2000/2000c Spectrophotometer, Thermo Scientific). Synthesis of cDNA was performed using random oligonucleotides and a reverse transcriptase (High capacity RNA to cDNA Kit, Applied Biosystem; Foster City, Calif., USA). Gene expression analysis by real-time RT-PCR was performed with resulting cDNA, using a heat-activated polymerase, TaqDNA (Taqman gene expression master mix, Applied Biosystem, Foster City, California, USA) and human-specific primers For RUNX2, (Hs00231692 m1) OPG (TNFRSF1 Hs00171068), VEGF-A (Hs00173626 m1) and VEGFR2 (Hs00176676 m1) (Applied Biosystem; Foster City, California, USA). After an initial incubation of 10 minutes at 25°C and another of 2 hours at 37°C, the samples were cycled at 4°C. The results were expressed as expression levels of each gene (once normalized to the 18s RNA as constitutive gene) in each experimental condition, relative to its corresponding control: $2^{-\Delta Ct}$, where Ct represents the PCR threshold cycle in the Which the program detects for the first time an appreciable increase of fluorescence on the basal signal. All determinations were performed in duplicate. ($\Delta Ct = Ct$ (gene of interest) - Ct (18S endogenous control)).

Statistical analysis

Results were expressed as mean $\pm$ Standard Deviation (SD). The non-parametric comparison between the means of two samples was performed by the Mann Whitney test. Non-parametric ANOVA was used to compare several samples (Kruskal-Wallis). All values with $p < 0.05$ were considered significant.

Results

Firstly, the characterization of ASCs from the primary culture of liposuction was carried out. Phenotypic analysis of ASC by flow cytometry revealed that 99.6% were CD90⁺, 91.6% CD44⁺, 90.4% CD 29⁺, 4.2% CD34⁺, 2.2% CD45⁺. In addition, the markers CD106, CD155, KDR and CD31, all negative for ASCs (Table 1), were analyzed.

To evaluate the potential for osteogenic differentiation of ASC, they were subjected to osteoblastic differentiation *in vitro*. As shown in Figure 1A, ALP activity revealed that ASCs in osteogenic differentiation medium had undergone such differentiation. The red cytochemical staining of alizarin (Figure 1B) also revealed the positive result of osteogenic differentiation.

Gene expression was evaluated for some of the markers of osteogenic differentiation such as OPG and Runx2^{12,13,24}. The expression of these two markers was significantly increased ($p < 0.05$) after 14 days of culture with the osteogenic differentiation medium (Figure 2A-B).

The gene expression of the VEGFA system and its receptor induced by osteogenic differentiation was evaluated. As can be seen in Figure 3, after 7 days of differentiation the cells significantly decrease the expression of VEGF and its type 2 receptor.

Cell proliferation assays with media supplemented with PTHrP and VEGF showed different results. A proliferation study of the ASCs in response to VEGF (160 pM) was performed for 24 hours under basal conditions (growth medium) or with osteogenic differentiation medium.

As shown in Figure 4, VEGF significantly increased the proliferation of ASCs in growth medium, but had no significant effect on proliferation of cultured cells in osteogenic differentiation medium ($p < 0.05$). The results indicated that VEGF increased the proliferation of ASCs, but provided they did not begin their differentiation process. In

response to PTHrP (1-36) (100 nM) for 24 hours, ASCs did not significantly alter their proliferation index (Figure 4A). When the above-described proliferation assay was performed on osteogenic differentiation medium, it was found to change the ASC phenotype, and altered its responsiveness to these factors. Thus, in this case, PTHrP increased proliferation index in cells already committed to osteoblastic differentiation (Figure 4B).

Discussion

In the present study, ASCs from healthy women were exposed to short treatments with PTHrP and VEGF under different differentiation conditions and their proliferative capacity was analyzed. Our results indicate that the more undifferentiated populations would be more sensitive to VEGF while the progenitors already committed to osteoblastic differentiation respond better to PTHrP (1-36), showing a greater expression of PTH/PTHrR (PTH1R) receptor 1. Human ASCs derived from liposuction as an *in vitro* model of osteogenic differentiation were used. During this study the characterization of the ASCs was carried out by a study of the cell surface markers. The data obtained were those expected according to previous studies^{21,22}. The capacity of osteoblastic differentiation of ASCs was also studied. Such differentiation was verified by the ALP activity of these cells after 14 days in osteogenic differentiation medium. Similarly, histochemical staining of alizarin red was used to verify such differentiation thereby complementing the analysis of ALP activity. In addition, the gene expression of some markers such as OPG and Runx2^{16,23} was evaluated. Runx2 is essential for osteoblastic differentiation, leading ASCs to the osteoblastic lineage and inhibiting their differentiation into adipogenic or chondrogenic lineage²³. In addition, Runx2 has been described as a factor that keeps osteoblasts in the imma-

Table 1. Characterization by flow cytometry of ASCs isolated from adipose tissue obtained by liposuction

Surface marker	Expected expression	% mean expression obtained
CD 90	(+) for mesenchymal cell	94.6%
CD 44	(+) for mesenchymal cell	91.6%
CD 29	(+) for mesenchymal cell	90.4%
CD 45	(-) for mesenchymal cell	2.2%
CD 34	(-) for mesenchymal cell	4.2%
CD 106	(+) for endothelial cells	7.6%
	(-) for mesenchymal cell	
CD 144	(+) for endothelial cells	0.6%
KDR	(+) for endothelial cells	0.5%
CD 31	(+) for endothelial cells	0.2%

ture state without differentiating into osteocytes²³. Likewise, previous studies consider OPG as a marker of early osteogenic differentiation in human mesenchymal cells²⁴. As expected, our data show that osteogenic differentiation induces an increase in the expression of these two markers, OPG and Runx2, with respect to the undifferentiated state.

Gene expression of the VEGFA system and its VEGFR2 receptor were also evaluated under conditions of osteogenic differentiation. After seven days in the presence of the osteogenic differentiation medium, a decrease in the expression of VEGF and its type 2 receptor was observed. In our study, we hypothesized that the effects of VEGF and PTHrP on cell proliferation of osteoprogenitors are dependent on the stage of differentiation Osteoblast. Thus, the more undifferentiated populations would be more sensitive to VEGF due to their proximity to the endothelial niche *in vivo*, whereas the already compromised progenitors towards osteoblastic differentiation would respond better to PTHrP (1-36) by showing the PTH1R receptor a more important role in osteoblasts Mature and osteocytes. After confirming the model's *in vitro* differentiation ability by Runx2 gene expression and mineralization, the pro-

liferative response to VEGF or PTHrP (1-36) stimuli of ASCs subjected to or not undergoing osteogenic induction was analyzed. VEGF, but not PTHrP (1-36), favored the proliferative capacity of uninduced ASCs, whereas PTHrP (1-36), but not VEGF, favored the proliferation of previously differentiated ASCs, confirming the differential role of these growth factors in different stages of differentiation. Previous studies indicate that secreted VEGF is critical in the differentiation of BMSCs into osteoblasts, hindering their differentiation to other cell lineages such as adipogenic^{16,17}. Likewise, Alonso et al.¹⁵ have shown how VEGF and PTHrP modulate the differentiation and survival of osteoblasts. Our results from the proliferation study of ASCs in response to VEGF for 24 hours in growth medium or with osteogenic differentiation medium show an increase in the proliferation of ASCs grown in normal medium but without significant effects on cell proliferation cultured in the middle of osteogenic differentiation, supporting the notion that VEGF exerts a preponderant role on the regulation of proliferation in early stages of differentiation, although previous studies also point to an implication of VEGF in the survival of osteoblasts¹⁵.

Figure 1. Primary ASC culture response to specific means of cell line differentiation. (A) Detection of alkaline phosphatase activity (ALP) and (B) detection of 14-day osteogenic differentiation by alizarin red staining to detect mineralization

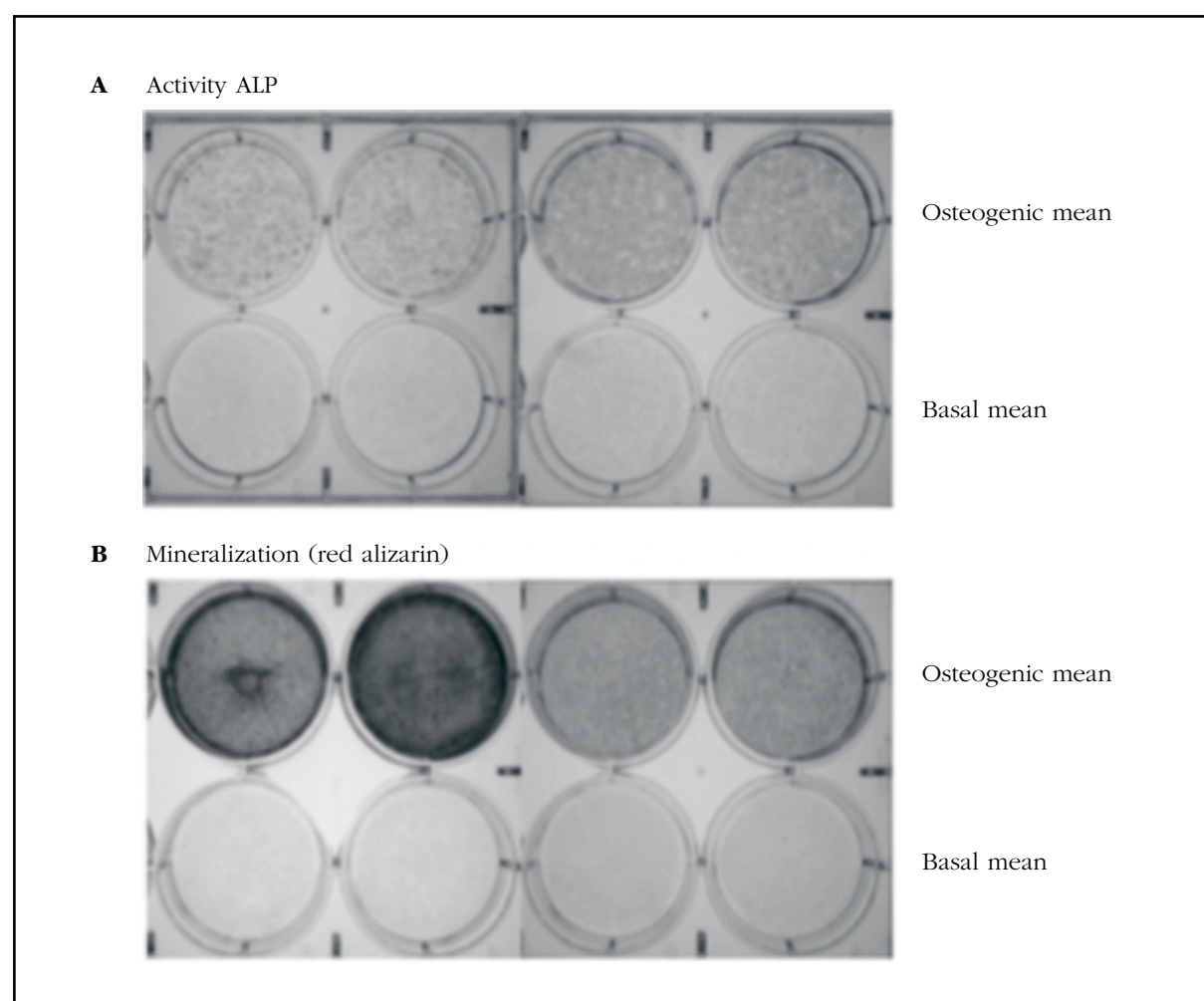
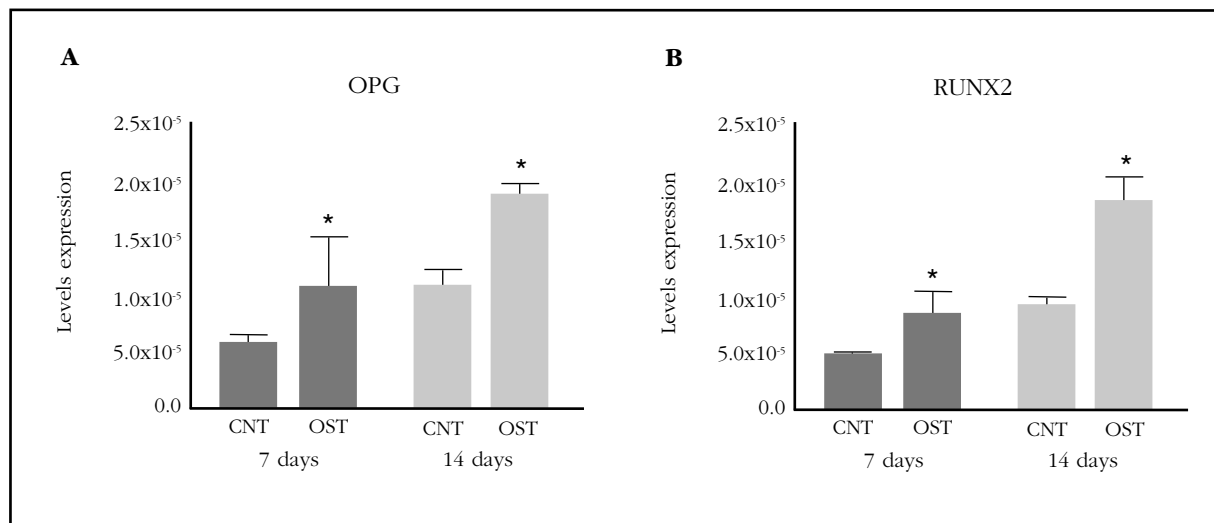
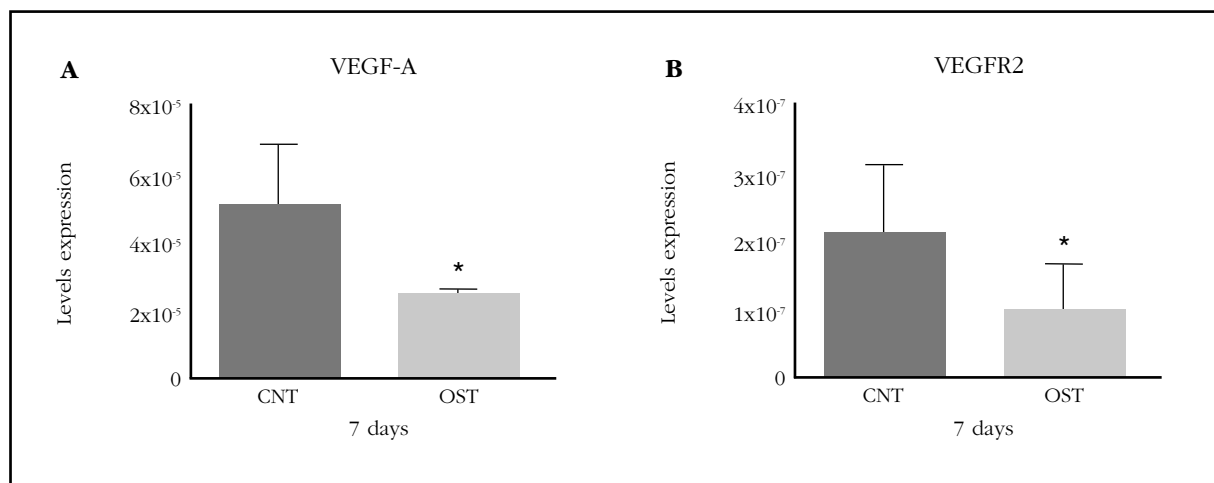


Figure 2. Changes in gene expression (analyzed by real-time PCR) of bone differentiation factors: (A) OPG, (B) Runx2, at different times of osteoblastic differentiation



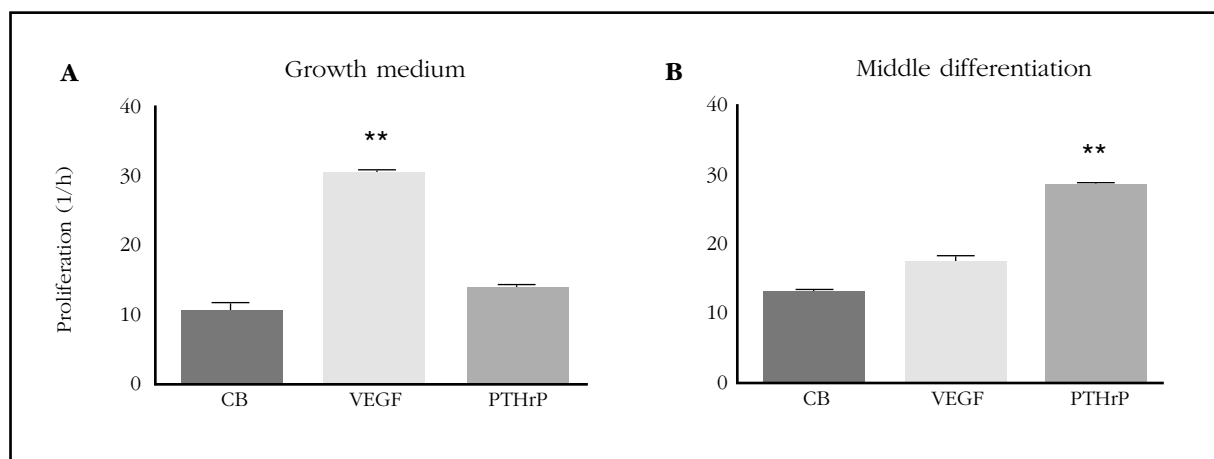
(*) $p < 0.05$ vs. corresponding basal value.

Figure 3. Changes in gene expression after 7 days of differentiation (analyzed by real-time PCR) of angiogenesis factors: (A) VEGF-A and (B) VEGFR2



(*) $p < 0.05$ vs. corresponding basal value.

Figure 4. Cell proliferation index (103) in ASC cultures undergoing stimuli with 160 pM VEGF165 and 100 nM PTHrP for 24 hours. (A) Proliferation in growth medium. (B) Proliferation in the middle of osteogenic differentiation



(**) $p < 0.05$ vs. corresponding basal value.

After 24 hours of treatment with PTHrP (1-36), undifferentiated ASCs did not alter their proliferation index. However, in the presence of osteogenic differentiation medium, PTHrP (1-36) significantly increased proliferation, pointing to the implication of this factor in the proliferation of BMSCs involved in osteogenic differentiation *in vivo*.

Thus, we may conclude that, although previous studies have shown that VEGF and PTHrP modulate the differentiation and survival of osteoblasts¹⁵, these factors could regulate the proliferation of osteoprogenitors depending on their commitment or not to the osteoblastic differentiation, with VEGF more involved in the proliferation of more undifferentiated progenitors close to the perivascular niche, whereas PTHrP (1-36) would stimulate cells more involved in osteoblastogenesis.

Conflict of interest: The authors declare they have no conflict of interest regarding this work.

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