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Methylsulfonylmethane enhances MSC chondrogenic commitment and promotes pre-osteoblasts formation

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Abstract

Background: Methylsulfonylmethane (MSM) is a nutraceutical compound which has been indicated to counteract osteoarthritis, a cartilage degenerative disorder. In addition, MSM has also been shown to increase osteoblast differentiation. So far, few studies have investigated MSM role in the differentiation of mesenchymal stem cells (MSCs), and no study has been performed to evaluate its overall effects on both osteogenic and chondrogenic differentiation. These two mutually regulated processes share the same progenitor cells.

Methods: Therefore, with the aim to evaluate the effects of MSM on chondrogenesis and osteogenesis, we analyzed the expression of SOX9, RUNX2, and SP7 transcription factors in vitro (mesenchymal stem cells and chondrocytes cell lines) and in vivo (zebrafish model). Real-time PCR as well Western blotting, immunofluorescence, and specific in vitro and in vivo staining have been performed. Student's paired t test was used to compare the variation between the groups.

Results: Our data demonstrated that MSM modulates the expression of differentiation-related genes both in vitro and in vivo. The increased SOX9 expression suggests that MSM promotes chondrogenesis in treated samples. In addition, RUNX2 expression was not particularly affected by MSM while SP7 expression increased in all MSM samples/model analyzed. As SP7 is required for the final commitment of progenitors to preosteoblasts, our data suggest a role of MSM in promoting preosteoblast formation. In addition, we observed a reduced expression of the osteoclast-surface receptor RANK in larvae and in scales as well as a reduced pERK/ERK ratio in fin and scale of MSM treated zebrafish.

Conclusions: In conclusion, our study provides new insights into MSM mode of action and suggests that MSM is a useful tool to counteract skeletal degenerative diseases by targeting MSC commitment and differentiation.

Keywords: Methylsulfonylmethane, Mesenchymal stem cells, Chondrocytes, Osteoblasts

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Background

Methylsulfonylmethane (MSM) is a popular dietary supplement, suggested for relieving joint/muscle pain, thanks to its anti-inflammatory properties. MSM is a natural member of the methyl-S-methane compounds, and it can be synthetically produced through the oxidation of DMSO with hydrogen peroxide (H₂O₂) [1]. In *in vitro* studies, it has been demonstrated that MSM inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) [2, 3] as well as p65-S536 phosphorylation [4]. Hence, NF- κ B inhibition results in the reduced expression of IL-1, IL-6, tumor, and necrosis factor- α (TNF- α) [5, 6]. In addition, MSM reduces the expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [7].

MSM has been suggested as a nutraceutical compound to counteract osteoarthritis [8]. Osteoarthritis (OA) is a degenerative disease characterized by pain, affecting cartilage homeostasis by inducing its degradation and impairing matrix neo-synthesis by chondrocytes [9]. Chondrocytes derive from mesenchymal stem cell (MSCs) condensation and differentiation induced by the transcription factor SOX9 [10, 11]. When the balance between degradation and synthesis of cartilage matrix proteins is affected, inflammation and OA disease arise [12]. Cheleschi et al. by performing an *in vitro* study demonstrated that MSM has beneficial effects on chondrocytes exposed to interleukin (IL)-1 β , most likely by modulating the NF- κ B pathway [8]. In another *in vitro* study, MSM was shown to increase osteoblast differentiation through the Jak2/STAT5b pathway [13]. It is well known that MSC osteogenic differentiation is induced by the upregulation of the master gene RUNX2, which in turn transactivates its downstream partner SP7 (Osterix), SP7 then promotes the expression of osteogenic genes such as SPARC (osteonectin), SSP1 (osteopontin), COL1A1 and COL1A2 (collagen type I), and BGLAP (osteocalcin) [14]. Other *in vitro* studies suggested that MSM increases osteogenic differentiation by inducing BMP2 [15] or transglutaminase-2 (TG2) [16]. MSM influence on the commitment and differentiation of mesenchymal cells appears evident on the basis of these findings. SOX9 and RUNX2 interact in progenitor cells and can affect their respective transactivation in a reciprocal way [17]. Regulation of RUNX2 and SOX9 occurs at the transcriptional and posttranscriptional levels: phosphorylation, acetylation, and ubiquitination can modulate their activity [18–20]. The temporal and spatial distribution and overlapping expression of RUNX2 and SOX9 occurring in embryogenesis show a coordinated interplay between these two genes in bone formation [21–23]. Importantly, it has been demonstrated that SOX9 induces RUNX2 degradation; hence, it plays an important role in controlling mesenchymal stem cells differentiation fate [17].

With the aim to deepen our knowledge about MSM effects on both chondrogenesis and osteogenesis, we have investigated the molecular modulation following MSM supplementation during the differentiation in both *in vitro* models (mesenchymal stem cells and chondrocytes) and *in vivo* models (larvae and adult zebrafish).

Methods

Methylsulfonylmethane

Methylsulfonylmethane (MSM) (Artrosulfur; Laborest S.r.l, Assago, MI, Italy) was used at the concentration of 20 mM as previously reported [24, 25].

XTT test

Cell viability was evaluated by using the Cell proliferation kit II (XTT Chemicon) as previously described [26]. Six replicates in three independent experiments were tested.

Cell cultures

Human mesenchymal stem cells (hMSC, PromoCell, Heidelberg, Germany) were plated at a density of 5×10^4 cells and cultured with mesenchymal stem cell growth medium (PromoCell) or osteogenic differentiation medium or chondrogenic differentiation medium (PromoCell, Heidelberg, Germany) and incubated at 37°C in a humidified atmosphere with 5% CO₂. Differentiating cells were then used for further analyses. Human chondrocytes (HCH, PromoCell, Heidelberg, Germany) were plated at a density of 5×10^4 cells and cultured with DMEM 10% FBS medium at 37°C with 5% CO₂. IL1 β (1 ng/mL) was added to HCH cells to mimic OA conditions, as previously reported [26]. Three independent experiments were performed for each condition.

Zebrafish

Zebrafish experiments were performed at the CIRSAL (Interdepartmental Centre of Experimental Research Service) of the University of Verona, Italy. The animal protocol was approved by the Italian Ministry of Health Directorate-General for animal health and veterinary medicinal products (authorization n. 662/2019-PR of 16/09/2019). The embryos were obtained from natural spawning of *nacre* adults (ZFIN database ID: ZDB-ALT-990423-22), according to standard protocols [27], and staged according to Kimmel [28]. Zebrafish embryos were grown at 33°C in water containing 20-mM MSM from 2 days post fertilization (dpf). Zebrafish embryos were treated with MSM up to 1 week (experimental endpoint, 9 dpf) or 2 weeks (experimental endpoint, 16 dpf). At the end of the treatment, zebrafish embryos were euthanized and collected for molecular analyses as described below. Calcein staining was performed as previously reported [29]. Imaging was performed using

Leica M205FA fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Stained areas were quantified by using the ImageJ software, as previously reported [29].

Adult zebrafish (15–20 months) were grown in water containing 20 mM of MSM for 14 weeks. At the experimental endpoint, zebrafish were euthanized and collected for staining and molecular procedures, as reported below.

Total RNA extraction and reverse transcription

Pellets from cell lines or zebrafish samples were collected, stored at -80°C and processed for RNA extraction with a “RNeasy” protect mini kit” (Qiagen, Hilden, Germany) following the manufacturer’s instruction. RNA samples were quantified using the Qubit™ RNA HS assay kit” (Invitrogen, Carlsbad, USA). RNA (1 mg) was reverse transcribed using the first-strand cDNA synthesis kit (GE Healthcare, Little Chalfont), according to the manufacturer’s instruction. miRNAs were extracted by using miRNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. First-strand complementary DNA (c-DNA) synthesis was performed using the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer’s protocol. RNA, microRNAs, and cDNA samples were stored at -80°C until use.

Real-time PCR

To investigate gene expression modulation, we performed real-time PCR analyses as reported previously [30].

Briefly, predesigned, gene-specific primers, and probe sets for each gene (RUNX2, hs00231692_m1; OSTERIX (SP7), hs00541729_m1; COLLAGEN, TYPE I, ALPHA 2 (COL1A2), hs01028956_m1; OSTEONECTIN (SPARC), hs00234160_m1; OSTEOPONTIN (SPP1), hs00167093_m1, SOX9, hs01107818_m1; COMP, hs004359-m1; COL2A1, hs00264051_m1; B2M, hs999999_m1 (housekeeping); GAPDH, 0802021 (housekeeping); sox9a, Dr03112282_m1; col2a1a, Dr03099270_m1; miR-146b-5p, 474220; RNU44, 001094) were obtained from assay-on-demand gene expression products (Thermo Fisher Corporation, Waltham, MA, USA). In addition, the following custom primer sets (Invitrogen, Carlsbad, CA, USA) were also used: runx2a (fw GACGGTGGTGACGGTAATGG, rv TGCGGTGGGTTCTGTAATA), runx2b (fw CGGCTCCTACCAGTTCTCCA, rv CCATCTCCCTCCACTCCTCC), rank (fw GCACGGTTATTGTTGTTA, rv TATTCAGAGGTGGTGTAT), and as housekeeping gene actb1 (fw CCCAAAGCCAACAGAGAGAA, rv ACCAGAAGCGTACAGAGAGA).

Ct values for each reaction were determined using TaqMan SDS analysis software (Applied Biosystems; Foster City, California, USA) as reported previously. To calculate relative gene expression levels between

different samples, we performed the analyses by using the $2^{-\Delta\Delta\text{CT}}$ method as previously reported [30].

Immunofluorescence

Immunofluorescence analyses were performed as previously reported [30]. Briefly, cells were fixed and processed according to the manufacturer’s protocols. Primary antibodies for RUNX2 and osteocalcin (sc74495, Santa Cruz, Dallas, Texas, USA) were diluted according to the manufacturer’s instruction in antibody dilution buffer and incubated overnight at 4°C . Slides were then incubated with the secondary antibodies goat mouse fluorescein conjugated (cat. Ap124f, Millipore, Burlington, Massachusetts, USA). Nuclear staining was performed by ProLong™ Gold Antifade Mountant with DAPI. The staining was analyzed using a Leica (Wetzlar, Germany) TCS SP5 AOBS microscope. To express data in a semiquantitative way, six different fields were analyzed for each sample, in three independent experiments with about 80–100 total cells.

Western blotting

Protein levels were separated by SDS Page and investigated by Western blot analyses as previously reported [31]. Briefly, proteins were extracted by RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) and protein concentration was established by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). After protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA). PVDF membranes were then probed with the primary (Aggrecan (BC-3), Thermo Fisher Scientific; β -actin (BA3R), Thermo Scientific; ERK (13F5), Cell Signaling; p_ERK (D13.14.4E), Cells Signaling) and secondary antibodies (Anti-mouse (Cell Signaling, 7076); Anti-rabbit (Cell Signaling 7074)). Signals were detected using a chemiluminescence reagent (ECL, Millipore, Burlington, MA, USA) according to the manufacturer’s instructions. Images were acquired by a LAS4000 Digital Image Scanning System (GE Healthcare, Little Chalfont, UK). The Densitometric analysis was performed by ImageQuant software (GE Healthcare, Little Chalfont, UK). Proteins optical density was normalized to β -actin.

Alizarin Red Staining in in vitro experiments

To evaluate calcium deposition in differentiating osteogenic cells, we performed Alizarin red staining as previously described [30]. Briefly, after 21 days of culture in osteogenic medium, cells were fixed with 70% ethanol and washed with water. Then, cells were stained with 40 mM Alizarin red S for 5 min at pH 4.1 and rinsed for 15 min with 1x phosphate-buffered saline. The stained area

was quantified by using ImageJ software (NIH, Bethesda, MD, USA) as previously reported [30]. Six independent experiments were performed.

Zebrafish staining

Calcein staining in zebrafish larvae was performed as previously reported [29]. Imaging was performed using Leica M205FA fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The stained area was quantified by using the ImageJ software, as previously reported [29].

Bone and cartilage staining was performed on adult (>1 year old) zebrafish according to Sakata-Haga et al. [32], strictly following their “RAP System” protocol. Briefly, euthanized fish were immersed into the FIXATIVE (5% formalin, 5% Triton X-100, 1% potassium hydroxide (KOH) and gently rocked for 12 h at room temperature (RT)). Then, we proceeded either to the cartilage staining step for cartilage-only/double staining or directly to the bone staining step for bone-only stain. For cartilage staining, specimens were immersed into C-STAINING SOLUTION (70% ethanol, 20% acetate, 0.015–0.02% alcian blue) overnight at 20°C, then washed in C-STAINING MEDIUM (containing 1% Triton X-100) overnight at 20°C and washed with 50–70% ethanol. For cartilage-only staining, the protocol proceeded directly to clearing and stock steps. For cartilage and bone double staining, the protocol proceeded to the bone staining step. Briefly, for bone staining, specimens were immersed into B-STAINING MEDIUM (20% ethylene glycol and 1% KOH) and then in “B-STAINING SOLUTION (0.05% alizarin red S, 20% ethylene glycol, 1% KOH) overnight at 20°C. The specimens were then washed with clearing solution (20% Tween 20, 1% KOH) while gentle rocking for 12h, and the stocking was performed in glycerol 100%. All needed solutions were prepared as indicated in Sakata-Haga et al. [32]. The stained area was quantified by using the Image J software, as previously reported [29].

Statistical analysis

Results were expressed as mean $\pm$ S.E. Student's paired t test was used in order to compare the variation in a variable between two groups. Differences were considered statistically significant with $p < 0.05$. The statistical analyses were performed using GraphPad Prism software program (version 8.3; GraphPad Software).

Results

MSM improves chondrogenesis in vitro and in vivo

To evaluate the effects of MSM in chondrogenesis, we cultured mesenchymal stem cells with and without MSM during chondrogenic differentiation. MSM addition to chondrogenic medium did not affect cells number during the differentiation period (Fig. 1A).

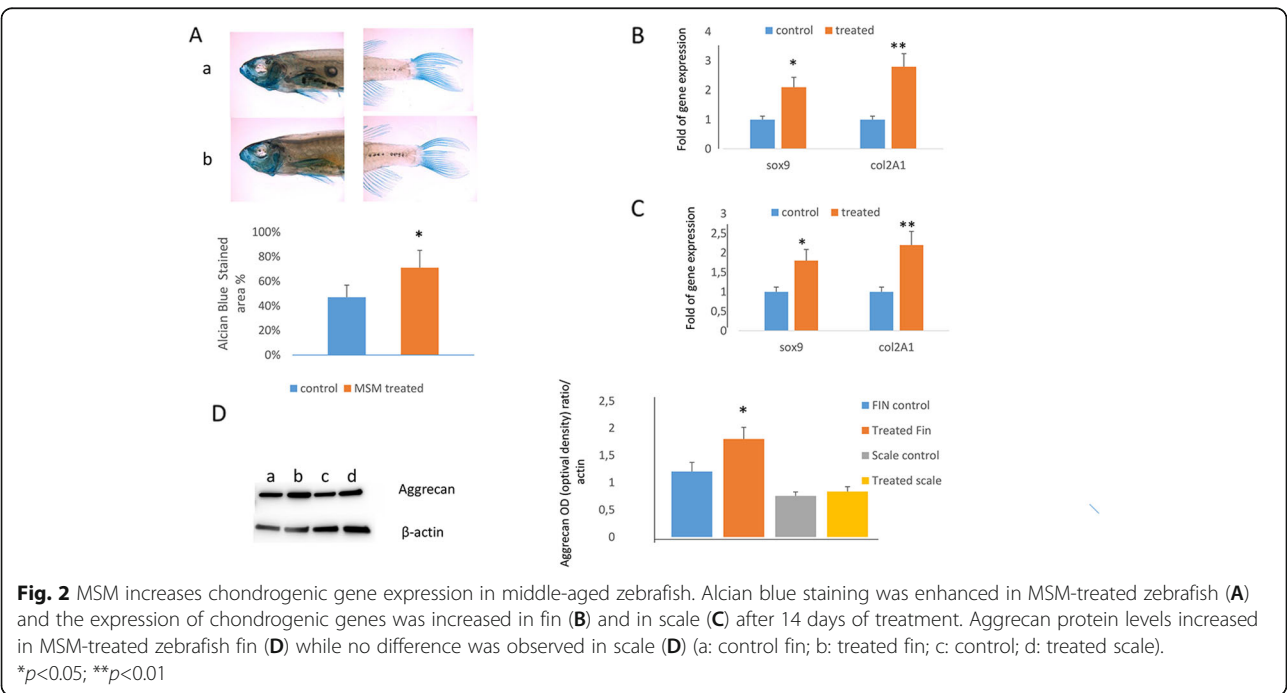
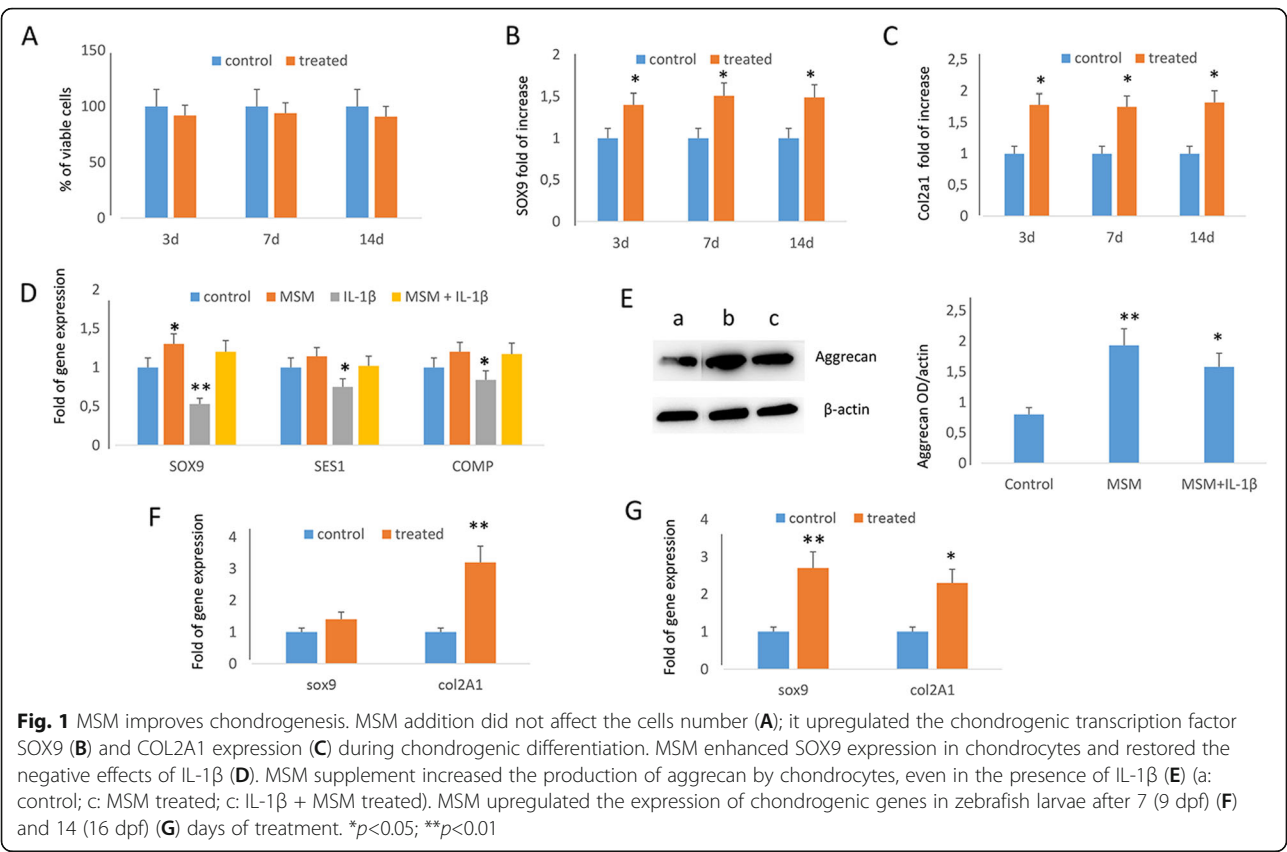
Instead, we observed the upregulation of the chondrogenic transcription factor SOX9 (Fig. 1B) and of COL2A1 (collagen type II) expression (Fig. 1C) after 3, 7, and 14 days of chondrogenic differentiation.

Increased SOX9 expression was also observed in chondrocytes cultured for 7 days in the presence of MSM (Fig. 1D). In addition, MSM supplementation restored the negative effects of IL-1 β on chondrocytes. In fact, MSM counteracted SOX9 downregulation as well as Sestrin 1 and COMP reduced expression induced by IL-1 β (Fig. 1D). Accordingly, the production of aggrecan, a cartilage specific proteoglycan protein, increased in chondrocytes in the presence of MSM, and this effect was appreciable also in chondrocytes treated with IL-1 β (Fig. 1E). The upregulation of chondrogenic genes was observed in MSM-treated zebrafish larvae after 7 (9 dpf) (Fig. 1F) and 14 (16 dpf) days (Fig. 1G) of treatment. We thereafter analyzed the effects of MSM treatment on middle-aged zebrafish (15–20 months corresponding to 36–48 years of human age) and observed increased chondrocyte-specific Alcian blue staining in treated zebrafish compared to control (Fig. 2A).

The upregulation of chondrogenic genes was observed after 14 days in fin (Fig. 2B) and in scale (Fig. 2C) of MSM-treated zebrafish. Therefore, as aggrecan plays an important role in cartilage maintenance, we investigated MSM effects on aggrecan production. In particular, we analyzed the protein levels in fin and we observed an increased production in MSM-treated zebrafish compared to controls (Fig. 2D). On the contrary, we did not observe a significantly increased production in zebrafish scale upon MSM treatment (Fig. 2D).

MSM promotes osteoblast maturation

Considering that chondrogenesis and osteogenesis are intertwining processes, we evaluated the effects of MSM in MSCs during osteogenic differentiation. As shown in Fig. 3A, after 3 and 7 days of osteogenic differentiation, respectively, we observed a reduced number of differentiating cells. Therefore, we analyzed the expression of osteogenic transcription factors RUNX2 and its downstream partner SP7. As shown in Fig. 3B, we observed the upregulation of SP7 in MSM treated cells, whereas RUNX2 expression levels were unchanged after 3 days of osteogenic differentiation. Subsequently, after 7 days of differentiation RUNX2 gene expression was reduced in MSM treated cells, while SP7 higher expression persisted. RUNX2 protein levels were consistently unchanged after 3 days of osteogenic differentiation (Fig. 3C) and significantly reduced after 7 days (Fig. 3D) in MSM-treated cell nuclei. MSM effects on osteogenic differentiation persisted also after 14 and 21 days. As shown in Fig. 4, the expression of RUNX2 was reduced and the expression of osteoblast maturation related



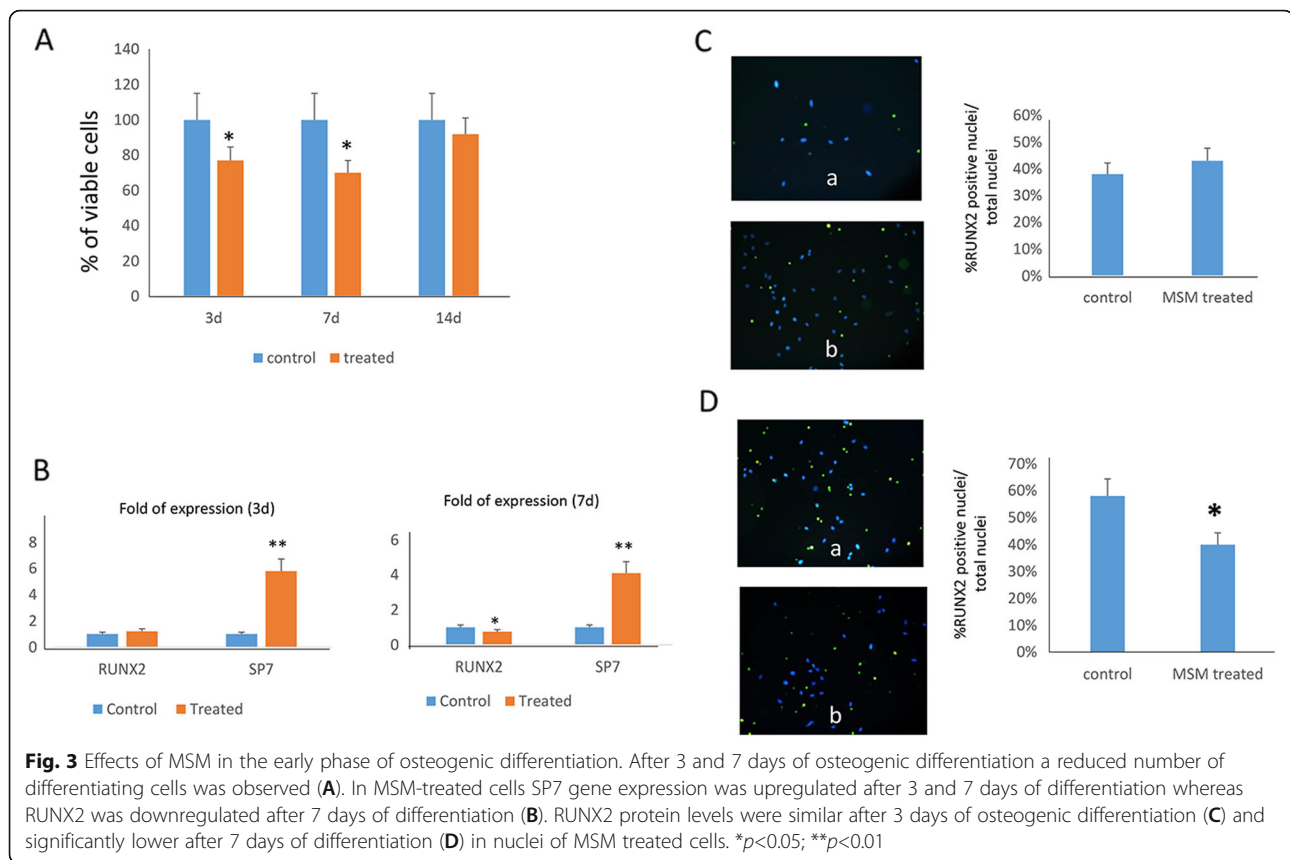


Fig. 3 Effects of MSM in the early phase of osteogenic differentiation. After 3 and 7 days of osteogenic differentiation a reduced number of differentiating cells was observed (A). In MSM-treated cells SP7 gene expression was upregulated after 3 and 7 days of differentiation whereas RUNX2 was downregulated after 7 days of differentiation (B). RUNX2 protein levels were similar after 3 days of osteogenic differentiation (C) and significantly lower after 7 days of differentiation (D) in nuclei of MSM treated cells. * $p < 0.05$; ** $p < 0.01$

genes, e.g., SPARC and SPP1, as well as the number of osteocalcin-positive cells increased in MSM-treated cells after 14 days of osteogenic differentiation (Fig. 4A, B). Therefore, after 21 days of differentiation, we observed an increased calcium deposition, evaluated by alizarin red staining, in cells treated with MSM (Fig. 4C).

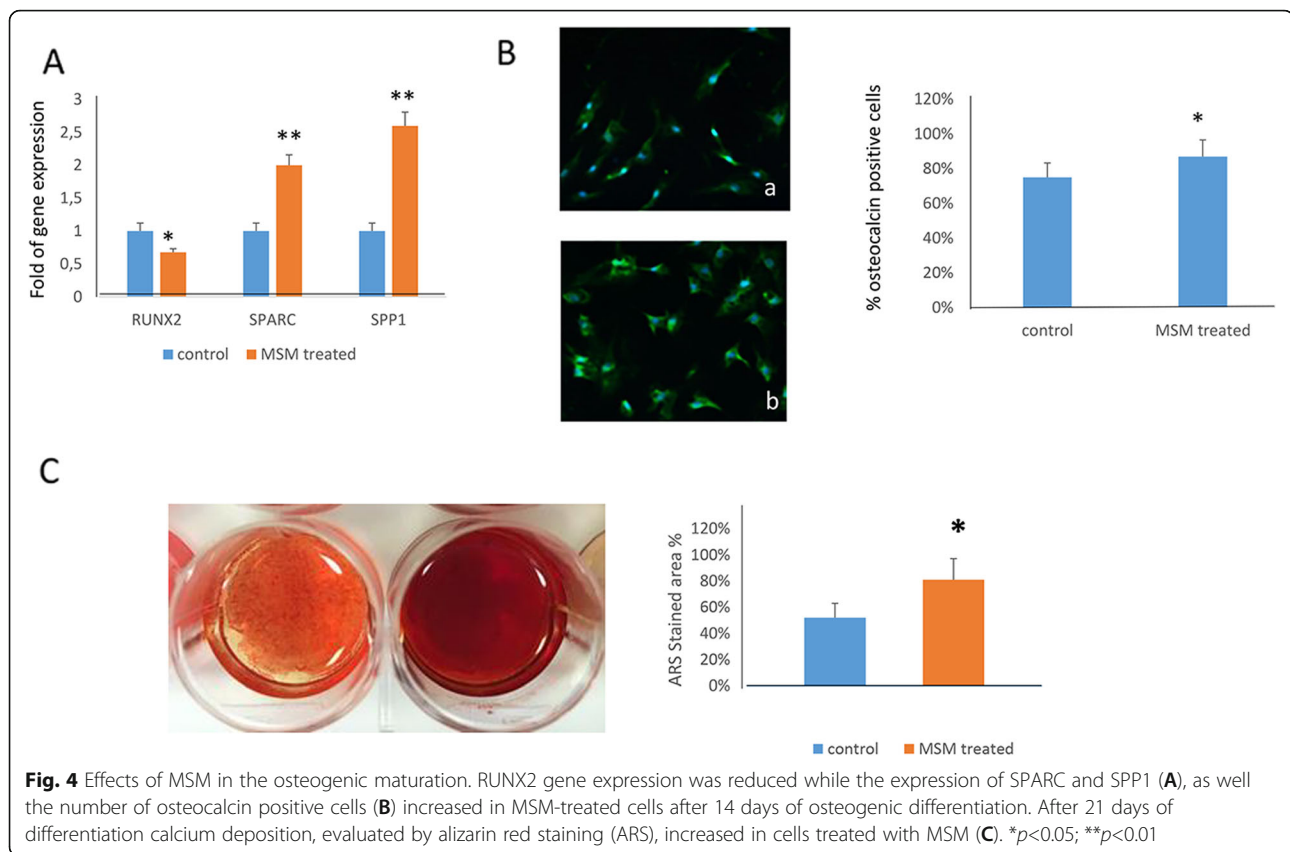
MSM enhances *runx2b* gene expression in zebrafish larvae

In order to confirm the effects of MSM on osteogenic maturation, we analyzed the osteogenic markers in zebrafish larvae after 7 and 14 days of treatment in the presence of 20-mM MSM. In particular, zebrafish larvae at 2 days after fertilization (dpf) were treated with or without MSM for 7 or 14 days. After 7 days of treatment, we observed increased expression of *runx2b* and *sp7* genes and reduced *tnfrsf1b* (homolog of human *RANK*) gene expression (Fig. 5A). To evaluate the direct effects of MSM on bone formation, we quantified bone mineralization by calcein staining. Fluorescence density was observed under microscopic inspection and analyzed by digital methods. Figure 5B shows higher bone mineral density in MSM-treated larvae compared to untreated larvae. Increased expression of *runx2b* and *sp7* and reduced expression of *tnfrsf1b* were confirmed after 14 days of MSM treatment (Fig. 5C). In addition, we also

observed the increased expression of *coll1a1* gene. Bone mineralization evaluated by calcein staining was higher in MSM-treated zebrafish compared to untreated zebrafish after 14 days of treatment too (Fig. 5D).

Increased bone mineralization, evaluated by alizarin red staining (ARS), was observed also in adult zebrafish treated with MSM for 14 days (Fig. 6A). As shown in Fig. 6B, a more intense ARS and an increased percentage of positive ARS staining area was observed in MSM-treated zebrafish compared to untreated zebrafish (Fig. 6C). In adult zebrafish, we also analyzed the osteogenic gene expression and we observed different gene modulation in fin and scale samples, respectively. As shown in Fig. 7A, *runx2a* was downregulated in fin of MSM-treated adult zebrafish. On the contrary, we observed increased *runx2b* and reduced *tnfrsf1b* gene expression in scales of MSM treated zebrafish (Fig. 7B).

To understand the different osteogenic genes modulation, we analyzed Alcian blue and alizarin red staining areas for the evaluation of cartilage and calcium deposition, respectively. As observed in Fig. 7C, a more pronounced Alcian blue area was present in fin of MSM-treated zebrafish compared to control. This finding is consistent with the increased aggrecan levels observed previously in fin of MSM-treated zebrafish. However, we also observed an increased alizarin red staining area in



them. On the contrary, Alcian blue staining was weak in scale of both control and treated zebrafish (Fig. 7D) while MSM increased calcium deposition, evaluated by alizarin red staining (Fig. 7E).

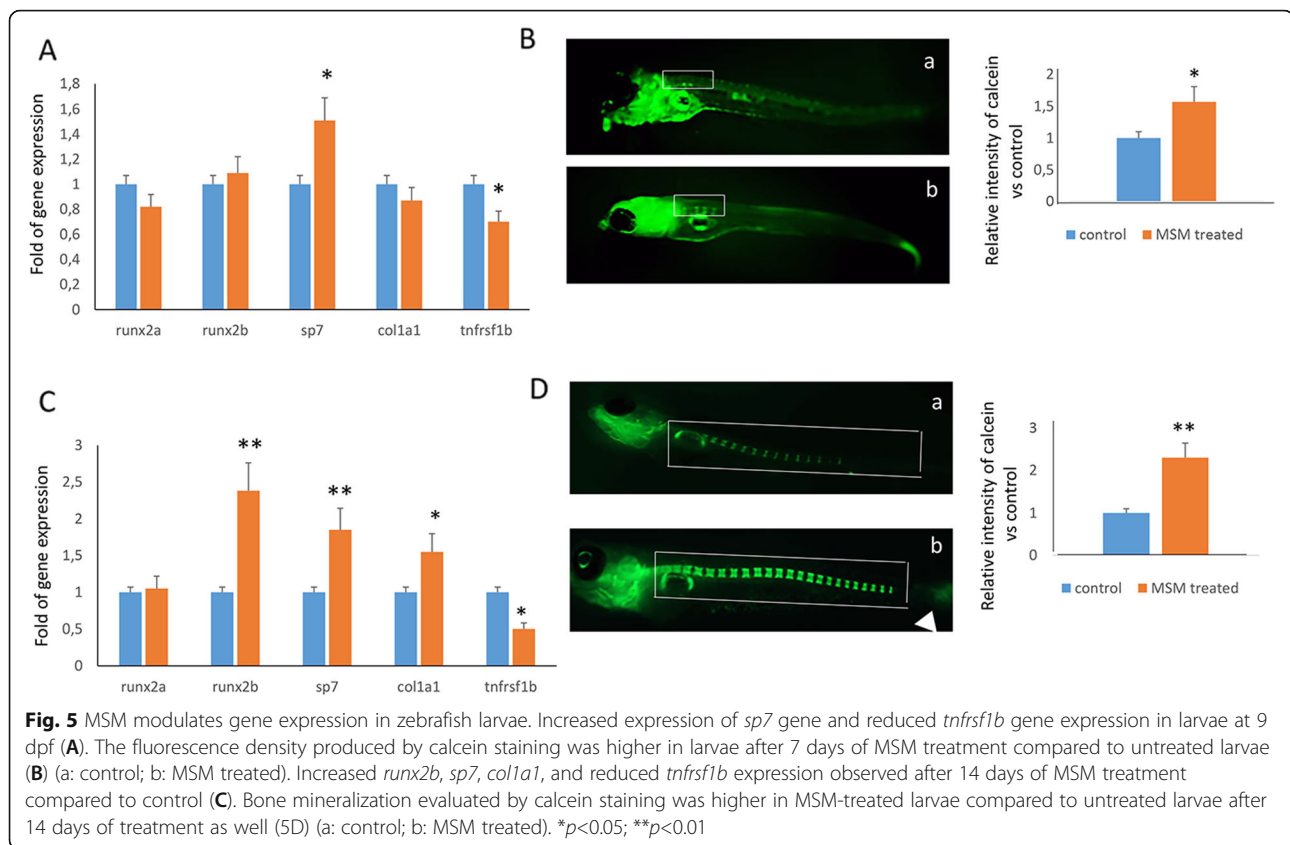
In order to evaluate a possible MSM-epigenetic effect, we analyzed the modulation of miR146b that in humans has been observed to be involved in mesenchymal stem cells differentiation. As shown in Fig. 8A, miR 146b expression was reduced in both fin and scale treated with MSM. Furthermore, as extracellular signal-regulated kinase (ERK) is involved in both osteogenic and chondrogenic commitment, we analyzed the ERK activation pathway in fin and scale of MSM-treated zebrafish. As shown in Fig. 8B, we observed increased levels of ERK and a reduction of pERK as well as of pERK/ERK ratio in both fin and scale of MSM-treated zebrafish.

Discussion

In the last decades, human lifespans have been increasing; consequently, aging-related diseases such as osteoporosis and osteoarthritis have been increasing in turn. Therefore, the characterization and testing of compounds capable of reducing the degenerative effects of aging-related disorders is an important challenge. In this context, some studies have indicated that MSM may be useful to counteract inflammation-related diseases

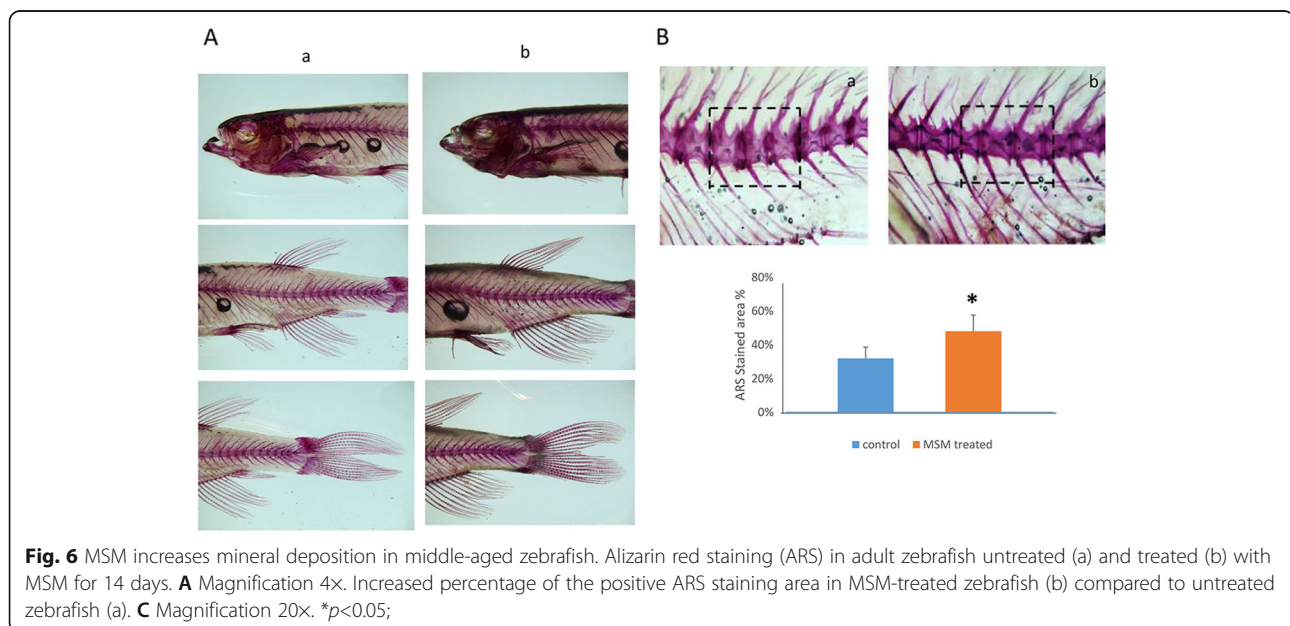
typical of the aging process. The interest in evaluating MSM effects on progenitor cells should be clear, since mesenchymal stem cells are the precursors of chondrocytes and osteoblasts, the cells which build the cartilage and bone, respectively, tissues which are mainly damaged by the aging process. Nevertheless, a few studies are available, which focused on evaluating the effects of MSM on osteogenic differentiation [24, 25, 33, 34] or on preventing cartilage degradation [35–38]. These studies showed that MSM is capable of enhancing osteogenic differentiation as well as of preventing chondrocytes degradation. However, no study has ever been performed to evaluate MSM overall effects on progenitor cells and to understand how MSM may promote both osteogenic and chondrogenic differentiation. This is a particularly relevant aspect, since osteogenesis and chondrogenesis originate from the same progenitor cells and are highly regulated processes [39].

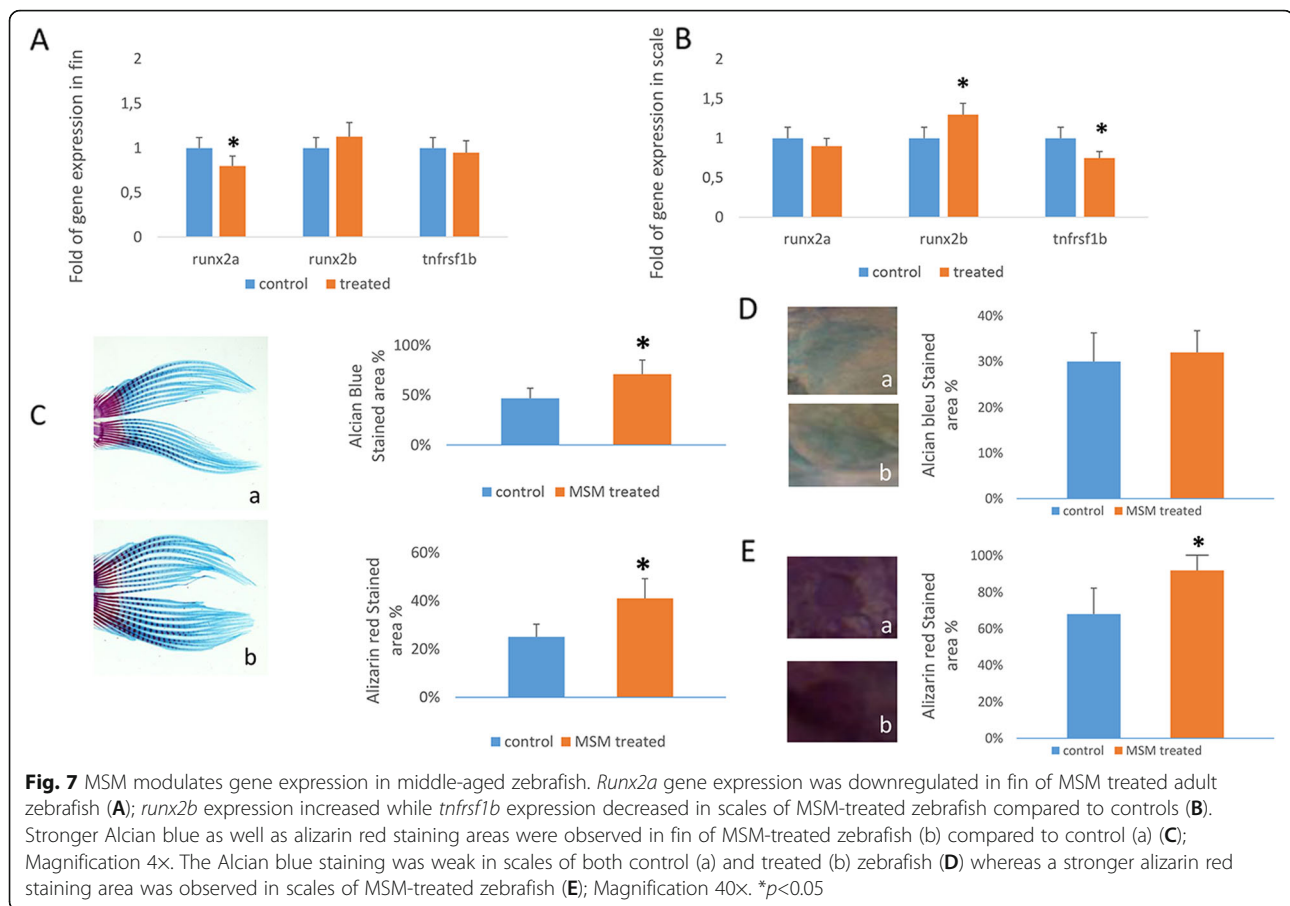
In our approach to understand the effects of MSM on both chondrogenic and osteogenic processes, we observed an increased chondrogenic differentiation in MSM-treated MSCs. In fact, we observed the upregulation of the chondrogenic transcription factor SOX9, as well as the increased expression of COL2A1, the cartilage ECM marker protein, upon differentiation. The upregulation of SOX9 was also observed in chondrocytes



treated with MSM. In addition, we observed the increased expression of SOX9, Sestrin 1, and COMP, the cartilage oligomeric matrix protein [40], also in chondrocytes treated with IL-1 β , thus confirming MSM protective role against chondrocyte degradation. It has been

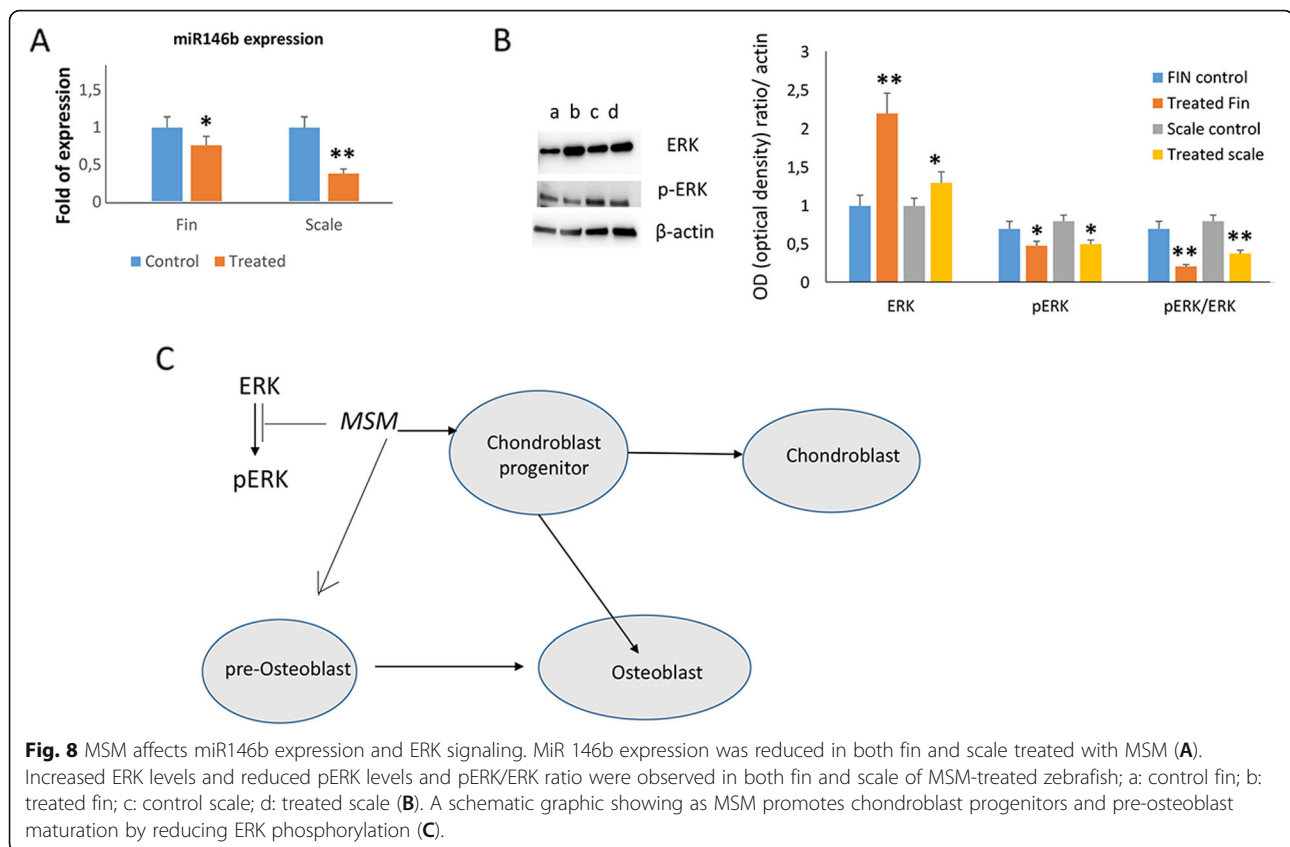
demonstrated that Sestrins (sesn) expression is suppressed in chondrocytes of osteoarthritic cartilage. Sestrins increase chondrocyte survival under stress conditions: sesn1 and sesn2 counteract oxidative stress damage by activating the Nrf2 (nuclear factor erythroid





2-related factor 2)-Keap1 (Kelch-like ECH-associated protein 1) pathway [41]. Accordingly, in our study, MSM increased the expression levels of ACAN (aggrecan) in chondrocytes even in the presence of IL- β 1, compared to controls. Such finding is noteworthy as aggrecan depletion predisposes to cartilage erosion [42]. In order to investigate whether MSM induces ACAN expression also in vivo, we evaluated the effects of MSM treatment in adult middle-aged zebrafish. Interestingly, we observed increased aggrecan levels in fin of treated zebrafish, but aggrecan expression was not likewise affected in scale. Therefore, our results confirmed that MSM induces chondrogenic differentiation in MSCs and that it counteracts IL β -negative effects on chondrocytes. In addition, increased aggrecan levels in MSM-treated adult zebrafish fins suggest that MSM enhances cartilage matrix synthesis. The more pronounced aggrecan synthesis in fin is likely due to a higher number of progenitor cells in fin compared to scale. It is well known that fin, which is composed by multiple bony rays, contains nerves, blood vessels, and mesenchymal cells [43] whereas scale contains bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) [44].

MSM treatment affected osteogenic differentiation both in vitro and in vivo. We observed increased expression of RUNX2-induced genes, whereas RUNX2 own expression was reduced in MSM-treated cells. The number of MSCs was reduced as well. SP7 expression increased during osteogenic route in MSM-treated samples. Since SP7 is mandatory for the final commitment of progenitors to preosteoblasts [45], our data suggest a role of MSM in promoting pre-osteoblast formation. The increased percentage of osteocalcin-positive cells, as well as calcium deposition increase evaluated by AR staining in MSM-treated cells, suggest MSM role in promoting osteoblasts maturation rather than osteogenic commitment. The induction of osteogenic maturation was observed also in the zebrafish model where increased calcein staining was observed after 7 and 14 days of treatment. Interestingly, we observed the increased gene expression of *runx2b* whereas *runx2a* was not affected after 14 days of MSM supplementation. Flores et al. demonstrated that *runx2b* is expressed in the vertebral column and in the fin in a juvenile fish and that *runx2a* is expressed in mesenchymal cells during caudal fin regeneration [46]. Therefore, the higher modulation of



runx2b versus *runx2a* observed in MSM-treated zebrafish suggests the effects of MSM on mature osteoblasts rather than on MSC commitment consistently with the RUNX2 downmodulation observed in in vitro experiments with mesenchymal stem cells.

Runx2b regulates SP7/osterix and osteocalcin expression indicating its central role in skeletal development [47, 48]. The increased osteogenic maturation was observed in MSM-treated adult zebrafish; a different gene expression pattern was observed in fin or scale of MSM-treated zebrafish. The increased *runx2b* and reduced *tnfrsf1b* expression observed in scale but not in fin reflect (as for aggrecan seen above) the different cellular composition of the two structures, i.e., the prevalence of osteoblasts and osteoclasts in scale. However, further more prolonged treatment, demonstrated increased *runx2b* expression even in fin. The reduced pERK and pERK/ERK ratio together with the increased ERK levels suggest the inhibition of Mitogen-activated protein kinases (MAPKs), the proteins involved in ERK phosphorylation [49]. As the reactive oxygen species (ROS) activate MAPK pathways [50], we speculate that the reduced ERK phosphorylation might be a consequence of ROS reduction due to MSM treatment. In fact, it is well known that MSM reduces mitochondrial ROS generation [51]. Interestingly, we observed a reduced miR146b

expression in MSM-treated fin and scale. The miR-146 family is highly conserved between fish and humans, and it shares the same seed sequence [52]. In humans, it has been demonstrated that miR146b is downregulated in both bone marrow stem cell (BMSC) chondrogenesis [53] and osteogenesis [54]. In addition, it has been demonstrated that miR146b promotes adipogenesis too [55]. Our finding therefore suggests an epigenetic effect of MSM. In addition, our study reports for the first time the involvement of miR146b in the differentiation of mesenchymal stem cells in zebrafish.

Ultimately, our results demonstrate that MSM modulates the expression of genes involved in chondrogenic and osteogenic differentiation, likely by acting on pathways involved in oxidation processes. Our findings also stimulate interesting ideas for future explorations such as evaluating MSM effects on adipogenesis, a process highly related to osteogenesis.

Conclusions

These findings provide new insights into the mechanism of action of MSM supplementation on bone and cartilage differentiation and strengthen the rationale for MSM supplementation in patients with osteoarticular disorders as a complementary therapeutic intervention targeting mesenchymal stem cells differentiation.

Abbreviations

MSM: Methylsulfonylmethane; MSCs: Mesenchymal stem cells; iNOS: Nitric oxide synthase; COX-2: Cyclooxygenase-2; IL-1 β : Interleukin; SPAR C: Osteonectin; SSP1: Osteopontin; COL1A1: Collagen type 2; COL1A2 : Collagen type 2; BGLAP: Osteocalcin; ARS: Alizarin red staining; Nrf2: Nuclear factor erythroid 2-related factor 2; Keap1: Kelch-like ECH-associated protein 1

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Authors' contributions

LDC, interpretation of data and statistical analysis, drafting of the manuscript; MM and NT, interpretation of data, data curation; JB, GM, SC, MD, and AM, generation, collection, and assembly of data; interpretation of data; MTV, conception and design, interpretation of data, critical revision of the manuscript, study supervision. The authors approved the final version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Zebrafish experiments were performed under ethical authorization n. 662/2019-PR of 16/09/2019.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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