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

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

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Abstract

Menaquinones, also known as Vitamin K2 family, regulate calcium homeostasis in a "bone-vascular cross talk" and recently received particular attention for their positive effect on bone formation.

Since the correlation between menaquinones and bone metabolism to date is still unclear, the objective of our study was to investigate the possible role of menaquinone-4 (MK-4), an isoform of the menaquinones family, in the modulation of osteogenesis.

For this reason we used a model of human Amniotic Fluid Mesenchymal Stem Cells (hAFMSCs) cultured both in two-dimensional (2D) and three-dimensional (3D, RCCS™bioreactor) *in vitro* culture systems. Furthermore, to mimic "bone remodeling unit" *in vitro*, hAFMSCs were co-cultured in the 3D system with human Monocyte (hMCs) as osteoclast precursors.

Results showed that in a conventional 2D culture system hAFMSCs were responsive to the MK-4 which significantly improved the osteogenic process through γ-Glutamyl Carboxylase (GGCX)-dependent pathway. The same results were obtained in 3D dynamic system where MK-4 treatment supported the osteoblast-like formation promoting the extracellular bone matrix deposition and the expression of the osteogenic-related proteins (alkaline phosphatase, osteopontin, collagen type-1 and osteocalcin). Worth noting is when the hAFMSCs were co-cultured in a 3D dynamic system with the hMCs, the presence of the MK-4 supported the cellular aggregate formation as well as the hAFMSCs osteogenic function but negatively affected the osteoclastogenic process.

Taken together our results demonstrate that MK-4 supported the hAFMSCs aggregate formation and increased the osteogenic functions. Specifically, our data could 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 leads to fragility as well as an increased risk of fractures. These chronic age-related diseases represent a major global health problem that affects people all over the world (Cauley, 2013). 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 recent knowledge, several drugs have been developed for the treatment of bone diseases even though to date, they do not lead to satisfying results because of the side effects and they do not completely reduce the incidence of fractures (Ito, 2014).

To date, the most efficient clinical practice for skeletal repair is the autologous bone graft. This approach may result invasive and does not lead to a complete structural integration of bone fragments. Today, 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, Amniotic Fluid (AF) represents a non-invasive and more easily obtainable source of MSCs. Amniotic Fluid Mesenchymal Stem Cells (AFMSCs) possess potentially important therapeutic properties for bone tissue regeneration. They are multi-potent, not tumorigenic and are not immunogenic stem cells with great osteogenic differentiation capability (De Coppi *et al.*, 2007, Pipino and Pandolfi, 2015). 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 herbal medicines Naringin and Curculigoside (Di Tomo *et al.*,

2013, Pipino *et al.*, 2014, Pipino and Pandolfi, 2015). It is of great interest to study natural agents that could potentially enhance bone health with a minimal number of side effects.

Menaquinones (MKs) is a family of natural molecules, also known as Vitamin K2, that in the last few years has received a lot of attention for their potential positive effect in bone metabolism (Hamidi and Cheung, 2014). MKs, whose main source is represented by fermented foods such as cheese and Natto (fermented soybeans) (Iwamoto, 2014), are lipid-soluble vitamins produced by bacteria and structurally characterized by the presence of a common ring of 2-methyl-1,4-naphthoquinones, called menadione as well as a side chain of different numbers of isoprenoid units which classify the different isoforms of MK (MKn, n=1-14). The longer the side chain, the more lipophilic MKs are (Willems *et al.*, 2014). In addition to their coagulation properties, known since 1929 (El Asmar *et al.*, 2014), MKs could play a key function in the regulation of calcium homeostasis in "bone-vascular cross-talk". Recently, it has been shown that they may be involved in this cross-talk by reducing calcium deposit in the arteries while increasing it in the bone tissue, a process known as "calcium paradox" (Flore *et al.*, 2013). In fact, MKs, act as co-factors for the enzyme γ-Glutamyl Carboxylase (GGCX) (Tie and Stafford, 2016) and through carboxylation of residues of glutamic acid in γ-carboxyglutamate cause the activation of specific Vitamin K-Dependent Proteins (VKDP) (Dalmeijer *et al.*, 2012). Particularly in bone tissue, the effect of MKs are explained through the activation of the VKDP known as Osteocalcin (OC). OC is a non-collagenous "*Bone Gla Protein*" specifically produced by osteoblasts and essential for the normal bone mineralization in its carboxylated form (cOC) (Iwamoto, 2014, Koitaya *et al.*, 2014). To date, the evaluation of the serum ratio between the cOC and the undercarboxylated form (ucOC) is considered a specific marker of both bone turnover as well as MKs status (Zhang *et al.*, 2016). Furthermore, high levels of ucOC are

correlated with a reduction of Bone Mineral Density (BMD) and an increase of hip fracture risk in elderly women (Szulc *et al.*, 1994). Interestingly, the MKs treatment seems to improve the cOC (Nakamura *et al.*, 2014) and plays 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 MKs as potential osteoinductive factors for bone tissue regeneration, little is known about their capability in modulating osteogenesis in MSCs (Atkins *et al.*, 2009, Yamaguchi and Weitzmann, 2011). The study carried out to date on MKs and their relation with bone were performed mainly on osteoblastic cells (Katsuyama *et al.*, 2007, Koshihara and Hoshi, 1997).

The purpose of our study was to investigate the possible role of MK-4, a specific isoform of MKs with four isoprenoid units, 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. In order to provide a particularly suitable model to understand the molecular mechanisms with which MK-4 supports osteogenic process, we analyzed the effect of the MK-4 treatment in a 3D co-culture system more closely to the bone physiological microenvironment represented by hAFMSCs and represented by human monocytes (hMCs) as osteoclast precursors.

2. Materials And Methods

2.1. Chemicals

Menaquinone 4, a powder (MK-4), was provided by Ibersan-Santiveri s.r.l. (Italy), re-suspended in Dimethyl Sulfoxide (DMSO) at 10 mM concentration and stored at -20 °C. DMSO, ascorbic acid-2-phosphate, β-glycerophosphate, dexamethasone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Alizarin Red S, propidium iodide, formaldehyde, triton X-100, pronase, haematoxylin, Calcein-AM assay and TRAP (no. 386) kit were purchased from Sigma-Aldrich (Saint Louis, USA). Trizol® reagent, CellTracker™ Green CMFDA, High Capacity cDNA Reverse Transcription Kit, Taqman Gene Expression assays and Universal Master mix were purchased from ThermoFisher Scientific (Waltham, MA USA). Vecstain ABC and the DAB solution were obtained from Vectorlabs (Burlingame, CA, USA). EIA kits (MK128 and MK118) were purchased from Takara (Shiga, Japan).

2.2. Cell Culture

hAFMSCs isolated from Amniotic Fluid (AF) of pregnant women (n=10) during routine amniocentesis between 16-18 weeks of gestation as previously described (Pipino *et al.*, 2014), were cultured for 2 days in complete Culture Medium (CM) composed of α-MEM (Sigma-Aldrich) containing 15% Fetal Bovine Serum (FBS, ThermoFisher Scientific), 1% penicillin/streptomycin and 1% L-glutamine (Sigma-Aldrich) supplemented with Chang Medium C Lyophilized Kit (IrvineScientific, Daimler St. Santa Ana, USA) (De Coppi *et al.*, 2007). The cells were grown in 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 then PBMCs were obtained

from diluted peripheral blood (1:2 in Hanks solution), separated by Histopaque®-1077 (Sigma-Aldrich).

hMCs were purified from PBMCs by the 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 out in order to remove non-adherent cells) (Piva *et al.*, 2005). The purity population was then verified by cytofluorimetric analysis employing 1×10^5 cells, incubated with Fluorescein Isothiocyanate (FITC) conjugated with the anti-human CD14 antibody (ImmunoTools GmbH, Friesoythe, Germany) for 15 minutes at 4 °C. A monoclonal antibody with no specificity was used as negative 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 positive CD14 samples were used. For the 3D dynamic co-culture, the hMCs ($0.5x10^6$) were treated with 5mM CellTracker ™ Green CMFDA (ThermoFisher Scientific), trypsinized and inoculated in High Aspect Ratio Vessel (HARV™; Synthecon™, Inc., Houston, TX, USA) in combination with hAFMSCs ($1x10^6$). After 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

As for the 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-LG), 0.05 mM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate and 100 nM dexamethasone supplemented with 5% FBS in presence (positive control) or absence (negative control) of the MK-4. In particular, preliminary data obtained using 1, 5 and 10% FBS

concentrations in OM showed that 5% FBS demonstrated the right balance between cellular proliferation and differentiation.

The MK-4 treatment started from the 7th day of osteogenic differentiation and 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 a 2D culture system was assessed by 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 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 according to the manufacter's instruction. The MTT crystals were solubilized with 200µL of DMSO and the spectrophotometric absorbance of each sample was then measured at 540nm by using a microplate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, CA, USA).

2.5. Alizarin Red S Assay

The effect of MK-4 on osteogenic differentiated hAFMSCs cultured in a monolayer or in 3D aggregates was assessed by Alizarin Red S (ARS) staining (Sigma-Aldrich). After being washed twice with PBS, the cells were fixed in 10% formaldehyde for 10 minutes at Room Temperature (RT). The cells were then stained with 40 mM of ARS solution (pH 4.2) for 20 minutes at RT to detect and to further quantify the calcium deposits by using the 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, Lafayette, CO, USA) 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) started from the 7^{th} day of osteogenic differentiation and continued for 7 days (treatment every 24 hours) as summarized in Figure 1B. The effects of the MK-4 treatment after the GGCX silencing was assessed by a quantitative reverse transcription polymerase chain reaction (qRT-PCR) and flow cytometry for the expression of specific osteogenic markers.

2.7. RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction

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

For reverse transcription, the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) was used. The equivalent of 0.1µg of cDNA was used for the reactions of qRT-PCR carried out with the ABI Prism 7900 Sequence Detection System (ThermoFisher Scientific). 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) and the TaqMan Universal PCR Master Mix (ThermoFisher Scientific) were used according to the manufacturer's instructions. The relative gene expression was calculated with the use of the comparative $2^{-\Delta\Delta CT}$ method.

2.8. Carboxylated and Undercarboxylated Osteocalcin

The carboxylated (cOC) and the undercarboxylated (ucOC) 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 (both MK128 and MK118 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 the total of ALP, RUNX2 and COL1A1 protein expression. Staining procedure followed a previous step of membrane fixation and permeabilization (Di Tomo *et al.*, 2013). The staining was performed by incubating $5x10^5$ cells/sample with primary monoclonal antibody against ALP (1:100, mouse anti-ALP antibody; ThermoFisher Scientific), RUNX2 (1:800, rabbit anti-RUNX2 antibody; Cell Signaling, Danvers, MA, USA) and COL1A1 (1:100, mouse anti-COL1A1 antibody; Santa Cruz Biotechnology, Dallas, TX, USA) for 30 minutes on ice, followed by staining with Alexa488-labeled secondary antibodies (1:1500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 minutes on ice. To evaluate cell viability, 7-aminoactinomycin D (7-AAD, 1:100) was used (BD Biosciences). To assess nonspecific fluorescence, samples stained with the corresponding secondary antibody alone were used. 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 a 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.). 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™ 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 4rpm, corresponding to the Ground Based dynamic culture. After 24 hours, the existence of aggregates was observed and the vessels were filled with osteogenic medium. Next to 7 days, the MK-4 (10 µM) was added into the HARV vessels and control samples were maintained in an osteogenic medium plus vehicle (DMSO) added with the same volumes.

At days 14/21 aggregates were collected, fixed in 4% of formaldehyde, embedded in paraffin, sectioned and processed for immunocytochemistry analysis.

2.11. Live/Dead Staining of Cells in 3D Culture

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. Histology and Immunocytochemistry

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 (Tris-Buffered Saline) 1X. 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) were added and incubated overnight (4°C). Cells were then incubated in Vecstain ABC (Vectorlabs, Burlingame, CA, USA) reagents (30 min) and stained with DAB solution (Vectorlabs). After washing, cells were mounted in glycerol and observed with the use of the Nikon Esclipse 50i optical microscope.

Histological sections (5µm) of 3D aggregates were used for immunohistochemistry. Thus, deparaffinized, rehydrated and enzymatic treated non-consecutive sections were incubated with pronase (1mg/mL, Sigma-Aldrich) for antigen retrieval and permeabilization. Slides were then immune-stained 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 counter-stained 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 reported (Piva *et al.*, 2005). The staining was 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 bright-field microscopy (Nikon Eclipse 50i; Nikon Corporation, Tokyo, Japan). For the analysis

of cells in monolayers and tissue sections obtained from aggregates, the positive immune-staining was expressed as a % percentage of the 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

Conventional 2D culture system was used to evaluate the dose-response effect of MK-4 on hAFMSCs in terms of cytotoxicity and extracellular matrix mineralization. As revealed by the MTT assay performed at day 3 (data not shown) and day 7 of the culture (Fig. 2A), increasing doses (0.1,1,10 μM) of the MK-4 were not cytotoxic. When osteogenic was induced, the hAFMSCs were exposed to daily MK-4 treatment with 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 evidence from studies, we chose to use the MK-4 at a concentration of 10 μM for the subsequent experiments.

The improvement of hAFMSCs osteogenic's ability by the MK-4 was validated by analyzing the expression of typical osteogenic markers. As reported in Figure 2C, the MK-4 treated cells showed a significant increase of the ALP, the RUNX2 and of the OC mRNA expression levels, in respect to the untreated cells after 14 days of the 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 the COL1A1 and of the OPN analyzed both at mRNA (Figure 2C) and immunocytochemical levels (Figure 2D) at the same time.

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

Given that the MK acts as a co-factor for the GGCX, we investigated as to whether the pro-osteogenic effect of MK-4 was mediated by a GGCX-dependent pathway in hAFMSCs. 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 the MK-4 treatment (21 days of osteogenic induction, see Figure 1B). The EIA quantifying showed a significant decrease of the undercarboxylated OC (ucOC) form and, on the contrary, an increase of carboxylated OC (cOC) levels in the MK-4 treated cells compared to untreated cells (Figure 3A-B). Accordingly, the ratio between cOC/ucOC significantly increased in the MK-4 treated cells (Figure 3C), indicating that hAFMSCs are effectively MK-4 responsive (scheme in Figure 3D).

In a second step the GGCX gene knockdown experiments were performed. The siRNA treatment was highly effective in the GGCX down-regulation 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 the siRNA treatment every 96 hours for 14 days of the 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, the GGCX silencing significantly affected the ability of the MK-4 to improve the osteogenic induction of hAFMSCs, in terms of the ALP, the RUNX2 and the COL1A1 expression levels as revealed by the qRT-PCR (Figure 4A) and by flow cytometry analysis (Figure 4B).

These evidences have demonstrated that the MK-4 pro-osteogenic ability occurs through a GGCX-dependent pathway.

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

Live/dead hAFMSCs in a 3D culture system was evaluated after 7 days of the MK-4 treatment (14 days of osteogenic induction, see Figure 1B) by Calcein AM/Propidium iodide (PI) double staining. As shown in Figure 5A and in Supporting Information

(Figure S2) the aggregates appeared sizeable and formed by viable cells both in presence or absence of the MK-4. The functional properties of the cells forming the aggregates were then investigated by immunohystochemical analysis. hAFMSCs maintained their ability to osteo-differentiate being COL1A1, OPN and ARS positive. Interestingly, the MK-4 treatment significantly improved the osteoblast-like formation after just 7 days of the 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 by the ARS positive areas respectively (Figure 5B). At the same time, the MK-4 maintained high COL1A1 protein levels, suggesting that it is overall positively affected by the extracellular bone matrix deposition (Figure 5B).

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

In a first step, the ability of the hAFMSCs to support the osteoclastogenesis of osteoclast precursors and the inhibition of this phenomenon by the MK-4 was demonstrated by the TRAP assay and by acting ring analysis in a conventional 2D transwell co-culture system (Supporting Information, Figure S3).

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

Calcein-AM/PI assay carried out after 14 days of co-culture both in presence and in absence of MK-4 (Figure 6A and Supporting Information, Figure S2) revealing that also in this case the aggregates appeared formed by viable cells.

As shown in Figure 6B, the TRAP assay clearly highlighted the ability of the hAFMSCs to sustain osteoclastogenesis in the 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 the positive TRAP areas and multinucleated cells was observed (Figure 6B). Conversely, an osteoblastic–like cell component was positively affected by the MK-4 given a significant increase of mineral matrix deposition, the OPN and the COL1A1 expression in the inner portion of aggregate was observed (Figure 6C). Notably, the analysis performed after 14 days of the MK-4 treatment (21 days of osteogenic induction, see Figure 1B) showed that the aggregate was maintained only in the presence of the 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 safety 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).

The Amniotic Fluid Mesenchymal Stem Cell (AFMSCs), obtained during pregnancy by routine amniocentesis, represents a less invasive and more primitive source of stem cells to keep up with the pace of growth and of demands of cells necessary for regenerative medicine. In fact, they possess a multi-potent differentiation ability, anti-inflammatory and immune-modulatory properties, non-tumorigenicity, and no ethical problems (Cananzi *et al.*, 2009). It has been demonstrated that the hAFMSCs have a great capability to differentiate in osteogenic cells (Chen *et al.*, 2010, De Coppi *et al.*, 2007, Pipino *et al.*, 2015), and several studies have shown that this process could 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).

Among natural molecules, a promising compound with potential osteoinductive properties may be menaquinones (MKs), a family of fat soluble vitamins that act as a co-factor of the enzyme ɣ-Glutamyl Carboxylase (GGCX) (Tie and Stafford, 2016).

To date, the MKs are used for the treatment of osteoporosis in Japan and several clinical trials have suggested their healthy effects on bone metabolism particularly in the maintenance and improvement of vertebral Bone Mineral Density (BMD) as well as 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, they have recently received major attention for their 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, the 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 recently demonstrated that the MKs in association with vitamin D3 could improve the hBMSCs osteogenic differentiation, today little is known about its possible osteogenic effect on MSCs from bone marrow and other sources (Zhang *et al.*, 2016).

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

In particular, a conventional two-dimensional (2D) cell culture system was used to demonstrate the hAFMSCs responsiveness to the 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, the GGCX silencing significantly affected the ability of the MK-4 to improve the osteogenic induction of the hAFMSCs, in terms of ALP, RUNX2 and COL1A1 expression levels as revealed by the 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 increased following treatment, thus further supporting the hypothesis that this vitamin in hAFMSCs, acts as a co-factor of the GGCX.

These evidences have demonstrated that the MK-4 pro-osteogenic ability occurs through the GGCX-dependent pathway. Other possible mechanisms, such 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 reinforced the knowledge on the positive effect of the MK-4 during the osteogenic process by using the 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 which supports the production of bone-like matrix in the cell aggregates even without exogenous scaffold employment, thus providing a more biologically relevant model rather 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 such as a "bone mimetic product" to study potential drugs such as the MK-4. In fact, as expected and in accordance with studies (Yamaguchi and Weitzmann, 2011), the MK-4 treatment negatively affects osteoclastogenesis but supports the osteoblastic–like cell component. Our data clearly demonstrates that the presence of the 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 observed.

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

For the first time, despite the possible prevalence in the hAF of one over the other cells' phenotype could explain the variability of the 3D cell-aggregate quality, the presence of the MK-4 supports and maintains bone aggregate formation and the 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 gives proof that combining the MSCs with appropriate molecules in a 3D culture system, makes it possible to reproduce a physiological microenvironment through which give informative answers on the properties of the cells and on the effects of a specific substance.

According to previous reports (Zhang *et al.*, 2016) we additionally showed in our study (Supporting Information, Figure S4) that the MK-4 treatment also improved *in vitro*

osteogenesis in human BMSCs. In recent times, it was demonstrated that 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 based on our results, it can be hypothesized that *in vivo*, besides the positive direct effects of this vitamin on osteoblastic cells (Katsuyama *et al.*, 2007, Koshihara and Hoshi, 1997), the administration of the MK-4 could 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, together our results suggest the ability of MK-4 to improve hAFMSCs osteogenic differentiation in both a 2D and a 3D culture system.

Together with the unique features of hAFMSCs and the possibility of cell banking, our results allow us to speculate the development not only of new strategies of cell-based therapy but also a possible engineered system for a 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 with the use of dietary supplements and our bone mimetic product based on the combination of the hAFMSCs with the OCs precursors could be proposed as a platform to test different bone anabolic or catabolic molecules.

Conflict of Interest

The authors declare no potential conflict of interest.

Acknowledgments

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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**)

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 10 µM (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 (10 µM) 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**) The evaluation of live/dead cells by Calcein-AM/PI assay. Green staining reveals 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

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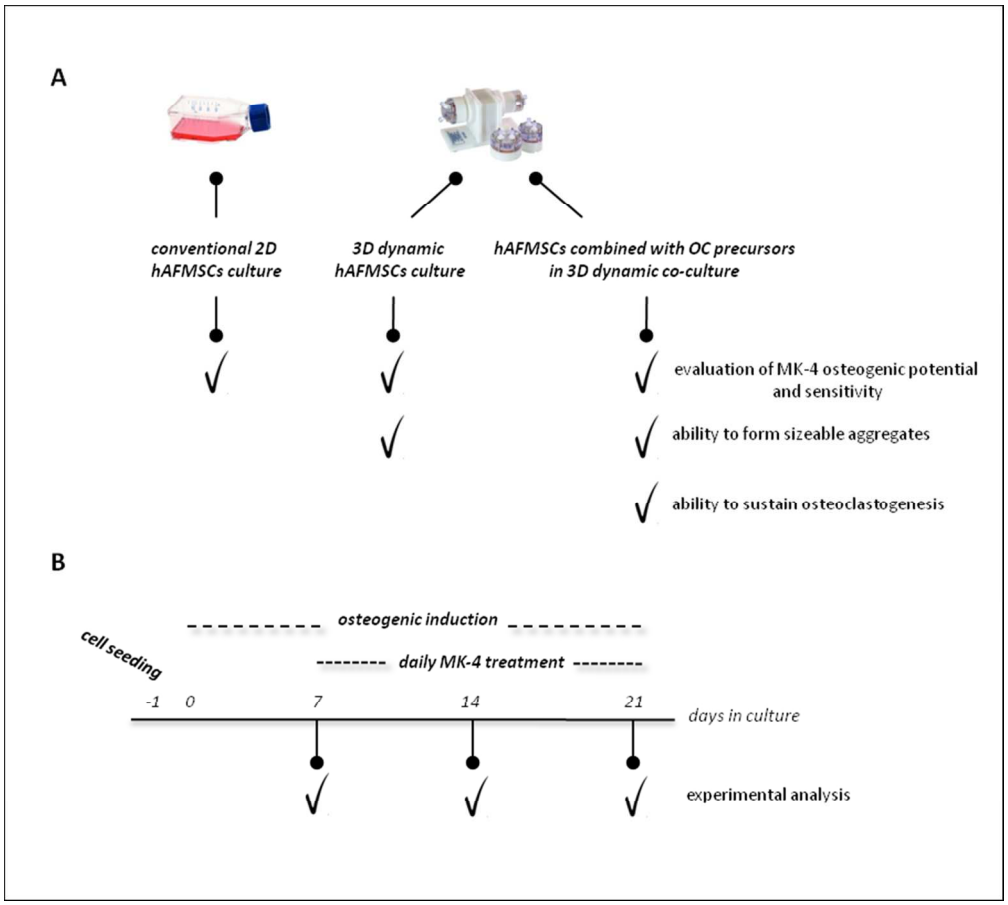
Figure 6. Effect of MK-4 on hAFMSCs and osteoclast precursors 3D dynamic co-culture system.

(**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. The evaluation of live/dead cells by Calcein-AM/PI assay was 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 ± 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 10 μM (OM+ MK-4 10 μM, black column). (*p<0.05 vs OM). Scale bar: 50 μm.



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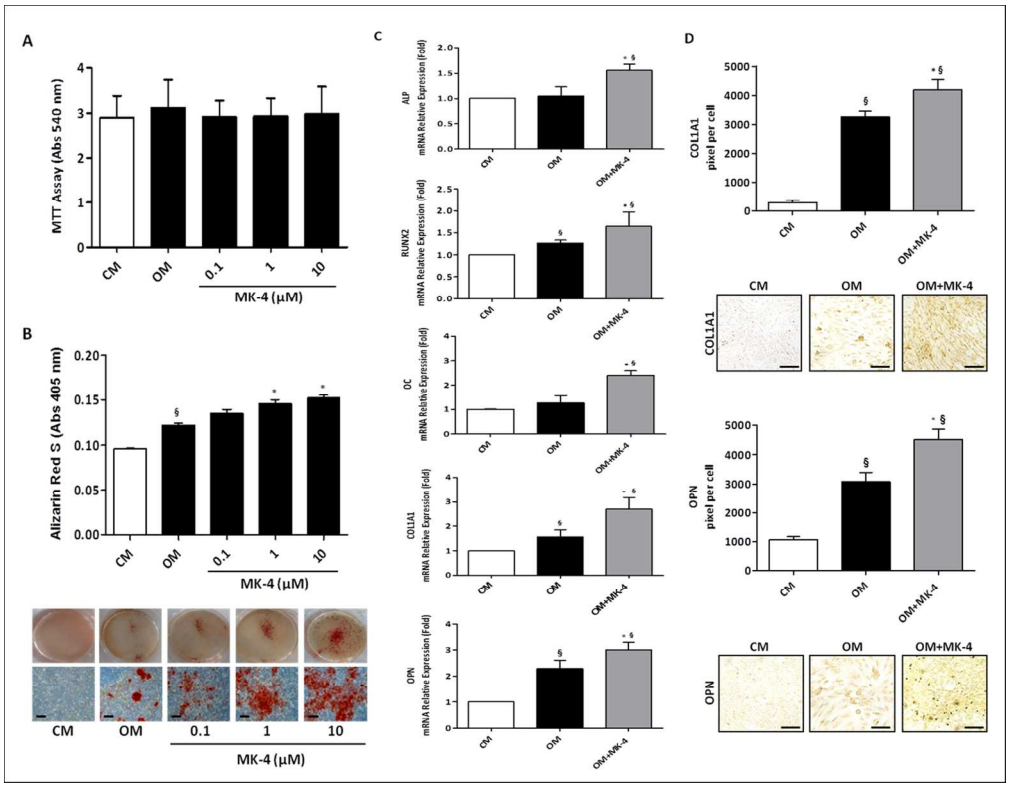

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Control medium (CM, white column), osteogenic medium (OM, black column) and OM added with MK-4 10 μM (OM+MK-4, grey column).
For all experiments results are expressed as mean±standard deviation (SD) (n=3) (§p< 0.05 vs CM; *p< 0.05 vs OM).

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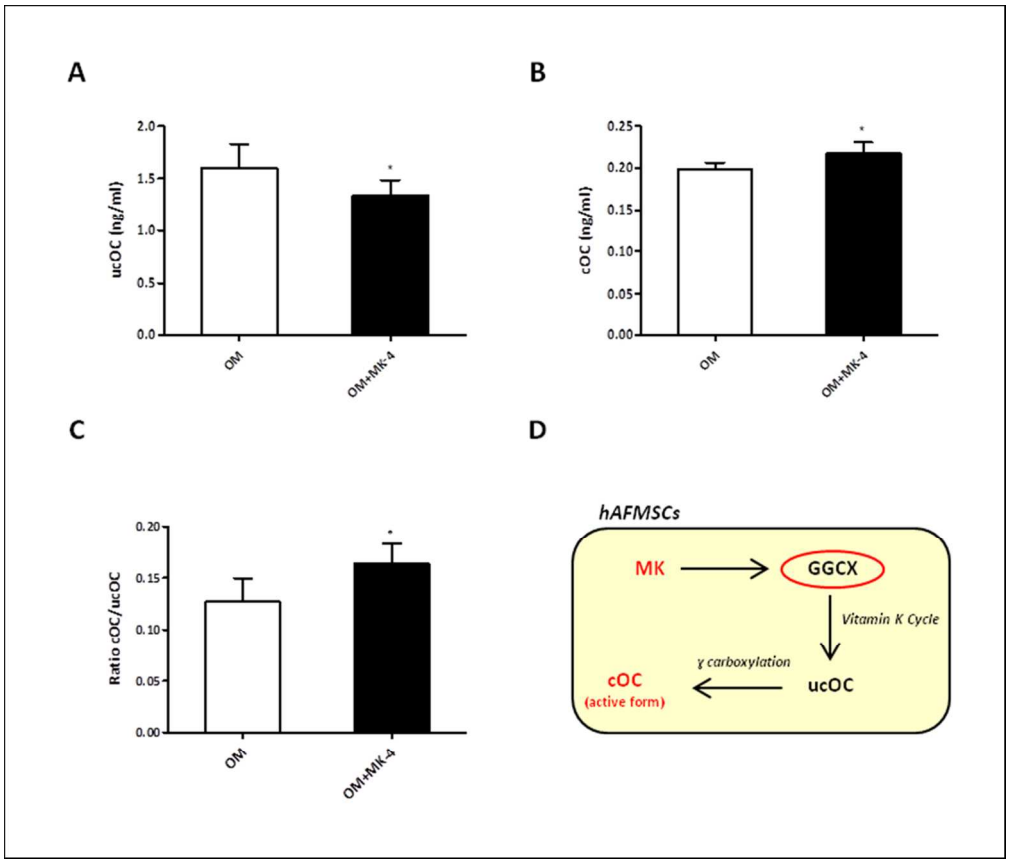

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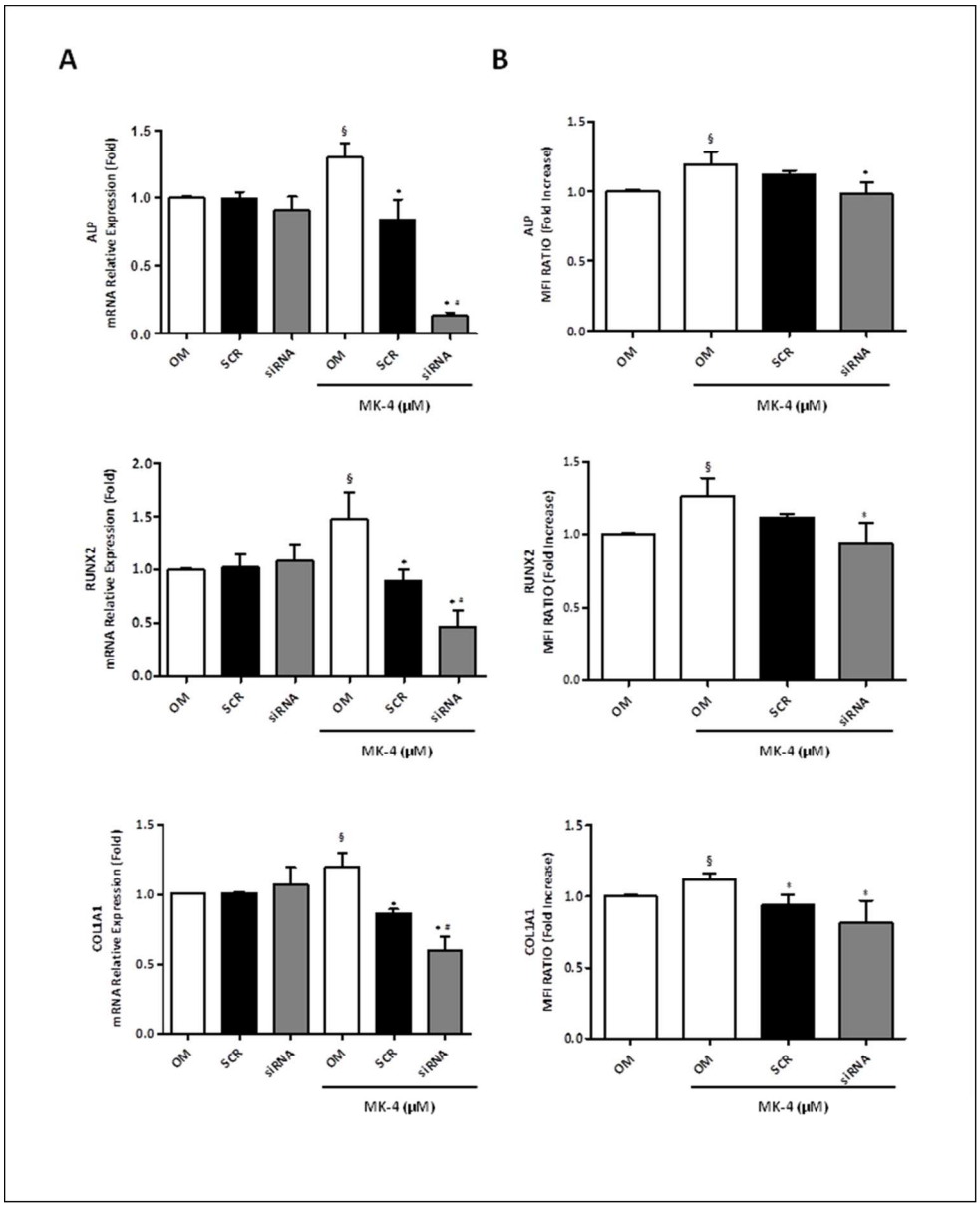

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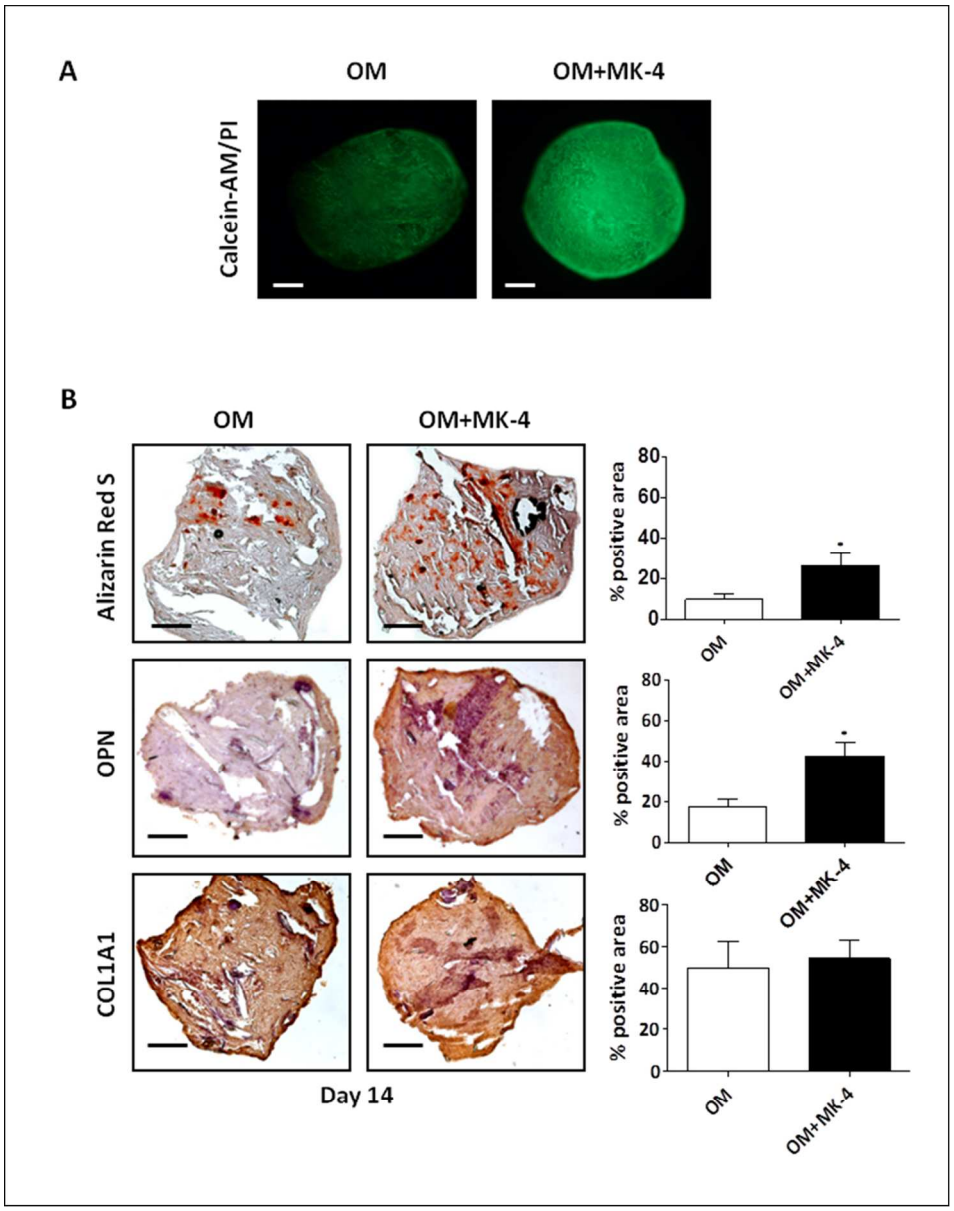

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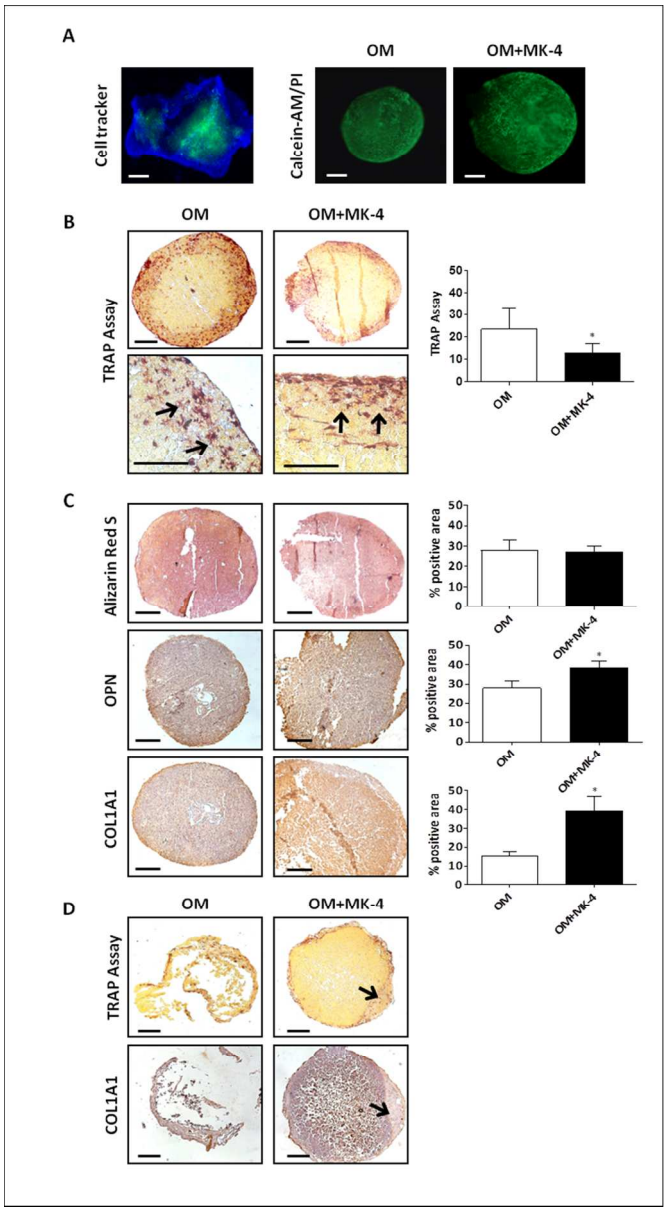

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