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Running head: Menaquinone-4 Enhances Osteogenic Differentiation

Menaquinone-4 enhances osteogenic potential of human Amniotic Fluid Mesenchymal Stem Cells cultured in 2D and 3D dynamic culture system.

Domitilla Mandatori[1], Letizia Penolazzi[3], Caterina Pipino[1], Pamela Di Tomo[1], Sara Di Silvestre[1], Natalia Di Pietro[2], Sara Trevisani[3], Marco Angelozzi[3], Mariangela Ucci[1], Roberta Piva[3], Assunta Pandolfi[1]

[1]Centro Scienze dell'Invecchiamento e Medicina Traslazionale (Ce.SI-MeT), Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" Chieti-Pescara, StemTeCh Group "G. d'Annunzio" University Foundation, Chieti.

[2]Centro Scienze dell'Invecchiamento e Medicina Traslazionale (Ce.SI-MeT), Department of Medicine and Aging Sciences, University "G. d'Annunzio" Chieti-Pescara.

[3]Department of Biomedical and Specialty Surgical Sciences, University of Ferrara, Ferrara, Italy.

Author Contributions

D.M.: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; L.P.: Conception and design, collection and assembly of 3D culture systems, data analysis and interpretation, manuscript writing, final approval of manuscript; C.P.: Conception and design, data analysis and interpretation, final approval of manuscript; P.D.T.: Collection and assembly of cell samples, final approval of manuscript; S.D.S.: Collection and

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characterization of cell samples, final approval of manuscript; N.D.P.: Data analysis and interpretation, final approval of manuscript; S.T.: collection and assembly of 3D culture systems, assembly of histological samples, final approval of manuscript; M.A.: Conception and design, data analysis and interpretation, final approval of manuscript; M.U.: Collection and characterization of cell samples, final approval of manuscript; R.P.: Conception and design, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript; A.P.: Conception and design, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript.

+Address correspondence to A. Pandolfi, PhD, Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio", Chieti-Pescara, Italy. Phone #39 0871 541425,

Email: assunta.pandolfi@unich.it

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Abstract

Menaquinone, also called Vitamin K2, regulates calcium homeostasis in a "bone-vascular cross talk" and recently received much attention for its positive effect on bone formation. To further understand the correlation between menaquinone and bone metabolism, the effects of menaquinone-4 (MK-4) on osteogenic differentiation of human amniotic fluid mesenchymal stem cells (hAFMSCs) were here investigated. Conventional 2D cell culture system was used to demonstrate the hAFMSCs responsiveness to MK-4 which significantly improved the cellular osteogenic potential through a γ-glutamyl carboxylase (GGCX)-dependent pathway. When hAFMSCs were cultured in a 3D dynamic system (RCCS™bioreactor) without exogenous scaffold, MK-4 supported the osteoblast-like formation already after 7 days, promoting the extracellular bone matrix deposition and the expression of osteogenic-related proteins, including alkaline phosphatase, osteopontin, collagen type 1 and osteocalcin. In order to explore the effect of MK-4 in a culture system more close to the bone physiological microenvironment, hAFMSCs were then co-cultured in the bioreactor with human monocytes, as osteoclast, precursors without osteoclastogenic inducers. Notably, the presence of MK-4 supported cellular aggregate formation and hAFMSCs osteogenic function that otherwise would not have been able to sustain this phenomenon for an extended period. This is the proof that combining a 3D culture system with appropriate molecules, we can meet the challenge of reproducing a physiological microenvironment through which give informative answers both on the properties of the cells and on the biologic effect of a specific substance. Specifically, our data may help to optimize bone regenerative medicine combining cell–based approaches with MK-4 treatment.

1. Introduction

Bone diseases such as osteoporosis and osteopenia are characterized by loss of bone mass and structural deterioration of tissue that lead to fragility and increased risk of fractures. These chronic age-related diseases represent a major global public health problem that affects worldwide people (Cauley, 2013). Consequently, in the past two decades, numerous studies have been developed in this field and there have been great advances in understanding the processes that regulate physiologic bone turnover. Based on these innovative knowledge, several drugs have been developed for the treatment of bone diseases even though they do not yet lead to satisfying results because of their side effects and also they do not reduce completely the cases of fractures (Ito, 2014).

To date, the most efficient clinical practice for skeletal repair is the autologous bone graft. Unfortunately, this approach may result invasive and does not lead to a complete structural integration of bone fragments. Thus, nowadays, there is a growing interest in the development of alternative approaches against bone injury such as the application of cell-based therapies.

Considering their physiological role in bone healing, bone marrow mesenchymal stem cells (BMMSCs) represent the most suitable cells for the treatment of traumatic and degenerative bone defects in regenerative medicine (Pantalone *et al.,* 2016). As an alternative, the amniotic fluid (AF) represents a non-invasive and more easily obtainable source of MSCs. Indeed amniotic fluid mesenchymal stem cells (AFMSCs) possess potentially important therapeutic properties for bone tissue regeneration. They are multipotent, not tumorigenic and not immunogenic stem cells with great osteogenic differentiation capability (De Coppi *et al.,* 2007; Pipino and Pandolfi 2015). Interestingly, recent evidences have demonstrated that their osteogenic differentiation process could be improved by the use of pharmacological molecules such as Simvastatin or calciomimetics as well as the herbal medicines Naringin and

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Curculigoside (Di Tomo *et al.,* 2013; Pipino *et al.,* 2014; Pipino and Pandolfi, 2015). In this regard, there is great interest in studying natural agents that could potentially enhance bone health with minimal side effects.

Menaquinone (MK), also called Vitamin K2, is one of these natural molecules that in the last few years received much attention for its potential positive effect in bone metabolism (Hamidi and Cheung 2014). MK, whose main source is represented by fermented foods such as cheese and Natto (fermented soybeans) (Iwamoto, 2014), is a lipid-soluble vitamin produced by bacteria and structurally characterized by the presence of a ring of 2-methyl-1,4-naphthoquinones, called menadione, and a side chain of different number of isoprenoid units (1-14, MK_n) (Shearer and Newman, 2008). In addition to its coagulation properties, known since 1929 (El Asmar *et al.,* 2014), MK may play a key function in the regulation of calcium homeostasis in "bone-vascular cross-talk". Recently, it has been shown that it may be involved in this cross-talk by reducing calcium deposit in the arteries while increasing it in the bone tissue, process known as "calcium paradox" (Flore *et al.,* 2013). MK, in fact, acts as cofactor for the enzyme γ-glutamyl carboxylase (GGCX) (Tie and Stafford 2016) and through carboxylation of residues of glutamic acid in γ-carboxyglutamate causes the activation of specific vitamin K-dependent proteins (VKDP) (Dalmeijer *et al.,* 2012). Particularly, in bone tissue, the effect of MK is explained through the activation of the VKDP osteocalcin (OC). OC is a non-collagenous "*Bone Gla Protein*" specifically produced by osteoblasts and essential for normal bone mineralization in its carboxylated form (cOC) (Iwamoto, 2014; Koitaya *et al.,* 2014). To date, the evaluation of serum ratio between cOC and undercarboxylated form (ucOC) is considered a specific marker of both bone turnover and MK status (Zhang *et al.,* 2016). Furthermore, high levels of ucOC is correlated with a reduction of Bone Mineral Density (BMD) and increased of hip fracture risk in elderly women (Szulc *et al.,* 1994). Interestingly, MK treatment seems to

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improve cOC (Nakamura *et al.,* 2014) and play a role in the maintenance and improvement of vertebral BMD and in the prevention of fractures in postmenopausal women with osteoporosis (Huang *et al.,* 2015). Although these evidences make MK a potential osteoinductive factor for bone tissue regeneration, little is known about its capability in modulating osteogenesis in MSCs (Gigante *et al.,* 2015; Zhang *et al.,* 2016). Indeed, the study carried out so far on MK and its relation with bone were performed mainly on osteoblastic cells (Katsuyama *et al.,* 2007; Koshihara and Hoshi, 1997). Thus, the purpose of our study was to investigate the possible role of MK-4 in the modulation of osteogenesis in a model of human amniotic fluid mesenchymal stem cells (hAFMSCs) both in two-dimensional (2D) and three-dimensional dynamic (3D) *in vitro* culture systems.

Moreover, in order to provide a particularly suitable model to understand the molecular mechanisms with which MK-4 supports osteogenic process (Atkins *et al.,* 2009; Yamaguchi and Weitzmann, 2011), we analyzed the effect of MK-4 treatment in a 3D co-culture system more closely to bone physiological microenvironment represented by hAFMSCs and human monocytes as osteoclast precursors (Knight and Przyborski, 2015; Penolazzi *et al.,* 2016).

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2. Materials And Methods

2.1. Chemicals

Powered Menaquinone 4 (MK-4) was provided by Ibersan-Santiveri s.r.l. (Italy), resuspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA) at 10 mM concentration and stored at -20 °C.

2.2. Cell culture

hAFMSCs, obtained from amniotic fluid as previously described (Pipino *et al.,* 2014), were cultured for 2 days in control medium (CM) composed of Chang Medium C Lyophilized Kit (IrvineScientific, Daimler St. Santa Ana, USA) supplemented with 15% Fetal Bovine Serum (FBS, Gibco-Life Technologies, Monza, Italy), 1% penicillin/streptomycin and 1% L-glutamine (Sigma-Aldrich, St. Louis, USA). The cells were growth under a controlled atmosphere (5% CO_2 and 37°C) and used for all experiments between passages 3 and 6.

For peripheral blood mononuclear cells (PBMCs) healthy volunteers (n=10, median age 37.5 years) were recruited after informed consent and PBMCs were obtained from diluted peripheral blood (1:2 in Hanks solution), separated by Histopaque®-1077 (Sigma-Aldrich).

Human monocytes (hMCs) were purified from PBMCs by adhesion selection on polystyrene plates (1×10^6 PBMCs/cm^2 were plated in T-25 culture flasks, allowed to settle for 4 hours at 37°C and flasks were then rinsed to remove non-adherent cells) (Piva *et al.,* 2005). Then, the purity population was verified by cytofluorimetric analysis employing 1×10^5 cells, incubated with fluorescein isothiocyanate (FITC) conjugated anti-human CD14 antibody (ImmunoTools GmbH, Friesoythe, Germany) for 15 minutes at 4 °C. A monoclonal antibody with no specificity was used as negative

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control. The fluorescence levels were evaluated using the FACS Scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and CELLQUEST software (Becton Dickinson European HQ, Erembodegem Aalst, Belgium). Only the samples CD14 positive $\geq$ 95% were used. For 3D dynamic co-culture, hMCs (0.5×10^6) were treated with 5mM CellTracker ™ Green CMFDA (Thermo Fisher scientific, Waltham, MA USA), trypsinized and inoculated in High Aspect Ratio Vessel (HARV™; Synthecon™, Inc., Houston, TX, USA) in combination with hAFMSCs (1×10^6). Following 24 hours of incubation the generated aggregates were observed under a fluorescence microscope (Nikon, Optiphot-2; Nikon Corporation, Tokyo, Japan) using the filter block for fluorescein.

2.3. *In Vitro* hAFMSCs 2D Osteogenic induction and treatment with MK-4

For osteogenic differentiation, hAFMSCs were seeded at a density of $3,000/cm^2$ in six-well plates and treated for 21 days with Osteogenic Medium (OM) containing Dulbecco's Modified Eagle's Medium low glucose (DMEM), 0.05 mM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate and 100 nM dexamethasone (all from Sigma-Aldrich, St. Louis, USA,) supplemented with 5% FBS in the presence or absence of MK-4. MK-4 treatment was started from 7th day of osteogenic differentiation and was continued for 14 days (treatment every 24 hours) as outlined in Figure 1B.

2.4. MTT Assay

The effect of MK-4 on hAFMSCs viability in 2D culture system was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich). The cells were seeded in 96-well plates at a density of $5600/cm^2$. The assay, based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells, provides a quantitative determination of viable

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cells. After 3 and 7 days of treatment, a solution of MTT in phosphate buffer saline (PBS) (Sigma-Aldrich) was added to each well (0.5 mg/ml) and the plate was incubated for 3 hours at 37°C. The MTT crystals were solubilized with 200 µL DMSO and the spectrophotometric absorbance of each sample was then measured at 540 nm by using a microplate reader (SpectraMAX 190, Molecular Devices).

2.5. Alizarin Red S Assay

The effect of MK-4 on hAFMSCs osteogenic differentiation cultured in monolayer or in 3D aggregates was assessed by Alizarin Red Staining (Sigma-Aldrich). After two washes with PBS, the cells were fixed in 10% formaldehyde for 10 minutes at room temperature (RT) . Then, cells were stained with 40 mM of Alizarin Red S solution (pH 4.2) for 20 minutes at RT to detect and then quantify the calcium deposits using established protocol (Pipino *et al.,* 2014).

2.6. GGCX siRNA transfection

To 2D GGCX silencing experiments, hAFMSCs were transfected with siGenome Human GGCX small interfering RNA (siRNA) or siGENOME Non-Targeting siRNA (both 70 nm; Dharmacon) using Hiperfect Reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Firstly, we set up the gene silencing experiments at 48, 72 and 96 hours to identify the optimum silencing time. Then, we silenced GGCX every 96 hours in hAFMSCs previously treated with OM alone for 7 days. The treatment with MK-4 (10 µM) was started from 7th day of osteogenic differentiation and was continued for 7 days (treatment every 24 hours) as summarized in Figure 1B. The effects of MK-4 treatment after GGCX silencing was assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and flow cytometry for the expression of specific osteogenic markers.

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2.7. RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from hAFMSCs, 2D cultured in CM or OM in presence or absence of MK-4, using Trizol® reagent (Invitrogen), according to the manufacturer's instructions. RNA concentration and quality was measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific).

For reverse transcription High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA) was used. The equivalent of 0.1 µg of cDNA was used for the reactions of qRT-PCR carried out with ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Commercially available TaqMan Gene Expression Assay human alkaline phosphatase (ALP, Hs01029144_m1), human runt-related transcription factor 2 (RUNX2, Hs00231692_m1), human osteopontin (OPN, Hs00959010_m1), human collagen type I alpha I (COLIAI, Hs00164004_m1), human osteocalcin (OC, Hs01587813_g1), β2 microglobulin (B2M, Hs99999907_m1; Applied Biosystems) and the TaqMan Universal PCR Master Mix (Applied Biosystems) were used according to the manufacturer's instructions. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method.

2.8. Carboxylated and Undercarboxylated Osteocalcin

Carboxylated (Gla) and undercarboxylated (Glu) osteocalcin form were quantified in cell conditioned media collected after 14 days of MK-4 treatment (21 days of hAFMSCs osteogenic induction) using commercially available enzyme immunoassay (EIA) kits (MK128 and MK118 respectively Takara, Shiga, Japan), according to the manufacturer's instructions.

2.9. Flow Cytometry

hAFMSCs silencing for GGCX as previously described were stained under permeabilizing conditions to visualize total ALP, RUNX2 and COL1A1 protein expression. Briefly, the staining procedure followed a previous step of membrane fixation and permeabilization, as previously described (Di Tomo *et al.,* 2013). The staining was performed incubating $5x10^5$ cells/sample with primary monoclonal antibody against ALP (1:100, mouse anti-ALP antibody; Pierce-Thermo Scientific), RUNX2 (1:800, rabbit anti-RUNX2 antibody; Cell Signaling) and COL1A1 (1:100, mouse anti-COL1A1 antibody; Santa Cruz Biotechnology) for 30 minutes on ice, followed by staining with Alexa488-labeled secondary antibodies (1:1500; Jackson ImmunoResearch Laboratories) for 30 min on ice. To evaluate cell viability, 7-aminoactinomycin D (7-AAD, 1:100) was used (BD Biosciences). To assess nonspecific fluorescence, we used samples stained with the corresponding secondary antibody alone. Samples were analyzed by flow cytometry. Data are indicated as a Mean Fluorescence Intensity (MFI) Ratio in live cells. The MFI Ratio was calculated by dividing the MFI of positive events by the MFI of negative events (MFI of secondary antibody).

2.10. hAFMSCs cultured in 3D dynamic system

The 3D dynamic culture condition was established by utilizing the RCCS-4TM bioreactor (Synthecon™, Inc., Houston, TX, USA), with a High Aspect Ratio Vessel (HARV™; Synthecon™, Inc., Houston, TX, USA). The HARV vessel contains a horizontally rotated culture chamber, where the cells are suspended, and a perfusion system with media continuously flowing through the culture chamber. hAFMSCs ($1x10^6$ cells/mL) alone and in combination with hMCs (2:1 cell ratio) were inoculated in HARV vessels (2 mL/10mL) filled with control medium, inserted into the RCCS-4^{TM}

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rotary bioreactor and placed in an incubator (37 °C and 5% CO2) for the indicated times. As previously reported (Penolazzi *et al.,* 2016), the rotation speed applied for the experiments was 4 rpm, corresponding to Ground Based dynamic culture. Following 24 hours, the existence of aggregates was observed, and the vessels were filled with osteogenic medium. Next to 7 days, MK-4 (10 µM) was added into the HARV vessels and control samples were maintained in osteogenic medium plus vehicle (DMSO) added at the same volumes.

At day 14/21 aggregates were collected, fixed in 4% formalin, embedded in paraffin, sectioned and processed for histochemical analysis.

2.11. Cell viability

According to the manufacturer's instructions, hAFMSCs and hMCs viability in 3D dynamic culture and co-culture systems was evaluated by double staining with propidium iodide and Calcein-AM assay (Sigma-Aldrich). Cells were examined under a fluorescence microscope (Nikon, Optiphot-2; Nikon Corporation, Tokyo, Japan) employing the filter block for fluorescein. Dead and viable cells were stained in red or in green, respectively.

2.12. Immunocytochemistry and histology

Immunocytochemistry was executed using the ImmPRESS (Vectorlabs, Burlingame, CA). hAFMSCs were fixed in cold 100% methanol and permeabilized 0.2% (v/v) Triton X-100 in TBS 1X (Tris-buffered saline). Following the incubation in blocking serum, polyclonal antibodies for OPN (LF-123) and COL1A1 (H-197) (rabbit anti-human, 1:200 dilution, Santa Cruz Biotech, Dallas, TX, USA) were added and incubated overnight (4 °C). Cells were then incubated in Vecstain ABC (Vectorlabs, Burlingame, CA) reagents (30 min) and stained with DAB solution (Vectorlabs, Burlingame, CA).

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After washing, cells were mounted in glycerol and observed using the Nikon Esclipse 50i optical microscope.

Histological sections (5 µm) of 3D aggregates were used for immunohistochemistry. Thus, deparaffinized, rehydrated and enzymatic treated non-consecutive sections were incubated with pronase (1 mg/mL, Sigma-Aldrich) for antigen retrieval and permeabilization. Slides were then immunostained overnight with the primary antibody against OPN (LF-123), COL1A1 (H-197) (rabbit anti-human, 1:100 dilution) in a humid chamber at 4 °C. The DAB staining was carried out as described above. The sections were counterstained with haematoxylin, mounted in glycerol and observed using the Nikon Esclipse 50i optical microscope. TRAP staining was carried out with the Acid Phosphatase Leukocyte (TRAP) Kit no. 386 (Sigma-Aldrich) according to the manufacturer's protocol as already reported (Piva *et al.,* 2005). The staining 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). For the analysis of cells in monolayer and tissue sections obtained from aggregates, the positive immunostaining was expressed as % of positive area (three replicates per donors were acquired; five sections per sample; n=3).

2.13. Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by the unpaired Student's t test or by the one-way Analysis of Variance (ANOVA) and Bonferroni post *hoc* test. A p value <0.05 was considered statistically significant.

3. Results

3.1. Conventional 2D Culture of hAFMSCs: Osteogenic Potential and Responsiveness to MK-4

hAFMSCs were obtained and characterized as previously described (Pipino *et al.,* 2014). In order to add new information on the role of MK-4 on bone cells and improve the knowledge on an interesting experimental model represented by hAFMSCs, the different culture conditions reported in the experimental plan have been adopted (Figure 1).

Conventional 2D culture system was used to evaluate the MK-4 dose-response by the cells in terms of cytotoxicity and extracellular matrix mineralization as *in vitro* endpoint reflecting advanced osteogenic differentiation. As revealed by MTT assay performed at day 3 (data not shown) and day 7 of culture (Fig. 2A), increasing doses (0.1-1-10 µM) of MK-4 were not cytotoxic. When osteogenic induced hAFMSCs were exposed to daily MK-4 treatment at the same doses for 14 days (21 days of osteogenic induction, see Figure 1B), a significant increase of mineral matrix deposition was observed, as shown by Alizarin Red staining (Figure 2B). Based on these data and in agreement with some evidences from literature, we chose to use MK-4 at concentration of 10 µM for the subsequent experiments.

The improvement of hAFMSCs osteogenic ability by MK-4 was validated by analyzing the expression of typical osteogenic markers. As reported in Figure 2C, MK-4 treated cells showed a significant increase of ALP, RUNX2 and OC mRNA expression levels, respect to untreated cells after 14 days of MK-4 treatment (21 days of osteogenic induction, see Figure 1B). The positive effect of this treatment was also confirmed by the increased expression of two extracellular matrix proteins such as COL1A1 and OPN analyzed both at mRNA (Figure 2C) and protein levels (Figure 2D) at the same time.

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3.2. MK-4 Pro-Osteogenic Ability Occurs Through a GGCX-Dependent Pathway

Since MK acts as cofactor for GGCX, we investigated whether in hAFMSCs the pro-osteogenic effect of MK-4 was mediated by a GGCX-dependent pathway. First of all, the vitamin K-dependent γ-carboxylation on osteocalcin (OC), a critical protein for bone mineral mineralization, was evaluated in conditioned media collected from hAFMSCs after 14 days of MK-4 treatment (21 days of osteogenic induction, see Figure 1B). The EIA quantifying showed a significant decrease of undercarboxylated OC (ucOC) form and, on the contrary, an increase of carboxylated OC (cOC) levels in MK-4 treated cells compared to untreated cells (Figure 3A-B). Accordingly, the ratio between cOC/ucOC significantly increased in MK-4 treated cells (Figure 3C), indicating that hAFMSCs are effectively MK-4 responsive (scheme in Figure 3D).

In a second step GGCX gene knockdown experiments were performed. siRNA treatment was highly effective in GGCX downregulation already after 48 hours, achieving > 90% inhibition of GGCX expression compared to scrambled-treated cells (SCR) and this was maintained up to 96 hours (Supporting Information, Figure S1). On the basis of these observations, osteogenically differentiated hAFMSCs were subjected to siRNA treatment every 96 hours up to 14 days of culture (corresponding to 7 days of MK-4 treatment, see Figure 1B), and monitored for the expression of osteogenic markers. As shown in Figure 4, GGCX silencing significantly affected the ability of MK-4 to improve the osteogenic induction of hAFMSCs, in terms of ALP, RUNX2 and COL1A1 expression levels as revealed by qRT-PCR (Figure 4A) and flow cytometry analysis (Figure 4B).

All together these evidences have demonstrated that MK-4 pro-osteogenic ability occurs through a GGCX-dependent pathway.

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3.3. Analysis of the MK-4 Effect in a 3D Dynamic Cell Culture System

In order to explore the effect of MK-4 in a cell culture system closer to the physiological condition, we cultured hAFMSCs in a 3D dynamic system represented by an horizontally rotated culture chamber High Aspect Ratio Vessels (HARV) applied to the Rotary Cell Culture System (RCCS). With this system the ability of the cells to generate self assembling aggregates, without the employment of exogenous scaffold, can be evaluated. hAFMSCs were cultured in osteogenic medium at the concentration of 1×10^6 cells/ml and subjected to 4 rpm (Penolazzi *et al.,* 2016). Macroscopic observations during the first seven days in culture revealed an initial formation of cell aggregates that over time assumed a spherical shape. After 7 days of MK-4 treatment (14 days of osteogenic induction, see Figure 1B) the aggregates were recovered and subjected to Calcein AM/Propidium iodide (PI) double staining for cell viability assessment. As shown in Figure 5A, the aggregates appeared sizeable with cells high viable in both conditions. In addition, the functional properties of the cells forming the aggregates were then investigated by immunohystochemical analysis. hAFMSCs maintained their ability to osteodifferentiate being COL1A1, OPN and ARS positive. Interestingly, MK-4 treatment significantly improved the osteoblast-like formation after just 7 days of MK-4 treatment (14 days of osteogenic induction, see Figure 1B), as highlighted by the 3-fold and 6-fold increase of the OPN expression levels and ARS positive areas respectively (Figure 5B). At the same time, MK-4 maintained high COL1A1 protein levels, suggesting that it overall positively affected the extracellular bone matrix deposition (Figure 5B).

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3.4. Effect of MK-4 on hAFMSCs and Osteoclast Precursors 3D dynamic Co-Culture System

In a first step, the ability of hAFMSCs to support osteoclastogenesis of osteoclast precursors and the inhibition of this phenomenon by MK-4 was demonstrated by TRAP assay and acting ring analysis in a conventional 2D transwell co-culture system (Supporting Information, Figure S2).

To assess the ability of MK-4 in modulating cell response more closely to the physiological condition of bone microenvironment, hAFMSCs and osteoclast precursors (hMCs) have been combined in a 3D dynamic co-culture system (2:1 cell ratio). We observed that cells were able to interact each other producing sizeable self assembled aggregates already after 24 hours. A representative image showing how CellTracker™-labeled hMCs clearly contribute to the cellular architecture of the aggregate is reported in Figure 6A.

Cell viability was also confirmed by Calcein-AM/PI assay carried out after 14 days of co-culture both in presence and in absence of MK-4 (Figure 6A).

As shown in Figure 6B, TRAP assay clearly highlighted the ability of hAFMSCs to sustain osteoclastogenesis in absence of osteoclastogenic inducers, allowing the formation of multinucleated cells mainly localized in the outer part of the aggregate. Interestingly, the MK-4 treatment for 7 days (14 days of osteogenic induction, see Figure 1B) negatively affected osteoclastogenesis since a significant decrease of TRAP positive areas and multinucleated cells was observed (Figure 6B). Conversely, osteoblastic–like cell component was positively affected by MK-4 since a significant increase of mineral matrix deposition, OPN and COL1A1 expression in the inner

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portion of aggregate has been observed (Figure 6C). Notably, the analysis performed after 14 days of MK-4 treatment (21 days of osteogenic induction, see Figure 1B) showed that the aggregate was maintained only in the presence of MK-4 (Figure 6D).

4. Discussion

The emerging field of bone regenerative medicine has intensified the demand for novel natural and/or pharmacological molecules acting on advantageous sources of stem cells with safe potential for therapy, as well as the discovery of new innovative approaches mimicking the *in vivo* bone environment.

To date, the use of mesenchymal stem cells (MSCs) represents the most investigated approach to treat traumatic and degenerative bone defects (Caplan, 2005). In addition to bone marrow mesenchymal stem cells (BMSCs), that are already clinically applied despite the invasive and painful procedure of bone marrow aspiration (Pantalone *et al.,* 2016), an innovative and advantageous source of MSCs for skeletal regeneration is represented by extra-embryonic tissues such as amniotic fluid (Pipino and Pandolfi, 2015).

Indeed, amniotic fluid mesenchymal stem cell (AFMSCs), obtained during pregnancy by routine amniocentesis, represent a less invasive and more primitive source of stem cells to keep pace with the growth and demands of cells necessary for regenerative medicine. In fact, they possess multipotent differentiation ability, anti-inflammatory and immunomodulatory properties, non-tumorigenicity, and no ethical problems (Cananzi *et al.,* 2009). We and others have demonstrated hAFMSCs great capability to differentiate in osteogenic cells (Chen *et al.,* 2010; De Coppi *et al.,* 2007; Pipino *et al.,* 2015), and several studies have showed that this process may be improved by using pharmacological and natural molecules such as simvastatin, calciomimetics, naringin and curculigoside (Di Tomo *et al.,* 2013; Pipino *et al.,* 2014; Pipino and Pandolfi, 2015).

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Among natural molecules, a promising compound with potential osteoinductive properties may be menaquinone (MK), a fat soluble vitamin that acts as a cofactor of the enzyme ɣ-glutamylcarboxylase (GGCX) (Tie and Stafford, 2016).

To date, MK is used for the treatment of osteoporosis in Japan and several clinical trials have suggested its healthy effects on bone metabolism particularly in the maintenance and improvement of vertebral Bone Mineral Density (BMD) and in the prevention of fractures in postmenopausal women (Huang *et al.,* 2015; Koitaya *et al.,* 2014; Ronn *et al.,* 2016; Shiraki *et al.,* 2000). Thus, since it recently received much attention for its positive effect on *in vivo* bone formation, several studies have been performed to better understand its possible mechanism of action (Atkins *et al.,* 2009). In particular, MK-4 has been studied *in vitro* mainly on osteoblastic cells (Katsuyama *et al.,* 2007; Koshihara and Hoshi, 1997) and, although Gigante and colleagues (Gigante *et al.,* 2015) have lately demonstrated that MK in association with vitamin D3 may improve hBMSCs osteogenic differentiation, today little is known about its possible osteogenic effect on MSCs from bone marrow and other sources (Zhang *et al.,* 2016).

Then, to further understand the relation between MK and bone metabolism, in this study we investigated the effects of MK-4, the most abundant form of this vitamin in the human body (Thijssen and Drittij-Reijnders, 1996), on osteogenic differentiation of hAFMSCs.

In particular, conventional two-dimensional (2D) cell culture system was used to demonstrate the hAFMSCs responsiveness to MK-4 which significantly improved the cellular osteogenic potential (evaluated through both mineralization levels and the expression of common osteogenic markers such as ALP, RUNX2, OC, COL1A1 and OPN) via a γ-glutamyl carboxylase (GGCX)-dependent pathway. In fact, GGCX silencing significantly affected the ability of MK-4 to improve the osteogenic induction of hAFMSCs, in terms of ALP, RUNX2 and COL1A1 expression levels as revealed by

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qRT-PCR and flow cytometry analysis. In addition, we also evaluated the effect of MK-4 on the ratio between carboxylated (cOC) and undercaboxylated (ucOC) levels, which was increased following treatment, thus further supporting the hypothesis that this vitamin in hAFMSCs acts as a cofactor of GGCX.

All together, these evidences have demonstrated that MK-4 pro-osteogenic ability occurs through GGCX-dependent pathway. Other possible mechanisms, as the activation of the steroid and xenobiotic receptor (SXR), cannot be excluded and will be investigated by further analysis (Azuma *et al.,* 2014; Ichikawa *et al.,* 2006).

Following these observations, we strengthened the knowledge on the positive effect of MK-4 on osteogenic process by using hAFMSCs cultured in a three-dimensional (3D) unconventional way in an effort to better recapitulate the *in vivo* bone situation.

We performed this experiment using a rotational bioreactor that supports the production of bone-like matrix in the cell aggregates even without exogenous scaffold employment, providing a more biologically relevant model than traditional 2D monolayer cultures (Penolazzi *et al.,* 2016). In such a dynamic condition, the differentiation process resembles more closely, what happens *in situ*, and the cells are further stimulated to display their intrinsic properties. This approach has allowed us to demonstrate the ability of hAFMSCs to organize themselves in clusters of cells that self-assemble, and to sustain the osteoclastogenesis without exogenous inducers when combined with osteoclast precursors. This provides a physiologically relevant model as "bone mimetic product" to study potential drugs such as MK-4. In fact, as expected and accordingly with literature (Yamaguchi and Weitzmann, 2011), MK-4 treatment negatively affects osteoclastogenesis but supports osteoblastic–like cell component. Notably, our data clearly demonstrate that the presence of MK-4 was essential for the maintenance of a well organized, long-lasting and functional cell aggregate. It will be important to investigate the quality of the extracellular matrix produced by the "bone mimetic

product" in order to identify potential new MK-4 targets that modulate the process we observed.

It is worth noting that hAFMSCs appeared difficult to manage in 3D conditions for long-term experiments respect to MSCs from other sources, and this affects the number of samples analyzable. Moreover, a certain marker expression variability was found in the "bone mimetic products" we obtained. This could be due to the fact that hAFMSCs may have a delayed, but in any case robust differentiation ability (Peister *et al.,* 2011). A possible reason could be the heterogeneity of AF samples where epithelial-like and fibroblast-like cells coexist (De Coppi *et al.,* 2007; Roubelakis *et al.,* 2011). In any case, a recent work by our group showed that both phenotypes of AFMSCs, probably stabilizing each other by a specific cross talk, differentiated well into mesenchymal lineages (Pipino *et al.,* 2015). This evidence may represent a benefit because allow the use of mixed cells without any immune selection method (Roubelakis *et al.,* 2011), thus reducing also cell manipulation for potential clinical purpose.

However, in this regard we showed here, for the first time, that despite the possible prevalence in hAF of one over the other cells' phenotype could explain the variability of the 3D cell-aggregate quality, the presence of MK-4 supports and maintains bone aggregate formation and osteogenic function of the hAFMSCs that otherwise would not have been able to sustain this phenomenon for an extended period.

As a whole our data give proof that combining MSCs with appropriate molecules in 3D culture system, make it possible to reproduce a physiological microenvironment through which give informative answers on the properties of the cells and on the effect of a specific substance.

Moreover, according to previous reports (Zhang *et al.,* 2016) we additionally showed in our study (Supporting Information, Figure S3) that MK-4 treatment also improved *in vitro* osteogenesis in human BMSCs. Notably, in recent times it was demonstrated that

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BMSCs transplantation prevents functional bone loss in mouse models of age-related osteoporosis (Kiernan *et al.,* 2016; Sui *et al.,* 2016). Based on these and our results, it can be hypothesized that *in vivo*, besides the showed positive direct effects of this vitamin on osteoblastic cells (Katsuyama *et al.,* 2007; Koshihara and Hoshi, 1997), the administration of MK-4 might improve the osteogenesis of both residential and/or transplanted BMSCs.

5. Conclusion

In summary, although further studies are needed to confirm and better explain the positive role of MK on osteogenesis, taken together our results suggest the ability of MK-4 to improve hAFMSCs osteogenic differentiation both in 2D and 3D culture system.

Together with the unique features of hAFMSCs and the possibility of cell banking, our results allow us to speculate the development of not only new strategies of cell-based therapy but also a possible engineered system for three dimensional application for bone repair in *vivo*.

As a final point, describing the mechanisms potentially involved, our investigation supports studies that promote the use of MK through dietary supplements and our bone mimetic product based on the combination of hAFMSCs with OCs precursors may be proposed as a platform to test different bone anabolic or catabolic molecules.

Conflicts Of Interest

The authors indicate no potential conflicts of interest.

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Figure Legends

Figure 1. Experimental plan.

(**A**) 2D and 3D culture systems. (**B**) Scheme of experimental protocol. MK-4 treatment was started from 7th day of osteogenic differentiation and carried out for 14 days (treatment repeated every 24 hours) in both 2D and 3D *in vitro* culture systems.

Figure 2. Conventional 2D culture of hAFMSCs: effect of MK-4 on cell viability and osteogenic potential.

(**A**) Effect of different concentrations (0.1-1-10 μM) of MK-4 on hAFMSCs cell survival evaluated by MTT assay. (**B**) ARS assay carried out after 14 days of MK-4 treatment (21 days of osteogenic induction, see scheme in Figure 1B). Scale bar: 100 μM. (**C**) qRT-PCR analysis of osteogenic markers ALP, RUNX2, OC, COL1A1 and OPN. (**D**) Immunocytochemical analysis of COL1A1 and OPN performed in hAFMSCs after 14 days of treatment with MK-4 (21 days of osteogenic induction, see Figure 1B). OPN and COL1A1 protein levels were quantified by densitometric analysis using ImageJ software and expressed as percentage of positive area. Quantification and representative images are reported Scale bar: 20 μm.

Control medium (CM, white column), osteogenic medium (OM, black column) and OM added with MK-4 10 μM (OM+MK-4, grey column).

For all experiments results are expressed as mean±standard deviation (SD) (n=3) (§$p <$ 0.05 vs CM; *$p <$ 0.05 vs OM).

Figure 3. Conventional 2D culture of hAFMSCs: effect of MK-4 on cOC/ucOC.

EIA quantifying (ng/ml) on hAFMSCs conditioned media collected after 14 days of treatment with MK-4 (21 days of osteogenic induction, see Figure 1B) for (**A**)

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undercarboxylated osteocalcin (ucOC) (**B**) carboxylated osteocalcin (cOC) and (**C**) ratio

between carboxylated and undercarboxylated form (cOC/ucOC). Osteogenic medium

(OM, white column), Osteogenic Medium added with MK-4 10µM (OM+MK-4, black

column). Results are expressed as mean±SD (n=3) (*p<0.05 vs OM). (**D**) Model of MK

and GGCX pathway (carboxylation signaling).

Figure 4. Conventional 2D culture of hAFMSCs: effect of GGCX gene silencing on the

expression of osteogenic markers.

(**A**) qRT-PCR and (**B**) flow cytometry analysis of the osteogenic markers ALP, RUNX2

and COL1A1. The cells were cultured in osteogenic medium (OM, white column) and

OM added with scrambled (SCR, black column) or small interfering RNA (siRNA, grey

column) in presence or absence of MK-4 (10µM) up to 14 days of culture (7 days of

treatment with MK-4 according to the scheme showed in Figure 1B). The transfection

was repeated every 96 hours. All data are presented as fold changes. Results are

expressed as mean±SD (n=3) (§p<0.05 vs OM; *p<0.05 vs OM+MK-4; #p< 0.05 vs

OM+MK-4+SCR).

Figure 5. Effect of MK-4 on hAFMSCs cultured in a 3D Dynamic Culture System.

(**A**) Viability Assay. Calcein-AM/PI assay reveals in green the presence of viable cells.

(**B**) Representative images and quantification of ARS assay and immunohistochemistry

for osteogenic markers OPN and COL1A1. hAFMSCs were cultured in 3D dynamic

system represented by RCCS™ (Rotary Cell Culture System) in osteogenic medium

(OM, white column) and OM added with MK-4 10 µM (black column) up to 14 days of

culture (7 days of MK-4 treatment, see Figure 1B). ARS staining, OPN and COL1A1

levels were quantified by ImageJ software and expressed as percentage of positive area.

Results are expressed asmean value±SD, 5 sections per sample, n=3). (*p<0.05 vs OM).

Scale bar: 50 μm.

Figure 6. Effect of MK-4 on hAFMSCs and osteoclast precursors 3D dynamic co-culture system.

(**A**) Fluorescently labeled osteoclast precursors (hMCs) (CellTracker ™ Green CMFDA) were co-cultured with hAFMSCs in 3D dynamic system represented by RCCS™ (Rotary Cell Culture System) to generate self-assembled aggregates. As reported in the microphotograph, after 24 hours the aggregate was sizeable and hMCs clearly evident as green stained area after DAPI counterstaining. Cell viability was confirmed by Calcein-AM/PI assay carried out after 7 days of MK-4 treatment (14 days of co-culture in OM, see Figure 1B). Representative images and quantification of (**B**) TRAP assay, (**C**) ARS assay and immunohistochemistry of the osteogenic markers OPN and COL1A1 on the histological sections. TRAP positive multinucleated osteoclasts are arrowed. TRAP activity, ARS staining, OPN and COL1A1 levels were quantified by ImageJ software and expressed as percentage of positive area (mean value $\pm$ SD, 5 sections per sample, n=3) after 7 days of MK-4 treatment (21 days of co-culture in OM, see Figure 1B). (**D**) Representative images of TRAP assay and immunohistochemistry of COL1A1 on histological sections after 14 days of MK-4 treatment (21 days of co-culture in OM, see Figure 1B): cellular aggregates are appreciable only in OM with the addition of MK-4 (10μM). Arrows indicate the histological localization of osteoclasts in the outer region of the aggregates. Osteogenic Medium (OM, white column), OM added with MK-4 10μM (OM+ MK-4 10μM, black column). (*p<0.05 vs OM). Scale bar: 50 μm.
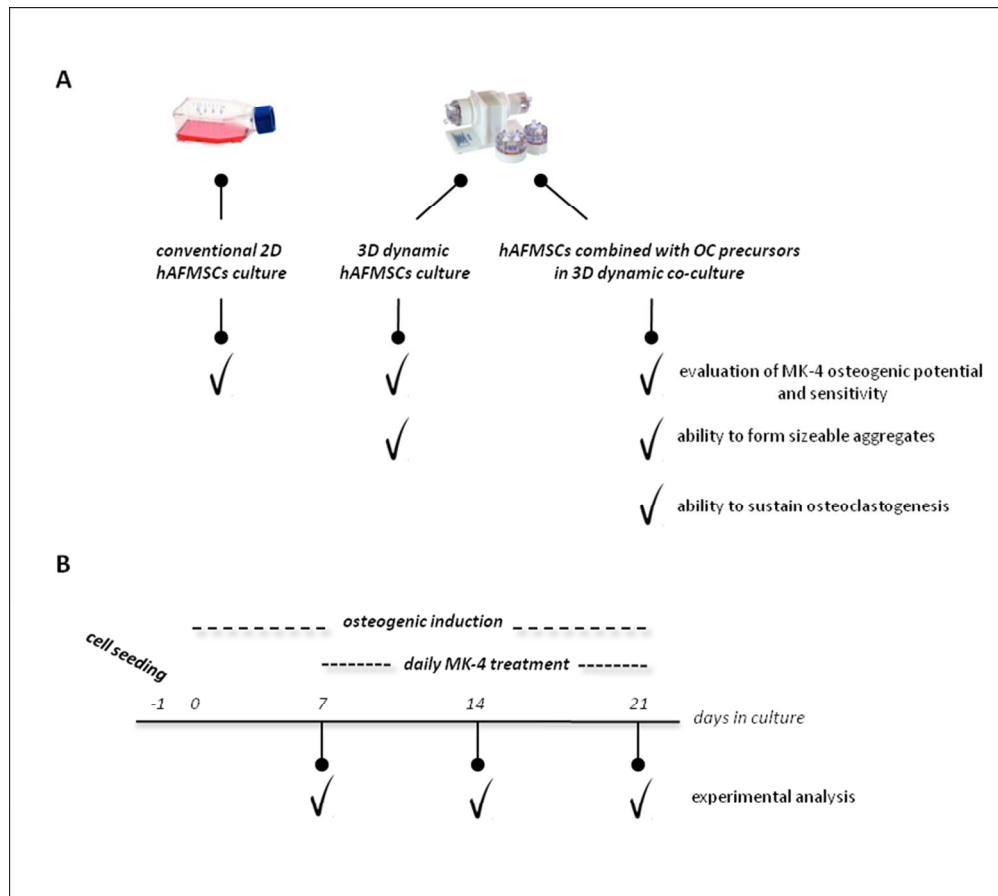
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Figure 1. Experimental plan.
(A) 2D and 3D culture systems. (B) Scheme of experimental protocol. MK-4 treatment was started from 7th day of osteogenic differentiation and carried out for 14 days (treatment repeated every 24 hours) in both 2D and 3D in vitro culture systems.
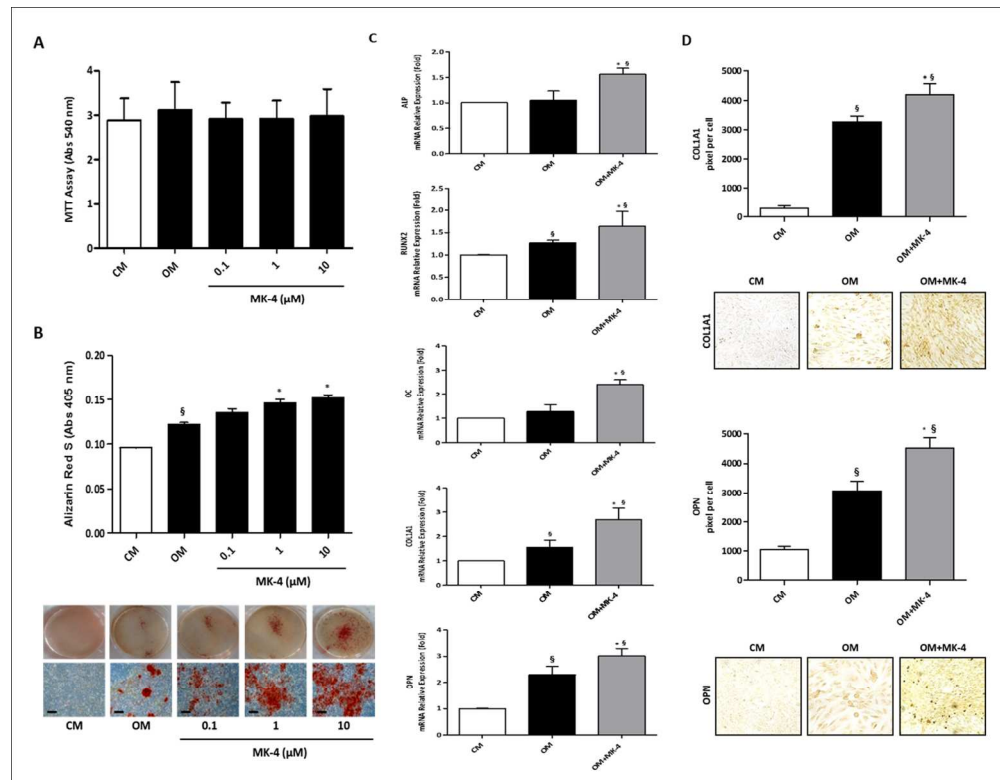
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Figure 2. Conventional 2D culture of hAFMSCs: effect of MK-4 on cell viability and osteogenic potential. (A) Effect of different concentrations (0.1-1-10 µM) of MK-4 on hAFMSCs cell survival evaluated by MTT assay. (B) ARS assay carried out after 14 days of MK-4 treatment (21 days of osteogenic induction, see scheme in Figure 1B). Scale bar: 100 µM. (C) qRT-PCR analysis of osteogenic markers ALP, RUNX2, OC, COL1A1 and OPN. (D) Immunocytochemical analysis of COL1A1 and OPN performed in hAFMSCs after 14 days of treatment with MK-4 (21 days of osteogenic induction, see Figure 1B). OPN and COL1A1 protein levels were quantified by densitometric analysis using ImageJ software and expressed as percentage of positive area. Quantification and representative images are reported Scale bar: 20 µm.
Control medium (CM, white column), osteogenic medium (OM, black column) and OM added with MK-4 10 µM (OM+MK-4, grey column).
For all experiments results are expressed as mean±standard deviation (SD) (n=3) (§p< 0.05 vs CM; *p< 0.05 vs OM).
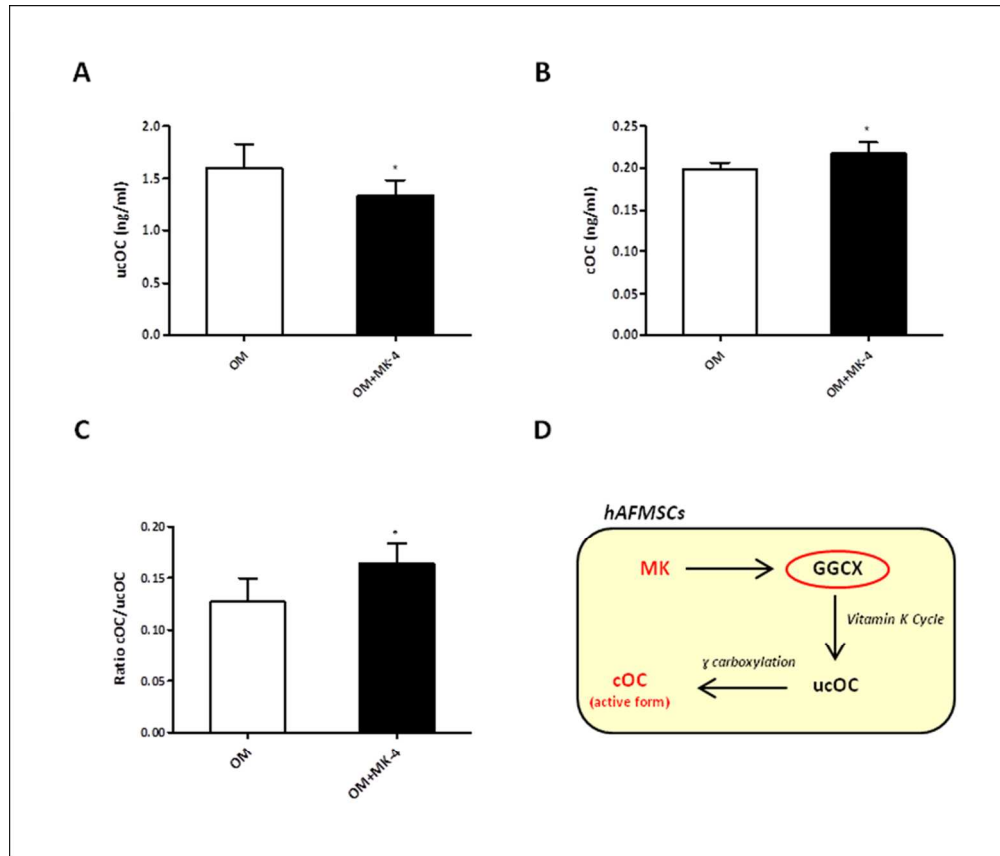
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Figure 3. Conventional 2D culture of hAFMSCs: effect of MK-4 on cOC/ucOC.
EIA quantifying (ng/ml) on hAFMSCs conditioned media collected after 14 days of treatment with MK-4 (21 days of osteogenic induction, see Figure 1B) for (A) undercarboxylated osteocalcin (ucOC) (B) carboxylated osteocalcin (cOC) and (C) ratio between carboxylated and undercarboxylated form (cOC/ucOC). Osteogenic medium (OM, white column), Osteogenic Medium added with MK-4 10μM (OM+MK-4, black column). Results are expressed as mean±SD (n=3) (*p<0.05 vs OM). (D) Model of MK and GGCX pathway (carboxylation signaling).
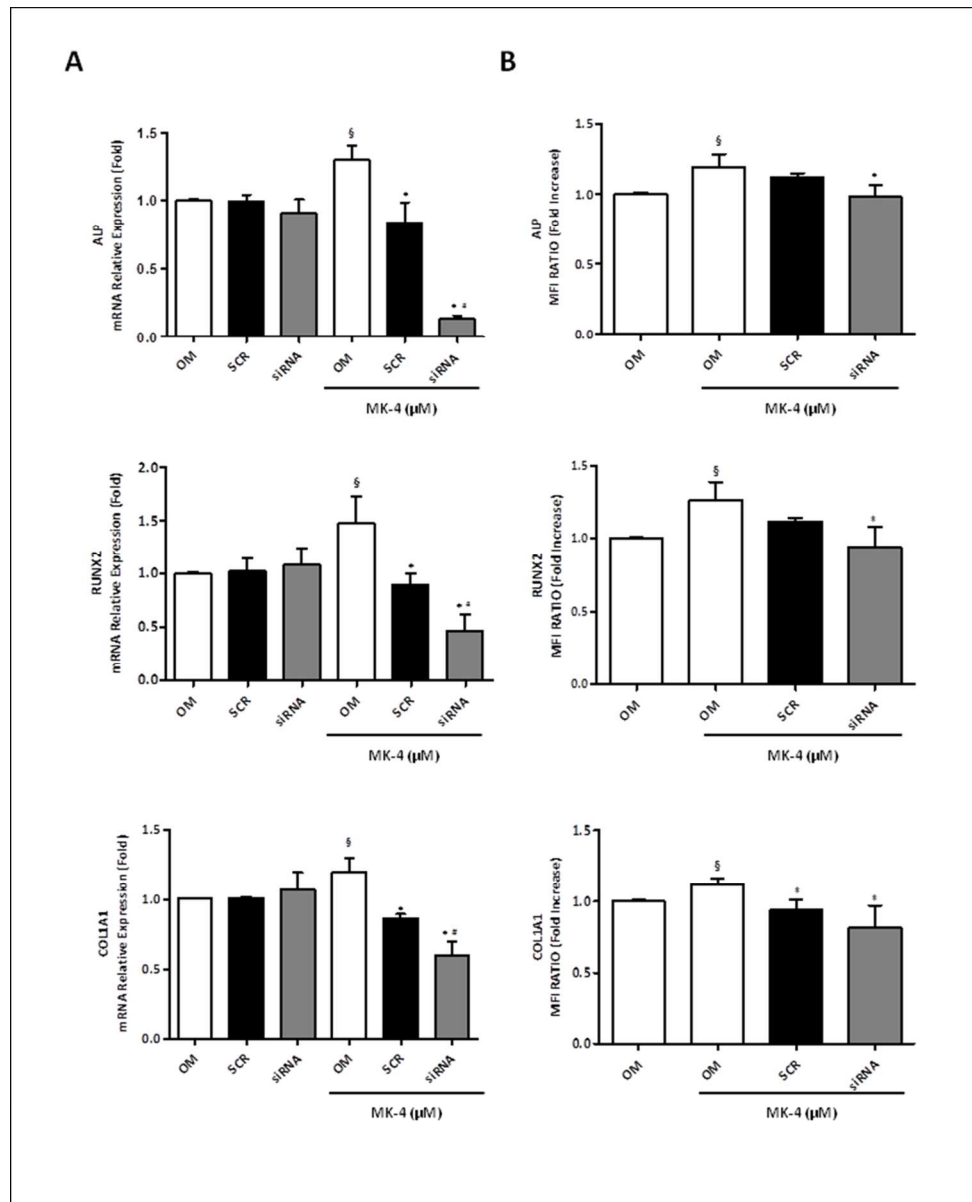
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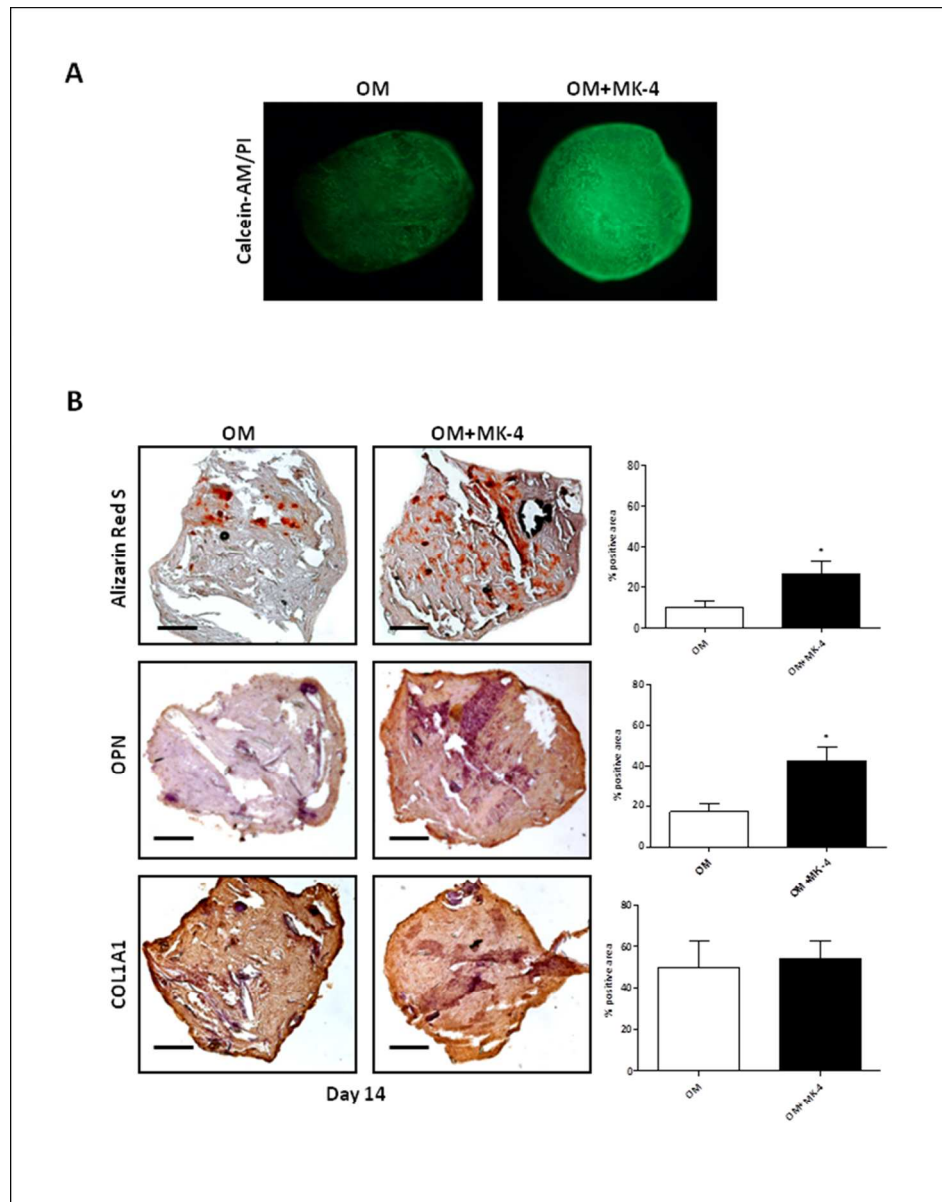51
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Figure 5. Effect of MK-4 on hAFMSCs cultured in a 3D Dynamic Culture System.
(A) Viability Assay. Calcein-AM/PI assay reveals in green the presence of viable cells. (B) Representative images and quantification of ARS assay and immunohistochemistry for osteogenic markers OPN and COL1A1. hAFMSCs were cultured in 3D dynamic system represented by RCCS™ (Rotary Cell Culture System) in osteogenic medium (OM, white column) and OM added with MK-4 10 µM (black column) up to 14 days of culture (7 days of MK-4 treatment, see Figure 1B). ARS staining, OPN and COL1A1 levels were quantified by ImageJ software and expressed as percentage of positive area. Results are expressed asmean value±SD, 5 sections per sample, n=3). (*p<0.05 vs OM). Scale bar: 50 µm.
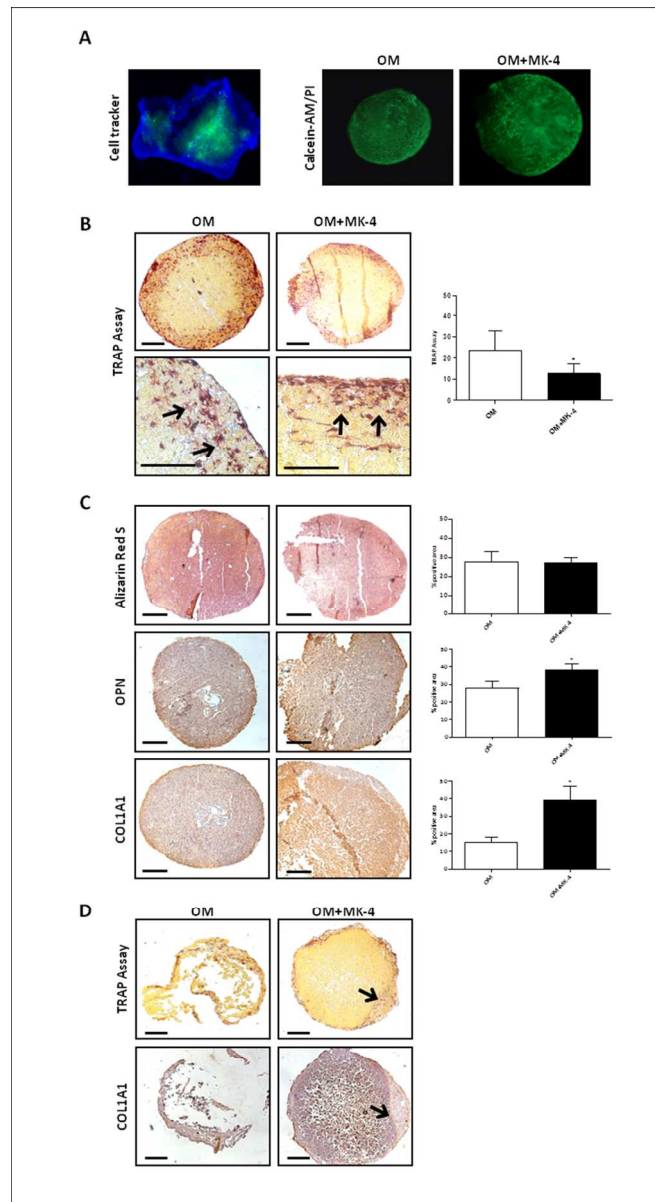
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MK-4 (10µM). Arrows indicate the histological localization of osteoclasts in the outer region of the aggregates. Osteogenic Medium (OM, white column), OM added with MK-4 10µM (OM+ MK-4 10µM, black column). (*$p<0.05$ vs OM). Scale bar: 50 µm.

130x236mm (300 x 300 DPI)