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Using osteogenic medium in the *in vitro* evaluation of bone biomaterials: artefacts due to a synergistic effect

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Abstract

In vitro tests using bone cells to evaluate the osteogenic potential of biomaterials usually employ the osteogenic medium (OM). The lack of correlation frequently reported between *in vitro* and *in vivo* studies in bone biomaterials, makes necessary the evaluation of the impact of osteogenic supplements on these results. This study analysed the proteomic profiles of human osteoblasts (HOb) cultured in the media with and without osteogenic agents (ascorbic acid and β-glycerol phosphate). The cells were incubated for 1 and 7 days, on their own or in contact with Ti. The comparative Perseus analysis identified 2544 proteins whose expression was affected by osteogenic agents. We observed that the OM strongly alters protein expression profiles with a complex impact on multiple pathways associated with adhesion, immunity, oxidative stress, coagulation, angiogenesis and osteogenesis. OM-triggered changes in the HOb intracellular energy production mechanisms, with key roles in osteoblast maturation. HOb cultured with and without Ti showed enrichment in the skeletal system development function due to the OM. However, differentially expressed proteins with key regenerative functions were associated with a synergistic effect of OM and Ti. This synergy, caused by the Ti–OM interaction, could complicate the interpretation of *in vitro* results, highlighting the need to analyse this phenomenon in biomaterial testing.

Keywords

Dental implants; osteoblasts; osteogenesis; ascorbic acid; cell protein expression; cell–material interaction

Declarations of interest: none

Author Contributions

Francisco Romero-Gavilán: Writing - Original Draft, Data Curation, Investigation, Formal analysis **Iñaki García-Arnáez**: Data Curation, Investigation **Andreia Cerqueira**: Investigation, Writing - Review & Editing **Carlos Arias-Mainer:** Data Curation, Investigation **Mikel Azkargorta:** Data Curation, Investigation **Félix Elortza:** Resources, Investigation **Raúl Izquierdo:** Resources, Funding acquisition **Mariló Gurruchaga:** Writing - Review & Editing, Funding acquisition, Project administration **Isabel Goñi:** Writing - Review & Editing, Funding acquisition, Project administration **Julio Suay:** Writing - Review & Editing, Funding acquisition, Project administration

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

1. Introduction

Dental implantation has become a standard procedure in oral rehabilitation; titanium (Ti) is widely employed in implantology because of its high biocompatibility [1]. Although this material presents an elevated success rate, Ti is considered bioinert. Therefore, Ti surface modifications have been employed to improve its bone regenerative properties and interaction with host tissues [2]. The appropriate evaluation of effectiveness is necessary for developing new surface treatments or biomaterials. For materials intended to be placed in contact with the bone, the osteogenic potential is usually studied in osteoblast cultures *in vitro* and, subsequently, by conducting *in vivo* experiments [3]. Osteoblasts go through a multi-step process during their maturation, from proliferation to differentiation to mineralisation [4]. In each stage, the cells undergo specific and well-established changes in the gene and protein expression and the cellular architecture. Therefore, characteristic markers associated with osteogenesis should be examined to select the best materials to improve the osseointegration of the implant [5].

To achieve optimal differentiation, osteoblastic cell lines are incubated *in vitro* in the culture media supplemented with ascorbic acid and β-glycerol phosphate [6]. Using this osteogenic medium (OM) in the *in vitro* characterisation of biomaterials intended for bone tissue regeneration has become standard practice. The procedure is included in the protocols for handling commercial osteoblast cell lines. The purpose of this supplementation is to accelerate osteoblast maturation and, thus, to carry out *in vitro* studies within affordable experimentation times. Hulsart-Billström *et al.* [7] conducted a multicentre analysis in which a surprisingly poor correlation between the *in vitro* and *in vivo* results was revealed in the characterization of biomaterials for bone regenerative medicine. The study highlighted the need to critically review procedures used in this type of research and develop new characterisation methods to improve the *in vitro–in vivo* correlation rates. Even though the use of OM is a common practice in many research groups, there is a remarkably lack of literature about the possible implications that its use can have in our results. Thus, the effect of osteogenic supplements on the results of *in vitro* studies of bone biomaterials should be thoroughly evaluated.

Many physiological phenomena in osteogenic cells are reflected at the molecular level [8]. Proteomics can supply a global view of molecular mechanisms of the regeneration processes, contributing to an improved understanding of the dynamic cell responses [9,10]. Lei *et al.* [11] used nLC-MS/MS to explore the effect of extracellular Ca^{2+} on the growth and differentiation of human mesenchymal stem cells (hMSCs). They found that these ions have an osteogenic effect on this cell line, especially on the MAPKs signalling pathway. Cerqueira *et al.* [12] conducted a proteomic characterisation of human osteoblasts affected by Mg-biomaterials. They reported that the improvement in cell adhesion caused by Mg is not just the consequence of its interaction with integrins; it is a complex process affecting the entire cell machinery, involving multiple adhesion pathways. Omidi *et al.* [13] reported the effects of both Mg and Ti implants on human primary osteoblasts using proteomics. Degrading Mg showed a significant larger and more diverse impact on these cells with respect to Ti, likely because of its higher bioactivity. Likewise, the proteomic analysis of osteogenic differentiation of MSCs *in vitro* under high glucose conditions revealed a correlation between the reduced osteogenesis *in vitro* and proteins dysregulated in the cells due to the elevated glucose levels [14].

Given the limited literature on the effect of osteogenic supplementation on cell cultures for evaluating bone biomaterials, the aim of the present study was to conduct a comprehensive analysis of the OM impact on human osteoblasts (HOb) using proteomics. For that, the HOb cells were cultured with the OM (supplemented with ascorbic acid and β-glycerol phosphate) or with a non-supplemented medium (for 1 and 7 days). Their proteomic profiles were analysed using nLC-MS/MS to identify the OM effects. To evaluate the OM impact on the *in vitro* testing of a biomaterial, the cells were incubated over sandblasted acid-etched Ti discs in the OM or non-supplemented medium. The proteomic profiles of different cultures were compared to examine the expression of relevant proteins and the key signal pathways. The results here presented can contribute to obtain a comprehensive understanding of how the OM effects influence the entire osteoblast machinery, being this knowledge key to improve the design of osteogenic *in vitro* evaluations of biomaterials and the interpretation of their results.

2. Materials & methods

2.1. Ti sample preparation

Grade-4 Ti discs (1-mm thick and 10 mm in diameter) were modified by sandblasting and acid-etching (SAE; GMI-Ilerimplant S.L., Lleida, Spain), following the procedures described previously [15]. The samples were sterilised by γ-irradiation before use.

2.2. Proteomic evaluation

2.2.1. Cell culture

Human osteoblasts (HOb) cell line derived from healthy bone (Cell Applications Inc., San Diego, CA, USA) were expanded in a low-glucose Dulbecco′s Modified Eagle′s Medium with 0.584 g L^{-1} L-glutamine (DMEM; Merck, Darmstadt, Germany), supplemented with foetal bovine serum (10%; FBS; Merck) and penicillin/streptomycin (100 U mL^{-1}; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Using FBS instead of human serum allows differentiating between human proteins produced by the cells and the bovine proteins from the serum. Cells were seeded at the density of 2.5×10^4 per cm^2 in empty wells or wells with Ti discs in 48-well plates. Half of the samples were cultured with non-supplemented (NM; DMEM, 1% penicillin/streptomycin, 10% FBS) and the other half with osteogenic medium (OM; DMEM, 1% penicillin/streptomycin, 10% FBS, 1% ascorbic acid (5 μg mL^{-1}; Merck) and β-glycerol phosphate (100 mM; Merck)). The osteoblasts were cultured for 1 or 7 days in a humidified atmosphere (37 °C, 5% CO_2), and the medium was changed every 3 days.

2.2.2. Protein extraction and digestion

The HOb cells were seeded and cultured as previously described. Phosphate-buffered saline (PBS; Merck) was used to wash the cells (four times). Then, the cells were lysed using 2M thiourea, 7M urea, 4% CHAPS, and 200 mM dithiothreitol buffer (Merck) under 280-rpm agitation for 30 min. The lysate was collected and centrifuged at 13 000 rpm for 30 min at 4 °C. Afterwards, the supernatant was stored at -80 °C until analysis. The experiments were performed in quadruplicate; each replica was obtained by pooling the lysate from three wells. Protein was digested following the filter-aided FASP protocol described by Wisniewski *et al.* [16], with minor modifications. Trypsin was added to a trypsin:protein ratio of 1:20, and the mixture was incubated overnight at 37 °C, dried in an RVC2 25 speedvac concentrator (Christ, Osterode/Harz, Germany) and resuspended in 0.1% FA. The peptides were desalted using C18 stage tips (Merk Millipore, Burlington, MA, USA) and resuspended in 0.1% FA.

2.2.3. Protein identification, quantification and bioinformatic data analysis

The proteomic analysis was performed following the protocol described by Cerqueira *et al.* [12], with small variations. Briefly, a 200-ng sample was loaded onto an Evosep One chromatograph (Evosep Biosystems, Odense C, Denmark) coupled to a hybrid trapped ion mobility spectrometer–quadrupole time-of-flight mass spectrometer (timsTOF Pro with PASEF; Bruker, Billerica, MA, USA). The Evosep 30 SPD protocol was employed (44-min gradient), using a 15-cm column (Evosep). The timsTOF Pro was operated in DDA mode with the standard 1.1-s cycle time. MaxQuant programme (http://maxquant.org/) was used to perform protein identification and quantification, and the Perseus software platform (https://www.maxquant.org/perseus/) was employed for label-free comparative analysis. To examine the effects of osteogenic supplementation on protein expression, proteomic profiles of HOb cells cultured with OM or with NM (with and without Ti discs) were compared. The functional analyses of dysregulated proteins were carried out using the PANTHER classification system (http://www.pantherdb.org/) and DAVID v6.8 (Database for Annotation, Visualisation and Integrated Discovery, https://david.ncifcrf.gov/). The pathway analysis of differentially expressed proteins was performed using Ingenuity Pathway Analysis software (IPA; Ingenuity System, Redwood City, CA). The statistical functional enrichment of the canonical pathways is shown in terms of -log (*p*-value).

2.2.4. Statistical Analysis

The proteomic experiments were performed in quadruplicate. The identification of differential proteins between conditions was performed using Perseus software. Proteins were considered differentially expressed at $p \leq 0.05$ (Student's *t*-test) and with the ratio of abundances for the compared conditions higher than 1.5 in either direction (UP: increased and DOWN: reduced). The canonical pathway functional enrichment analysis was performed using IPA software. The IPA results are shown in terms of -log (*p*-value). *p*-values show the probability that the association between a set of proteins and a given pathway is due to random chance (right-tailed Fisher's exact test). A pathway is considered statistically enriched at -log (*p*-value) > 1.3, which is obtained for *p*-value < 0.05.

3. Results

3.1. HOb proteomic profile analysis

MaxQuant software was used to identify and quantify the proteins in the lysed HOb samples. Then, the comparative analyses were carried out using the Perseus system. First, the proteomic profile of HOb cells cultured with OM (C-OM) was compared with the profile obtained for the NM (C-NM) cultures to identify the specific effects of osteogenic supplementation **(Supplementary Table 1).** Then, to examine the impact of the supplements on the results of the *in vitro* cultures with Ti, the proteomic profiles of HOb cells cultured on Ti discs in the OM (Ti-OM) were compared to those cultured on Ti and with NM (Ti-NM) **(Supplementary Table 2)**. **Table 1** shows the number of upregulated and downregulated proteins differentially expressed after 1 and 7 days, revealed in the two comparative analyses. In the osteoblasts cultured without the Ti material, 597 and 890 proteins were deregulated after 1 and 7 days, respectively, due to the presence of OM. In the HOb cells incubated with Ti, 602 and 1029 proteins were deregulated after 1 and 7 days, respectively, because of the osteogenic supplementation. Moreover, the number of downregulated proteins increased with the culture time. To examine the effect of Ti on the cells, the

proteomic profiles of HOb cultured without and with Ti (in the non-supplemented medium) were compared **(Supplementary Table 3**).

Table 1. The number of proteins upregulated (UP), downregulated (DOWN), and the total number of proteins differentially expressed in HOb cells as a result of the osteogenic supplementation (Perseus comparatives analyses; n=4).

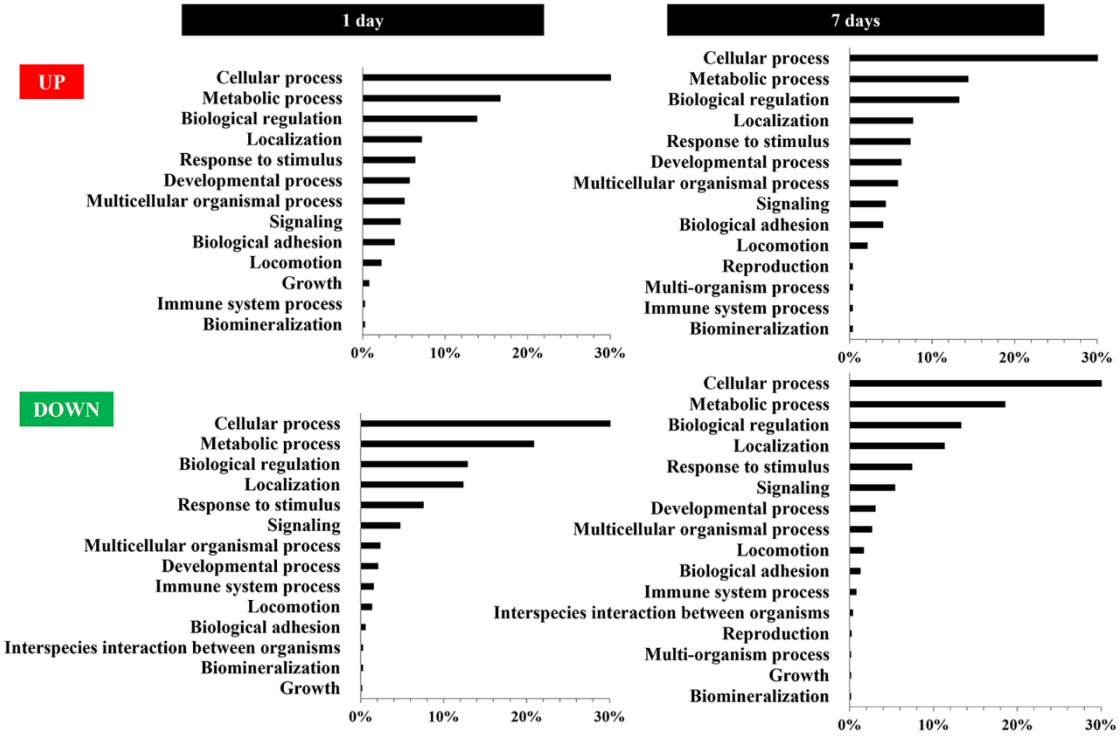
	C-OM vs C-NM		**Ti-OM vs Ti-NM**	
	1 day	**7 days**	**1 day**	**7 days**
UP	224	177	137	119
DOWN	373	713	465	910
TOTAL	597	890	602	1029

3.2. Functional analyses

3.2.1. PANTHER classification

The PANTHER programme was employed to classify the differentially expressed proteins according to their associated functions in biological processes. **Fig. 1** shows that such proteins found by comparing C-OM and C-NM cultures are mainly related to cellular, metabolic, biological regulation, localisation, response to stimulus, developmental, multicellular organismal signalling, biological adhesion, locomotion, immune system and biomineralisation processes. After day 1, the growth-related functions were also detected, and after 7 days, the reproduction and multi-organism process functions were enriched among the deregulated proteins. In addition, interspecies interaction between organisms was associated with the downregulated proteins.

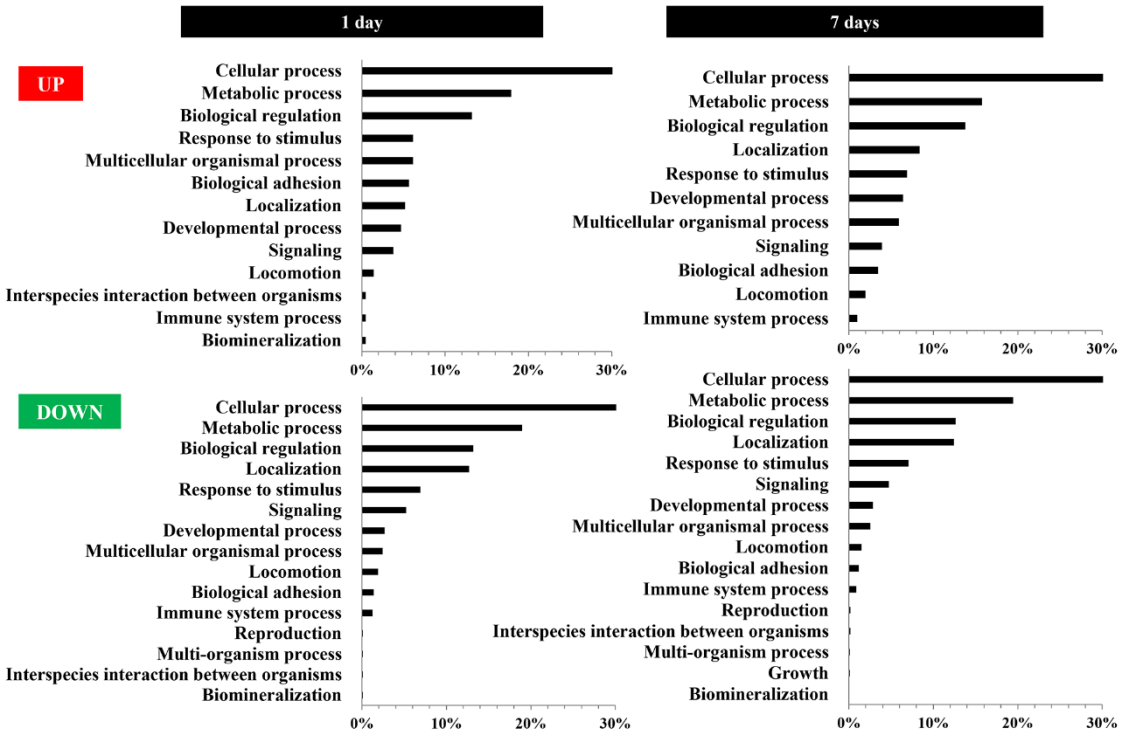
Figure 1. PANTHER diagram of biological processes related to the functions of proteins differentially expressed in HOb (cells cultured with OM compared with those cultured with NM).



205

206 **Fig. 2** shows the PANTHER functional classification of proteins differentially expressed in cells incubated
207 with Ti discs as a result of adding the osteogenic supplements. The data are similar to the results for HOb
208 cultured without Ti and the same biological processes were altered due to OM. However, in this case,
209 growth process functions were not found for the proteins upregulated on day 1. In contrast, the interspecies
210 interaction between organisms was found for the upregulated and downregulated proteins in cells cultured
211 with Ti; in the cells grown without Ti, this function was only seen for downregulated proteins. The
212 biomineralisation, reproduction and multi-organism process functions were associated with upregulated
213 proteins after 7 days in the comparison of cultures without Ti (C-OM vs C-NM); they appear only in the
214 downregulated group in the cultures with Ti discs.

215 **Figure 2.** PANTHER diagrams of biological processes related to the functions of proteins differentially
216 expressed in HOb cells cultured on Ti with OM compared to those cultured with Ti and NM.



3.2.2. Ingenuity pathway analysis (IPA)

The IPA pathway analysis of deregulated proteins was performed to identify canonical pathways significantly altered in cells cultured with OM in comparison with NM. **Fig. 3** shows the pathways enriched due to the protein expression changes in the C-OM vs C-NM comparative analysis. Eighty-four pathways were altered in the HOb cells exposed to OM. These pathways were classified, according to their functions, into four sets: adhesion, immunity and oxidative stress, coagulation and angiogenesis, and osteogenesis. Most affected functions are associated with osteogenesis (49 of 84). Fourteen pathways were related to adhesion, 15 to inflammation and oxidative stress, and six to coagulation and angiogenesis. Among the adhesion-related pathways, the strongest alterations were found after 7 days for integrin, actin cytoskeleton and RAC signalling (**Fig. 3a**). The sirtuin signalling, associated with antioxidant and anti-inflammatory effects, was the top enriched function related to immunity and oxidative stress (**Fig. 3b**). Among the coagulation and angiogenesis functions, vascular endothelial growth factor (VEGF) pathway showed the most elevated -log (*p*-value) after 1 day of culture. After 7 days, the hepatocyte growth factor (HGF), the hypoxia-inducible factor 1 (HIF-1) and thrombin signalling were the pathways most affected by the OM (**Fig. 3c**). In the osteogenesis group, the oxidative phosphorylation showed the highest level after 1 day; the EIF2 and mTOR signalling were the pathways most enriched after 7 days (**Fig. 3d**).

Figure 3. Heatmap distribution of enriched pathways in IPA analysis of C-OM vs C-NM comparison. The statistical functional enrichment of the canonical pathways for the differentially expressed proteins is shown in terms of -log (*p*-value). A pathway is considered statistically enriched for -log (p-value) > 1.3. The enriched signals were classified into four functional categories: a) adhesion, b) immunity and oxidative stress, c) coagulation and angiogenesis and d) osteogenesis.

The pathways enriched in osteoblasts cultured on Ti discs as a result of osteogenic supplementation are displayed in **Fig. 4**. Eighty-eight pathways were affected by the differences between the proteomic profiles found in the Ti-OM vs Ti-NM comparative analysis. The OM mainly impacted the pathways associated with osteogenesis (52 of 88). Moreover, 15 pathways related to cell adhesion, 15 to immunity and oxidative stress and 6 with key roles in coagulation and angiogenesis were also enriched. Thus, integrin, actin nucleation and RAC signalling were the adhesion-related pathways most affected by OM when evaluating the *in vitro* responses to Ti (**Fig. 4a**). The sirtuin signalling was the most enriched pathway within the immune function group (**Fig. 4b**). Among the coagulation and angiogenesis functions, VEGF was the enriched pathway with highest -log (p-value) after 1 and 7 days, followed by HGF and HIF-1 signalling (**Fig. 4c**). The osteogenesis-related pathways with the highest enrichment were the oxidative phosphorylation, EIF2, FAT10 and the estrogen receptor (**Fig. 4d**).

Figure 4. Heatmap distribution of enriched pathways in the IPA analysis performed for the differentially expressed proteins found in the comparison Ti-OM vs Ti-NM. A pathway is considered statistically enriched at -log (p-value) > 1.3. Enriched pathways were classified into four functional categories: a) adhesion, b) immunity and oxidative stress, c) coagulation and angiogenesis and d) osteogenesis.

3.2.3. DAVID functional annotation

A clustering annotation analysis was performed to identify the enriched functions associated with the HOb proteins upregulated or downregulated due to OM. **Supplementary Tables 4** and **5** display the enriched Gene Ontology (GO) function terms. They also show the clusters of differentially expressed proteins associated with these enriched functions for the cells cultured without and with Ti discs. **Table 2** contains the GO terms of the functions enriched for the upregulated proteins obtained in comparisons C-OM vs C-NM and Ti-OM vs Ti-NM. The functions enriched in the presence of OM, with and without titanium, were associated with adhesion, coagulation and angiogenesis, oxidative stress, immunity, osteogenesis, and extracellular matrix (ECM) formation and organisation.

Table 2. Functions detected as enriched in the DAVID analysis of differentially upregulated proteins found in the comparisons C-OM vs C-NM and Ti-OM vs NM. The identified Gene Ontology function terms (GO) were classified into 6 groups: adhesion, oxidative stress, immunity, coagulation and angiogenesis, osteogenesis and extracellular matrix.

	C-OM vs C-NM	Ti-OM vs Ti-NM
Adhesion	GO:0005913~cell-cell adherens junction GO:0098609~cell-cell adhesion GO:0098641~cadherin binding involved in cell-cell adhesion GO:0007155~cell adhesion GO:0005178~integrin binding GO:0003779~actin binding	GO:0005913~cell-cell adherens junction GO:0098609~cell-cell adhesion GO:0098641~cadherin binding involved in cell-cell adhesion GO:0007155~cell adhesion GO:0005178~integrin binding GO:0003779~actin binding GO:0005925~focal adhesion GO:0007160~cell-matrix adhesion
Coagulation and angiogenesis	GO:0048407~platelet-derived growth factor binding GO:0001568~blood vessel development GO:0030168~platelet activation GO:0002576~platelet degranulation	GO:0048407~platelet-derived growth factor binding GO:0001568~blood vessel development GO:0030168~platelet activation GO:0002576~platelet degranulation
Oxidative stress	GO:0055114~oxidation-reduction process GO:0000302~response to reactive oxygen species GO:0004601~peroxidase activity	GO:0055114~oxidation-reduction process
Immunity	GO:0050776~regulation of immune response	-
Osteogenesis	GO:0001501~skeletal system development GO:0043491~protein kinase B signalling GO:0005520~insulin-like growth factor binding GO:0007179~transforming growth factor beta receptor signalling pathway GO:0001558~regulation of cell growth GO:0009611~response to wounding	GO:0001501~skeletal system development GO:0043491~protein kinase B signalling GO:0005520~insulin-like growth factor binding GO:0030154~cell differentiation GO:0008284~positive regulation of cell proliferation GO:0009611~response to wounding
Extracellular matrix (ECM)	GO:0031012~ECM GO:0030198~ECM organisation GO:0005201~ECM structural constituent GO:0030199~collagen fibril organisation GO:0030574~collagen catabolic process GO:0005581~collagen trimer	GO:0031012~ECM GO:0030198~ECM organisation GO:0005201~ECM structural constituent GO:0030199~collagen fibril organisation GO:0030574~collagen catabolic process GO:0005581~ collagen trimer

3.3. Identification of synergistic effects

The upregulated proteins from the Ti-OM vs Ti-NM analysis, related to key regenerative functions by DAVID **(Supplementary Table 5**), were compared to the overexpressed proteins with the same functions detected in the comparison C-OM vs C-NM **(Supplementary Table 4**). From this analysis, **Table 3** shows a group of proteins differentially expressed in the cells cultured with Ti due to the OM, which were not upregulated in cell cultures without Ti discs. The group contained collagens (CO2A, CO6A2),

thrombospondins (*e.g.*, TSP2 and TSP4), coagulation factors (FA10 and F13A) and proteins related to adhesion (*e.g.*, FHOD1, PI4KA, RAN).

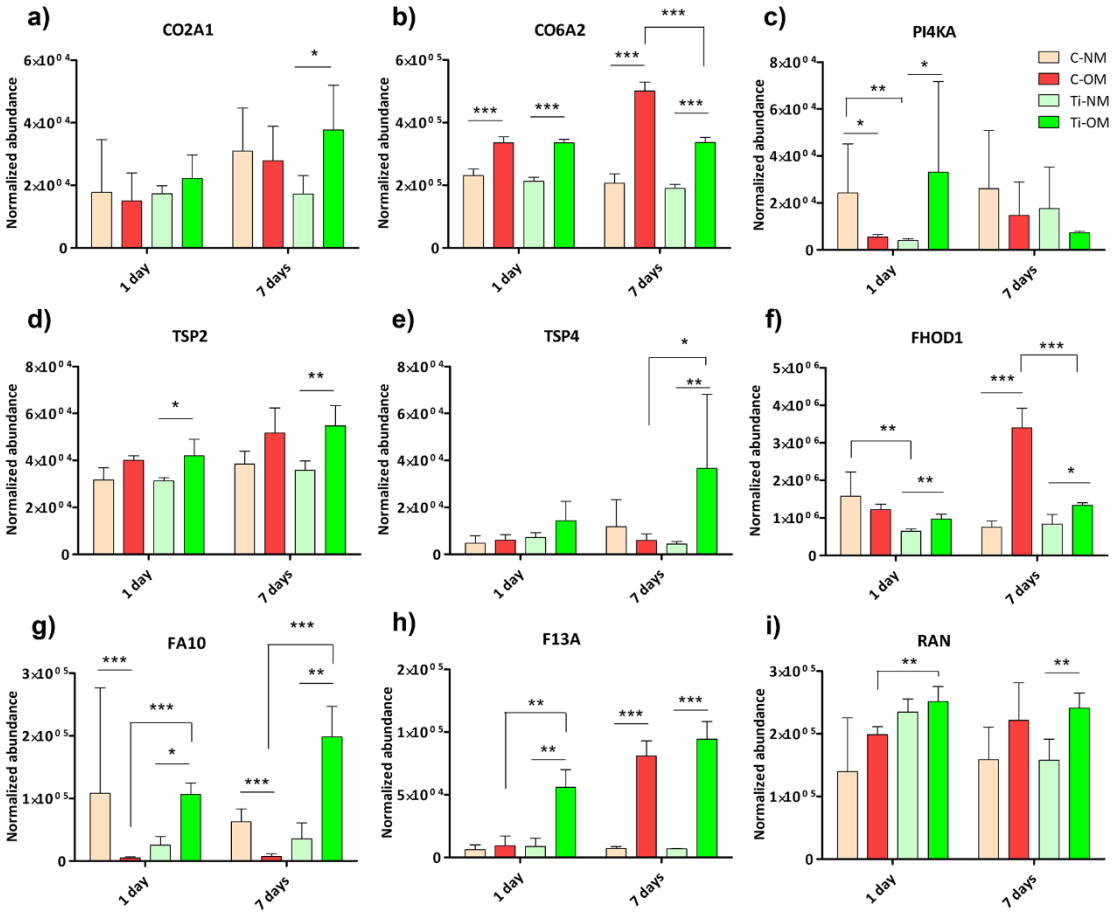
Table 3. Proteins related to regenerative functions by DAVID, overexpressed in cells cultured with Ti in the presence of OM but not upregulated in the C-OM vs C-NM comparison.

Extracellular matrix		Osteogenesis		Coagulation and angiogenesis		Adhesion		Oxidative stress	
1 d	**7 d**	**1 d**	**7 d**	**1 d**	**7 d**	**1 d**	**7 d**	**1 d**	**7 d**
CASPE	CO2A1	CO6A2	CO2A1	F13A	CO2A1	CO6A2	DOCK7	PLOD2	-
CO6A2	LOXL1	CRIP2	DHCR7	QSOX1	DHCR7	E41L2	FBLI1	PDPR	
DCD	RAN	FA10	FA10	FA10	TSP1	EMIL1	FHL1	SERA	
H4	RL35A	FHL1	NEMO		TSP2	ES8L2	IF4H	QSOX1	
RL35A	RS25	FLNB	TSP1		FA10	FHOD1	PDLI2	ADRO	
RS19	TSP1	HEG1	TSP2			K1C18	RAN	QORX	
RS20	TSP2	KBP	TSP4			LAP2A	RL22	P4HA1	
S10A9	TSP4	MCTS1				NOP56	RS8		
						PDLI1	TSP1		
						PI4KA	TSP2		
						PODXL	TSP4		
						POSTN			
						PTPRK			

To examine the effect of using OM in the evaluation of Ti as a biomaterial, the normalised abundances of representative proteins from **Table 3** were obtained (shown in **Fig. 5)**. No differences were found between the amounts of CO2A1 detected in C-NM and C-OM cultures (**Fig. 5a**). Similarly, no such differences were detected between C-NM and Ti-NM cultures. However, the cells exposed to Ti and the OM expressed more CO2A1 than those incubated with Ti and the NM (after 7 days), revealing a synergistic effect of the Ti–OM combination. **Fig. 5b** shows that the HOb expressed more CO6A2 in the presence of OM than in the non-supplemented medium. The cells cultured in the NM with Ti expressed the CO6A2 at the same levels as those cultured without the discs (null Ti effect). However, in the C-OM experiments, more CO6A2 was detected than in the Ti-OM cultures (after 7 days), showing a negative synergistic effect of the Ti–OM interaction. As a result of this Ti–OM interplay, the level of CO6A2 decreased in cells incubated on Ti-OM respect C-OM, while the cells cultured in Ti-NM and C-NM showed the same expression. In the examination of the Ti effect on the HOb protein profiles, this result can be considered an artefact. The PI4KA protein reduced its expression on day 1 due to the OM (C-OM vs C-NM; **Fig. 5c**) and in the cells incubated on Ti discs with NM with respect to C-NM. In contrast, its levels increased in Ti-OM cultures compared with Ti-NM. No differences were detected between the normalised abundances of TSP2 in C-OM vs C-NM and Ti-NM vs C-NM comparisons. However, the expression of this protein was higher in Ti-OM than in Ti-NM cultures (after 1 and 7 days) (**Fig. 5d**). The normalised abundance of TSP4 increased in Ti-OM in comparison with Ti-NM experiments after 7 days (suggesting an interaction between Ti and the OM) (**Fig. 5e**). Since, no differences could be attributed to Ti or the OM individually (Ti-NM vs C-NM and C-NM vs C-OM). This synergistic effect caused a discrepancy in the amounts of this protein found in the cultures with and without Ti, depending on the type of medium employed. **Fig. 5f** shows that after 1 day, the expression of FHOD1 was not affected by OM (C-OM vs C-NM) but decreased in the presence of Ti (Ti-NM vs C-NM). However, the level of this protein was higher in Ti-OM than in Ti-NM, suggesting a synergy. After 7 days, the osteoblasts cultured with the OM showed higher expression of FHOD1 than those cultured with NM. Although Ti did not affect the levels of this protein in cultures without osteogenic

supplementation, the expression of FHOD1 was lower in Ti-OM than in C-OM. The FA10 level was lower in the cells cultured with OM than with NM (after 1 and 7 days) (**Fig. 5g**). In contrast, no differences were found due to Ti for cultures using NM (Ti-NM vs C-NM). However, the abundance of this protein was significantly higher in Ti-OM than in Ti-NM samples at both time points. Thus, Ti-OM cultures showed an increase in the FA10 level (compared with C-OM) due to the synergistic effect between Ti and OM. The same phenomenon was observed for F13A after 1 day (**Fig. 5h**). After 7 days, the production of F13A increased in OM cultures in both Ti-OM vs Ti-NM and C-OM vs C-NM comparisons. Moreover, no differences were found due to Ti discs (Ti-NM vs C-NM and Ti-OM vs C-OM), therefore, synergistic effects were not observed. Thus, the results for F13A (after 7 days) obtained using OM, although showing higher abundances, correlated with data from incubations without osteogenic supplements. Fig. **5i** shows no changes in the expression of RAN that could be ascribed to the individual effect of the medium or the tested material. However, an increase in the abundance of this protein was detected in the Ti-OM in comparison with the Ti-NM cultures after 7 days.

Figure 5. The normalised abundance of differentially expressed proteins related to regenerative functions in the Ti-OM compared with Ti-NM cultures. Results are shown as mean ± SE. The asterisks ($p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***); n=4) indicate statistically significant differences.



4. Discussion

The low correlation between the *in vitro* and *in vivo* results in evaluating biomaterials for bone tissue regeneration highlights the need for a critical review of cell culture studies conducted with such materials [7]. *In vitro* parameters, such as the type of cell line or the composition of the culture medium, could be related to this problem. An appropriate cell culture medium is key for cell survival, proliferation and various cellular functions. In the trials evaluating the osteogenic potential of materials, the skeletal cells are cultured with supplemented medium to favour their maturation. This is a controversial procedure due to the potential effect of the osteogenic supplements on the outcome [17]. Thus, the present work focuses on analysing the impact of these supplements on the results of *in vitro* tests with HOb cells. The aim is to evaluate the possible relationship between this practice and the poor effectiveness associated with such experiments. To achieve that, we characterised the proteome profiles of HOb cells cultured with and without SAE-Ti for 1 and 7 days, using a normal culture medium and the same medium supplemented with osteogenic compounds.

Proteomic label-free relative quantification detected 2544 proteins differentially expressed in HOb cultured in OM and NM, with and without Ti discs. The results demonstrated that osteogenic supplements clearly affected the osteoblast proteomic profiles. It seems that the impact of the OM on the cells increases with culture time; this effect was stronger for cells cultured with Ti discs than those incubated without this material. The differences between the protein levels in Ti and C (no Ti discs) samples reflect the different material–cell interactions conditioning the osteoblasts responses [18], independent of OM. The differentially expressed proteins found in each comparison were classified, using PANTHER, according to their functions. Many proteins were associated with cellular, metabolic, regulation, localisation, and response to stimuli biological processes (in both C-OM vs C-NM and Ti-OM vs Ti-NM comparisons). In smaller quantities, the proteins with functions in processes related to tissue regeneration, such as development, biological adhesion, growth, immunity or biomineralisation, were also identified. Moreover, regardless of the use of OM, differences in the distribution of these functions were found for the C and Ti samples. For example, 5.7% of the proteins differentially overexpressed in HOb cultured with Ti (for 1 day) were related to the adhesion processes; in the C cultures, this function was only associated with 3.9% of overexpressed proteins.

The IPA relative functional analysis identified the regeneration-related pathways altered by the OM. Most of these pathways were involved in adhesion, immunity and oxidative stress, coagulation and angiogenesis, and osteogenesis in HOb cultured with and without Ti. Moreover, it seems that the osteogenic supplements have a complex impact on the osteoblast machinery since they simultaneously affect each of these processes through different signals. This result highlights the simplification inherent to *in vitro* experimentation, where only some markers are evaluated to predict the regenerative response to a material. Such tests cannot encompass the complexity of the mechanisms of skeletal cells that can be observed in proteomic studies [19]. The analysis of protein expression of HOb cultured with Mg-based biomaterials has shown that the promotion of cell adhesion by this type of material is due to its effect on multiple adhesion pathways [12]. Similarly, in our study, we demonstrated that the use of OM altered the integrin-related signals (integrin, ILK or Gaq signalling) but also the signals associated with the Rho family of GTPases and with the cytoskeleton regulation. Moreover, these pathways are altered faster in the cultures exposed to Ti. Considering the effect of OM on pathways associated with osteogenesis (both in the C and Ti samples),

oxidative phosphorylation and oestrogen receptor signalling were the most affected pathways at day 1. However, after 7 days, the EIF2 signalling showed the highest -log (p-value) in C samples, and oxidative phosphorylation continued to be the most affected signal in cultures with Ti. Oxidative phosphorylation, as well as glycolysis, generate ATP during osteoblast differentiation [20]. Here, the IPA results showed that the OM had a clear impact on the intracellular energy metabolism of HOb with an important alteration in the oxidative phosphorylation capacity at day 1. However, after 7 days, the effect on glycolysis I signal increases in C samples; in the Ti cultures, the oxidative phosphorylation mechanism continues showing higher -log (p-values) than glycolysis. The shift of biological energy production of the bone progenitor cell from glycolysis to oxidative phosphorylation increases their osteogenic differentiation potential [21]. Thus, the OM effects on the glycolysis and oxidative phosphorylation pathways could be related to its osteogenic induction, and the distinct behaviours of the C and Ti cultures might be caused by the different osteogenic responses triggered by the biomaterial. The oestrogen receptor signalling also contributes to bone healing by stimulating energy production, osteoblast maturation and angiogenesis [22]. The oestrogen-related receptor α is a regulator of the Wnt-signalling pathway during osteoblast differentiation, using a cell-intrinsic mechanism that does not affect the nuclear translocation of b-catenin [23]. The eukaryotic translation initiation factor 2 (EIF2) signalling is associated with osteoblast differentiation and bone formation [24]. The eukaryotic initiation factor proteins (EIFs) play a key role during translation initiation, allowing cells to conserve resources while a new gene expression programme is adopted to prevent stress damage [25]. The pathways such as the mechanistic target of rapamycin (mTOR), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), extracellular signal-regulated kinase (ERK)- mitogen-activated protein kinase (MAPK) and transforming growth factor-β (with a key role in osteoblast maturation [26]) were also affected by the osteogenic supplements in cells cultured with and without Ti. However, some other pathways related to osteogenesis, such as bone morphogenesis protein (BMP) [26], angiopoietin [27], and dopamine receptor signalling [28], were only affected by OM in cultures with Ti discs. Therefore, differential activation of osteogenic pathways due to the Ti effect (independent of the OM) was detected by the IPA.

The enriched functions among both the upregulated and downregulated proteins in HOb cells due to the OM were studied by DAVID analyses. The GO functions detected as enriched were related to adhesion, coagulation and angiogenesis, oxidative stress, immunity, osteogenesis and ECM in C-OM vs C-NM and Ti-OM vs Ti-NM comparisons. The OM tends to downregulate the expression of HOb proteins with functions in the immune response. It alters the osteogenesis, showing enrichment in the skeletal system development functions in cells cultured with Ti and without this material. Moreover, the proteins that are part of the ECM or have a key role in its organisation were upregulated.

Among the differentially upregulated proteins associated with regenerative functions in DAVID analysis, 52 were found in cells cultured with Ti due to the use of OM (Ti-OM vs Ti-NM), but they did not show this behaviour when comparing the C-OM and C-NM cultures at the same time point. Therefore, the upregulation of these proteins in HOb could not be attributed to the OM itself but must have resulted from the interaction of Ti with osteoblasts. One would expect to find these proteins also overexpressed in the comparison between Ti and C cultures without osteogenic supplements (Ti-NM vs C-NM). However, none of these proteins was seen among the proteins overexpressed in Ti-NM compared to C-NM. Some of them

(*e.g.*, FHOD1 and PI4KA) were even differentially less expressed in cells cultured with Ti. Therefore, the overexpression of this cluster could not be due to the biomaterial on its own but was a result of synergy between the osteogenic effect of the medium supplements and Ti. The analysis of normalised abundances of affected proteins showed that this synergistic effect depended on the culture duration. Thus, on the intensity of this effect, a lack of correlation between the protein expression in the Ti and C samples was observed as a consequence of using the OM. It is likely that the proteins produced by adhering cells ultimately determine the cell fate and, therefore, the efficacy of regeneration [29]. The synergetic behaviour affecting the proteome profiles of osteoblasts, caused by the interactions between the OM and the tested material, could alter markers employed during traditional *in vitro* testing.

The OM effect should not hamper the *in vitro* testing of biomaterials since all samples are cultured under the same conditions. However, the synergistic interactions between biomaterials and the OM identified in the present study can cause some problems, since the synergistic effect on the HOb protein expression could alter the *in vitro* results giving misleading signals. Although we are all aware of the limitations associated with traditional *in vitro* experimentation, the existence of synergistic effects between the OM and the materials under study had not been taken into account before. These interactions should be considered in the future development of new *in vitro* methodologies for the evaluation of bone biomaterials. More studies are necessary to assess the scope of the impact of this phenomenon and its effect on *in vitro* outcomes; proteomics could be an effective tool to address this issue.

5. Conclusion

Since each cell type has specific growth requirements, choosing the right cell culture medium is crucial as it will directly affect the cell performance and, therefore, the study results. For efficient bone biomaterial evaluation, one should examine the effect of using an osteogenic culture medium on the molecular mechanisms of osteoblasts. The proteomic analyses conducted here showed that the OM affects the entire cellular mechanism of HOb cells, including multiple adhesion and osteogenic processes. This effect is exerted not just by altering the specific pathways but also by modulating intracellular energy production during the osteoblast maturation process. Despite the effect of OM on the cellular machinery of HOb, it was possible to detect differences in signalling in the osteoblasts cultured with and without Ti. This implies that the OM can be used in tests examining distinct osteogenic potentials under different conditions. This is not surprising as this medium has been employed in *in vitro* studies finding different responses between biomaterials. A summative effect is usually expected when the OM and osteogenic materials are combined. However, the synergistic effects caused by interactions between the medium and the tested material were seen here, based on the HOb protein expression profiles. This observation suggests that some artefact data might be produced in such experiments, which might distort the interpretation of the *in vitro* outcome.

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