

Comparison between curcumin and all-trans retinoic acid in the osteogenic differentiation of mouse bone marrow mesenchymal stem cells

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Abstract. The use of bone marrow mesenchymal stem cells (BMSCs) has great potential in cell therapy, particularly in the orthopedic field. BMSCs represent a valuable renewable cell source that have been successfully utilized to treat damaged skeletal tissue and bone defects. BMSCs can be induced to differentiate into osteogenic lineages via the addition of inducers to the growth medium. The present study examined the effects of all-trans retinoic acid (ATRA) and curcumin on the osteogenic differentiation of mouse BMSCs. Morphological changes, the expression levels of the bone-associated gene markers bone morphogenetic protein 2, runt-related transcription factor and osterix during differentiation, an *in vitro* mineralization assay, and changes in osteocalcin expression revealed that curcumin supplementation promoted the osteogenic differentiation of BMSCs. By contrast, the application of ATRA increased osteogenic differentiation during the early stages, but during the later stages, it decreased the mineralization of differentiated cells. In addition, to the best of our knowledge, the present study is the first to examine the effect of curcumin on the osteogenic potency of mouse embryonic fibroblasts (MEFs) after reprogramming with human lim mineralization protein (hLMP-3), which is a positive osteogenic regulator. The results revealed that curcumin-supplemented culture medium increased hLMP-3 osteogenic potency compared with that of MEFs

cultured in the non-supplemented medium. The present results demonstrate that enrichment of the osteogenic culture medium with curcumin, a natural osteogenic inducer, increased the osteogenic differentiation capacity of BMSCs as well as that of MEFs reprogrammed with hLMP-3.

Introduction

Bone is a calcified connective tissue formed via the differentiation of osteoprogenitor cells into mature osteoblasts. Osteoblasts, the bone forming cells, are characterized by their cuboidal appearance, and their association with bone matrix formation (1). Several reports have demonstrated the importance of bone marrow mesenchymal stem cells (BMSCs) in cell-based therapy for bone tissue regeneration and the treatment of skeletal tissue damage (2-5). BMSCs have a number of advantages, such as their great potential for proliferation and differentiation. BMSCs exhibit plasticity, and the ability to differentiate into chondrocytes, osteocytes and adipocytes *in vitro* and *in vivo* (6). In addition, BMSCs have the ability to secrete biological factors with paracrine regenerative and anti-inflammatory effects (7). The differentiation of BMSCs *in vitro* is reliant on the culture conditions; for example, osteogenic medium (OM) supplemented with dexamethasone, ascorbic acid and β -glycerol phosphate induces the differentiation of BMSCs into an osteogenic lineage (8).

All-trans retinoic acid (ATRA) is derivative of vitamin A, which is vital for important physiological processes and functions (9). A number of previous studies have reported the key role of ATRA in the regulation of bone cell function (10-12); ATRA may promote physiological bone remodeling (11). The ability of ATRA to influence osteoblast differentiation has been observed in numerous cell systems (13-19). In addition, ATRA has been reported to enhance *in vitro* osteogenesis in multiple cell types, including preosteoblasts (20), calvarial osteoblasts (16) and mesenchymal stem cells (MSCs) (14,18,21,22). However, ATRA does not induce the osteogenic differentiation of BMSCs; dexamethasone is primarily used to initiate osteogenesis, whereas ATRA inhibits osteoblast gene

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expression and mineralization (23-25). The effect of ATRA on osteoblastogenesis and osteoclastogenesis is dependent on the differentiation marker examined, as well as the cell system employed (26). In a previous study, ATRA exerted divergent effects on osteoblastogenesis and adipogenesis in MSCs (13). ATRA may reduce the osteogenic differentiation capacity and promote the adipogenesis of mouse embryonic palate mesenchymal cells via its influence on bone morphogenetic protein (BMP) signaling (27). Studies performed *in vitro* and *in vivo* have suggested that bone may be a main target of retinoid action (10).

Curcumin has become a subject of scientific interest as a potential therapeutic agent in orthopedic fields. Curcumin is a phenolic natural product extracted from the rhizome of *Curcuma longa* (turmeric). It has been widely used as a dietary spice and traditional medicine for many centuries in Eastern populations as a treatment for numerous diseases (28-30). In addition, curcumin supplementation has been demonstrated to be efficient in the prevention and management of osteopenia (31). Several studies have reported its useful effects on bone health and fat metabolism (32-34). It was previously demonstrated that curcumin protects against ovariectomy-induced bone loss and decreased osteoclastogenesis in animal models (35-38). In addition, curcumin improved bone microarchitecture and increased mineral density in mice (39,40). Furthermore, the therapeutic effect of curcumin has also been reported in arthritis (41). Jain *et al.* (42) revealed that a curcumin-eluting tissue scaffold increased the mRNA and protein expression of known osteogenic markers.

At the cellular level, curcumin modulates important molecular targets that participate in the regulation of bone remodeling (43-46). The effects of curcumin on bone cells *in vitro* have been reported (47-52). The action of curcumin on osteoblast cells is controversial (49,53). Curcumin enhanced the osteogenic differentiation of MSCs *in vitro* (53). By contrast, curcumin attenuated the osteogenic differentiation and calcification of rat vascular smooth muscle cells (54). Curcumin has also been revealed to activate the Wnt/ β -catenin signaling pathway (55-57); therefore, curcumin is considered an effective treatment for osteoporosis. However, other studies have demonstrated that curcumin suppresses this pathway (58,59). Curcumin is able to improve bone health in patients with osteoporosis by acting on multiple steps in the activation and differentiation of osteoclasts, and improving mineral density and mechanical properties. The potential mechanisms that have been proposed include inhibition of nuclear factor (NF)- κ B, receptor activator of NF- κ B ligand (RANKL), nitric oxide production, the generation of reactive oxygen species and inflammatory cytokine synthesis (38,39,47,50,51,60,61).

Transdifferentiation is a process in which adult, mature and fully differentiated cells differentiate into another specific terminal cell type via the induction of lineage-specific transcription factors. Previous studies have revealed that fibroblasts can be converted into several lineages, including neurons (62), cardiomyocytes (63), hepatocytes (64) and osteoblasts (65,66) via the ectopic expression of multiple lineage-specific transcription factors or microRNAs (67). Importantly, this approach has also been applied *in vivo* using a number of lineage-specific transcription factors (68). Several studies have reported that the growth factor human limb mineralization protein (hLMP)

is directly associated with osteoblastic differentiation, and appears to be a positive regulator of bone formation (69-72). The production of multiple osteogenic growth factors via the administration of a single therapeutic molecule amplifies osteoinductive signaling, and thus may be highly advantageous when using LMP. The different types of secreted BMPs may be mixed and potentially form heterodimeric BMPs that may have more potent osteoinductivity than homodimeric BMPs (73).

The aim of the present study was to evaluate and clarify the efficiency of the natural osteogenic modulators curcumin and ATRA during the osteogenic differentiation of mouse BMSCs, as there are numerous contradictory opinions regarding their roles during this process. Therefore, the present study tested the effect of curcumin on another osteogenic transdifferentiation model to elucidate its wide range of effects on different cell types. Mouse embryonic fibroblasts (MEFs), undergoing osteogenic reprogramming using hLMP3, as a positive regulator of osteoblast differentiation, were cultured in curcumin-enriched medium. The results revealed a significant difference in bone markers between the curcumin-enriched MEFs and the control MEFs. These findings highlight the role of curcumin in osteogenic differentiation in different cell lines.

Materials and methods

All reagents were purchased from Gibco; Thermo Fisher Scientific, Inc., (Waltham, MA, USA) unless stated otherwise in the text.

Experimental animals. A total of 25 male BALB/c mice (5-6 weeks old; body weight, 15-21 g) and 2 pregnant female C57/BL mice (12-13 weeks old; body weight, 23-26 g) at 13 days post-coitum were used throughout the present study. Animals were obtained from the Laboratory Animal Centre, Jiangsu University (Zhenjiang, China). The animals were housed in cages in a temperature-controlled room (20-25°C and 40-0% humidity), with a 12-h light/dark cycle and free access to commercial food and water. All procedures involving animals and their care conformed to the USA National Institutes of Health guidelines (NIH Pub. No. 85-23, revised 1996). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of School of Animal Science and Technology, Yangzhou University (Yangzhou, China; approval no. YZUDWSY2017-0029). The procedures were performed in accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals (China, 1988), and the Standards for the Administration of Experimental Practices (Jiangsu, China; 2008).

Isolation and culture of mouse BMSCs. The isolation and culture of BMSCs was conducted as previously described (74). In brief, 5-6-week-old male BALB/c mice were sacrificed via the cervical dislocation method. The animals were then rinsed with 70% ethanol for few min. The hind limbs were excised from the trunk of the body, and the bones were kept in PBS for the subsequent steps under a sterile hood. The bones were placed on sterile gauze and were gently rubbed to remove any attached soft tissue. The epiphysis was cut, a 26-gauge syringe needle was inserted into the bone marrow cavity and

the marrow was flushed out using Dulbecco's modified Eagle's medium (DMEM); flushing was continued until pale white bone was observed. The cell suspension was filtered through a 70- μ m filter mesh to remove any bony spicules, muscle or cell clumps. The number of viable cells was counted via trypan blue staining. Cells were cultured in 95-mm dishes using 1 ml complete culture medium (CCM) at a density 25×10^6 cells/ml, and then kept at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. The CCM comprised DMEM containing 15% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 2 mM L-glutamine and 1% penicillin-streptomycin (Beyotime Institute of Biotechnology, Haimen, China). Cells were cultured with frequent medium changes as described previously (74). When primary cultures became nearly confluent, the culture was treated with 0.5 ml 0.25% trypsin EDTA for 2 min at room temperature. The cells that were lifted within 2 min were harvested and cultured in a 60-mm plate. Once the culture reached 70-80% confluence, the cells were harvested for successive passages. The subsequent experiments were performed with cells from passages 3 and 4.

Isolation and culture of MEFs. Pregnant female mice (C57/BL) at 13 days post-coitum was sacrificed via the cervical dislocation method. Uterine horns were removed, washed with PBS and opened. Embryos were harvested and the head and the visceral organs were removed. After washing with PBS, the embryos were minced, and then incubated at 37°C for 15 min in 0.25% trypsin EDTA with gentle shaking. Trypsin was neutralized with an equal amount of MEF medium and cells were collected by centrifugation at 500 x g for 7 min at room temperature (20-25°C). Cells were then resuspended and cultured on gelatin-coated dishes with MEF growth medium containing high glucose DMEM mixed with 10% FBS, 4 mM L-glutamine and 1:100 penicillin-streptomycin at 37°C with 5% CO₂. The cells were examined daily using an inverted microscope (Olympus TH4-200; Olympus Corporation, Tokyo, Japan) to assess their general appearance, and to identify any signs of microbial contamination. Once confluent, cells were passaged 1:3 and the second passage cells were trypsinized and frozen at -80°C. In the present experiments, MEFs within three passages were used to avoid replicative senescence.

Lentiviral vector. The codon-optimized hLMP-3 cDNA sequence obtained from Pola *et al* (75), was synthesized, and cloned into T-Vector pMD19 (Takara Biotechnology, Co., Ltd., Dalian, China) by GenScript (Project ID. 7162905-1; GenScript, Jiangsu, China). cDNA sequencing and the primer design of hLMP-3 were also conducted by GenScript. The construction of the viral expression vector containing the transcription factors hLMP-3 was performed by Genomeditech (Shanghai, China; Project ID. GM-Lc-01147). The primers used for hLMP-3 (Accession number: AAK30569.1) were as follows: Forward, 5'-CCCTCGAGGGCTTGCCATGGATGTTTCAAGGTGGTC-3' and reverse, 5'-GCGTTCGACGTTACGCCACTTGAGGCGGGCATCTG-3' (restriction recognition sites are underlined).

Characterization of mouse BMSCs. BMSCs (passage 3) were used for MSC cell surface marker experiments. The MSC surface markers cluster of differentiation (CD)44 and CD90,

and also the hematopoietic marker CD45 were examined by flow cytometry as previously described (76,77). Briefly, cells at passage 3 were harvested using trypsin/EDTA. Following cell counting, cell suspensions were stained with the following fluorescence-conjugated antibodies: Fluorescein isothiocyanate (FITC)-anti-mouse/human CD44 antibody (1:100; cat. no. 103021; BioLegend, Inc., San Diego, CA, USA), FITC anti-mouse CD45 monoclonal antibody (1:100; cat. no. 11-0451; eBioscience; Thermo Fisher Scientific, Inc.) and FITC anti-mouse CD90 monoclonal antibody (1:100; cat. no. 11-0903; eBioscience; Thermo Fisher Scientific, Inc.) for 1 h at 4°C. Cells stained with FITC-labeled rat anti-mouse immunoglobulin G (IgG; 1:100; cat. no. 406001; BioLegend, Inc.) served as controls. Cells were pelleted, washed twice with PBS and fixed with 1% paraformaldehyde in PBS. Cell surface antigens were detected with a flow cytometer using BD Accuri C6 Plus software (BD Biosciences, Franklin Lakes, NJ, USA).

In vitro osteogenic induction of BMSCs. BMSCs were seeded at a density of 50 cells/cm² and cultured until they reached 70% confluency. The CCM was then changed to OM (fresh complete medium with 15% FBS) supplemented with 50 μ g/ml ascorbic acid (Sigma Aldrich; Merck KGaA, Darmstadt, Germany), 10 mM β -glycerol phosphate disodium salt (Sigma Aldrich; Merck KGaA), and 100 nM dexamethasone (Sigma Aldrich; Merck KGaA). Cells cultured in osteogenic medium only comprised the OM group; while in the curcumin group (CR group), 15 μ M curcumin was added to the OM. This concentration was selected based on previous studies that used curcumin in cell culture to induce osteogenic differentiation with minimal cytotoxicity (53,78,79). Curcumin was prepared according to the method previously described by Gu *et al* (53). Briefly, curcumin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was dissolved in dimethyl sulfoxide and stored at -20°C. In the ATRA group, 1 μ M ATRA (Sigma-Aldrich; Merck KGaA) was added to the OM. This concentration has repeatedly been used to study the effects of ATRA on *in vitro* osteogenic differentiation; since the normal physiological level of ATRA is ≤ 0.01 μ M and the effective pharmacological concentration is > 0.1 μ M, the 1 μ M concentration was selected to induce osteogenic differentiation (27,80-82).

In vitro osteogenic induction of MEFs. The protocol used was a modified version of that described by Yamamoto *et al* (83); the preparation and dilution of the OM components were conducted according to the protocol provided by the supplier. MEFs were re-suspended in CCM, and seeded onto 35-mm dishes (Corning®; 5×10^4 cells/dish) or a 24-well plate (Corning®; 1.2×10^4 cells/well) on day 1. The next day, transduction of MEFs was performed using the supernatant containing the pGMLVPE1-hLMP-3 expression vector (multiplicity of infection=4), and supplemented with 4 μ g/ml polybrene (Genomeditech). Following culture for 24 h, the virus-containing medium was replaced with an OM composed of fresh complete culture medium (10% FBS) supplemented with 50 μ g/ml ascorbic acid, 10 mM β -glycerol phosphate disodium salt and 100 nM dexamethasone. In the curcumin-supplemented group, 15 μ M curcumin was added to the medium.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cell samples using TRIZOL[®] reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following isolation, 1 μ g RNA was reverse-transcribed into cDNA using FastQuant RT kits with gDNase (cat. no. KR106; Tiangen Biotech Co., Ltd., Beijing, China). The reverse transcription reaction was performed at 42°C for 15 min, followed by 95°C for 3 min. The cDNA samples were analyzed by RT-qPCR in a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using Super Real Premix plus (SYBR Green; cat. no. FP205; Tiangen Biotech Co., Ltd.). The thermocycling conditions for qPCR were as follows: 95°C for 15 min, followed by 40 amplification cycles of 95°C for 10 sec and 60°C for 32 sec. The sequences of the RT-qPCR primers are listed in Table I. The primers were designed and manufactured Takara Biotechnology Co., Ltd. Relative quantification was calculated with $2^{-\Delta\Delta C_q}$ (84) and normalized to β -actin. Data are presented as levels relative to the expression level in the control cells.

In vitro mineralization assay. Early osteogenic differentiation was evaluated by alkaline phosphatase staining (ALP). Matrix mineralization was evaluated by Alizarin red (ALZ) and von Kossa (VK) staining. The staining intensity was quantified using Fiji in ImageJ software, version 1 (National Institutes of Health, Bethesda, MD, USA) (85).

ALP staining. Histochemical staining was performed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) ALP color development kits (cat. no. C3206; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The cells were washed with PBS buffer, then fixed with 4% formalin at room temperature for 2 min. The cells were then washed 3 times with PBS. Following the last wash, an appropriate amount of BCIP/NBT staining solution was added, ensuring that the sample was fully covered. After the addition of the working solution, cells were incubated in the dark at 37°C for 5-30 min in an incubator until the desired color developed. The BCIP/NBT stain working solution was then removed, and cells were washed once or twice with distilled water to stop the color reaction.

ALZ staining. The cells were washed with 1X PBS, and fixed in 10% formaldehyde in 1X PBS for 15 min. Following fixation, the cells washed with distilled water 2 or 3 times. Cells were incubated in 40 mM ALZ staining solution for 5 min in the dark. Finally, cells were washed with distilled water 4 times to remove the excess stain. The ALZ staining solution was prepared by diluting the ALZ staining powder (cat. no. A5533; Sigma-Aldrich; Merck KGaA) in distilled water. The pH was adjusted to 4.1-4.3 with 10% ammonium hydroxide using a pH Meter (Mettler Toledo, Columbus, OH, USA).

VK staining. For VK staining, the cells were washed with PBS, fixed with 10% formalin and stained with freshly prepared 5% silver nitrate solution for 30 min with exposure to UV light. After washing with distilled water 3 times, the cells were treated with 5% sodium thiosulfate solution for 3 min

to remove any remaining silver nitrate. Final washing with distilled water was repeated 3 times. The silver nitrate solution (5%) was prepared by diluting silver nitrate powder (cat. no. GB12595-90; Beijing HenGye Zhongyuan Chemical Co., Ltd., Beijing, China) in distilled water. The same diluent was used for the preparation of a solution of sodium thiosulfate (cat. no. 217263; Sigma-Aldrich; Merck KGaA).

Fluorescent immunocytochemistry. The cells were rinsed briefly with PBS, and then fixed for 20 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. The cells were permeabilized for 10 min with 0.1% TritonX-100 in PBS, to allow for specific antibody entrance into the cells, and then blocked for 45-60 min with 4% bovine serum albumin (BSA) in PBS at room temperature. Cells were incubated overnight (16-18 h) at 4°C with mouse monoclonal OCN antibody (1:500; cat. no. sc-376726; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The cells were then washed 3 times with washing buffer. This was followed by 1 h incubation at room temperature with the secondary antibody Alexa Fluor 647-labeled goat anti-mouse IgG (1:500; cat. no. ab150115; Abcam, Cambridge, MA, USA). Following a final round of 3 washes with the wash buffer, nuclei were counterstained using DAPI at room temperature for 5 min (1 mg/ml PBS; Invitrogen; Thermo Fisher Scientific, Inc.). Cells were examined using inverted fluorescence microscopy (Olympus TH4-200).

Western blot analysis. All steps were conducted according to the manufacturer's protocol, using a Bio-Rad system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell lysates were collected with RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented with phenyl methane sulfonyl fluoride (Beyotime Institute of Biotechnology). Following centrifugation at 12,000 \times g at 4°C for 10 min, the supernatant was collected and the protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Protein samples were heated for 10 min at 95°C, and then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (20 μ g protein/lane). Samples were then blotted onto polyvinylidene fluoride membranes (Beijing Solarbio Science & Technology Co., Ltd.) using a Trans-Blot SD system (Bio-Rad Laboratories, Inc.). The membranes were blocked with BSA blocking buffer (cat. no. CW2143S; CWBIO, Beijing, China) at room temperature for 2 h, and then incubated with the primary mouse monoclonal osteocalcin (OCN) antibody (1:500; cat. no. sc-376726; Santa Cruz Biotechnology, Inc.) at 4°C overnight. After washing three times, the corresponding secondary antibody horseradish peroxidase-labeled goat anti-mouse IgG (1:2,000; cat. no. 665739; Merck KGaA) was added. The membranes were incubated at 37°C for 2 h and then washed with TBST (cat. no. CW0043S; CWBIO). Bands were visualized with enhanced chemiluminescence method western blot kits (cat. no. CW0048M; CWBIO) using the FluorChem Q system (ProteinSimple, San Jose, CA, USA). β -actin (1:1,000; cat. no. AA128; Beyotime Institute of Biotechnology) was used as a loading control with overnight incubation at 4°C. The protein band intensity was quantified using Fiji in ImageJ version 1 (85).

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β -actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA
Runx2	TGCAAGCAGTATTTACAACAGAGG	GGCTCACGTCGCTCATCTT
OSX	GCGACCACTTGAGCAAACATC	CGGCTGATTGGCTTCTTCTT
BMP-2	TGATGTGGGGTGGAATGACT	CAGCAAGGGGAAAAGGACAC

Runx2, runt-related transcription factor 2; OSX, osterix; BMP-2, bone morphogenetic protein 2.

Statistical analysis. All quantitative data are expressed as the mean \pm standard deviation (n=3). Statistical analyses were performed using SPSS software version 21 (IBM Corp., Armonk, NY, USA). Statistical significance was determined using one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. For some experiments the statistical significance was determined using paired sample t-tests ($P < 0.05$ and $P < 0.01$). GraphPad Prism software version 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used to produce the graphs.

Results

Morphological and immunotyping characterization of mouse BMSCs. Bone marrow was harvested from BALB/c mice, and P3 cells were seeded into 35-mm culture dishes at a density of 25×10^6 cells/ml. BMSCs are recognized as the adherent cells derived from bone marrow and are capable of extensive proliferation, producing a fibroblastic shape. Following 15 days of culture, almost homogeneous populations of fibroblast-like cells were observed (Fig. 1A). To confirm cell identity, immunofluorescent staining was used to identify the BMSCs with the surface markers CD90 and CD44, while the surface marker CD45 was used to detect hematopoietic cell contamination. The cultured cells were positive for the MSC markers CD44 and CD90, and negative for the hematopoietic cell marker CD45 (Fig. 1B).

Morphological changes during the osteogenic differentiation of BMSCs. BMSCs were induced to differentiate into an osteogenic lineage, and were able to proliferate *in vitro*. The morphology of BMSCs changed following the first week of culture in OM (OM group) from their spindle-like fibroblast shape to the characteristic cuboidal morphology of primary osteoblasts. Comparable results were observed in the CR group. By contrast, the BMSCs cultured in CCM did not differentiate into the osteoblast lineage, and cells in the ATRA group displayed a more elongated shape, and dendrites were clearly visible (Fig. 2).

Osteogenic differentiation capacity of BMSCs following induction with curcumin and ATRA. The present study performed an *in vitro* mineralization assay to determine the effects of curcumin and ATRA on the onset of the osteogenic differentiation of BMSCs. ALP, ALZ and VK staining were conducted during the differentiation period. ALP activity was analyzed as an early indicator of osteogenic differentiation.

The results revealed that OM induced ALP activity in the mouse BMSCs after 7 and 14 days. The ALP staining intensity was significantly increased in the CR group ($P < 0.01$ vs. the OM and ATRA groups); while no significant difference was detected between the OM and ATRA groups. Matrix mineralization was detected by ALZ and VK staining. Mineralization was not clearly detected after 7 days, and was slowly developing after 14 days. However, after 21 and 28 days, the OM and CR groups exhibited positively stained mineralized nodules. Mineralization was significantly stronger in the CR group compared with the OM group. By contrast, the ATRA group did not show any signs of mineralization (Fig. 3).

Effects of curcumin and ATRA on the expression levels of bone-associated gene markers during BMSC osteogenic differentiation. In order to evaluate the molecular changes following the supplementation of the OM with curcumin and ATRA, the present study examined the expression levels of the main genes associated with osteoblastic differentiation, namely runt-related transcription factor 2 (Runx2), osterix and BMP2. These markers are well known for their roles in numerous osteoblast differentiation pathways. The expression levels of these bone markers were quantified at 7, 14 and 21 days (Fig. 4). The results revealed that curcumin-supplemented OM induced a significant upregulation of osteo-specific markers when compared with the ATRA and OM groups, and this upregulation appeared to be time-dependent. After 7 days, the CR group exhibited induced expression of osteo-specific markers when compared with the OM and ATRA groups. After 14 days, the CR group had significantly higher expression levels of the three tested markers when compared with the OM and ATRA groups ($P < 0.01$). The expression levels of the markers had begun to decline in the ATRA group, and were not significantly different when compared with the OM group for Runx2 and BMP2. Notably, the OM group exhibited significantly higher ($P < 0.01$) osterix expression when compared with the ATRA group. These results were consistent with the results of the mineralization assay, which revealed the inhibitory effect of ATRA during the differentiation process. Finally, after 21 days, the results revealed that the CR group had upregulated levels of the three markers when compared with the OM and ATRA groups ($P < 0.01$). In addition, the expression levels of osteo-specific markers in the ATRA group appeared to decline further compared with those in the OM group. These RT-qPCR results demonstrated the positive effect of curcumin-supplemented medium on the osteogenic differentiation of BMSCs. By contrast, ATRA supplementation exhibited an inhibitory effect.

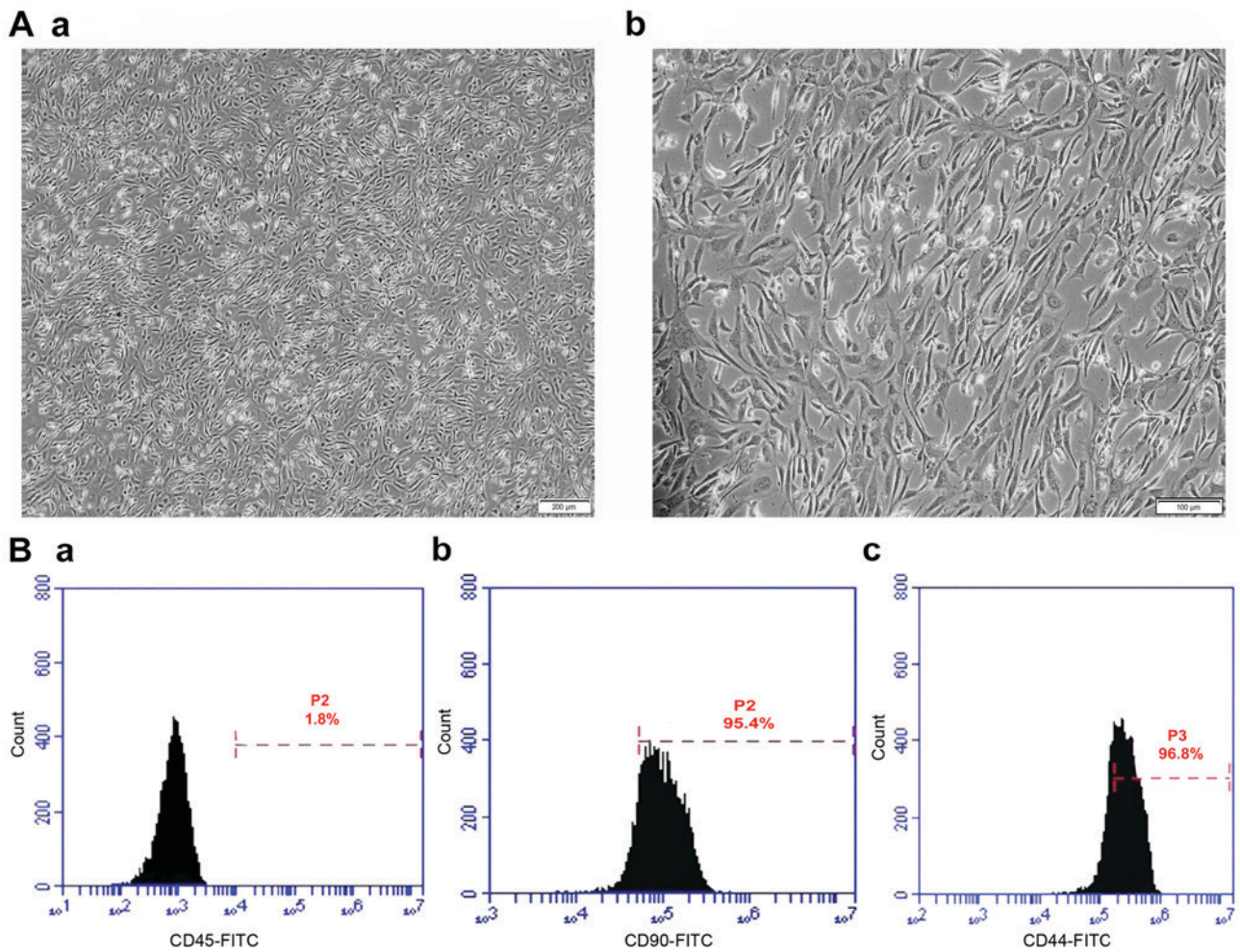


Figure 1. Characterization of mouse BMSC culture. (A) BMSCs at P3 were characterized by their fibroblastic shape: (a) Magnification, x40; (b) magnification, x100. (B) Flow cytometric analysis of mouse BMSCs at P3: Cells were (a) negative for the CD45 surface marker of hematopoietic cells, (b) positive for the MSC surface marker CD90 and (c) positive for the MSC surface marker CD44. BMSC, bone marrow mesenchymal stem cell; MSC, mesenchymal stem cell; P3, cells at passage 3; CD, cluster of differentiation; FITC, fluorescein isothiocyanate.

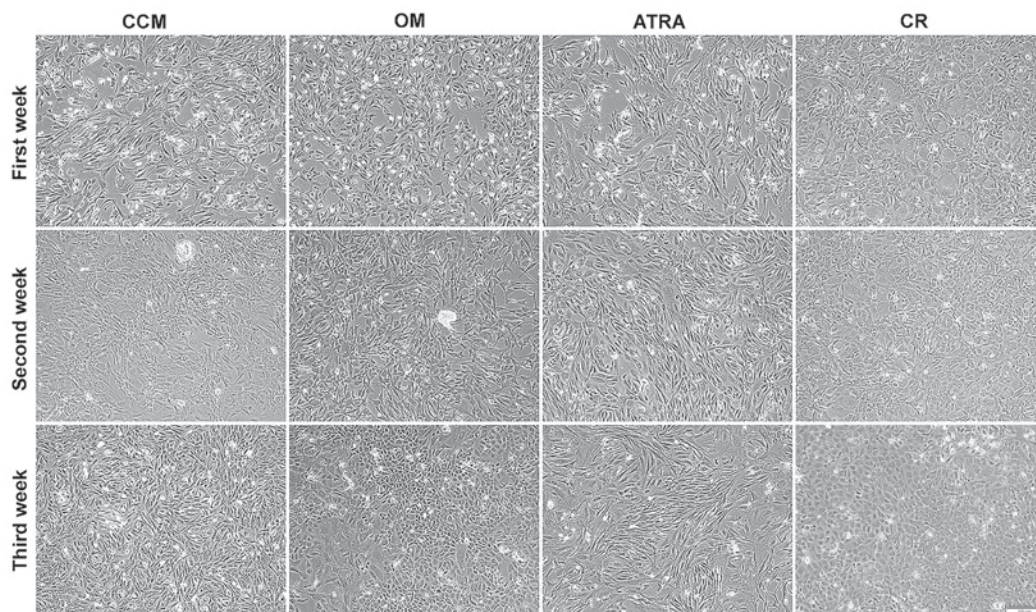


Figure 2. Morphological changes of BMSCs during osteogenic differentiation. BMSCs were divided into four groups: CCM, OM, ATRA and CR. BMSCs, bone marrow mesenchymal stem cells; CCM group, BMSCs cultured in complete culture medium; OM group, BMSCs cultured in osteogenic medium; ATRA group, BMSCs cultured in osteogenic medium supplemented with 1 μ M all-trans retinoic acid; CR group, BMSCs cultured in osteogenic medium supplemented with 15 μ M curcumin (Scale bar: 100 μ m).

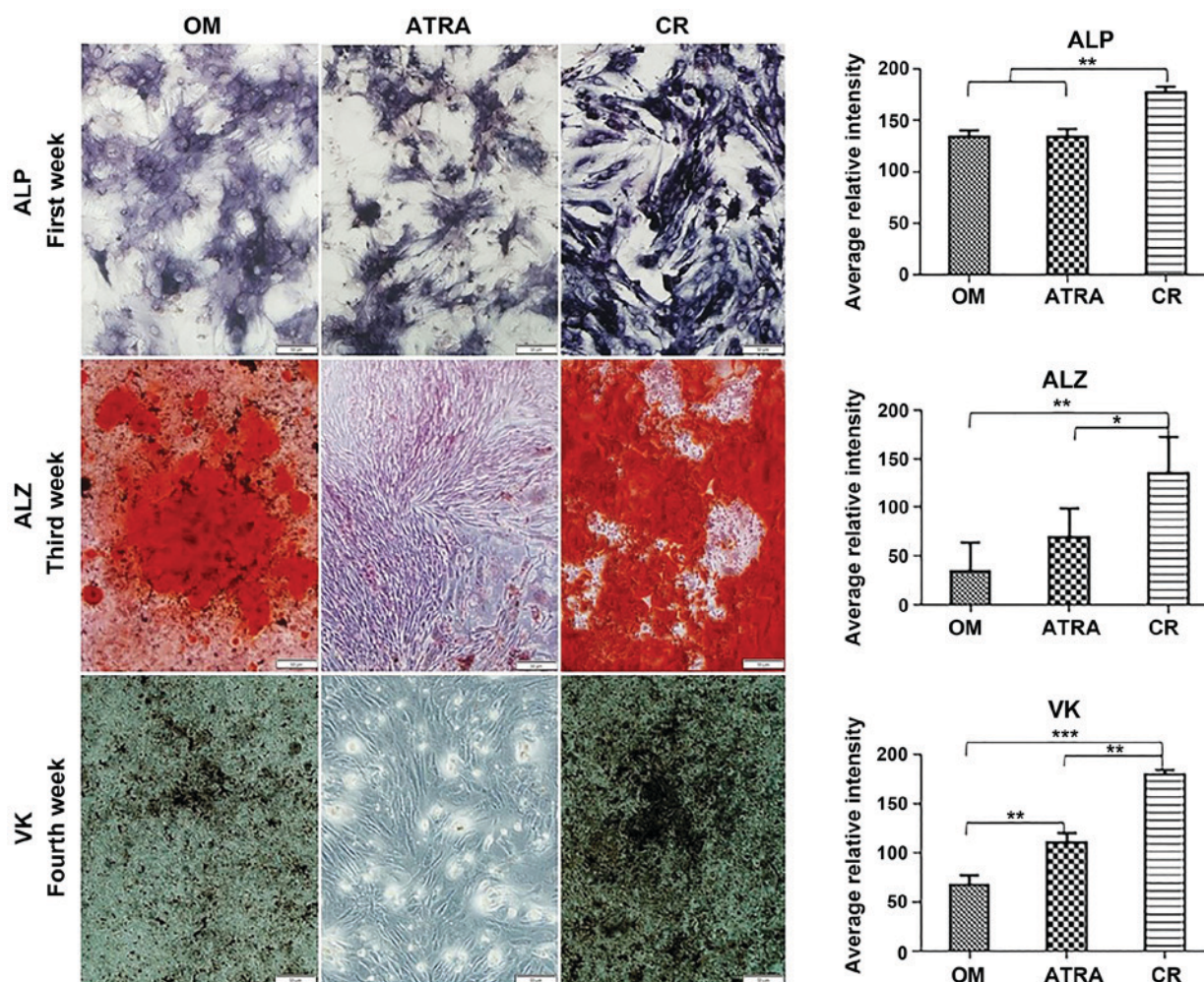


Figure 3. *In vitro* mineralization assay of BMSCs during osteogenic differentiation following induction with curcumin and ATRA. ALP staining results were obtained 1 week post-induction, ALZ staining was performed 3 weeks post-induction and VK staining was conducted 4 weeks post-induction. The level of calcium deposition and, in turn, the extent of mineralization was higher in the CR group than in the OM and ATRA groups. By contrast, the ATRA group did not present any symptoms of mineralization. Quantification of ALP, ALZ and VK staining intensity was performed with Fiji in ImageJ software. Quantitative data are expressed as the mean \pm standard deviation ($n=3$). Statistical significance was determined using one-way analysis of variance, followed by Tukey's post hoc test for multi-group comparisons. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. BMSCs, bone marrow mesenchymal stem cells; OM group, BMSCs in osteogenic medium; CR group, BMSCs in osteogenic medium + curcumin; ATRA group, BMSCs in osteogenic medium + ATRA; ATRA, all-trans retinoic acid; ALP, alkaline phosphatase; ALZ, Alizarin red; VK, von Kossa.

Expression of OCN during the osteogenic differentiation process. OCN, a marker of mature osteoblasts, was evaluated during the differentiation process using immunostaining and the protein levels of OCN were determined by western blot analysis. In line with the previous results, the CR group exhibited a distinct increase in the expression level of OCN when compared with the OM and ATRA groups. Furthermore, the addition of ATRA inhibited the induction of OCN expression (Fig. 5).

Effect of curcumin-supplemented OM on the osteogenic differentiation of lentivirus-transduced MEFs with hLMP-3. The aforementioned results reveal the positive role of curcumin-supplemented medium on the osteogenic differentiation of BMSCs. Therefore, the present study investigated whether this positive role could be applied in other cell types. In a previous study, the present research team studied the reprogramming of MEFs to osteoblast cells using hLMP-3 (86). The results revealed that the transduction of MEFs with a lentiviral vector expressing the osteogenic factor

hLMP-3 induced osteoblast cell formation *in vitro* (86). The present study examined the effect of curcumin enrichment on the osteogenic differentiation of MEFs reprogrammed with the osteogenic factor hLMP-3. MEFs were successfully transduced with pGMLVPE1-hLMP-3, as previously reported (86). At 2 days post-transduction, the culture medium was changed to OM supplemented with curcumin. The effect of curcumin-supplemented medium was compared with that of the non-supplemented medium using RT-qPCR to detect the difference in the expression of bone markers between the two groups. The RT-qPCR results revealed that the addition of curcumin to the OM upregulated the expression of the bone markers Runx2, BMP and osterix at 7, 14 and 21 days post-transduction (Fig. 6).

Discussion

In the present study, the effects of two natural compounds on the *in vitro* osteogenic differentiation of BMSCs were

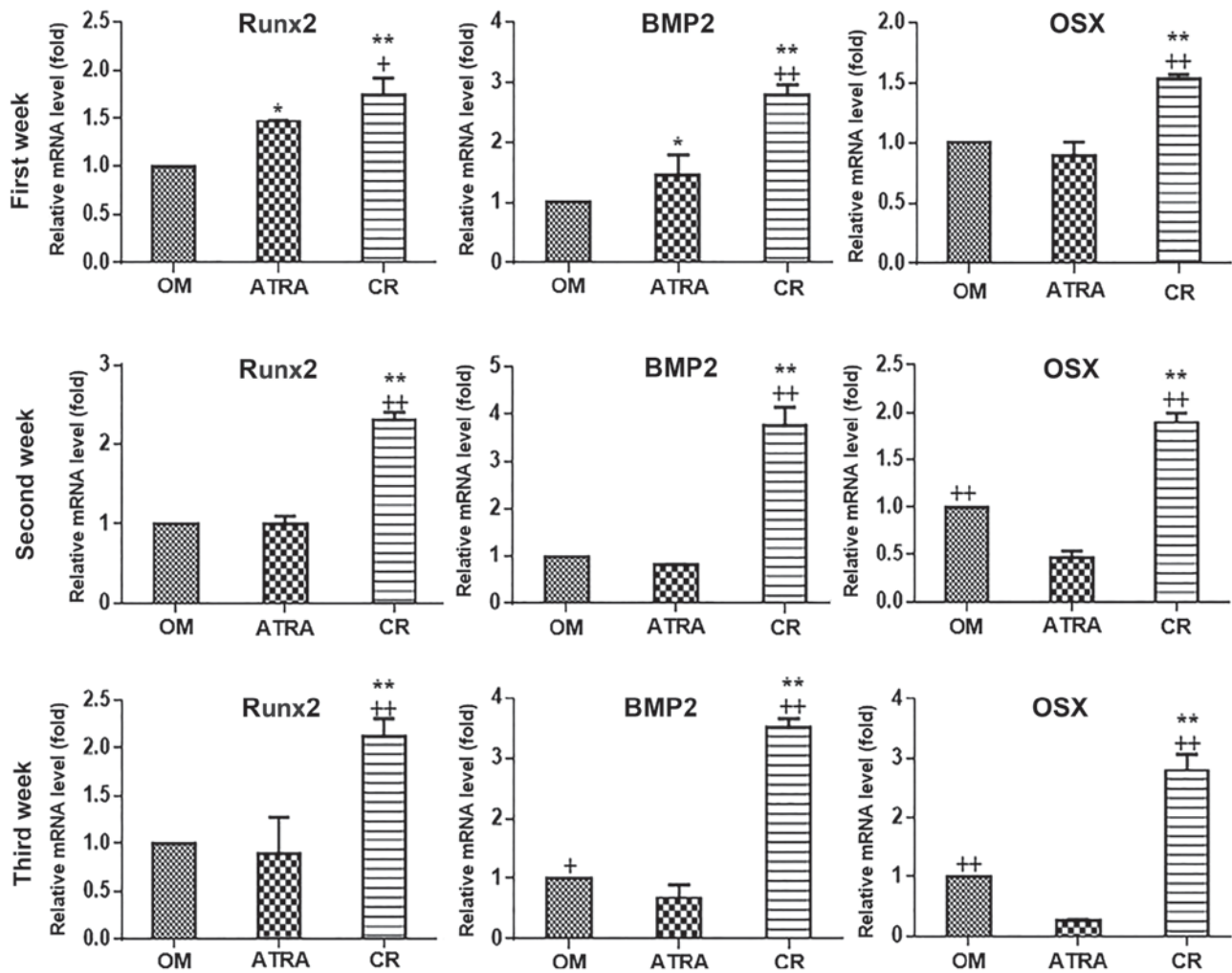


Figure 4. Expression level changes in bone specific markers during the differentiation of BMSCs to osteoblast cells. Quantification of the bone specific factors determined by reverse transcription-quantitative polymerase chain reaction at different time points. Quantitative data are expressed as the mean \pm standard deviation ($n=3$). Statistical significance was determined using one-way analysis of variance followed by Tukey's post hoc test for multi-group comparisons. * $P<0.05$ and ** $P<0.01$ vs. OM group; + $P<0.05$ and ++ $P<0.01$ vs. ATRA group. BMSCs, bone marrow mesenchymal stem cells; OM group, BMSCs in osteogenic medium; CR group, BMSCs in osteogenic medium + curcumin; ATRA group, BMSCs in osteogenic medium + ATRA; ATRA, all-trans retinoic acid; Runx2, runt-related transcription factor 2; BMP2, bone morphogenetic protein 2; OSX, osterix.

investigated. BMSCs were isolated from the bone marrow of BALB/c mice, and the osteogenic differentiation of the BMSCs to osteoblasts was induced using a standard protocol. In certain treatment groups, the OM was supplemented with 15 μM curcumin (the CR group) or 1 μM ATRA (the ATRA group). Cells were examined for osteogenic differentiation to determine the effect of curcumin or ATRA. Curcumin supplementation was revealed to increase the osteogenic differentiation capacity of BMSCs, as detected by the mineralization assay and RT-qPCR analysis of bone markers and OCN expression. By contrast, ATRA downregulated the osteogenic differentiation of BMSCs. To determine if the positive effect of curcumin also occurred in other cell types, curcumin was added to the culture medium during MEF reprogramming to osteoblasts using the osteogenic factor hLMP-3. Again, an elevated expression of all the bone markers was observed when compared with hLMP-3 transduced cells cultured in OM without curcumin supplementation.

The effect of ATRA on osteogenic differentiation is currently unclear in the literature, and whether its effect

is pro-osteogenic, involved in delaying osteogenesis, or even anti-osteogenic is unknown (12,14,16,20,87). In the present study, ATRA increased ALP expression during the mineralization assay in the first week. Later, during the differentiation process, a decreased expression of the bone markers was observed, as well as the inability to develop matrix mineralization. All these findings indicate that ATRA served a negative role during the osteogenic differentiation of BMSCs. These results are consistent with those of previous studies (27,82), which suggested that this inhibitory effect of ATRA is mediated via BMP signaling. They reported that ATRA upregulated the expression of BMP-receptor IA which is responsible for adipogenic differentiation, and reduced the expression of BMP-receptor IB, which is responsible for the osteogenic differentiation of BMSCs. Another explanation posed by Green *et al.* (88) is that retinoic acid receptor (RAR) agonists, such as ATRA, impair osteogenesis through RAR α and RAR γ , which may explain why high intake and serum levels of retinol are associated with fracture risk. By contrast, several studies have demonstrated the positive role

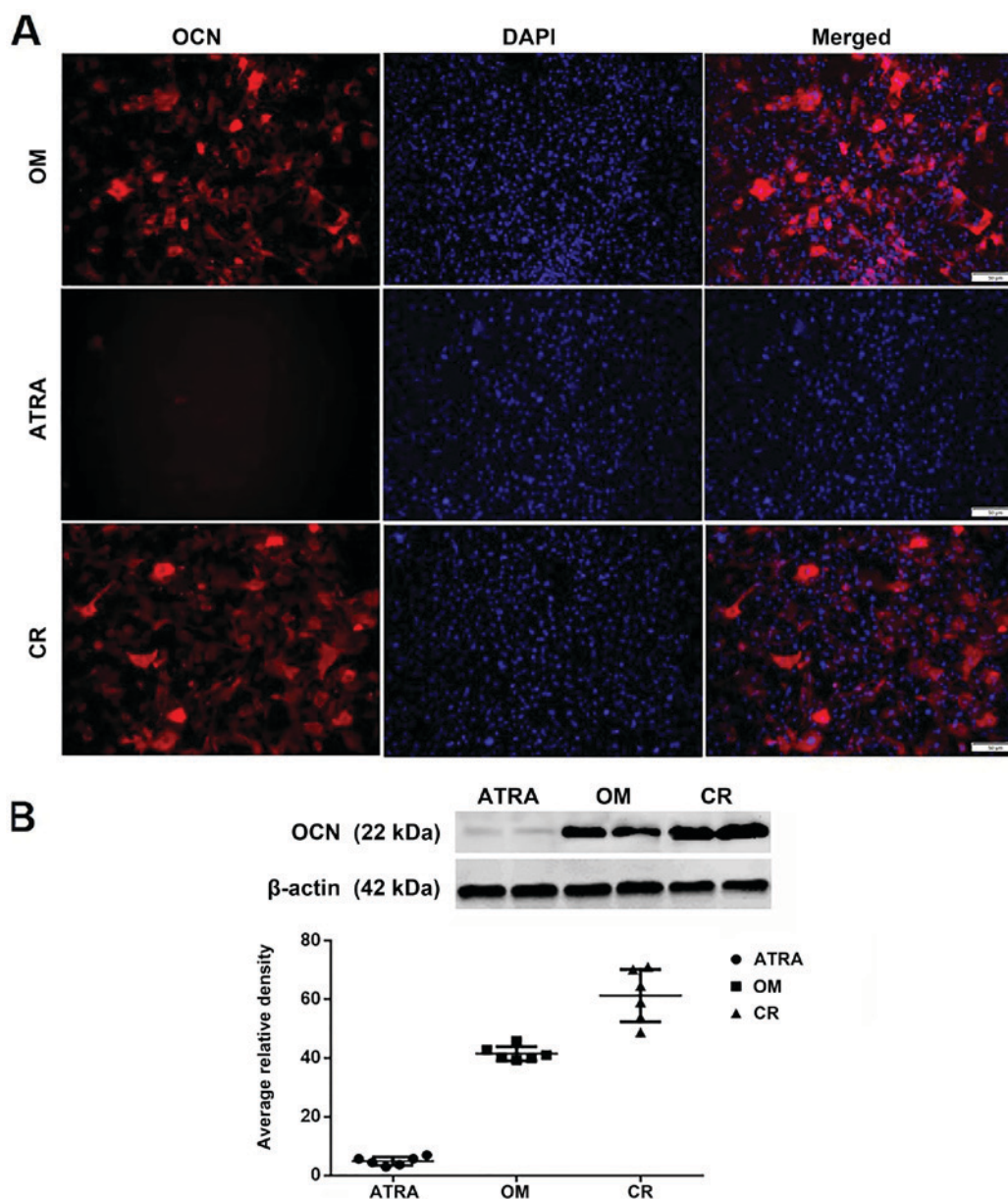


Figure 5. Effect of curcumin and ATRA on OCN expression in BMSCs at 28 days post-osteogenic differentiation. (A) BMSCs cultured in different media were immunostained with the specialized marker of late osteoblast differentiation, OCN. Scale bar, 50 μ m. (B) The protein expression of OCN was evaluated using western blot analysis. β -actin served as an internal control. The mean relative density of the OCN bands was measured using Fiji in ImageJ software. The mean and standard deviation are presented. BMSCs, bone marrow mesenchymal stem cells; OM group, BMSCs in osteogenic medium; CR group, BMSCs in osteogenic medium + curcumin; ATRA group, BMSCs in osteogenic medium + ATRA; ATRA, all-trans retinoic acid; OCN, osteocalcin.

of ATRA during the osteogenic differentiation of cell types other than BMSCs (18,89,90). In the present study, BMSCs treated with ATRA did not express OCN, which is the main non-collagenous protein of bone. This may be explained by interactions between ATRA and the OCN promoter (91).

In the present study, curcumin upregulated the osteogenic differentiation of BMSCs. Several studies have examined the role of curcumin in the orthopedic field (35-38). The present results were consistent with those of Gu *et al* (53), who reported that curcumin increased the osteoblast differentiation of rat MSCs with a reduction in adipocytes. The authors considered that this increase occurred due to an increase in HO-1 expression induced by curcumin, which in turn promoted osteoblast differentiation. In the same context, Son *et al* (92) reported that curcumin increased the expression of genes such as Dlx5,

Runx2, ALP and OCN, which subsequently induced osteoblast differentiation in C3H10T1/2 cells. These findings were interpreted through a new hypothesis, which is that curcumin induced mild ER stress, similar to BMP2 functioning, in osteoblast cells. Furthermore, Son *et al* (92) reported that curcumin is similar to BMP2 as it induces the phosphorylation of Smad 1/5/9. By contrast, another study revealed that the administration of curcumin did not efficiently improve bone mineralization in ovariectomized rats, and indicated that curcumin affected the level of osteogenesis commitment, not osteoblast maturation (36). It has also been revealed that curcumin reduces the expression of RANKL and inhibits osteoclastogenesis by acting on BMSCs (61). This could be explained by the activity of curcumin as a scavenger of reactive oxygen species. Upregulation of RANKL induced by estrogen

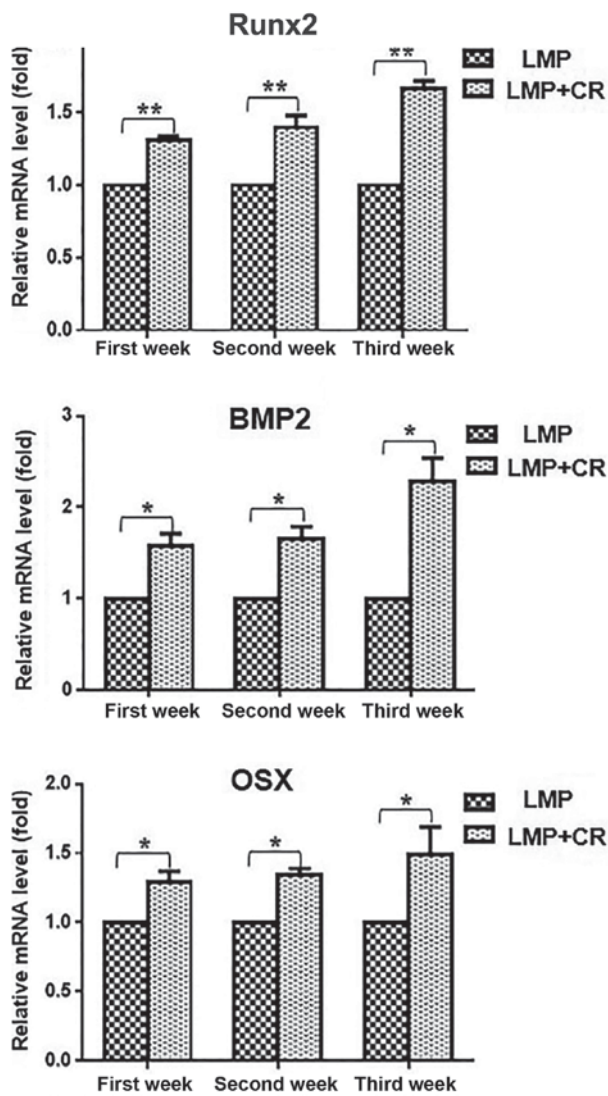


Figure 6. Expression level changes of bone-specific markers during the osteogenic reprogramming of mouse embryonic fibroblasts using the osteogenic factor hLMP-3 with and without curcumin enrichment. Quantification of the bone specific factors by reverse transcription-quantitative polymerase chain reaction at different time points (1, 2 and 3 weeks) post-transduction. The quantitative data are expressed as the mean \pm standard deviation ($n=3$). Statistical significance was determined using a paired sample t-test. * $P<0.05$ and ** $P<0.01$. hLMP-3, human lim mineralization protein 3; CR, curcumin; LMP + CR group, MEFs reprogrammed with hLMP-3 and cultured in medium supplemented with $15 \mu\text{M}$ curcumin; LMP group, MEFs reprogrammed with hLMP-3 without curcumin supplementation; Runx2, runt-related transcription factor 2; BMP2, bone morphogenetic protein 2; OSX, osterix.

deficiency in humans results in increased bone resorption (93). In addition to RANKL inhibition, curcumin also inhibits NF- κ B (47), which is associated with impaired bone formation in osteoporosis, and inhibits the differentiation and mineralization of mature osteoblasts. The inhibition of NF- κ B results in stimulation of the differentiation and mineralization of primary murine BMSCs and pre-osteoblasts (94,95). Thus, curcumin may enhance osteogenesis through its interactions with NF- κ B. Curcumin has also been demonstrated to affect other signaling pathways associated with bone remodeling, including the Wnt (96) and transforming growth factor- β signaling pathways (97).

In conclusion, the results of the present study highlight the potential use of some natural osteogenic inducers in the orthopedic field. The positive role of curcumin during the osteogenic differentiation of BMSCs was demonstrated, and curcumin was revealed to be capable of enriching the osteogenic differentiation of other cell types, namely, MEFs reprogrammed with hLMP-3. By contrast, the use of ATRA inhibited the osteogenic differentiation process of BMSCs rather, which is contrary to its important role in the osteogenic induction of other cell types.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MFA and AKE have performed the experiments, contributed to data analysis and wrote the manuscript. HC substantially contributed to the interpretation of data and revision of the manuscript. RZ and QZ contributed to the study conception, and analysis and interpretation of data. MSY, YZ and BL conceived and designed the study, and approved the final version to be published.

Ethics approval and consent to participate

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of School of Animal Science and Technology, Yangzhou University (Yangzhou, China; approval no. YZUDWSY2017-0029).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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