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Complex effects of Mg-biomaterials on the osteoblast cell machinery: A proteomic study

Andreia Cerqueira [a,1], Iñaki García-Arnáez [b,1], Francisco Romero-Gavilán [a,*], Mikel Azkargorta [c], Félix Elortza [c], José Javier Martín de Llanos [d,e], Carmen Carda [d,e], Mariló Gurruchaga [b], Isabel Goñi [b], Julio Suay [a]

[a] Department of Industrial Systems Engineering and Design, Universitat Jaume I, Av. Vicent Sos Baynat s/n, 12071 Castellón de la Plana, Spain
[b] Department of Science and Technology of Polymers, University of the Basque Country, P. M. de Lardizábal, 3, 20018 San Sebastián, Spain
[c] Proteomics Platform, CIC bioGUNE, Basque Research and Technology Alliance (BRTA), CIBERehd, ProteoRed-ISCIII, Bizkaia Science and Technology Park, 48160 Derio, Spain
[d] Department of Pathology Medicine and Odontology, Medicine Faculty, University of Valencia, Av Blasco Ibáñez, 13, 46010, Valencia, Spain
[e] Research Institute of the University Clinical Hospital of Valencia (INCLIVA), C. de Menéndez y Pelayo, 4, 46010, Valencia, Spain

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ABSTRACT

The cell–biomaterial interface is highly complex; thousands of molecules and many processes participate in its formation. Growing demand for improved biomaterials has highlighted the need to understand the structure and functions of this interface. Proteomic methods offer a viable alternative to the traditional *in vitro* techniques for analyzing such systems. Magnesium is a promoter of cell adhesion and osteogenesis. Here, we used the LC-MS/MS to compare the protein expression profiles of human osteoblasts (HOb) exposed to sol-gel coatings without (MT) and with Mg (MT1.5Mg) for 1, 3, and 7 days. PANTHER, DAVID, and IPA databases were employed for protein identification and data analysis. Confocal microscopy and gene expression analysis were used for further characterization. Exposure to MT1.5Mg increased the HOb cell area and the expression of *SP7*, *RUNX2*, *IBP3*, *COL3A1*, *MXRA8*, and *FBN1* genes. Proteomic analysis showed that MT1.5Mg affected the early osteoblast maturation (PI3/AKT, mTOR, ERK/MAPK), insulin metabolism, cell adhesion (integrin, FAK, actin cytoskeleton regulation) and oxidative stress pathways. Thus, the effects of Mg on cell adhesion and osteogenesis are rather complex, affecting several pathways rather than single processes. Our analysis also confirms the potential of proteomics in biomaterial characterization, showing a good correlation with *in vitro* results.

1. Introduction

Magnesium (Mg) is essential in many cellular processes; it is important in bone formation and metabolism [1]. It enhances matrix mineralization and collagen type X expression in mesenchymal stem cells (MSCs) [2] and stimulates osteoblast differentiation, ALP activity, and mineral deposition in osteoblast cells [3]. Moreover, Mg increases cell adhesion by mediating in the function of membrane-associated adhesion receptors, such as integrin receptors α5β1, β1 and α3β1 [4]. The potential of Mg-doped biomaterials has been shown for bioactive glasses [4], biodegradable alloys [5] and sol-gel coatings for titanium (Ti) surfaces described by our group [6]. We have demonstrated that Mg-doped materials improve cellular adhesion, reduce inflammation, and increase osteogenic marker expression in a dose-dependent manner.

The growing demand for improved biomaterials for medical applications has increased the urgency of deepening our understanding of cell–material interactions. The difficulties translating *in vitro* results into patient outcomes have strengthened the interest in developing alternative early-stage assays to predict the *in vivo* response and optimize the biomaterial testing [7]. The application of *omics* in the analysis of biomaterials could tackle the complexity of their interactions with biological systems [8]. Our group has already used proteomics to examine serum protein adsorption patterns on the materials doped with Mg [6]. This demonstrated the potential of proteomics for the characterization of biomaterials and studying the profiles of proteins expressed by cells exposed to Mg-based materials can be an interesting alternative to

* Corresponding author.
E-mail address: gavilan@uji.es (F. Romero-Gavilán).
[1] Co-autorship.

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explore. The proteomic methods have been employed to analyze the effects of biomaterials in cell cultures, revealing unique cellular protein expression profiles [9]. In recent years, these techniques helped in understanding how different biomaterials modulate the expression of proteins associated with cell adhesion, proliferation, and differentiation in osteosarcoma [10] and mesenchymal stem cells (MSCs) [11,12]. Even though the number of such studies is still quite limited, they demonstrate that proteomics can be used to predict the outcomes of real-life biomaterial applications and point the way to further exploration.

This article presents a first study of the effects of Mg-doped sol-gel coatings on the cell–biomaterial interactions employing proteomics. *In vitro* assays were carried out using proteomics to examine the cell adhesion and osteogenic responses. The proteomic profiles of cells exposed to coatings without (MT) and with Mg (MT1.5Mg) were compared. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to identify the proteins, and computational methods were used to evaluate protein interactions. Our results show that proteomic methods can be useful for *in vitro* characterization of biomaterials and help further the knowledge of Mg effects on cellular behavior.

2. Materials and methods

2.1. Material synthesis

Methyltrimethoxysilane (MTMOS; Merck, Darmstadt, Germany) and tetraethyl orthosilicate (TEOS; Merck) were used as precursors (molar ratio of 7:3) to obtain hybrids by the sol-gel route. The percentage of $MgCl_2$ (1.5 wt.%) was selected based on results reported in Cerqueira et al. [6], and the materials were synthesized following the methods described in that study. Grade-4 Ti discs (10-mm diameter, 1-mm thickness) were sandblasted, acid-etched and used as the substrate for the coatings. The discs were immersed in the sol-gel solutions at 60 cm min^{-1} for 1 min and removed at 100 cm min^{-1} employing a dip-coater (KSV DC; KSV NIMA, Espoo, Finland).

2.2. Cell culture

Human osteoblasts (HOb) derived from healthy bone were purchased from Cell Applications Inc. (San Diego, CA, USA) and expanded in a proliferation medium consisting of low-glucose Dulbecco's Modified Eagle's Medium enriched with L-glutamine (DMEM; Merck), 100 U/mL penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA) and 10% foetal bovine serum (FBS; Merck). The cells were seeded on the discs at a density of $2.5 \times 10^4/cm^2$ and cultured for 1, 3, and 7 days in an osteogenic medium consisting of a low-glucose DMEM supplemented with 1% pen/strep, 10% FBS, 1% ascorbic acid (5 μg mL^{-1}; Merck), and 100 mM β-glycerol phosphate (Merck). The osteogenic medium was chosen to enable comparisons with previous studies. Instead of human serum, the FBS was used. The human serum might be more clinically relevant; however, FBS it is the standard for HOb culturing. It also helps distinguish between the human proteins produced by the cells and the bovine proteins adsorbed onto the surface. Cell culture was maintained at 37 °C in a humidified atmosphere (95%) with 5% CO_2. The cell culture medium was refreshed every three days.

2.3. Cytoskeleton arrangement

For cytoskeleton arrangement evaluation, the HOb cells were seeded on the materials and cultured for 7 days. Then, the samples were treated as described in Cerqueira et al. [6]. Briefly, after washing with PBS, the samples were fixed with 4% paraformaldehyde (PFA) for 20 min. Then, were permeabilized with 0.1% Triton X-100 for 5 min and incubated with phalloidin (1:100; Abcam, Cambridge, UK) diluted in 0.1% *w/v* bovine serum albumin (BSA)-PBS for 1 h. For nuclei staining, the samples were incubated for 5 min in a mounting medium with DAPI (Abcam). Fluorescence was detected with a Leica TCS SP8 Confocal Laser Scanning Microscope with 20× (dry) lenses. The images were obtained with LAS X software (Leica) and analyzed using the Image J software (National Institutes of Health, Maryland, USA). A total of three samples of each material were studied and 30 photos were taken for image analysis.

2.4. Relative gene expression: RNA extraction, cDNA synthesis and qRT-PCR

Quantitative real-time PCR (qRT-PCR) was employed to examine the effect of Mg on gene expression. Total RNA extraction, cDNA synthesis and qRT-PCR were carried out as described by Cerqueira et al. [6]. The targets (Supplementary Table 1) were designed using the *Primer3Plus* software tool and purchased from Thermo Fisher Scientific. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method, and the data were normalized to control wells (without materials). The experiment was carried out in quadruplicate.

2.5. Statistical analysis

The Student's *t*-test was carried out using the GraphPad Prism 5.04 software (GraphPad Software Inc., La Jolla, CA) to determine the changes in cell area and gene expression. The differences between MT and MT1.5Mg cultures were considered statistically significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Data were expressed as means ± standard error (SE).

2.6. Proteomic profile characterization

2.6.1. Protein extraction

At each time point (4 samples were prepared, each made up by pooling lysates obtained from 3 culture wells), the cells were washed four times with phosphate-buffered saline (PBS; Merck) and lysed with cell lysis buffer (CLB). The CLB consisted of 2 M thiourea, 7 M urea, 4% CHAPS and 200 mM dithiothreitol (DTT). All reagents were purchased from Merck. The samples were incubated in CLB under agitation (280 rpm) for 30 min at room temperature. Next, the lysate was collected and centrifuged (13,000 rpm, 4 °C, 30 min). Finally, the supernatant was recovered and frozen at −80 °C until further analysis.

2.6.2. Protein identification and functional classification

For protein identification, an approximately 200-ng aliquot of the sample was directly loaded onto an Evosep ONE (Evosep, Odense C, Denmark) chromatograph and acquired in a hybrid trapped ion mobility spectrometry – quadrupole time-of-flight mass spectrometer (timsTOF Pro with PASEF, Bruker Daltonics, Billerica, MA, USA). Each sample was analyzed in quadruplicate, and the obtained results were processed using the MaxQuant (http://maxquant.org/) and Perseus (https://www.maxquant.org/perseus/) software. The Student's *t*-test was conducted to determine which proteins were differentially expressed on MT1.5Mg in comparison with MT. Protein expression differences were considered statistically significant for $p \leq 0.05$ and the ratio higher than 1.5 in either direction (underexpressed or overexpressed). The functional classification of the statistically significant proteins was performed employing PANTHER (http://www.pantherdb.org/), DAVID (https://david.ncifcrf.gov/) and UniProt (https://www.uniprot.org/). The canonical pathways analysis, upstream regulator analysis, and molecule activity prediction (MAP) were performed using QIAGEN Ingenuity® Pathway Analysis (QIAGEN IPA®, Hilden, Germany) software. In the IPA®, the right-tailed Fisher's exact test was employed to calculate statistical differences. Differences between the expression of molecules, proteins and associated pathways were considered significant for $p < 0.05$ ($-\log(p\text{-value}) > 1.3$). Data analysis focused on the proteins and pathways associated with osteoblast metabolism and maturation.

3. Results

3.1. Cytoskeleton arrangement & relative gene expression

The cell cytoskeleton organisation was evaluated by staining with phalloidin after 7 days of culture (Fig. 1A-B). The cells cultured on MT1.5Mg had elongated shapes and more protruding lamellipodia than those grown on MT (Fig. 1B). Cell area measurements showed that the cells in contact with MT1.5Mg significantly increased their surface area in comparison with those cultured on the MT discs (Fig. 1C). The relative gene expression of target markers was examined to understand further the effects of the studied materials on osteogenesis (*SP7*, *RUNX2, IBP3,* and *COL3A1*) and cell adhesion (*MXRA8* and *FBN1*). The *SP7* expression of cells grown on MT1.5Mg increased after 1 day but was downregulated after 7 days of incubation (Fig. 2A). In contrast, MT1.5Mg significantly increased the expression of *RUNX2*, *IBP3*, *COL3A1*, *MXRMA8* and *FBN1* after 7 days of culture (Fig. 2B-F).

3.2. Functional classification using PANTHER

A total of 2545 proteins were identified on the MT and MT1.5Mg materials. The comparison between the MT and MT1.5Mg determined that HObs seeded on MT1.5Mg differentially expressed 144 proteins after 1 day and 180 and 44 proteins after 3 and 7 days, respectively (Supplementary Table 2). The PANTHER analysis of the biological functions revealed that MT1.5Mg regulated the expression of proteins associated with the cellular, metabolic, reproductive, and developmental processes, biological adhesion, signaling, response to stimulus, biological regulation, and localization (Fig. 3A). The protein class analysis categorized the proteins into 19 groups (Fig. 3B). The most dominant protein class was the metabolite interconversion enzyme (for all time points). Other classes such as membrane traffic protein, nucleic acid metabolism, protein-binding activity modulator, cytoskeletal protein, cell adhesion protein and extracellular matrix protein were also detected.

3.3. IPA analysis

3.3.1. Canonical pathway analysis

The IPA analysis determined that MT1.5Mg mainly regulated the expression of pathways associated with osteogenesis, cell adhesion and oxidative stress. Among those involved in osteogenesis, 21 pathways were significantly affected by MT1.5Mg (Fig. 4A). The changes were observed for most detected pathways after 1 day of culture, except for ascorbate recycling and fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), growth hormone and JAK/STAT signaling pathways. Among the most affected at this time point were the regulation of eukaryotic initiation factor 4F (eIF4) and p70S6K ($-\log(p\text{-value}) = 5.87$), paxillin signaling (3.31), mechanistic target of rapamycin (mTOR) (3.53), insulin receptor signaling (2.93), and insulin growth factor (IGF)-1 signaling (2.45). After 3 days, all the pathways, except for insulin secretion, macropinocytosis signaling, phospholipase C signaling and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), were significantly regulated by culturing with MT1.5Mg. The effect was strongest for the eukaryotic initiation factor 2 (EIF2) (5.08), mTOR (2.93), platelet glycoprotein VI (GP6), 14–3-3 (1.80) and IGF-1 signaling (1.78). After 7 days, only the EIF2 signaling (3.09), mTOR (2.14), regulation of eIF4 and p70S6K signaling (2.34), and IGF-1 signaling (1.55) showed statistically significant differences between the two materials. Sixteen pathways associated with cell adhesion were affected by culturing with the MT1.5Mg material (Fig. 4B). After 1 day, all pathways associated with cell adhesion were significantly regulated by MT1.5Mg, except for Gαq and sphingosine-1-phosphate signaling. At this time point, the effect of this material was more evident for pathways linked to actin arrangement (regulation of actin, actin cytoskeleton signaling, actin nucleation) with values of $-\log(p\text{-value})$ from 2.44 to 3.17. p21-activated kinase (PAK) signaling (4.10) and tight junction signaling (3.14) were also among the most affected pathways. After 3 days, five pathways were unaffected (CXCR4 signaling, Gα12/13, integrin-linked kinase (ILK), RAC and tight junction signaling). Pathways associated with integrin signaling (3.63) and paxillin signaling (3.31), regulation of actin (2.72) and actin cytoskeleton signaling (2.72) showed the biggest changes at this time point. After 7 days, the effects of the MT1.5Mg on adhesion subsided, and only the ephrin receptor signaling (2.20) was affected. Four pathways associated with oxidative stress were altered by the exposure to MT1.5Mg (Fig. 4C). After 1 day, the oxidative phosphorylation was significantly affected (2.36). The pathways associated with antioxidant action of vitamin C (1.99) and vitamin C transport (1.88), and superoxide radical degradation (1.82) showed significant differences after 3 and 7 days, respectively.

3.3.2. Upstream regulator analysis and MAP

The upstream regulator analysis was used to find the upstream molecules that might have caused the observed changes in protein expression. The IPA® predicted 1202 molecules (Supplementary Table 3). The MAP tool was used to simulate the downstream consequences of upregulating or downregulating a molecule and infer the upstream activity in the pathway. Two proteins (TGFB1 and IGF1) from the upstream regulator analysis were selected as main regulators of the molecules obtained in IPA analysis. The selection was based on their prominent roles in osteogenesis and cell adhesion. Fig. 5 shows the obtained network, with the interactions between the molecules; the common activity predicted was the binding of osteoblasts.

3.3.3. Protein and function association

Proteins affected by the exposure to MT1.5Mg were associated with

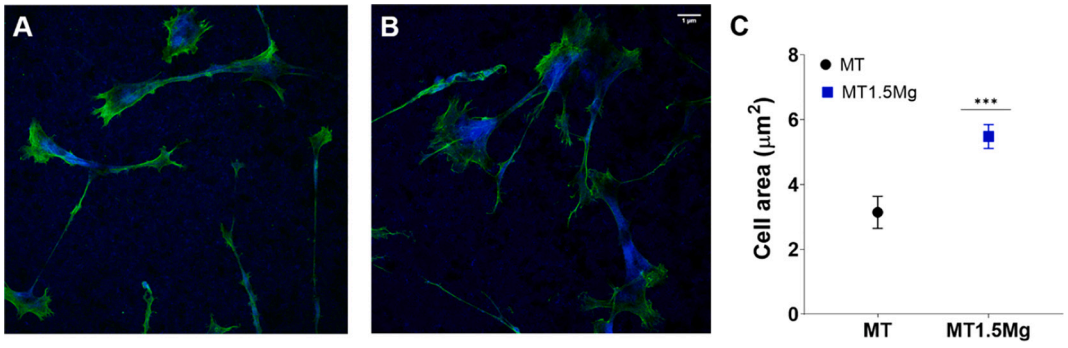

Fig. 1. Confocal fluorescence images of cytoskeleton arrangement for HObs on (A) MT and (B) MT1.5Mg. (C) Area of the cells adhering to the different materials. Actin filaments were stained with phalloidin (green) and nuclei with DAPI (blue). Scale bar: 1 μm. Results are shown as means ± SE. The asterisks ($p \leq 0.001$ (***)) indicate statistically significant differences between the MT and MT1.5Mg. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

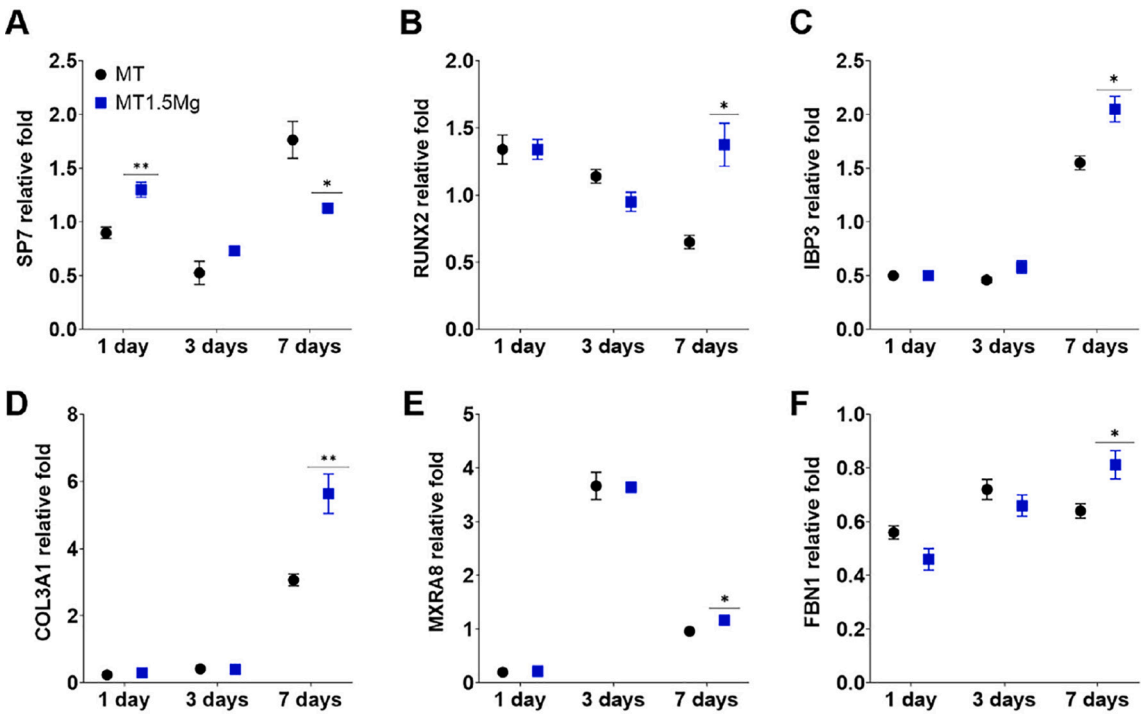

Fig. 2. Gene expression of (A) osterix (SP7), (B) runt-related transcription factor 2 (RUNX2), (C) insulin binding protein (IBP3), (D) collagen type III alpha 1 chain (COL3A1), (E) matrix remodeling-associated protein 8 precursor (MXRA8) and (F) fibrillin-1 (FBN1) in HOb cultures at 1, 3 and 7 days. Gene expression was normalized to blank wells (without any material) using the $2^{-\Delta\Delta Ct}$ method. Results are shown as means ± SE. The asterisks ($p \leq 0.05$ (*) and $p \leq 0.01$ (**)) indicate statistically significant differences between MT and MT1.5Mg.

osteogenesis, cell-matrix and focal adhesion and oxidative stress functions (Table 1).

Among the proteins linked to osteogenesis, we detected differential expression of proteins associated with Wnt, ERK/MAPK, PI3K/AKT, insulin signaling, Ras and mTOR pathways. After 1 day of culture, the exposure to MT1.5Mg increased the expression of seven proteins (PI4KA, PLCB4, RAB9A, RAB35, EIF3M, CO3A1 and CEMIP). Six proteins (CSK22, CUL1, PAIP1, 2A5D, RALB, and CO5A2) were downregulated. After 3 days of culture with MT1.5Mg, the MK01, IBP3, ST38L, STK24, and PP1B proteins were upregulated, and the GBB1, PLCG1, CO2A1 and CO4A2, downregulated. No proteins were upregulated after 7 days of incubation with MT1.5Mg (compared to MT), but the MP2K2 expression was significantly reduced. In the cell-matrix and focal adhesion group, some proteins associated with cytoskeletal regulation, actin, cadherin and microfibril binding and integrin, FAK and PAK signaling were differentially expressed. After 1 day of culture, the cells seeded on MT1.5Mg overexpressed seven proteins (PI4KA, ITA5, LPXN, ADSV, VNN2, MYO5A and CDC42) and underexpressed 1433S, ARHG2, CAD13, ITB5, PAXI, MYL3, RALB and YKT6. The cells cultured with MT1.5Mg for 3 days showed increased expression of MXRA8, PCDGL, MYLK, PTN11, ST38L, STK24, MA7D1 and MYO9B. In contrast, other seven proteins (LEG3, TES, FBLI1, LTBP2, VASP, RHOG and ITA6) were downregulated. After 7 days, no proteins were upregulated, and the expression of LTBP2 and RAB10 was reduced. The proteins associated with oxidative stress are involved in the antioxidant action, oxidative phosphorylation and superoxide radical degradation pathways. None of these proteins was upregulated after incubation with MT1.5Mg. However, two proteins (PRDX2 and GPX1) were downregulated after 1 day, three proteins (GSTO1, SODM and UCRI) after 3 days, and one protein (CATA) after 7 days of the assay.

4. Discussion

The complex interactions between biomaterials and biological systems are difficult to understand in their entirety. Using *omics* applications to obtain proteomic profiles of cells exposed to the biomaterials can overcome the problems associated with traditional methods of biological characterization. In this study, we characterized the proteome of HOb cells cultured in contact with sol-gel coatings doped with Mg. This element is involved in many physiological processes, from metabolic reactions to the cell membrane, DNA and protein structure maintenance.

As demonstrated by Martínez-Sánchez et al. [13], Mg extracts affect the expression of proteins associated with cell attachment, growth, differentiation, and survival in human umbilical cord perivascular (HUCPV) cells undergoing chondrogenesis. Thus, it was unsurprising to find (PANTHER analysis) that the MT1.5Mg mainly affected the metabolic reactions, nucleic acid metabolism, cytoskeleton, binding and transport functions. However, the observed effects on the proteomics profiles by Mg-doped coatings can be due to the internalization of Mg by the cell, but also due the surface-cell interaction since these two processes are concurrent.

Bone regeneration is a well-orchestrated process that involves numerous cell types and intracellular and extracellular molecular signaling pathways [14]. The PI3K/AKT pathway has been described as the central nexus between the signaling pathways responsible for osteoblast differentiation and homeostasis [15]. Mg is known for activating PI3K/AKT in rat calvarial osteoblasts [16]. Incubation with MT1.5Mg affected PI3K/AKT expression and other associated paths such as PTEN signaling, regulation of eIF4 and p70S6K, and p70S6K signaling. PI3K/AKT is a known controller of many osteogenic markers, such as collagen [17]. The expression of collagen alpha-1 (III) (CO3A1), collagen alpha-2 (V) (CO5A2), collagen alpha-1 (II) (CO2A1) and collagen alpha-2 (IV) (CO4A2) was significantly regulated by MT1.5Mg. COL3A1 was upregulated by MT1.5Mg after 7 days of culture. In bone, the major collagenous components are collagen type I, III, V and X. CO3A1 is essential for early osteoblast growth and the commitment of these cells to differentiation [18] and was upregulated after 1-day culture with the Mg-

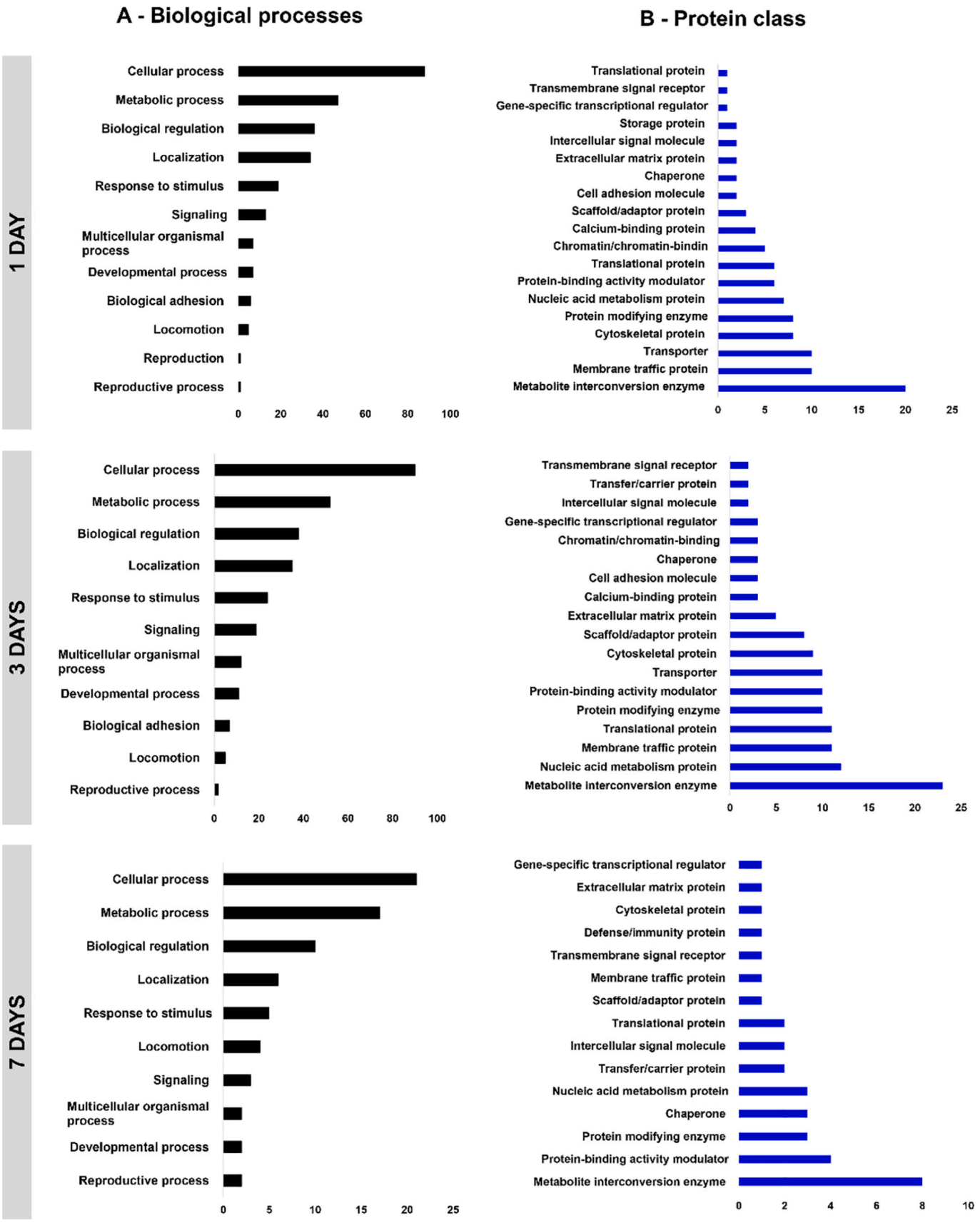

Fig. 3. Biological processes (A) and protein class (B) associated with the proteins differentially expressed by HOb cells seeded onto MT1.5Mg, after 1, 3 and 7 days of culture.

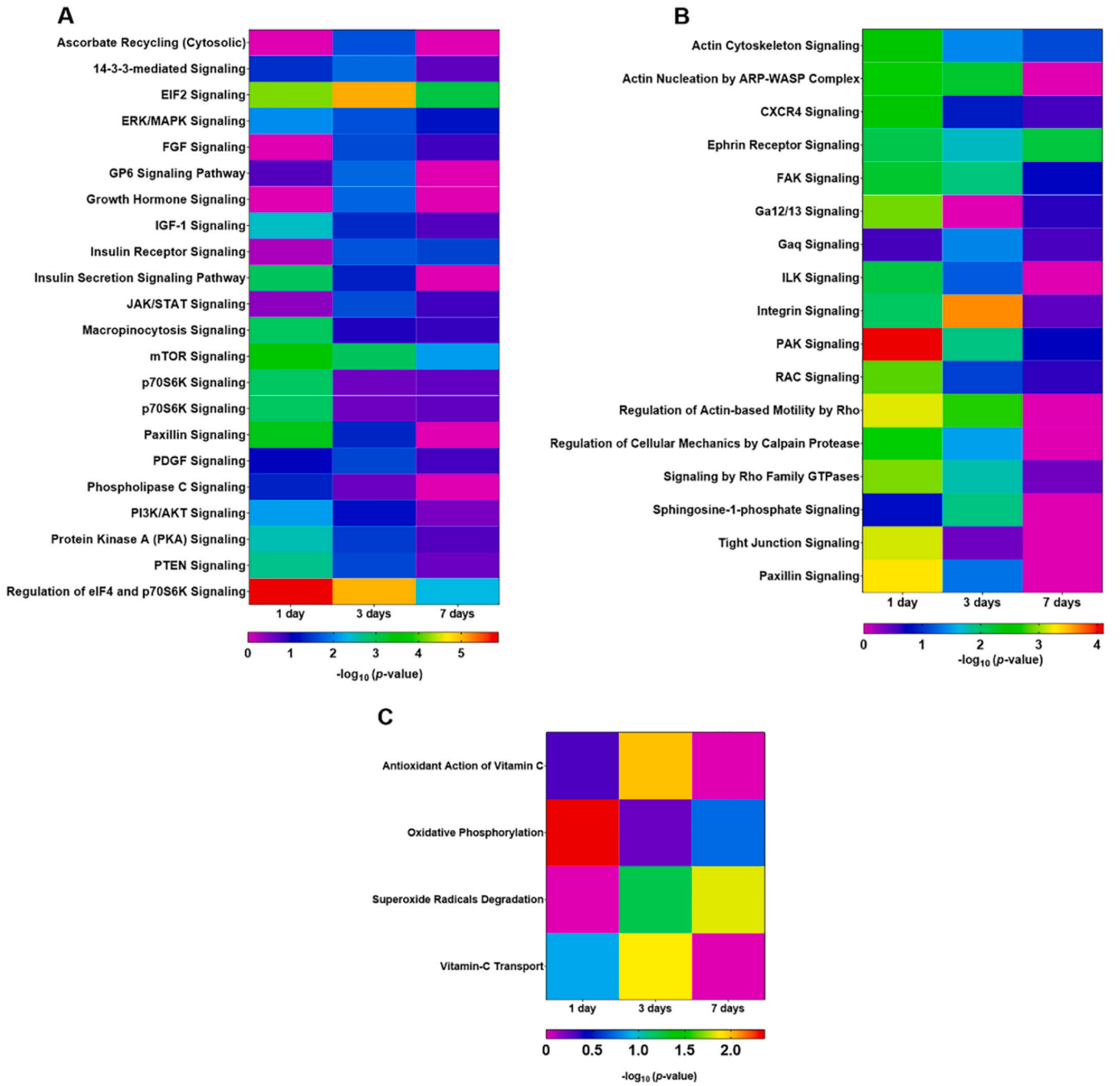

Fig. 4. Heat map of enriched canonical pathways associated with (A) osteogenesis, (B) cell-matrix and focal adhesion and (C) oxidative stress in HOb cells seeded onto MT1.5Mg after 1, 3 and 7 days of culture. Pathways were considered significantly regulated when −log(*p*-value) was bigger than 1.3.

doped material.

The IPA pathway analysis also showed that MT1.5Mg regulated mTOR expression at all time points. This pathway is associated with PI3K/AKT, and its activation *via* the p70S6K phosphorylation and eIF4 translation is a key step in initiating protein synthesis and cell growth [19]. The mediation of Wnt and IGF-1 signaling by mTOR is essential for osteoblast maturation and differentiation during bone formation [20,21], and RICTOR/mTOR loss in primary osteoblasts significantly decreases bone mass [22]. Liu et al. [23] have described mTOR expression enhancement in the presence of Mg, while Cappadone et al. [24] have shown that Mg depletion inhibits this pathway and reduces cell proliferation. Curiously, the eukaryotic initiation factor 3 (eIF3) was upregulated after a 1-day incubation with MT1.5Mg. Holz et al. [25] have described this protein as the mediator between mTOR/Raptor, eIF4 and S6K phosphorylation, acting as a scaffold in response to stimuli that promote efficient protein synthesis. Moreover, the pathway analysis revealed that MT1.5Mg significantly affected IGF-1 signaling, insulin receptor, and insulin secretion signaling. Insulin and IGF-1 are two important hormones regulating metabolism and growth. These two ligands activate insulin and IGF-1 receptors, respectively. This, in turn, will activate AKT and MAPK and regulate proliferation, differentiation, apoptosis, and metabolism [26]. IGF-1 is the most abundant growth factor in the bone matrix and regulates new bone formation by acting as a differentiation factor for osteoblasts [21]. In our study, after 3 days of culture with MT1.5Mg, the insulin-like growth factor binding protein-3 (IGFBP3) was significantly upregulated, and its gene expression increased after 7 days. The IGFBP3 is the third most abundant IGFBP protein found in osteoblasts; it binds to IGF-1. The binding of IGFBP3 to

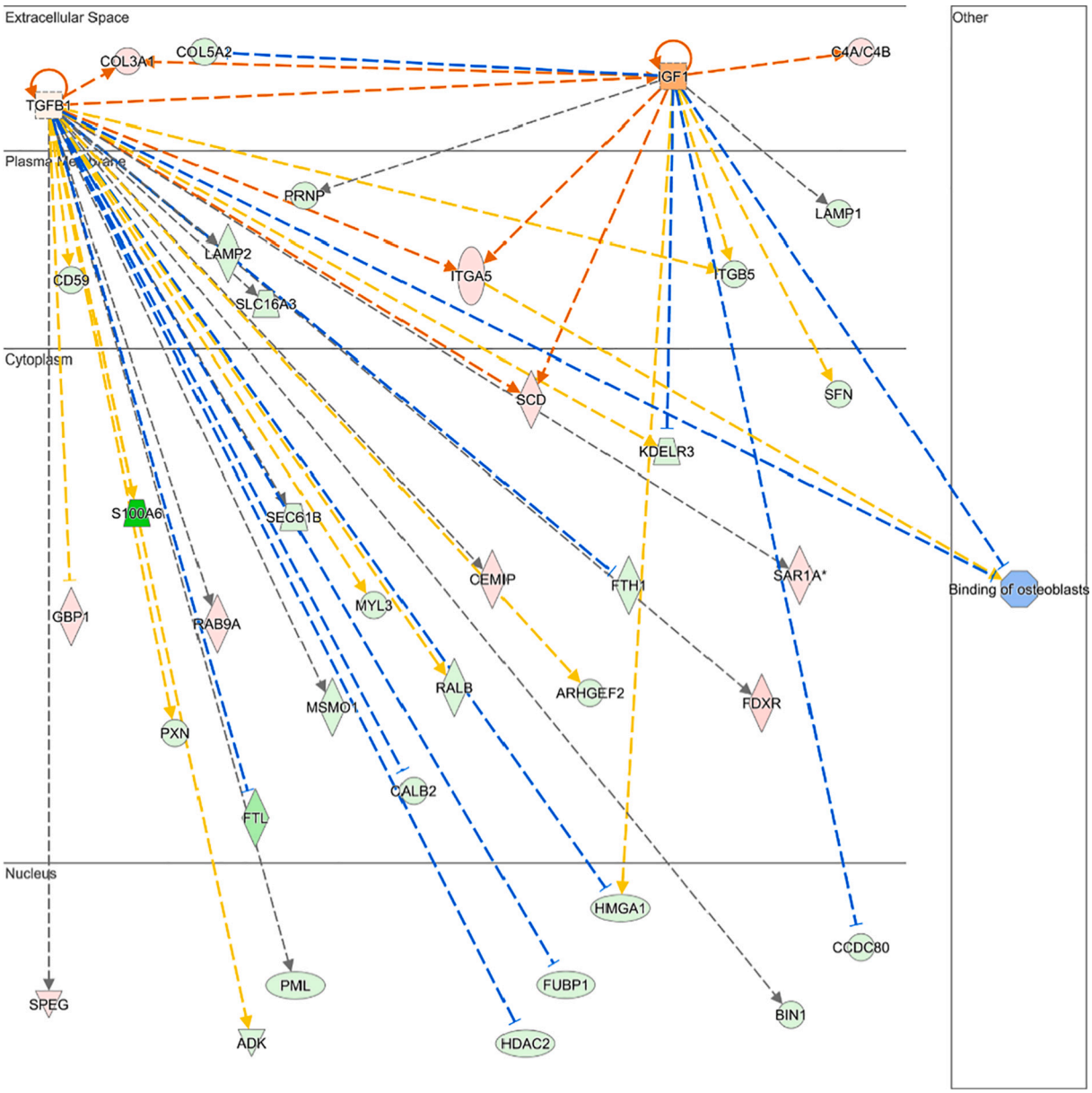

Fig. 5. The IPA® network analysis for associations among upstream regulators linked to TFGB1, IGF1, binding of osteoblasts and the relationships with proteins obtained from HOb seeded on MT1.5Mg (after 1 day of culture) that justify the predictions. Upregulated proteins are shown in red and downregulated in green. Orange lines indicate the *predicted activation*, and blue lines, the *predicted inhibition*. Yellow lines show the *findings inconsistent with the state of the downstream molecule*, and grey lines signify the *effect not predicted*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

IGF-1 leads to an increment in bone formation [21]. It has been shown that the local delivery of IGFBP3 using chitosan gold nanoparticles improves bone healing *in vivo* and enhances bone morphogenic protein (BMP) 2/7 and osteopontin (OPN) expression [27]. Moreover, the IGFBP3 is associated with transforming growth factor (TGF)-β, predicted here as upregulated by MAP. The TGF-β is a superfamily of ligands vital for cell growth, differentiation, apoptosis, cell motility and cell adhesion; IGFBP3 has been associated with its latency and amplification cascade [28]. TGF-β regulates several cell types directly involved in bone remodeling and fracture healing, and MT1.5Mg has been shown to increase its gene expression after 7 days *in vitro* [6]. Interestingly, the JAK/STAT pathway was also affected by MT1.5Mg. STAT, which MT1.5Mg also regulated, is a transcriptional promoter of IGF-1, vital in osteoblast proliferation and differentiation *via* the growth hormone signaling [29]. The growth hormone stimulates the production and secretion of IGF-1 and has well-described roles in incrementing bone density and length [30]. Mitogen-activated protein kinases (MAPKs) are a set of signal transducers of external stimuli that regulate cell proliferation, differentiation, and apoptosis [14]. The osteoblasts essentially express two isoforms of canonical extracellular signal-regulated kinases (ERK) MAPKs (MAPK1 and MAPK3); the ERK/MAPK pathway is known for positively regulating osteoblast activity and bone formation [31]. Wang et al. [32] have reported that Mg enhances MC3T3-E1 cell proliferation by increasing ERK phosphorylation. Moreover, stem cell differentiation can be augmented by Mg *via* the selective activation of ERK/MAPK and consequent upregulation of cAMP-responsive element-binding protein 1 (CREB1) and osterix (SP7) [33]. In our study, the ERK/MAPK expression was affected after 1 and 3 days of culture with MT1.5Mg. The MAPK1 (MK01) expression was enhanced after 3 days. Upon the ERK/MAPK activation, MAPK1 phosphorylates RUNX2 to increase its transcriptional activity during early osteoblast maturation [34]. Here, MT1.5Mg induced the expression of *SP7* and *RUNX2* after 1

Table 1
Proteins differentially expressed by cells grown on MT1.5Mg, with functions in osteogenesis, cell-matrix and focal adhesion and oxidative stress. The expression changes with $p \leq 0.05$ and a ratio higher than 1.5 in either direction (UP: increased and DOWN: reduced) were considered statistically significant.

Function	Pathways		1 day	3 days	7 days
Osteogenesis	PI3K/AKT mTOR Wnt MAPK Insulin signaling Ras	UP	PI4KA, PLCB4, RAB9A, RAB35, EIF3M, CO3A1, CEMIP	MK01, IBP3, ST38L, STK24, PP1B	–
	PI3K/AKT MAPK Wnt mTOR	DOWN	CSK22, CUL1, PAIP1, 2A5D, RALB, CO5A2	PLCG1, CO2A1, CO4A2	MP2K2
Cell-matrix and focal adhesion	Cadherin binding Integrin signaling Actin binding FAK signaling PAK signaling Cytoskeletal regulation	UP	PI4KA, ITA5, CDC42, LPXN, ADSV, VNN2	MXRA8, PCDGL, MYLK, PTN11, ST38L, STK24, MA7D1, MYO9B	–
	Cadherin binding Integrin signaling Actin binding PAK signaling FAK signaling Microfibril binding	DOWN	1433S, ARHG2, CAD13, ITB5, PAXI, MYL3, RALB, YKT6	LEG3, TES, FBLI1, LTBP2, VASP, ITA6, RHOG	RAB10, LTBP2
Oxidative stress	– Antioxidant action Oxidative phosphorylation Superoxide radicals degradation	UP DOWN	– PRDX2, GPX1	– GSTO1, SODM, UCRI	– CATA

and 7 days of culture. In similarity with these results, Cerqueira et al. [6] have demonstrated that MT1.5Mg has an increased affinity to VTNC and CYTA, which correlates with *RUNX2* and *SP7* expression induction in osteoblasts. The ERK/MAPK regulation can explain these results. Based on these results, we can conclude that the MT1.5Mg material affects osteogenesis through multiple cellular processes essential for osteoblast maturation.

Biomaterials used in bone tissue regeneration must act as substrates for cell adhesion, triggering signals that regulate cell differentiation, migration, and survival. The integrins, responsible for primary cell adhesion, link the intracellular actin and extracellular matrix (ECM) and regulate cellular responses and cytoskeleton arrangement [35]. Mg is known for its effects on the cytoskeleton arrangement and integrin expression [4]. The MT1.5Mg material studied here increases the cell area and integrin gene expression of MC3T3-E1 cells. It has enhanced affinity to proteins associated with cell adhesion, which might explain the well-known effects of Mg [6]. The current study found that this material induced protruding lamellipodia and filopodia and increased the HOb cell area. We demonstrated that MT1.5Mg affected several pathways associated with the cytoskeleton, such as the regulation of actin-based motility by Rho. Our results showed that the cell division control protein 42 homolog (Cdc42), belonging to the Rho GTPase family, was upregulated by MT1.5Mg. This protein is active in the lamellipodial regions of cells and is the main contributor to filopodium formation [36]. In addition, PAK signaling presented effects by MT1.5Mg at 1 and 3 days. PAKs are effectors of Cdc42, and the action of these proteins promotes polymerized actin structures formation (*i.e.*, lamellipodia and filopodia), integrin-based adhesion turnover, the bond to the ECM, and cell motility and adhesion [37]. PAKs also control myosins, actin-based motor proteins that allow the cytoskeletal regulation and contraction and cellular force maintenance [38]. Incubation with MT1.5Mg upregulated the expression of myosin-Va (MYO5A), myosin-IXb (MYO9B) and myosin light chain kinase (MYLK). The effect on myosin light chain 3 (MYL3) expression was reduced. These motor proteins need ATP to fuel their movement during their conformational changes. It has been demonstrated that Mg is an essential cofactor for ATP binding and hydrolysis in myosins. Mg^{2+} modulates the motor activity of five myosins, including MYO5A and MYO9B [39]. Moreover, Omidi et al. [40] have shown that pure Mg materials affect myosin expression.

Here, the IPA pathway analysis identified changes in the expression of integrin and ILK signaling. The effect of Mg on integrins has been reported by Zreiqat et al. [4]. These proteins are the membrane-associated adhesion receptors thought to be responsible for mediating cell–biomaterial interactions. In our earlier publication, we have reported that MT1.5Mg induces the integrin gene expression in MC3T3-E1 osteoblasts [6]. In the current study, we discovered that MT1.5Mg not only modulated the expression of integrin-β5 (ITB5), integrin-α5 (ITA5) and integrin-α6 (ITA6) but also affected the vascular non-inflammatory molecule 2 (VNN2), galectin-3 (LEG3) and matrix remodeling-associated protein 8 (MXRA8). In the cultures with MT1.5Mg, the expression of MXRA8 increased 47.5-fold. This protein, also known as DICAM, suppresses osteoclastogenesis *via* integrin-αVβ3 pathway attenuation and p38 MAPK inhibition [41]. It also promotes cell adhesion through specific binding to integrin-αvβ3 [42]. The integrin signaling is one of the most potent inducers of focal adhesion (FA) formation. FAs are protein assemblies essential for extracellular signal transduction into intracellular responses, acting as a bridge between the integrins and actin [43,44]. MT1.5Mg affected the focal adhesion kinase (FAK) signaling by downregulating paxillin (PAXI) and upregulating leupaxin (LPXN). These major components of FA are homologous and responsible for FAK binding to integrins [45]. Even though the PAXI and LPXN both target the FA, they have distinct roles in cell adhesion and spreading [45]. The expression of these proteins seems to depend on the cell lineage; it has been shown that the LPXN is responsible for cytoplasmic projections at osteoclast adhesion sites [46]. So far, the function of these proteins in HOb cells (or the effect of Mg on their expression) has not been described. Our results indicate that Mg effects on cell adhesion might go beyond integrin expression, allowing to hypothesize that Mg affects the whole process.

Oxidative stress is a normal response to biomaterial implantation, and the production of reactive oxygen species (ROS) occurs naturally during normal cellular metabolism [47,48]. ROS act as a signal in many intracellular signaling pathways; our results show that MT1.5Mg can regulate oxidative phosphorylation and superoxide radicals degradation. Proteins associated with the antioxidant system (PRDX2, GPX1, SODM, GTSO1 and CATA) were downregulated in the presence of MT1.5Mg. However, redox reactions are essential for cell adhesion. For example, Chiarugi et al. [49] have demonstrated that redox signaling is crucial for FAK and MAPK phosphorylation, focal adhesion formation and cell spreading. Fernandes et al. [47] have shown that an increment in ROS production and FAK phosphorylation accompanies the early stages of cell adhesion (30 min to 2 h). Moreover, Fiaschi et al. [50] have proved that ROS produced by integrins are essential for actin fiber formation during cell spreading. Thus, the downregulation of the antioxidant system can be a consequence of cell adhesion, especially considering that the expression of oxidative enzymes remains unaltered.

Our results show the potential of proteomics in biomaterial characterization and the effect of Mg-doped sol-gel coating on cellular responses. Even though more studies are needed to ensure that we can extrapolate the effects here observed to another types of Mg-based materials, it was possible to demonstrate that the effects of Mg on

osteoblasts are complex, regulating several pathways associated with osteogenesis, cell-matrix and focal adhesion and oxidative stress. This indicates that the observed changes result from overall, combined effects rather than alterations in individual processes.

5. Conclusions

The aim of this study was to analyze the effects of exposure of HOb cells to Mg-enriched coating. The cells were cultured for 1, 3, and 7 days *in vitro*. Their proteome was examined using mass spectrometry methods. Confocal microscopy and gene expression analysis were used for further characterization. The results showed that MT1.5Mg mainly regulated pathways of early osteoblast maturation (PI3/AKT, mTOR, ERK/MAPK), insulin metabolism (IGF-1, insulin secretion and receptor signaling, JAK/STAT), cell adhesion (integrin and FAK signaling, actin cytoskeleton regulation) and oxidative stress (oxidative phosphorylation and superoxide radicals degradation). We conclude that the proteomic methods are valuable tools for biomaterial characterization. The effects of Mg on the cell are rather complex, affecting the whole cellular machinery. The observed osteogenesis and cell adhesion modulation by Mg seem to be an overall effect rather than a result of alterations in single processes. Further biomaterial studies employing proteomics are needed to widen our understanding of responses induced by the biomaterials and improve their design.

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CRediT authorship contribution statement

Andreia Cerqueira: Formal analysis, Investigation, Data curation, Writing – original draft. **Iñaki García-Arnáez:** Investigation, Data curation. **Francisco Romero-Gavilán:** Investigation, Data curation, Writing – review & editing. **Mikel Azkargorta:** Investigation, Data curation. **Félix Elortza:** Resources, Investigation. **José Javier Martín de Llano:** Resources, Funding acquisition. **Carmen Carda:** Resources, Funding acquisition. **Mariló Gurruchaga:** Conceptualization, Writing – review & editing, Funding acquisition. **Isabel Goñi:** Conceptualization, Writing – review & editing, Funding acquisition. **Julio Suay:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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