Research Paper

Curcumin Promotes the Proliferation and Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells by Up-Regulating microRNA-148a

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To probe curcumin action on the proliferation and osteogenic differentiation of human bone marrow mesenchymal stem cells and its possible molecular mechanism. Human bone marrow mesenchymal stem cells were osteogenic induced for 14 d. Real-time quantitative reverse transcription-polymerase chain reaction and Western blotting analysis were utilized for the detection of microRNA-148a, alkaline phosphatase, osteocalcin and osteopontin levels in human bone marrow mesenchymal stem cells after curcumin incubation for 14 d. After curcumin treatment, the proliferation of human bone marrow mesenchymal stem cells was notably rose, and contents of alkaline phosphatase, osteocalcin and osteopontin in osteogenic induced-human bone marrow mesenchymal stem cells were elevated. Curcumin treatment elevated microRNA-148a expression. Overexpression of microRNA-148a could boost human bone marrow mesenchymal stem cells proliferation, and increased alkaline phosphatase, osteocalcin and osteopontin contents in human bone marrow mesenchymal stem cells during osteogenesis induction. Down-regulation of microRNA-148a reversed the promoting effect of curcumin on human bone marrow mesenchymal stem cells proliferation and the levels of alkaline phosphatase, osteocalcin and osteopontin in human bone marrow mesenchymal stem cells during osteogenesis induction ($p<0.05$). Curcumin promoted the proliferation and osteogenic differentiation of human bone marrow mesenchymal stem cells by up-regulating microRNA-148a.

Key words: Curcumin, microRNA-148a, human bone marrow mesenchymal stem cells, proliferation, osteoporosis

Osteoporosis (OP) is characterized by a decrease in bone mass per unit volume and changes in bone microstructure, and is more likely to occur in elderly men and postmenopausal women[1]. Bone homeostasis is related to various factors[2,3]. Postmenopausal women face a high risk of OP due to estrogen deficiency and net bone loss, leading to faster bone turnover. Bone Marrow Mesenchymal Stem Cells (BMSCs) can differentiate into osteoblasts, cartilage, adipocytes and so on, and are key to new bone formation[4]. Targeting BMSCs may be a promising method for OP prevention.

Research has found that curcumin, a natural polyphenol compound isolated from *Curcuma* root, has anticancer, anti-inflammatory, antibacterial, antioxidant, and regulatory activity in bone metabolism[5,6]. The injection of curcumin in rats could improve bone microstructure and bone formation, and exerted protective effects to prevent OP[7]. Curcumin could alleviate the symptoms of postmenopausal OP[8,9]. Currently, increasing evidence has manifested the impact of epigenetic modifications on OP[10]. MicroRNAs (miRNAs) are one of epigenetic regulators and can modulate various cell biological processes by affecting mRNA expression[11]. Research has shown that miR-148a enhanced the differentiation of pluripotent stem

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cells into cardiomyocytes[12]. miR-148a expression was elevated in human BMSCs (hBMSCs) during myogenic differentiation, the decrease of miR-148a reduced myocardial cell specific markers Alpha-Myosin Heavy Chain (α-MHC), and miR-148a boosted hBMSC myocardial differentiation[13]. However, the role of miR-148a in OP and whether curcumin affected OP progression by miR-148a still need further exploration.

Here, this study mainly probed the action of curcumin on the proliferation and osteogenic differentiation of hBMSCs, and explored its regulatory effect on miR-148a.

MATERIALS AND METHODS

Materials and reagents:

HBMSCs, Fetal Bovine Serum (FBS), trypsin and α-Minimum Essential Medium (MEM) (American Type Culture Collection, United States of America (USA)); curcumin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) kit, dexamethasone, ascorbic acid, Beta (β)-glycerol phosphate and Dimethyl Sulfoxide (DMSO) (Beyotime, Beijing, China); Radioimmunoprecipitation Assay (RIPA) lysis buffer and Bicinchonic Acid (BCA) reagent kit (Shanghai Yanjing Biotechnology Co., Ltd); TRIzol, reverse transcription, and quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) reagents (Takara, Japan); Alkaline Phosphatase (ALP), Osteocalcin (OCN), Osteopontin (OPN) antibodies, and Horseradish Peroxidase (HRP)-labeled Immunoglobulin G (IgG) antibodies (Santa Cruz, USA); miR-148a mimic, inhibitor (anti-miR-148a) and the contrasts (miR-NC or anti-miR-NC) (Geenseed, Guangzhou, China). The primers of qRT-PCR.

Cell culture and osteogenic differentiation:

hBMSCs were cultured in α-MEM containing 10 % FBS with 5 % Carbon dioxide (CO2) at 37°. For osteogenic differentiation, 100 μmmol/l-1 dexamethasone, 100 μg/ml ascorbic acid and 10 mm β-glycerol phosphate were added into α-MEM medium.

Experimental grouping:

hBMSCs, underwent osteogenic differentiation or not, were exposed to 1, 2, or 4 μg/ml curcumin for 48 h, namely 1, 2, or 4 μg/ml curcumin group. The untreated cells were used as the control group. hBMSCs, underwent osteogenic differentiation or not, were transfected with miR-NC or miR-148a for 48 h, namely miR-NC or miR-148a group. hBMSCs with or without osteogenic differentiation were transfected with anti-miR-NC or anti-miR-148a for 48 h, followed by treating with 4 μg/ml curcumin, namely 4 μg/ml+anti-miR-NC group or 4 μg/ml+anti-miR-148a group.

Cell transfection:

hBMSCs were inoculated into the 6-well plate (1×10^5/well), then as per the protocol of Lipofectamine 2000, cell transfection were conducted based on the experimental grouping using the Lipofectamine 2000.

MTT assay:

hBMSCs at 2.5×10^4 per well were inoculated overnight on a 96 well plate. After 24, 48, and 72 h of cultivation, adding 20 μl MTT (5 g/l) into per well, followed by incubating for 4 h and 150 μl DMSO for 2 h, then Optical Density (OD) value at 490 nm was assayed.

qRT-PCR:

Total Ribonuclic Acid (RNA) was extracted from the cells of each group and the purity of RNA were detected using NanoDrop 2000c spectrophotometer. Then complementary Deoxyribonucleic Acid (cDNAs) were produced by reverse transcript and qRT-PCR reaction were performed. The fold changes of miR-148a, ALP, OCN and OPN mRNA were calculated by $2^{-\Delta\Delta Ct}$ method. U6 or Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as the internal reference. miR-148a forward: 5'-ACACTCCAGCTGGGTCA GTGCACTACAGAA- 3', reverse: 5'-TGGTGTCGTGGAGTCG-3'; U6 forward: 5'-CTCGCTTCGGCAGCACATA-3', reverse: 5'-CGAATTTGCGTGTCATCCT-3'; ALP forward: 5'-AGACCTTCATAGCGCACGTC-3', reverse: 5'-ACCTTTGGCTCTCGACCAG-3'; OCN forward: 5'-ACCGAGACACCATGAGAG-3', reverse: 5'-CTGGGTCTCTTCACTACCT-3'; OPN forward: 5'-AATATAAGCGCGAGGCCAAT-3', R5'-AATTCACGGCTCTGGGATTC-3; GAPDH forward: 5'-CACGTCTGCCACGATAACAC-3' and reverse: 5'-AGGCCTGTGATGGATTGTCT-3'.

Western blot:

The total protein of each group of cells was extracted and quantified by a BCA method.

After transferring to a Polyvinylidene Difluoride (PVDF) membrane, the antibody diluents including ALP (1:1000), OCN (1:1000), OPN (1:500) and GAPDH (1:3000) were incubated overnight at 4° with membranes, and then incubated at 37° for 1 h with the secondary antibodies. The protein bands were analyzed by Enhanced Chemiluminescence (ECL) incubation.

Statistical analysis:

The data were presented by $\bar{x}\pm s$. Statistical Package for the Social Sciences (SPSS) 21.0 software was used to analyze the data. The comparison of the two groups or multiple groups was conducted using t-test or Analysis of Variance (ANOVA). $p<0.05$ suggested significant difference.

RESULTS AND DISCUSSION

After culturing hBMSCs in a basic medium with 1, 2, and 4 μg/ml curcumin, it was found that OD values of hBMSCs in the 2 μg/ml and 4 μg/ml curcumin group were markedly increased relative to the control group (Table 1), indicating the increased proliferation. After osteogenic differentiation for 14 d, we also found ALP, OCN, and OPN contents in hBMSCs of the 4 μg/ml curcumin group were notably increased relative to the control group (Table 2 and fig. 1).

After osteogenic differentiation for 14 d, hBMSCs were incubated with 1, 2, and 4 μg/ml curcumin, and then we observed that the expression of miR-148a in hBMSCs at the 2 μg/ml and 4 μg/ml curcumin group were markedly increased relative to the control group (Table 3).

Transfection of miR-NC and miR-148a into hBMSCs suggested miR-148a levels in hBMSCs in the miR-148a group was significantly higher than those in the miR-NC group (Table 4), indicating successful transfection. Moreover, miR-148a overexpression increased the OD values of hBMSCs in the miR-148a group (Table 4). After osteogenic differentiation for 14 d, we also found that levels of ALP, OCN, and OPN in hBMSCs of miR-148a group were elevated (fig. 2 and Table 5).

Comparison with the 4 μg/ml+anti-miR-NC group, miR-148a levels in hBMSCs of the 4 μg/ml+anti-miR-148a group were declined, indicating successful transfection (Table 6). Besides, the OD values of hBMSCs in the 4 μg/ml+anti-miR-148a group were markedly decreased (Table 6). After the osteogenic differentiation, the levels of ALP, OCN, and OPN were notably reduced in hBMSCs of 4 μg/ml+anti-miR-148a group were down-regulated (fig. 3 and Table 7).

TABLE 1: CURCUMIN PROMOTES THE PROLIFERATION OF hBMSCs ($\bar{x}\pm s$, n=3)

Group	OD values (490 nm)		
	24 h	48 h	72 h
Control	0.39±0.03	0.61±0.05	0.95±0.08
1 μg/ml	0.43±0.03	0.67±0.07	1.03±0.10
2 μg/ml	0.49±0.05*	0.75±0.06*	1.19±0.09*
4 μg/ml	0.58±0.06*	0.86±0.06*	1.37±0.13*
F	10.367	9.610	10.643
p	0.004	0.005	0.004

Note: In comparison to control group, *p<0.05

TABLE 2: CURCUMIN PROMOTES OSTEOGENIC DIFFERENTIATION OF hBMSCs ($\bar{x}\pm s$, n=3)

Group	mRNA			Protein		
	ALP	OCN	OPN	ALP	OCN	OPN
Control	1.01±0.11	0.99±0.13	1.02±0.10	0.43±0.04	0.21±0.03	0.27±0.03
1 μg/ml	1.32±0.17	1.28±0.14	1.23±0.15	0.52±0.05	0.27±0.03	0.34±0.04
2 μg/ml	1.68±0.19*	1.52±0.21*	1.78±0.23*	0.68±0.07*	0.49±0.06*	0.51±0.05*
4 μg/ml	2.26±0.28*	1.89±0.26*	2.52±0.31*	0.85±0.08*	0.63±0.08*	0.72±0.07*
F	22.232	11.752	29.674	26.649	38.644	48.727
p	0.000	0.003	0.000	0.000	0.000	0.000

Note: In comparison to control group, *p<0.05

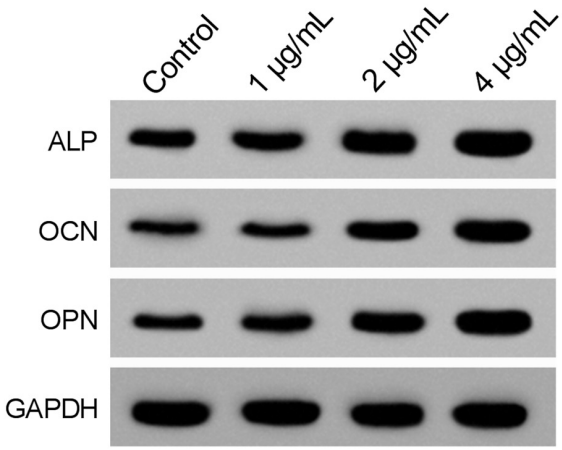

Fig. 1: Expression of ALP, OCN, and OPN protein

TABLE 3: CURCUMIN PROMOTES miR-148A EXPRESSION IN hBMSCs (x̄±s, n=3)

Group	miR-148a
Control	1.01±0.13
1 μg/ml	1.35±0.19
2 μg/ml	1.83±0.23*
4 μg/ml	2.72±0.32*
F	31.740
p	0.000

Note: In comparison to control group, *p<0.05

TABLE 4: miR-148A PROMOTES THE PROLIFERATION OF hBMSCs (x̄±s, n=3)

Group	miR-148a	OD values (490 nm)		
		24 h	48 h	72 h
miR-NC	1.01±0.09	0.35±0.04	0.57±0.05	0.89±0.07
miR-148a	2.83±0.36*	0.54±0.06*	0.81±0.08*	1.33±0.13*
t	8.495	4.564	4.406	5.162
p	0.001	0.010	0.012	0.007

Note: In comparison to miR-NC group, *p<0.05

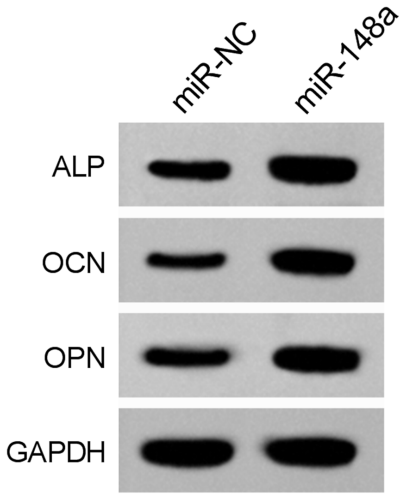

Fig. 2: Expression of ALP, OCN, and OPN protein

TABLE 5: miR-148A PROMOTES OSTEOGENIC DIFFERENTIATION OF hBMSCs (x̄±s, n=3)

Group	mRNA			Protein		
	ALP	OCN	OPN	ALP	OCN	OPN
miR-NC	1.02±0.12	1.01±0.10	1.01±0.08	0.39±0.05	0.23±0.02	0.31±0.03
miR-148a	2.17±0.34*	1.82±0.21*	2.35±0.28*	0.82±0.07*	0.59±0.07*	0.75±0.08*
F	5.524	6.032	7.970	8.658	8.565	8.92
p	0.005	0.004	0.001	0.001	0.001	0.001

Note: In comparison to miR-NC group, *p<0.05

TABLE 6: miR-148A SILENCING REVERSES CURCUMIN-EVOKED PROLIFERATION IN hBMSCs (x̄±s, n=3)

Group	miR-148a	OD values (490 nm)		
		24 h	48 h	72 h
Control	1.01±0.08	0.36±0.04	0.58±0.05	0.87±0.09
4 μg/ml	2.81±0.31*	0.59±0.06*	0.83±0.08*	1.40±0.16*
4 μg/ml+anti-miR-NC	2.75±0.36	0.62±0.07	0.80±0.07	1.45±0.19
4 μg/ml+anti-miR-148a	1.56±0.18#	0.45±0.05#	0.69±0.05#	1.08±0.11#
F	36.115	14.127	9.546	11.028
p	0.000	0.001	0.005	0.003

Note: In comparison to control group, *p<0.05 and relative to 4 μg/ml+anti-miR-NC group, #p<0.05

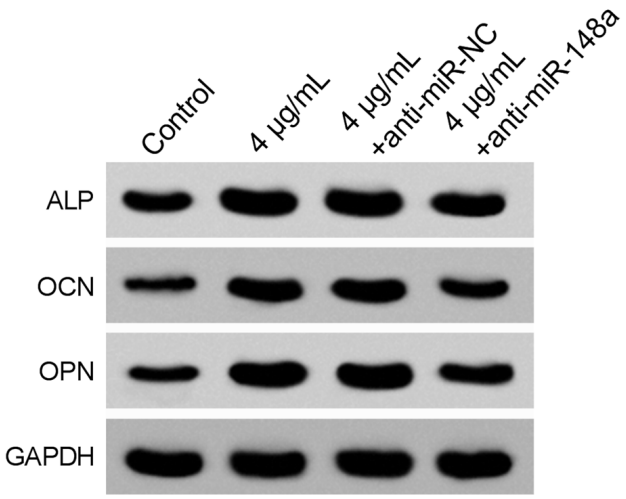

Fig. 3: Expression of ALP, OCN, and OPN protein

TABLE 7: miR-148A SILENCING REVERSES CURCUMIN-EVOKED OSTEOGENIC DIFFERENTIATION IN hBMSCs (x̄±s, n=3)

Group	mRNA			Protein		
	ALP	OCN	OPN	ALP	OCN	OPN
Control	1.02±0.10	1.00±0.08	1.01±0.12	0.41±0.04	0.22±0.02	0.29±0.03
4 μg/ml	2.23±0.26*	1.91±0.21*	2.39±0.32*	0.83±0.08*	0.61±0.07*	0.71±0.08*
4 μg/ml+anti-miR-NC	2.31±0.22	1.85±0.25	2.47±0.28	0.80±0.07	0.64±0.05	0.69±0.06
4 μg/ml+anti-miR-148a	1.46±0.21#	1.36±0.19#	1.65±0.17#	0.57±0.06#	0.36±0.04#	0.42±0.05#
F	27.299	14.932	25.310	28.818	52.117	38.112
p	0.000	0.001	0.000	0.000	0.000	0.000

Note: In comparison to control group, *p<0.05 and relative to 4 μg/ml+anti-miR-NC group, #p<0.05

OP is the most common skeletal disease among elderly patients of all races and genders worldwide, especially in developed and aging societies[14]. The prevalence rate of the elderly over 60 y old in China is 36 %, and OP is an asymptomatic disease, there are no obvious symptoms in the early stage, which is called "silent disease", therefore both diagnosis and treatment are insufficient. Owing to impaired bone strength, decreased bone mass, and deteriorating microstructure, the fractures are prone to occur in OP patients[15,16]. Mesenchymal Stromal Cells (MSCs) is an important cell bank involved in tissue regeneration. Under the action of special signals caused by tissue injury, MSC migrate to the damaged site, accumulate and proliferate locally, and differentiate along different pathways according to different injury signals. MSC is easy to isolate and expand, has strong *in vitro* multiplication ability, and can maintain its multidirectional differentiation ability even if amplified 100 million times. In addition, transplantation of MSCs can change the hematopoietic microenvironment, rebuild the immune system, and promote the recovery of hematopoietic function. Therefore, MSC is a practical seed cell for tissue repair, and BMSCs play a significant role in bone repair processes[17,18].

Curcumin is the main component of curcumin compounds extracted from the roots and stems of *Curcuma*, and multiple studies have found that curcumin can improve OP. For instance, curcumin could improve bone biomechanical properties and preserved bone microstructure through Transforming Growth Factor-Beta (TGF-β)/ Mothers against Decapentaplegic Homolog (SMAD) 2/3 pathway protects OP in type 2 diabetes rats, and protected type 2 diabetes rats against OP[19]. The dysfunction of osteoblasts caused by oxidative stress could be improved by curcumin through Glycogen Synthase Kinase-3β (GSK-3β)-Nuclear Factor Erythroid 2-related factor 2 (Nrf2) pathway[20]. The bone microstructure of glucocorticoid-evoked secondary OP mice could be ameliorated by curcumin through activating miR-365 *via* Matrix Metallopeptidase 9 (MMP-9)[21]. Curcumin improved bone metabolism and trabecular microstructure in ovariectomized OP model rats, and inhibit bone resorption[22]. Curcumin ameliorated bone microstructure and prevented bone loss by downregulating Enhancer of Zeste Homolog-2 (EZH2) expression[23]. Curcumin blocked Nuclear Factor Kappa B (NF-κB) activation to promote osteogenic differentiation in BMSCs[24]. ALP is a key marker in early differentiation of osteoblasts, OCN functions in bone remodeling and mineralization, OPN has been shown to be implicated in bone accelerated hBMSC proliferation, and induced osteogenic differentiation of hBMSCs by elevating ALP, OCN, and OPN levels.

Research has shown that miR-148a can promote cell differentiation. In an *in vitro* rabbit induced differentiation model, miR-148a-3p enhanced precursor adipocyte differentiation by down-regulating PTEN[25]. miR-148a promoted MSC differentiation into cardiomyocytes by targeting DNA Methyltransferase 1 (DNMT1) [26]. In our work, we found curcumin increased miR-148a expression in hBMSCs. Functionally, miR-148a I overexpression promoted hBMSCs proliferation, and elevated ALP, OCN, and OPN levels in cells. Furthermore, the downregulation of miR-148a reversed the effects of curcumin on hBMSCs.

In all, curcumin could promote the proliferation and osteogenic differentiation of hBMSCs, which might be linked with the upregulation of miR-148a, providing a theoretical basis for curcumin treatment of OP.

Conflict of interests:

The authors declared no conflict of interests.

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