# Human Osteoclasts/Osteoblasts 3D Dynamic Co-Culture System to Study the Beneficial Effects of Glucosamine on Bone Microenvironment

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# Abstract

**Background:** Glucosamine (GlcN) functions as a building block of the cartilage matrix and its multifaceted roles in promoting joint health has been extensively investigated. On the contrary, the role of GlcN in osteogenesis and bone tissue is poorly understood, mainly due to the lack of adequate experimental models. Consequently, the benefit of GlcN in bone disorders remains controversial. In order to broaden the pharmacological relevance and potential therapeutic/nutraceutic efficacy of GlcN, we investigated the effect of GlcN on human primary osteoclasts (hOCs) and osteoblasts (hOBs) that were grown with 2D traditional methods or co-cultured in a more complex culture system one step closer to the in vivo bone microenvironment, consisting in a three-dimensional (3D) dynamic system.

**Methods:** Osteoclasts derived from peripheral blood monocytes (hMCs) of either healthy or osteoarthritis (OA) donors, were used and kept in culture with osteoclastogenic inducers. hMCs were also combined with hOBs in a 3D dynamic co-culture system by using RCCS-4TM bioreactor in osteogenic medium without osteoclastogenic inducers. In this condition osteoclastogenesis was supported by hOBs and sizeable self assembling aggregates were obtained. The differentiated osteoclasts were evaluated by the tartrate-resistant acid phosphatase assay, osteogenic differentiation was monitored by analyzing mineral matrix deposition through Alizarin Red staining, and expression of osteogenic markers through RT-qPCR. The cells were treated with DONA® Crystalline Glucosamine Sulfate (i.e. the original GlcN sulfate product).

**Results:** DONA® was effective in decreasing the hOCs differentiation and function. Osteoclasts from OA donors were more sensitive than those from healthy donors. At the same time, DONA® showed anabolic effects on osteoblasts both in 2D conventional cell culture and in osteoclasts/osteoblasts 3D dynamic co-culture system.

**Conclusions:** Here we demonstrated the effectiveness of a 3D dynamic co-culture system to provide useful information on the spectrum of action of GlcN on bone microenvironment. This can pave the way to better define the potential applications of a compound such as GlcN which is positioned between pharmaceuticals and nutraceuticals. Therefore, based on our observations, we hypothesize that GlcN could have potential benefits either in the treatment of osteopenic diseases such as osteoporosis, or in the bone health maintenance.


# Introduction

Glucosamine (GlcN) is an aminosaccharide that acts as a preferred substrate for the biosynthesis of glycosaminoglycans and, subsequently, for the production of aggrecan and other proteoglycans in the connective and cartilage tissues [1]. GlcN supports the joint structure functioning as a building block of the cartilage matrix, and maintains joint health by preventing the tissue degradation, reducing the inflammation and oxidative stress, improving the autophagy response of the chondrocytes, and increasing the chondrogenic potential of mesenchymal stem cells resident in the niche [1, 2]. Moreover,

GlcN is an essential substrate for the synthesis of glycosylated proteins and lipids [3]. For its biological properties, GlcN is prescribed as a drug or a dietary supplement in the management of one of the most common joint disorders such as osteoarthritis (OA) to delay the progression of tissue degeneration and to diminish the symptoms in humans [1, 4, 5]. Moreover, GlcN is recommended for joint health to prevent sports-related cartilage injuries in athlete [6]. To date, GlcN preparations are the most widely used nutraceutical for OA [7, 8]. There are three commonly forms of GlcN supplements in the market: glucosamine hydrochloride, glucosamine sulfate, and N-acetyl glucosamine. The chondroprotective action of these glucosamine compounds, is supported both by in vitro evidences by using different in vitro and in vivo experimental models, and clinical trials [1]. Currently, the prescription of crystalline GlcN sulfate 1500 mg once daily is recommended by the majority of clinical practice guidelines in the OA management [9].

Considering that during the evolution of OA and disease progression there is a substantial subchondral bone metabolic alterations and remodeling [10], and that OA in the elderly is often accompanied by osteoporosis [11], it is critical to also consider how bone tissue may be affected by GlcN. To date, limited data are available about the action of GlcN on human osteoclasts and osteoblasts that populate the bone microenvironment [12–14]. It would be important to have information in this regard, in order to broaden the pharmacological relevance and potential therapeutic efficacy of GlcN in the broad scenario of skeletal diseases.

The primary purpose of the present pilot study was to examine the effects of GlcN on human osteoclasts (hOCs) we cultured both in conventional 2D monolayer and in a more complex culture system one step closer to the in vivo bone microenvironment, consisting in an osteoclasts/osteoblasts 3D dynamic co-culture system [15]. The employment of this in vitro model mimicking the process of bone matrix deposition and remodelling allows us to have information on the cell population of osteoclasts and osteoblasts at the same time.

We examined the effects of DONA® Crystalline Glucosamine Sulfate, (i.e. the original glucosamine sulfate product), on osteoclastogenesis which was achieved both by treatment with osteoclastogenic inducers or by the presence of osteoblasts. As osteoclast progenitors source, monocytes (hMCs) from peripheral blood of healthy or OA donors were used.

Here we demonstrated for the first time, as far as we know, that DONA® was effective in decreasing the osteoclastic cell differentiation and function. At the same time, DONA® appeared to have anabolic effects on osteoblasts both in 2D conventional cell culture and in osteoclasts/osteoblasts 3D dynamic co-culture system.

These findings may help elucidate the mechanisms underlying the effect of GlcN on human bone context, which are still unclear today. This can pave the way to better define the potential applications of a compound such as GlcN which is positioned between pharmaceuticals and nutraceuticals. Therefore, based on our observations, we hypothesize that GlcN could have potential benefits either in the treatment of osteopenic diseases such as osteoporosis, or in the bone health maintenance.

# Materials And Methods

## Reagents

DONA® Crystalline Glucosamine Sulfate was provided by Mylan S.r.l (Italy), resuspended at 50 mg/mL concentration and stored at 4°C. Histopaque®-1077, Ascorbic acid-2-phosphate, β-glycerophosphate, dexamethasone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Alizarin Red S (ARS), paraformaldehyde, Triton X-100, and Acid phosphatase, leukocyte (TRAP) Kit (no. 386), Fetal Calf Serum (FCS), L-Glutamine, antibiotics (penicillin and streptomycin) were purchased from Sigma-Aldrich (Saint Louis, USA). High Capacity cDNA Reverse Transcription Kit, TaqMan Gene Expression assays, Universal Master mix II and Alexa Fluor 488 Phalloidin (#A12379) were purchased from ThermoFisher Scientific (Waltham, MA, USA). Antibodies for human Runx2 (#sc-10758), COL1a1 (#sc-28657), NFATc1 (#sc-13033), Cathepsin K (#sc-48353), were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and OPN (LF-123) is a generous gift from dr L. Fisher (NIH, Bethesda, USA). Dulbecco's Modified Eagle's Medium High glucose (DMEM), Ham's F12 and PBS were purchased from Euroclone (Milan, Italy).

## Cell isolation and culture

Patients with osteoarthritis (OA patients, n=7, mean age 60 years, female) and healthy volunteers (n=4, mean age 45 years, female) were enrolled during routine medical check-up at "Centro di Medicina" (Ferrara, Italy) after informed consent, according to the Institution's research protocol approved (n. 172201). Briefly, PBMCs (Peripheral Blood Mononuclear Cells) were obtained from 20 mL of peripheral blood and separated by Histopaque®-1077 as previously described [16]. Monocytes (hMCs) were purified from PBMCs by adhesion selection on polystyrene plates. $1 \times 10^6$ PBMCs/$cm^2$ were plated, allowed to settle for 4 h at 37° and flasks were then rinsed to remove non-adherent cells. In order to confirm the ability of isolated hMCs to differentiate into mature osteoclasts (hOCs), M-CSF (25 ng/mL) and RANKL (30 ng/mL) (PeproTech EC Ltd, London, UK) were added to the culture medium and after 14 days, TRAP staining was carried out. The expression levels of the osteoclast-specific markers Cathepsin K, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) were assessed by immunocytochemistry.

Human osteoblasts (hOBs) were obtained from vertebral lamina, discarded during spinal surgery. Bone fragments were obtained from 4 donors (mean age 45 years, 2 males and 2 females) using research protocol approved by Ethics Committee of the University of Ferrara and S. Anna Hospital (protocol approved on November 17, 2016). Briefly, the bone fragments were placed in sterile phosphate buffered solution (PBS) at 4°C and dissected within 16 hours after removal. Bone chips were minced into smaller pieces as previously reported [17], washed twice with PBS, plated in T-25 culture flasks (Sarstedt, Nümbrecht, Germany) and cultured in 50% DMEM high-glucose/50% Ham's F12, supplemented with 10% FCS, 1 mM L-Glutamine, antibiotics (penicillin 100 µg/mL and streptomycin 10 µg/mL). Upon detection of a cell colony from the bone fragments (after 7 days), the cells were expanded until confluent (passage zero, P0). The cells were then harvested after treatment with 0.05 % trypsin ethylenediamine tetraacetic acid (Sigma-Aldrich), washed, counted by hemocytometric analysis, and used for further experiments

(passage 1 to passage 3). During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and the medium was changed every 3 days. hOBs (P0) were characterized for the presence of the following specific bone markers: Osteopontin (OPN), RUNT related transcription factor 2 (Runx2) and collagen type 1 alpha (COL1a1) by immunostaining.

For osteogenic differentiation, hOBs were seeded at a density of 10000/$cm^2$ in 12-well plates and cultured up to 14 days in osteogenic medium (OM) consisting in DMEM high-glucose 10% FCS, 10 mM β-glycerophosphate, 100 nM dexamethasone and 100 µM ascorbate, in the presence or absence of 200 µg/mL GLcN (treatment every 3 days).

### Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining of the cells was performed as previously reported [18]. Briefly, the cells were fixed in 4% paraformaldehyde (PFA) with 0.1 M cacodilic buffer, pH 7.2 (0.1 M sodium cacodilate, 0.0025% $CaCl_2$) for 15 min, extensively washed in the same buffer, and stained for TRAP (Acid Phosphatase Leukocyte Kit) according to the manufacturer's protocol. After washing with distilled water and drying, mature TRAP positive multinucleated cells containing more than three nuclei were counted as osteoclasts.

### Immunocytochemistry

Immunocytochemical analysis was performed using an ImmPRESS Universal Reagent kit (Vector Laboratories, Inc., Burlingame, CA, USA). The hOCs or hOBs were seeded in 24-well plates, fixed in cold 100% methanol at room temperature (RT) for 10 min and permeabilized with 0.2% (vol/vol) Triton X-100, in Tris-buffered saline (TBS 1X). Then the cells were treated in 0.3% $H_2O_2$ in TBS 1X for 10 min at RT, and subsequently incubated with ready-to-use (2.5%) normal horse serum blocking solution (ImmPRESS reagent kit; Vector Laboratories, Inc.) for 15 min at room temperature.

After the incubation in blocking serum, the different primary antibodies were added and incubated at 4 °C overnight: rabbit anti-human polyclonal antibodies for Runx2 (1:200 dilution), COL1a1 (1:100 dilution), NFATc1 (1:300 dilution), Cathepsin K (1:200 dilution) and OPN (1:200 dilution). After rinsing in TBS 1X, the cells were incubated for 30 min at room temperature with ImmPRESS reagent (ImmPRESS reagent kit; Vector Laboratories, Inc.) and then stained with substrate/chromogen mix (ImmPACT™ DAB; Vector Laboratories, Inc.). After washing, the cells were mounted in glycerol/PBS 9:1 and observed with a Nikon Eclipse 50i optical microscope (Nikon Corporation, Tokyo, Japan).

### MTT assay

The effect of GLcN on hOCs and hOBs viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The cells were seeded in 96-well plates and treated with increasing concentrations of GLcN (10-100-200 µg/mL). After 72 hours of treatment, a solution of MTT in PBS was added to each well and the plate was incubated for 3 hours at 37°C. The MTT crystals were solubilized with 200 µL lysis buffer (10% Sodium dodecyl sulfate, SDS).

Spectrophotometric absorbance of each sample was then measured at 570 nm by using a microplate reader (Sunrise™ Absorbance Reader, Tecan Group Ltd., Männedorf, Switzerland). Live cells were calculated as a percentage of control (untreated cells).

### Apoptosis (TUNEL assay)

At the end of osteoclastogenic induction mature hOCs were treated with GLcN (100 and 200 µg/mL) for 72 hours. The cells were then rinsed twice with PBS and fixed for 25 min in 4% PFA at room temperature. Apoptotic cells were detected by the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Moreover, all cells were subjected to hematoxylin staining, showing blue stained nuclei. The cells were mounted in glycerol/PBS 9:1 and observed under a Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany). The measurement of apoptosis was calculated as a percentage of apoptotic nuclei (dark brown nuclei) vs total nuclei of multinucleated TRAP positive cells, evaluated in triplicate from each experimental sample.

### Phalloidin staining

For analysis of F-actin organization, hOCs were fixed with 4% PFA for 10 min, permeabilized with 0.1 % Triton X-100 for 15 min and stained with Alexa Fluor 488 Phalloidin (1:500 dilution in PBS) for 30 min at room temperature. Nuclei were counterstained with 4',6- diamidino-2-phenylindole (DAPI). Fluorescent Images were obtained with a fluorescence microscopy (Nikon Eclipse 50i).

### RNA isolation and RT-qPCR

Total RNA was isolated from hOBs (2D cultured in OM in the presence or absence of GLcN 200 g/mL), by using the RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentration and quality was measured using a NanoDrop ND1000 UV-VIS spectrophotometer (Isogen Life Science, de Meern, the Netherlands). cDNA was synthesized from total RNA in a 20 µL reaction volume using the High Capacity cDNA Reverse Transcription Kit. Finally 100 ng of cDNA was used for RT-qPCR analysis. The TaqMan Universal Master Mix II and probes for human ALP (Hs01029144_m1), RUNX2 (Hs00231692_m1), OPN (Hs00959010_m1), COL1A1 (Hs00164004_m1), OCN (Hs01587813_g1), BSP (Hs00913377_m1). were used according to the manufacturer's instructions. RPL13a (Hs04194366_g1) was used for normalization of mRNA abundance. The gene expression was assessed using the CFX96TM PCR detection system (Bio-Rad, Hercules, CA, USA). Relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method (expressed as fold change). All reactions were performed in triplicate, n=4.

### hOBs/hMCs cultured in 3D dynamic system

The 3D dynamic culture condition was set up by using the RCCS-4TM bioreactor (Synthecon™, Inc., Houston, TX, USA), with a High Aspect Ratio Vessel (HARV™; Synthecon™, Inc., Houston, TX, USA). The HARV consists of a horizontally rotated culture chamber, where the cells are suspended, and a perfusion

system with media continuously flowing through the culture chamber. The culture chamber can rotate in the X-axis at certain speeds (rpm): higher rpm is associated to lower gravity. The rotation speed applied for the experiments was 4 rpm corresponding to Ground Based dynamic culture in which aggregates were in continuous falling rotation close to the bottom of the vessel (3D-DycC condition).

hOBs/hMCs aggregates were generated in the absence of exogenous scaffolds as previously reported [15, 18]. Briefly, $1x10^6$ cells/mL hOBs and $500x10^3$ cells/mL hMCs (2:1 cell ratio) were inoculated in 2 mL HARV filled with DMEM high glucose supplemented with 10% FCS and all air bubbles were removed from the culture chamber. HARV was then inserted into the RCCS-4$^{TM}$ rotary bioreactor and placed in an incubator at 37 °C, in a humidified atmosphere with 5% $CO_2$. After 24 hours, the presence of aggregates was observed, and the vessels were filled with osteogenic medium alone (OM) or in the presence of 200 µg/mL GlcN (OM/ GlcN). Osteogenic medium and treatment with GlcN was refreshed twice a week. After 14 days the aggregates were collected, fixed in 4% PFA, embedded in paraffin for further analysis.

## Histology

Immunohistochemistry was performed employing the ImmPRESS (Vectorlabs, Burlingame, CA). Histological sections (5 µm) of aggregates were subjected to immunohistochemistry. Non-consecutive sections were deparaffinized, rehydrated and enzymatic treated with 1 mg/mL protease K (Sigma-Aldrich) for antigen retrieval and permeabilization. Slides were then immunostained overnight with the primary antibody against osteopontin (OPN; rabbit anti-human, 1:100 dilution), in a humid chamber at 4 °C, followed by treatment with Vecstain ABC reagent (Vectorlabs) for 30 min. The reaction were developed using DAB solution (Vectorlabs); the sections were counterstained with haematoxylin, mounted in glycerol and observed using the Nikon Esclipse 50i optical microscope.

For Alizarin Red Staining (ARS) the sections were deparaffinized and stained with 40 mM Alizarin Red S solution (pH 4.2) at room temperature for 20 min. TRAP staining was carried out with the Acid Phosphatase Leukocyte (TRAP) Kit according to the manufacturer's protocol. The stainings were quantified by a computerised video camera-based image analysis system (NIH, USA ImageJ software, public domain available at: http://rsb.info.nih.gov/nih-image/) under brightfield microscopy (Nikon Eclipse 50i; Nikon Corporation, Tokyo, Japan). Colour TIFF file images were converted to 32-bit images and inverted so that the background could be set to the lower threshold limit. After applying the image threshold, the background was removed and not counted toward mean pixel intensity. Mean pixel intensity per area was used for the histological images quantification of OPN, (five sections per sample; n= 3). The percentage of positive area was applied for the quantification of ARS and TRAP staining, considering the tears/holes within matrix of samples.

## Statistical analysis

All data were presented as mean ± standard deviation (SD). Two-tailed Student’s t-tests were used to determine statistical significance. All tests were done using GraphPad Prism 5 program (La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

# Results

### GlcN induces apoptosis and decreases differentiation of osteoclasts from OA patients

Monocytes (hMCs) from peripheral blood of healthy or OA donors were used as osteoclast progenitors source, as described in the Material and Methods section. The ability of hMCs to differentiate into mature multinucleated osteoclasts (hOCs) was demonstrated by analysing the presence of typical osteoclast markers such as tartrate resistant acid phosphatase (TRAP), Cathepsin K and Nuclear factor of activated T-cells, cytoplasmic 1 (NFAT) during osteoclastogenic induction. After showing that the exposure to different GlcN concentrations (10-200 g/mL) did not affect osteoclast viability (Supplemental Fig. 1A), the below reported experiments were performed.

To investigate the effect of GlcN on hOCs, we planned the experimental strategy according to the scheme reported in Fig. 1, taking into account that the low cell number achievable from each patient will reduce the experimental analysis that can be performed.

Microscopic observations revealed that the number of multinuclear hOCs both from healthy or OA donors remains substantially unchanged after GlcN treatment (data not shown). At the end of the differentiation, the apoptosis level was assessed by Tunel assay (Fig. 2). The results demonstrated that GlcN treatment induced dose-dependent cell apoptosis in hOCs from OA patients, while hOCs from healthy donors underwent GlcN-induced DNA fragmentation only after exposure to a concentration of 200 g/mL.

Considering that cytoskeletal rearrangements are a prerequisite for bone resorption by osteoclasts [19], we next investigated the effect of GlcN on hOCs differentiation, by staining with FITC-conjugated phalloidin to evaluate actin ring formation. As shown in Fig. 3, GlcN treatment significantly decreased the polymerization of F-actin in a circular fashion in hOCs from OA patients, but not in hOCs from healthy donors.

### GlcN positively affects the osteoblast activity in osteoclasts/osteoblasts three-dimensional co-culture system

We then moved to investigating osteoclast response to GlcN when combined with osteoblasts (hOBs) in a three-dimensional culture system. The aim was on the one hand to validate the hOCs responsiveness to GlcN in an experimental condition which more closely resembles the in vivo bone microenvironment and, on the other, to try to understand if human osteoblasts, in turn, could represent a GLcN target. Osteoclast precursors (hMCs) were combined with hOBs in a 3D dynamic co-culture system, according to a previous protocol, in presence of osteogenic medium without osteoclastogenic inducers [18]. In this condition osteoclastogenesis was supported by hOBs and the cells were able to produce sizeable self assembling aggregates (Fig. 4). After exposure to GlcN we observed that the extent of TRAP positive area significantly decreased (Fig. 4).

Interestingly, GlcN showed a positive impact on osteoblast activity. In fact, a significant increase of both mineral matrix deposition and expression of a typical osteogenic marker such as osteopontin (OPN), was

found in cellular aggregates GlcN treated (Fig. 4). These results demonstrate that GlcN is effective not only in inhibiting the activity of osteoclasts, but also in enhancing the activity of osteoblasts.

This last aspect has been further explored and a larger number of osteogenic markers have been analyzed by expanding the hOBs in 2D conventional culture. As shown in Fig. 5, GlcN induced a general increase in early and middle stage osteogenic markers such as RUNT related transcription factor 2 (Runx2), collagen type I (COL1a1), alkaline phosphatase (ALP), osteopontin (OPN), and bone sialoprotein (BSP) [20]. In particular, a significant increase of expression was observed for Runx2 transcription factor which is considered the master regulator of osteogenesis [20] and OPN. No significant variations were observed for osteocalcin (OCN), the late differentiation marker.

# Discussion

Despite considerable knowledge of the biological activities of glucosamine (GlcN) including chondroprotective and anti-inflammatory action [1–7], its role in osteogenesis and bone tissue remains to be investigated in detail. The evidence collected so far on bone cells is mainly based on the use of monolayered non-human cell lines such as mouse MC3T3-E1 [14] and RAW 264.7 [21], fetal osteoblastic cell line hFOB1.19 [22], or animal models such as rat, mice or rabbit receiving GlcN oral administration [23–25].

Here we focused our attention on cells from human bone microenvironment, being we able to rely, in a first step, on peripheral blood samples of patients with OA. Since these patients were outpatients who did not require surgery, it was possible to obtain only a limited amount of peripheral blood, sufficient in any case to produce the osteoclast precursors for a comparative study with osteoclasts from healthy donors. Our data demonstrated that OA and healthy osteoclasts are differently susceptible to GlcN treatment which mostly decreased cell differentiation and function of OA osteoclasts.

These evidences led us to investigate the effects of GlcN in a more complex culture system one step closer to the in vivo bone microenvironment, consisting in an osteoclasts/osteoblasts three-dimensional (3D) dynamic co-culture system. With this approach we validated the catabolic effect of GlcN on osteoclast behavior, but above all we were able to investigate the responsiveness of human osteoblasts. After GlcN treatment, osteoblasts increased mineral matrix deposition and expression of specific differentiation markers such as OPN, demonstrating the ability of GlcN to exert anabolic effects. When GlcN treatment was performed on 2D conventional osteoblast culture these evidences were confirmed, demonstrating that GlcN supports favourable conditions for osteogenic differentiation and maintenance of osteoblastic phenotype.

The opposite responsiveness of bone cell populations certainly deserves further study, however, our data candidate GlcN to be evaluated with particular interest in a broader treatment/therapeutic plan regarding both joint and bone.

It is important to underline that identifying molecules capable of modulating simultaneously the activity of osteoblasts and osteoclasts is an important benefit for patients affected by bone loss, as it means being able to control a complex balance [26, 27]. It is in fact well known that the bone deposition by osteoblasts and resorption by osteoclasts are tightly coupled, and their balance defines both mass and quality of bone tissue [26]. Setting up culture conditions one step closer to the in vivo bone microenvironment as we here reported, represents an interesting perspective for at least two aspects: i. obtaining informative data on still controversial efficacy of biological agents such as GlcN, and ii. carrying out a patient-oriented research. This last aspect is based on the possibility of generating autologous osteoclasts/osteoblasts 3D co-cultures with cells from the same patient who needs orthopedic surgery and, in addition to peripheral blood, may provide bone fragments. Therefore, the employment of such an approach may further help both in the understanding the role of GlcN in bone tissue homeostasis, and in the development of patient-tailored nutraceuticals and pharmaceuticals treatments [28, 29].

The effects of GlcN we demonstrated here are in agreement with the only study, as far as we know, on human bone microenvironment by Tat et al [12]. In this paper, the authors studied the effect of chondroitin sulfate, glucosamine sulfate, and vitamin D3 on altered osteoblast metabolism of subchondral bone of OA patients, demonstrating that GlcN decreased the OA osteoblast pro-resorptive activity by modulating OPG/RANKL signaling [12].

To date, understanding how the altered bone remodeling that supports the development of both osteoarthritis and osteoporosis can be counteracted by adequate glucosamine treatment in terms of doses and intake, remains an open question. For this reason, clinical studies have to be accompanied by the development of suitable preclinical experimental models that provide useful information on exact mechanisms of action underlying GlcN beneficial effects.

It is worth mentioning that, in addition to the well-known role of its participation in the synthesis of components of the extracellular matrix [1, 30], GlcN is the precursor of N-Acetyl-glucosamine which is added to serine and threonine residues of nuclear and cytoplasmic proteins in the O-GlcNAcylation post-translational modification [31]. O-GlcNAcylation plays a critical role in the regulation of both cellular homeostasis, in response to nutritional or hormonal cues, but also in response to stress or damage [32]. A recent study reported that an increase of global O-GlcNAcglycosylation occurs during the early stages of osteoblast differentiation in MC3T3-E1 cells, but not during osteoclastic differentiation of RAW264 cell line [33]. Considering that acute and chronic alterations in the amount of O-GlcNAcylated proteins have been associated with different human diseases [34], a key point that need to be paid attention will be to clarify the involvement of O-GlcNAc glycosylation in the altered bone metabolism and in the modulation of osteogenic gene expression in human bone cells.

As a whole, our observations provide evidence that compounds such as GlcN that can be positioned between pharmaceuticals and nutraceuticals deserve to be further studied for developing new approaches for bone health maintenance and treatment of bone diseases.

# Abbreviations

ALP
alkaline phosphatase; ARS:Alizarin Red Staining; BSP:bone sialoprotein; COL1a1:collagen type I; DMEM:Dulbecco's Modified Eagle's Medium; FCS:Fetal Calf Serum; GlcN:glucosamine; HARV:High Aspect Ratio Vessel; hMCs:human monocytes; hOCs:human osteoclasts; hOBs:human osteoblasts; MTT:3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OA:osteoarthritis; OCN:osteocalcin; OPN:osteopontin; PBMC:Peripheral Blood Mononuclear Cells; Runx2:RUNT related transcription factor 2; SD:Standard deviation; TRAP:Tartrate-resistant acid phosphatase

# Declarations


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### Authors' contributions

LP designed the research, performed the experiments and analyzed the data. EL designed the research, performed the experiments and analyzed the data. AP analyzed the data and reviewed the manuscript. DM analyzed the data and reviewed the manuscript. VS coordinated the study and helped with interpretation of data. RP designed the research, wrote and edited the manuscript. All authors have read and approved the final manuscript submitted for publication.


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### Availability of data and materials

All data generated and analyzed in this study are disclosed in this article.

### Ethics approval and consent to participate

This study was approved by Ethics Committee of the University of Ferrara and S. Anna Hospital (protocol approved on November 17, 2016), and by "Centro di Medicina" (Ferrara, Italy) after informed consent, according to the Institution's research protocol approved (n. 172201).

### Consent for publication

Informed consent was obtained from all participants.

### Competing interests

The authors declare that they have no competing interests.

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# Figures

FIGURE 1

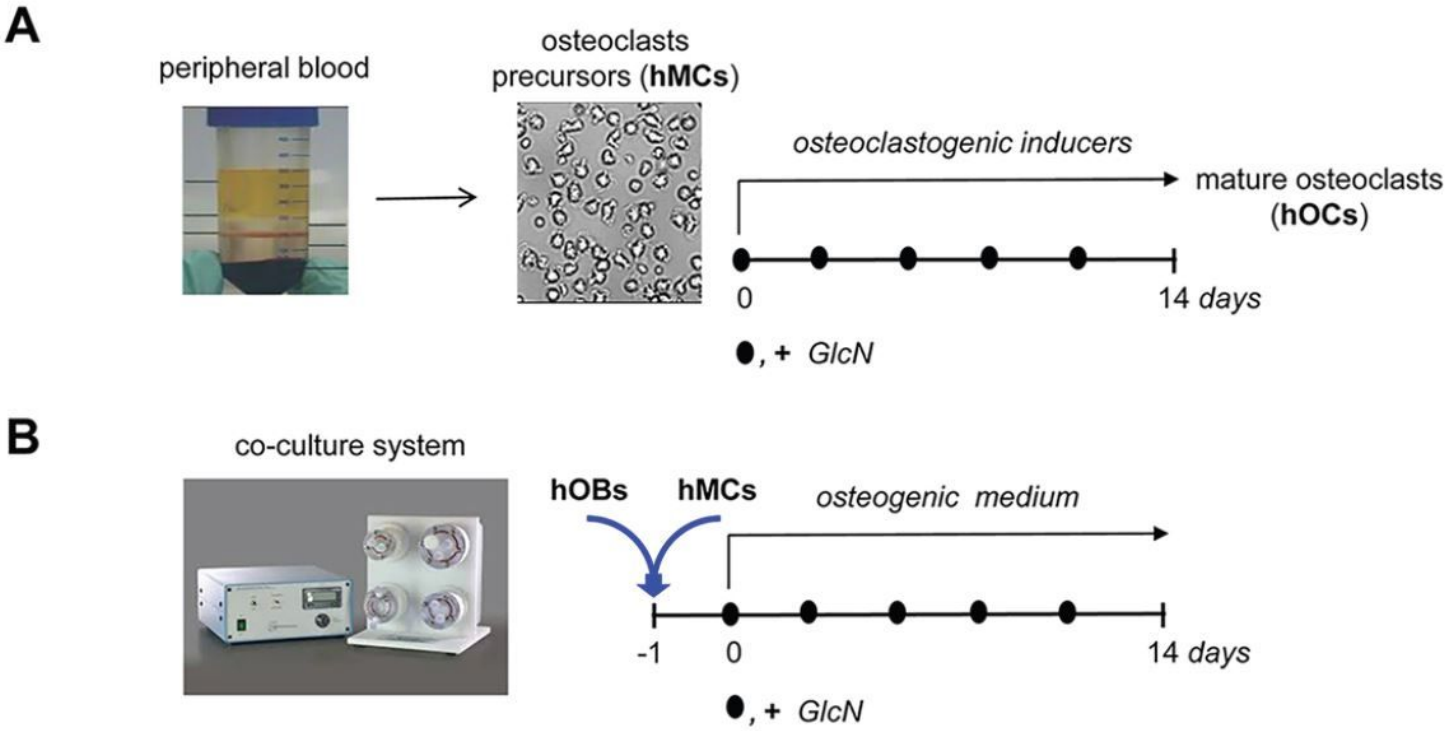



**Figure 1**

Experimental set-up. (a) mature osteoclasts (hOCs) were obtained after culturing for 14 days in osteoclastogenic medium the osteoclast precursors (human monocyte cells; hMCs) from healthy donors or OA patients peripheral blood. (b) hMCs were co-cultured with human osteoblasts (hOBs) in a 3D dynamic system represented by RCCS™ (rotary cell culture system) to generate self-assembled aggregates in osteogenic medium. In both cases cell cultures were also exposed to GlcN with a renewed treatment every three days.

FIGURE 2

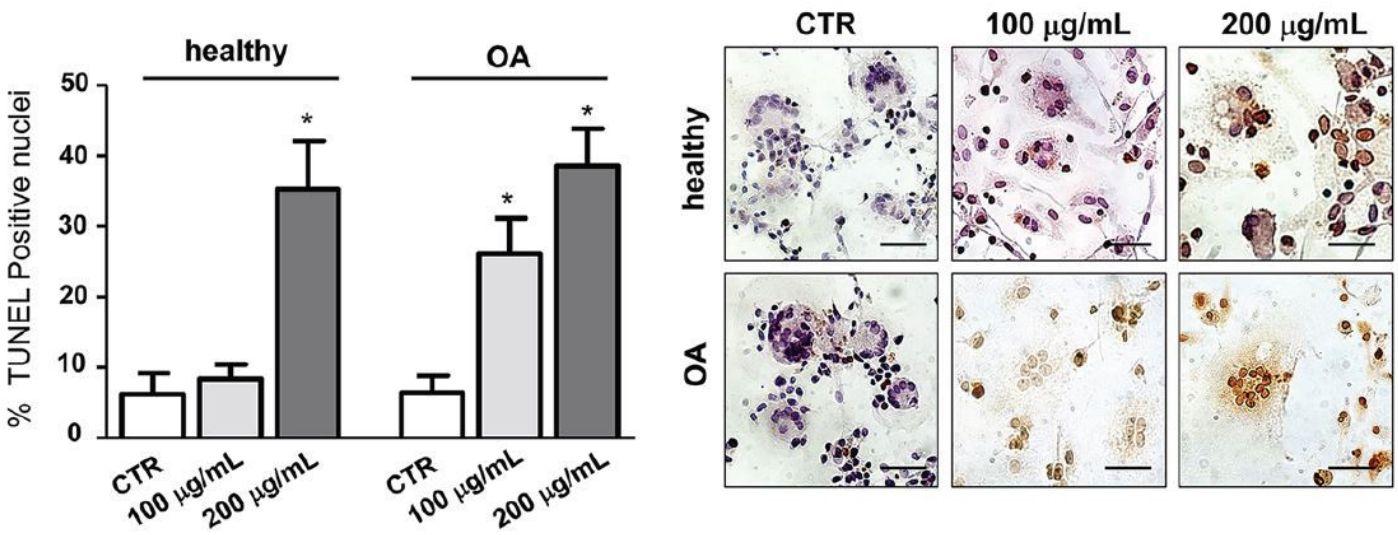



**Figure 2**

Effect of GlcN on apoptosis. hOCs were incubated with 100 and 200 g/mL of GlcN for 72 h or remained untreated (CTR) and then subjected to TUNEL assay to detect apoptosis. The cells were counterstained with ematoxylin. Quantitative results are reported in the graphs and presented as a percentage of TUNEL-positive nuclei (dark brown) vs total nuclei. Results are expressed as mean ± SD. Healthy donors, n = 3; OA patients, n = 7. *p < 0.05 vs CTR. Representative images of the cells after treatment are reported. Scale bars: 50 µm.

FIGURE 3

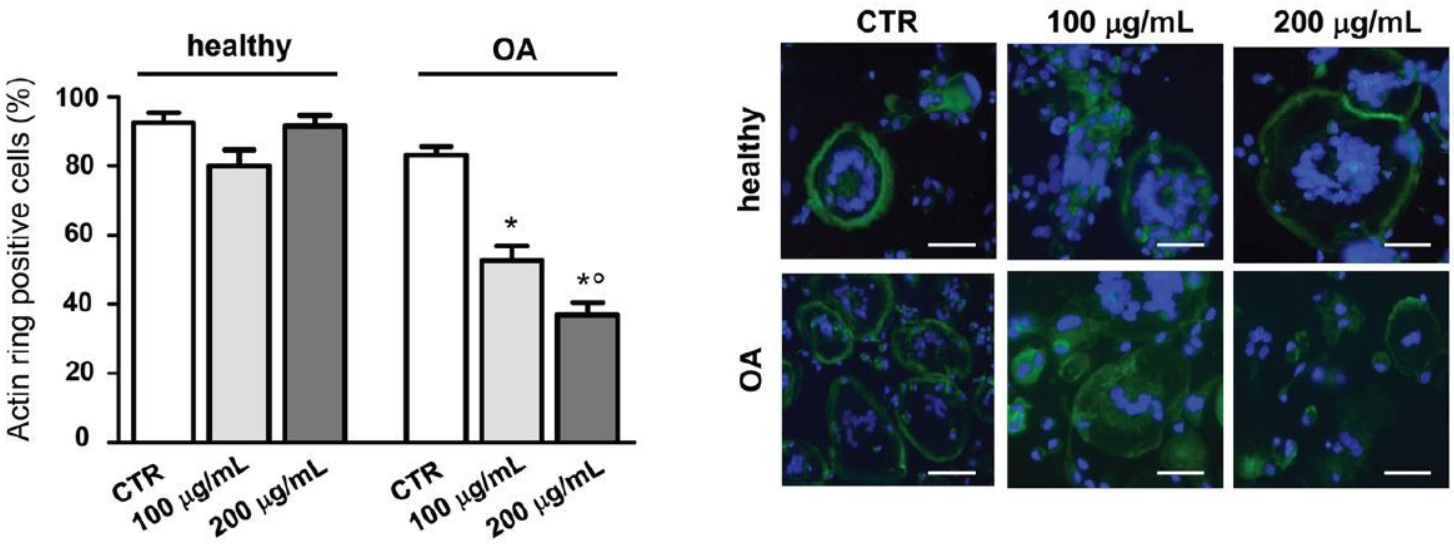
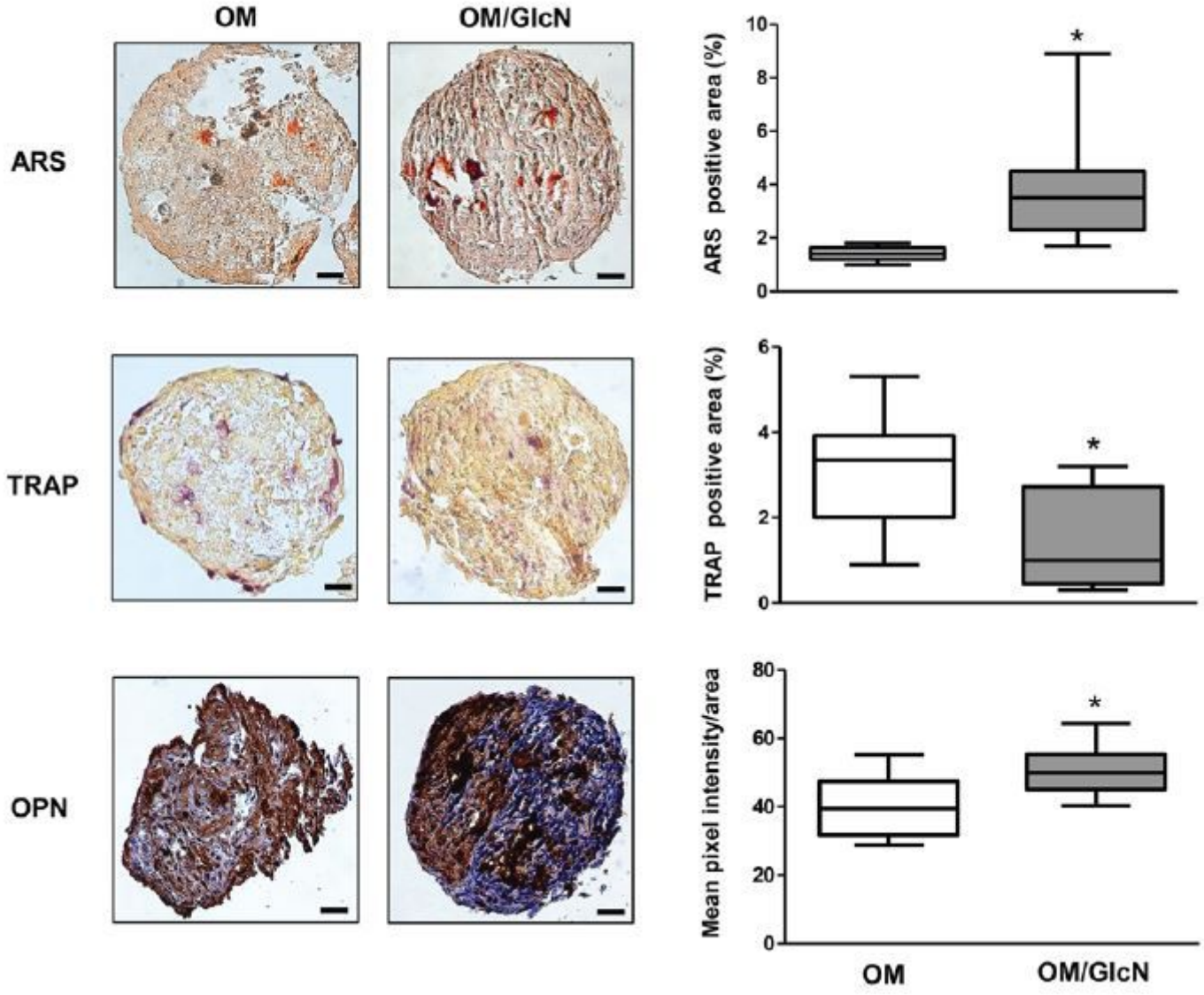



**Figure 3**

Effect of GlcN on actin ring formation. hMCs were cultured in osteoclastogenic medium in the absence (CTR) or in the presence of GlcN (100 and 200 □g/mL) for 14 days. Osteoclast actin rings were analyzed by using Alexa Fluor 488 Phalloidin (1:50 dilution) staining and fluorescence microscope (Nikon Eclipse 50i). Data are expressed as percentage of actin ring positive cells relative to total amount of cells, and evaluated by two independent investigators on 10 randomly selected optical fields. Results are expressed as mean ± SD. Healthy donors, n = 3; OA patients, n = 7 (*p<0.05 vs CTR; ° p< 0.05 vs 100 □g/mL). Representative images of the cells after treatment are reported. Nuclei were counterstained with DAPI (blue). Scale bars: 50 μm.

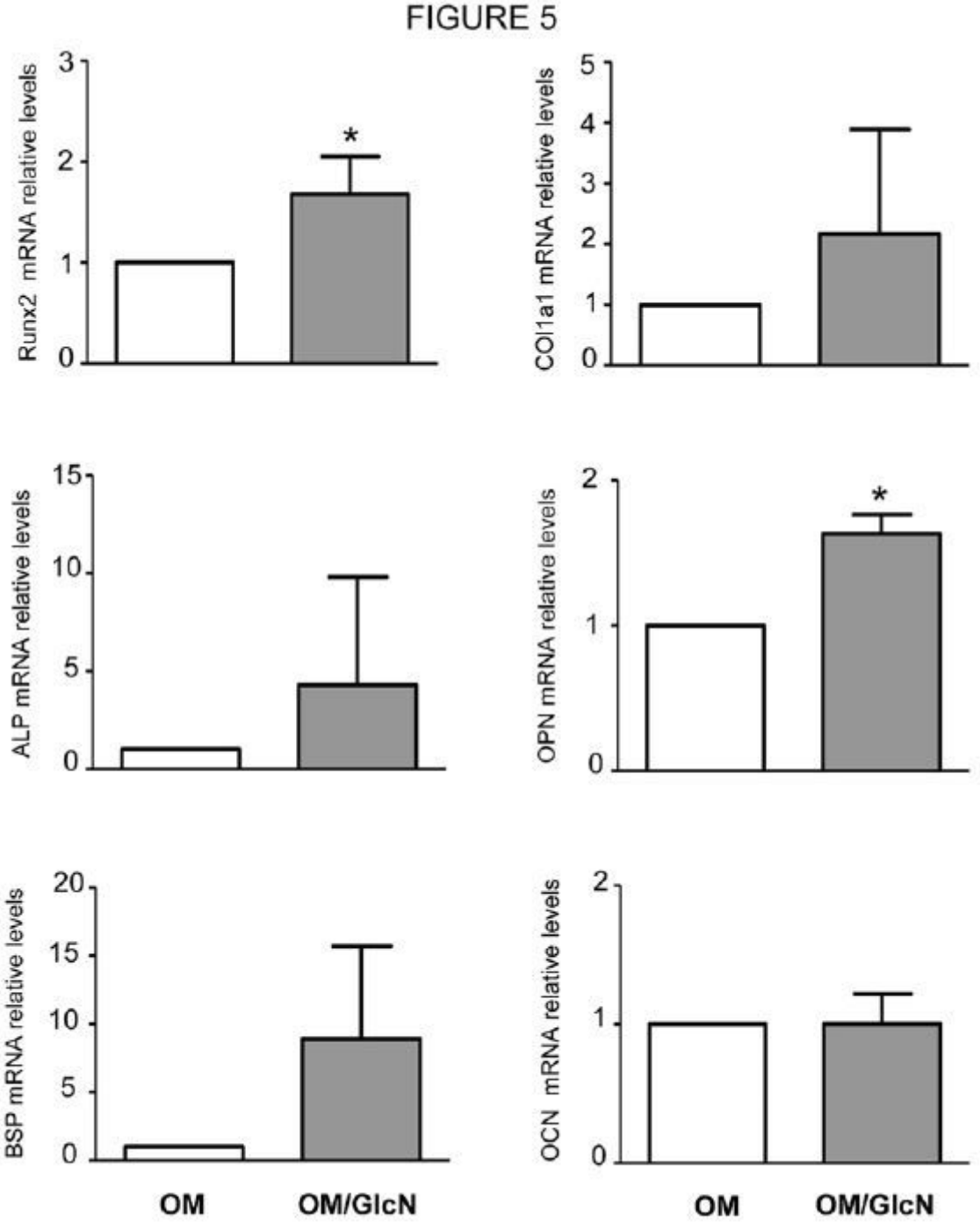
FIGURE 4




## Figure 4

Responsiveness to GlcN of osteoclasts and osteoblasts in the 3D dynamic co-culture system. hMCs were co-cultured with hOBs for 14 days in the 3D dynamic system, represented by RCCS™ (rotary cell culture system) in osteogenic medium alone (OM) or added with 200 μg/mL of GlcN (OM/GlcN). GlcN treatment was repeated every 3 days. Representative microphotographs of Alizarin Red (ARS, red staining), TRAP

assay (TRAP, purple staining) and osteopontin (OPN, dark brown staining) are reported. TRAP activity and ARS were quantified by ImageJ software and expressed as percentage of positive area (mean value ± SD, five sections per sample, n = 4). OPN levels were quantified by ImageJ software and expressed as mean pixel intensity per area (mean value ± SD, five sections per sample, n = 3). OM (white column), OM added with GlcN 200 □g/mL (OM/GlcN, grey column). *p < 0.05 vs OM. Scale bars: 50 μm.




## Figure 5

Effect of GlcN on hOBs in 2D conventional culture. The expression of typical osteogenic markers was analyzed in hOBs cultured in osteogenic medium alone (OM) or added with 200 μg/mL of GlcN

(OM/GlcN) for 14 days. GlcN treatment was repeated every 3 days. Total RNA was purified, and the mRNA expression of RUNT related transcription factor 2 (Runx2), collagen type I (COL1a1), alkaline phosphatase (ALP), osteopontin (OPN), bone sialoprotein (BSP) and osteocalcin (OCN) were evaluated by RT-qPCR. Relative expression levels are shown with regard to control cells mantained in osteogenic medium (OM) (value = 1) and normalized against the reference gene RPL13a. All reactions were performed in triplicate. Results represent means ± SD (n = 4). *p <0.05.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFIG.S1.pdf