Exosome derived from bone marrow mesenchymal stem cells pre-treated with curcumin alleviates osteoporosis by promoting osteogenic differentiation via regulating the METTL3/microRNA-320/RUNX2 signaling pathway

Type
Research paper

Keywords
curcumin, osteoporosis, bone marrow stem cells, Exosome, Runx2, METTL3, miRNA-320

Abstract

Introduction
Curcumin (CUR) was reported to stimulate the expression of methyltransferase-like 3 (METTL3), a potential new target for the replacement therapy of osteoporosis. Besides, marrow stem cells (MSC)-derived exosomes (EXO) were proven to improve osteoporosis by promoting the proliferation of osteoblasts. In this study, we aimed to study the effect of CUR and BMSC-derived exosomes in the treatment of osteoporosis.

Material and methods
Microscopy was used to characterize exosomes derived from bone marrow stem cells (BMSCs). MicroCT was carried out to analyze the parameters of bone formation. Western blot was carried out to analyze the expression of surface markers on BMSC-derived exosomes and METTL3/Runt-related transcription factor 2 (RUNX2) proteins under different conditions. Real-time PCR was used to assess the gene expression under different circumstances.

Results
The exosomes derived from CUR-treated BMSCs showed an enhanced therapeutic effect on osteoporosis mice compared with exosomes derived from untreated BMSCs. Mechanistically, the effect of BMSC-derived exosomes on restoring the de-regulated expression of METTL3, miR-320 and RUNX2 was significantly enhanced by CUR treatment upon BMSCs. Besides, CUR treatment upon BMSCs showed an obvious effect on enhancing the stimulatory role of BMSC-derived exosomes in the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs. Furthermore, luciferase assay demonstrated that miR-320 was capable of suppressing the expression of RUNX2 through binding to the 3’ UTR of RUNX2.

Conclusions
Our study demonstrated that BMSC-derived exosomes could modulate the METTL3/miRNA-320/RUNX2 axis to attenuate osteoporosis by promoting osteogenic differentiation of BMSCs. Moreover, the CUR treatment upon BMSCs promoted the therapeutic effect of BMSC-derived exosomes.
Exosome derived from bone marrow mesenchymal stem cells pre-treated with curcumin alleviates osteoporosis by promoting osteogenic differentiation via regulating the METTL3/microRNA-320/RUNX2 signaling pathway

Yunhui Zhang¹⁺, Naiguo Wang ²⁺, Chenglin Zhang ³*

1. Department of Orthopedics, Baoshan Hospital, Shuguang Hospital Affiliated to Shanghai University of Chinese Medicine, Shanghai 201999, China.
2. Department of Spinal surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong, China.
3. Department of Orthopedics, Shanghai General Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 201620, China.

* Correspondence to: Chenglin Zhang,
Affiliation: Department of Orthopedics, Shanghai General Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 201620, China.
Address: 1878 N. Sichuan Rd, Shanghai, China, 200081
Email: boneresearch@126.com

+ Yunhui Zhang and Naiguo Wang contributed equally to this study.

Abstract

Background: Curcumin (CUR) was reported to stimulate the expression of methyltransferase-like 3 (METTL3), a potential new target for the replacement therapy of osteoporosis. Besides, marrow stem cells (MSC)-derived exosomes (EXO) were proven to improve osteoporosis by promoting the proliferation of osteoblasts. In this study, we aimed to study the effect of CUR and BMSC-derived exosomes in the treatment of osteoporosis. Methods: Microscopy was used to characterize exosomes derived from bone marrow stem cells (BMSCs). MicroCT was carried out to analyze the parameters of bone formation. Western blot was carried out to analyze the expression of surface markers on BMSC-derived exosomes and METTL3/Runt-related
transcription factor 2 (RUNX2) proteins under different conditions. Real-time PCR was used to assess the gene expression under different circumstances. **Results:** The exosomes derived from CUR-treated BMSCs showed an enhanced therapeutic effect on osteoporosis mice compared with exosomes derived from untreated BMSCs. Mechanistically, the effect of BMSC-derived exosomes on restoring the de-regulated expression of METTL3, miR-320 and RUNX2 was significantly enhanced by CUR treatment upon BMSCs. Besides, CUR treatment upon BMSCs showed an obvious effect on enhancing the stimulatory role of BMSC-derived exosomes in the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs. Furthermore, luciferase assay demonstrated that miR-320 was capable of suppressing the expression of RUNX2 through binding to the 3’ UTR of RUNX2. **Conclusion:** Our study demonstrated that BMSC-derived exosomes could modulate the METTL3/miRNA-320/RUNX2 axis to attenuate osteoporosis by promoting osteogenic differentiation of BMSCs. Moreover, the CUR treatment upon BMSCs promoted the therapeutic effect of BMSC-derived exosomes.

**Running title:** CUR promoted the therapeutic effect of BMSC-derived exosomes

**Key words:** osteoporosis, curcumin, exosome, bone marrow stem cells, METTL3, miRNA-320, RUNX2

**Introduction**

As a bone condition featured by a weakening bone strength, lowered bone mass, damaged bone microstructures, as well as elevated brittleness of the bone, osteoporosis increases the danger of fracture. The risk of osteoporosis is increased over age [1, 2]. Along with the aging of the population worldwide, osteoporosis has turned into one of the most costly illness worldwide [3]. Osteoporosis can be classified into 2 groups, i.e., primary osteoporosis as well as secondary osteoporosis.

As a type of yellow polyphenolic substance originated from the extract of Curcuma longa, CUR was shown to possess anti-tumor, anti-inflammatory, anti-oxidant as well as anti-bacterial properties [4, 5]. Specifically, CUR was demonstrated to exhibit its anti-inflammatory effect via promoting the production of IL-10 and thus accordingly enhancing pathological interactions mediated by IL-10 [6]. Previous researches have presented that CUR may impair the function of
the tissue factors related to thrombotic diseases by inhibiting the aggregation of platelets or by decreasing platelet activity [7]. In addition, many studies have found that CUR exerts a beneficial effect in alleviating osteoporosis in both clinical as well as experimental models [8]. Previous researches indicated that the combined administration of CUR and alendronate could enhance bone mineral density (BMD) and modulate bone turnover markers in postmenopausal women with osteoporosis [9]. And unlike all-trans retinoic acid (ATRA) which inhibited the mineralization of differentiated cells during the later stages, CUR was proved to increase the osteogenic differentiation capacity of BMSCs [10]. Moreover, the pre-administration of CUR into the BMSCs could facilitate cell therapy in tissue repair treatment by improving mitochondrial function and destabilizing HIF-1α, leading to the obstruction of potential hypoxia/reoxygenation injury [11, 12]. It was found that CUR prevented LPS-induced augmentation of SCD-1 and SREBP-1c mRNA expression in the liver. Especially, CUR in the diet impacts the METTL14, METTL3, FTO, ALKBH5, as well as YTHDF2 mRNA expression while increasing the content of m6A in piglet liver [13].

BMSC are recognized to release exosomes containing the molecules of over 150 types of different miRNAs [14]. Numerous researches have revealed that exosomes play a primary role in the therapeutic functions of BMSCs. In the cardiovascular system, the medium conditioned by BMSCs improved heart functions [15]. Other studies illustrated that the mice treated with BMSC-derived exosomes showed a reduced size of cardiac infarct ex vivo as well as in vivo [14, 16, 17]. The BMSC exosomes derived from rat, human, as well as mouse have shown curative effects in a range of various diseases [18]. It was also shown that hFOB 1.19 cell proliferation was enhanced by Mesenchymal Stem Cells (MSC)-Exo, thus alleviating osteoporosis [19].

METTL3 was initially recognized as a primary methyltransferase in the process of methylation and was later verified to exert an effect on the progression of certain cancers [20, 21]. It was found that the METTL3 deletion in MSCs interrupts the normal cell cycle in mice to lead to osteoporosis. In addition, the gain-of-function of METTL3 protects against postmenopausal osteoporosis induced by estrogen deficiency [22]. Another study found that the methylation of pre-miR-320 mediated by METTL3 promoted bone formation as well as the osteogenic differentiation of BMSCs [23]. It was reported that by acting as a pro-osteogenic or an anti-osteoporotic factor, METTL3 could maintain a high level of RUNX2 expression via direct
methylation of RUNX2 as well as indirect up-regulation of RUNX2 expression through pre-miR-320 methylation [23]. And RUNX2, as previously reported, acts as a transcript factor specific to osteoblast which plays a critical role in the differentiation of MSCs into osteoblasts [24, 25]. RUNX2 could regulate osteoblast differentiation by triggering the expression of several major bone matrix genes during the early stages of osteoblast differentiation [24].

The over-expression of METTL3 has been reported as a potential approach of replacement therapy for the treatment of osteoporosis [23]. Moreover, it was also demonstrated that CUR affected the expression of METTL3 in the pathogenesis of LPS-induced liver injury [13]. Moreover, MSC-derived exosomes were proven to improve osteoporosis by promoting the proliferation of osteoblasts [19]. It has been shown that encapsulated with exosome will improve the delivery of curcumin [26]. On the other hand, combination with curcumin will enhance the therapeutic effect of exosomes [27]. Based on the above-mentioned evidences, we hypothesized that curcumin may promoted expression of METTL3 which subsequently downregulated expression of miR-320 by hypermethylation the promoter region and as a result, the expression of RUNX3, a direct target gene of miR-320, could be upregulated. Furthermore, pretreatment with curcumin will enhance the therapeutic effect of BMMSCs-derived exosomes in the treatment of osteoporosis. To test the hypothesis, we studied the effect of CUR and BMSC-derived exosomes in rat and cellular models of osteoporosis. Expression of METTL3, miR-320 and RUNX2 was investigated in vivo and in vitro to unveil the signaling pathway underlying the therapeutic effect of CUR on osteoporosis.

Materials and Methods

Animal and treatment

C57BL/6 mice with an average age of 8 weeks old were acquired from our animal center. All mice were kept in a specific pathogen-free (SPF) environment in the animal facility of our institution. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee and were conducted in strict compliance with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH). After 7 days of adaptation, the mice were divided into 4 groups, i.e., 1. Sham group (mice undergoing sham...
operation); 2. Osteoporosis group (mice induce of osteoporosis); 3. Osteoporosis + EXO group (mice induced of osteoporosis and then treated with EXO extracted from untreated BMSCs); and 4. Osteoporosis + EXO-CUR group (mice induced of osteoporosis and then treated with EXO extracted from BMSCs treated with CUR).

**Mouse Model of Osteoporosis**

The C57BL/6 mice with an average age of 8 weeks old were used to create the osteoporosis model. In brief, the model mice were subject to bilateral ovariectomy by exposing the bilateral ovaries and removing nearby adipose tissues. The mice in the sham group also underwent the ovariectomy operation without removing tissues. After the operation, all mice in the groups were kept alive for 8 weeks before they were killed to harvest tissue samples for subsequent analyses. The animal in each group were evaluated by microCT.

**Primary Cell Extraction and Culture**

In this study, BMSCs were isolated from femur and tibia bones collected from Sprague Dawley (SD) rats under aseptic conditions. In brief, before the collection of tibia and femur bones, the rats were killed via cervical vertebra dislocation. Then, BMSCs were isolated via rinsing the cavity of bone marrow with a Modified Eagle Medium (MEM, Gibco, Rockville, MD) containing a higher glucose content. Subsequently, the collected BMSCs which cultured in a Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Rockville, MD) added with 10% of fetal bovine serum, 1% penstrep as well as 1% L-glutamine (HyClone, South Logan, UT). The culture conditions were 37 °C, 5% CO₂ and saturated humidity. After the confluence of BMSCs reached 80%, they were trypsinized (Beyotime, Shanghai, China) for sub-culture. BMSCs between 2-5 passages were used for further analyses. In the EXO-CUR group, the BMSCs were incubated with CUR (1 μM) for 24 hours before the isolation of EXO.

**Exosome Extraction**

To remove the residual cells, the cell supernatants of BMSCs were collected and centrifuged at 300 g for 10 min and 2000 g for 15 min at 4°C. To remove the cell debris, a subsequent centrifugation at 12000 g for 30 min at 4°C was conducted. Particles larger than 200 nm was
removed by 0.22 μm filtration. And the cell suspensions were centrifuged at 100000 g for 2 h at 4°C again before the cell supernatants were discarded. For the final re-suspension step, PBS buffer were applied before a preservation at -80°C.

**RNA isolation and real-time PCR**

In this study, real-time PCR was utilized to assay the relative expression of METTL3, miR-320, RUNX2 mRNA, BGLAP mRNA, and TOUR mRNA in each sample. In brief, the collected tissue and cell samples were lysed using a Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the standard protocols provided in the manufacturer’s instruction manual to isolate total RNA, which was then converted to cDNA by using a cDNA RT assay kit (TAKARA, Tokyo, Japan) in accordance with the standard protocols provided in the manufacturer’s instruction manual. In the next step, the cDNA was used as the template for real time PCR reactions, where were done by utilizing a SYBR Premix EX Taq assay kit (TAKARA, Tokyo, Japan) on a PRISM 7900HT real time PCR instrument (ABI, Foster City, CA) in accordance with the standard protocols provided in the manufacturer’s instruction manual. Finally, the relative expression of METTL3, miR-320, RUNX2 mRNA, BGLAP mRNA, and TOUR mRNA in each sample was calculated by utilizing the $2^{-\Delta\Delta Ct}$ method.

**Cell culture and transfection**

BMSCs and MG63 cells were bought from ATCC and cultured in DMEM under conditions suggested by the manufacturer. Then, after the cell confluence reached 80%, the cells were divided into 3 groups, i.e., 1. NC group (cells treated with PBS only); 2. CUR group (cells treated with CUR); and 3. EXO-CUR group (cells treated with CUR-carrying EXO). Then, the cells were treated under corresponding conditions for 48 h before the cells were harvested for target gene analysis. Similarly, BMSCs and MG63 cells were divided into 2 groups, i.e., 1. NC siRNA group (cells treated with a scramble control siRNA); 2. METTL3 siRNA group (cells treated with METTL3 siRNA), and the target gene analysis was done after the cells were transfected with corresponding siRNAs for 48 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the standard protocols provided in the manufacturer’s instruction manual.

**Vector construction, mutagenesis, and luciferase assay**
Our binding site screening found that miR-320 could potentially bind to the 3’ UTR of RUNX2. Therefore, to clarify the regulatory relationship between the expression of miR-320 and RUNX2, luciferase vectors containing wild type and mutant RUNX2 were established: First, the wild type 3’ UTR of RUNX2 containing the miR-320 binding site was amplified and cloned into a pcDNA3.1 vector (Promega, Madison, WI) downstream of the luciferase gene to create the wild type vector for the 3’ UTR of RUNX2. At the same time, mutagenesis was carried out to generate one site-directed mutation in the miR-320 binding site located in the 3’ UTR of RUNX2, and the mutant fragment of 3’ UTR of RUNX2 was also amplified and cloned into another pcDNA3.1 vector to generate the mutant type vector for the 3’ UTR of RUNX2. In the next step, BMSCs and MG63 were co-transfected with mutant type/wild type vectors for the 3’ UTR of RUNX2 together with miR-320 mimics or a control using Lipofectamine 2000, and the luciferase activity of transfected cells was measured at 48 h after the start of co-transfection with a Dual-luciferase assay kit (Promega, Madison, WI) on a luminometer in accordance with the standard protocols provided in the manufacturer’s instruction manual.

**Western blot analysis**

Cell and tissue samples were first lysed using a RIPA lysis system (Beyotime, Shanghai, China) in accordance with the standard protocols provided in the manufacturer’s instruction manual. Then, 20 μg of proteins from each sample were fractionated by using a 10% SDS-PAGE gel, and the fractionated proteins were electro-transferred onto a polyvinylidene fluoride membrane (Millipore, Burlington, MA), which was blocked by using 5% non-fat, washed with TBST, and treated overnight (4°C) with anti-METTL3 and anti-RUNX2 primary antibodies (Abcam, Cambridge, MA) in accordance with the standard conditions provided in the manufacturer’s instruction manual, followed by further incubation against HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at ambient temperature for 2 h. Finally, the protein bands were developed by making use of an enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA) in accordance with the standard protocols provided in the manufacturer’s instruction manual, and the relative expression of METTL3 and RUNX2 proteins in each sample was semi-quantified by utilizing a ChemiDoc XRS+ machine (Bio-Rad laboratories, Hercules, CA) and the ImageJ 1.40 software.
**Immunohistochemistry**

For immunofluorescence assays, the samples were made into 4 \( \mu m \) thick slides and blocked with 5% BSA for 1 h before they were incubated successively with anti-METTL3 primary antibodies, and suitable AlexaFluor 568-conjugated secondary antibodies (all antibodies were obtained from Abcam, Cambridge, MA and used in accordance with the standard conditions shown in the manufacturer’s instruction manual). After counterstaining with DAPI, the positive expression of METTL3 protein in each sample was evaluated under an Axio Observer Z1 microscope coupled with a Zeiss LSM 5 imaging system (Carl Zeiss, Oberkochen, Germany).

**Statistical analysis**

All data analyses were done by making use of GraphPad Prism 6.0 software (GraphPad, San Diego, CA). Two-tailed Student’s t-tests were used for the comparison between two groups. All data were presented as mean ± standard deviation (SD) and each observation was repeated in triplicate. P-value < 0.05 was taken into consideration as statistically significant.

**Results**

**Isolation and characterization of BMSC-derived exosomes.**

BMSCs were cultured for three days and their morphology exhibited a vortex distribution and a spindle like shape under a microscope (Fig.1A). Exosomes were isolated from BMSCs and the shape of exosomes was evaluated using electron microscopy, and the results showed exosomes as round bubbles with a 40 nm diameter (Fig.1B). Furthermore, Western blot was carried out to analyze the expression of CD9, CD63 and CD81 in the supernatant of BMSCs as well as in BMSC-derived exosomes. The expression of CD9, CD63 and CD81 was significantly elevated in BMSC-derived exosomes when compared with that in the supernatant of BMSCs (data not shown).

**CUR treatment remarkably enhanced the therapeutic effect of BMSC-derived exosomes in mice with osteoporosis.**

In order to evaluate the therapeutic effect of CUR upon BMSC-derived exosomes, mice models of osteoporosis was established. MicroCT was used to measure the Bone volume/Tissue volume
ratio (BV/TV), BMD, Trabecular number (Tb.N), Trabecular thickness (Tb.Th) and Trabecular separation (Tb.Sp) of the osteoporosis mice treated with different therapeutic strategies. Accordingly, the BV/TV, BMD, Tb.N and Tb.Th were remarkably decreased in osteoporosis mice, while BMSC-derived exosomes exerted a notable effect on restoring the values of BV/TV, BMD, Tb.N and Tb.Th in osteoporosis mice. Moreover, the exosomes derived from CUR-treated BMSCs more significantly exerted an promoting effect on BV/TV (Fig.2A, Fig.2F), BMD (Fig.2B, Fig.2F), Tb.N (Fig.2C, Fig.2F), and Tb.Th (Fig.2D, Fig.2F) in osteoporosis mice. On the contrary, CUR treatment obviously enhanced the effect of BMSC-derived exosomes on suppressing the abnormally increased Tb.Sp value in osteoporosis mice (Fig.2E, Fig.2F).

**CUR treatment effectively strengthened the capability of BMSC-derived exosomes in maintaining the expression of METTL3, miR-320 and RUNX2 in osteoporosis mice.**

To gain a deep insight into the molecular mechanism underlying the therapeutic effect of CUR and BMSC-derived exosomes, we performed PCR, Western blot and immunohistochemistry to analyze the expression of METTL3, RUNX2 and miR-320 in osteoporosis mice treated under different conditions. Accordingly, the expression of METTL3 mRNA and protein was apparently suppressed in osteoporosis mice. And CUR treatment upon BMSCs obviously promoted the capability of BMSC-derived exosomes in maintaining the the suppressed expression of METTL3 mRNA (Fig.3A) and protein (Fig.3B, Fig.4) in osteoporosis mice. On the contrary, the activated expression of miR-320 in osteoporosis mice was effectively repressed by the exosomes derived from CUR-treated BMSCs (Fig.3C). Besides, the repressed expression of RUNX2 mRNA (Fig.3D) and protein (Fig.3E) in osteoporosis mouse was effectively maintained by the exosomes derived from CUR-treated BMSCs.

**BMSC-derived exosomes remarkably enhanced the effect of CUR on regulating the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs cells.**

Furthermore, we treated BMSCs cells with CUR alone or in combination with BMSC-derived exosomes to evaluate their effect on the expression of METTL3, miR-320, RUNX2, BGLAP and LAP. As shown in Fig.5, BMSC-derived exosomes showed a remarkable potential in enhancing the effect of CUR on increasing the expression of METTL3 mRNA (Fig.5A), METTL3 protein (Fig.5B),
RUNX2 mRNA (Fig.5D), RUNX2 protein (Fig.5E), BGLAP mRNA (Fig.5F) and LAP mRNA (Fig.5G) as well as on decreasing the expression of miR-320 in BMSCs cells (Fig.5C).

**METTL3 siRNA notably repressed the expression of METTL3, RUNX2, BGLAP, and LAP, and activated the expression of miR-320 in BMSCs cells.**

BMSCs cells were treated with METTL3 siRNA and the expression of METTL3, miR-320, RUNX2, BGLAP and LAP was analyzed using real-time PCR and Western blot. The expression of METTL3 mRNA (Fig.6A), METTL3 protein (Fig.6B), RUNX2 mRNA (Fig.6D), RUNX2 protein (Fig.6E), BGLAP mRNA (Fig.6F) and LAP mRNA (Fig.6G) was evidently suppressed by METTL3 siRNA in BMSCs cells. The expression of miR-320 was increased in BMSCs cells treated with METTL3 siRNA (Fig.6C).

**MiR-320 inhibited the expression of RUNX2 through binding to the 3’ UTR of RUNX2.**

Binding site screening found that miR-320 could potentially bind to the 3’ UTR of RUNX2 (Fig.7A). Luciferase vectors containing wild type and mutant RUNX2 were established and transfected into BMSCs and MG63 cells along with miR-320. The luciferase activity of wild type RUNX2 vector was significantly inhibited in BMSCs (Fig.7B) and MG63 (Fig.7C) cells, indicating that miR-320 suppressed the expression of RUNX2. Moreover, we examined the expression of RUNX2 in BMSCs and MG63 cells treated with different concentrations of a miR-320 precursor. The expression of RUNX2 mRNA was apparently suppressed in miR-320-treated BMSCs (Fig.7D) and MG63 (Fig.7E) cells.

In summary, our study demonstrated that BMSC-derived exosomes and CUR jointly enhanced the expression of METTL3, which down-regulated the expression of miR-320, resulting in the up-regulation of RUNX2. As a result, osteogenic differentiation was enhanced to alleviate osteoporosis (Fig.8).

**Discussion**

In this study, we harvested exosomes from bone marrow stem cells and treated osteoporosis mice with the combination of CUR and BMSC-derived exosomes. The therapeutic effect of CUR on osteoporosis was remarkably elevated by BMSC-derived exosomes. In addition, we performed real-time PCR, Western blot and IHC to examine the expression of METTL3, miR-320 and RUNX2.
at both the mRNA and protein levels in osteoporosis mice treated under different conditions. BMSC-derived exosomes strengthened with CUR significantly restored the expression of METTL3, miR-320 and RUNX2 in osteoporosis mice. As a type of phenolic substance separated from Curcuma longa, CUR exhibits certain anti-inflammatory, anti-tumor and anti-mutagenic effects [28, 29]. Studies have suggested that CUR affects both fat accumulation as well as bone health in the body. In addition, CUR may cause the apoptosis of osteoclasts while preventing the growth of osteoclasts by decreasing RANKL expression in BMSCs [29, 30]. It was also found that CUR reduced in vivo osteoporosis induced by DXM [31].

It was revealed that BMSC proliferation on modified surfaces of LC was significantly increased by 0.05 mg/ml of BMP2. Similarly, osteonectin expression was increased by BMP2 on LC as well as UDMA surfaces [32]. Surprisingly, a recent research suggested that the accumulation of m6A in both mouse and human was regulated by mRNAs through their selective binding to METTL3 [13, 33]. Consistently, Vashisht et al. recently suggested that CUR loaded in exosomes is not only resistant to enzymatic digestion, but also shows elevated permeability in the digestive tract [26, 34, 35]. Certain evidence suggested that MSC-derived exosomes as well as exosome-like nanoparticles derived from edible plants can also resist enzymatic digestion [26, 36].

A previous research presented that exosomal MALAT1 in BMSCs might contribute to increased osteogenic activity as well as relieved osteoporosis symptoms through functioning as a sponge of miR-34c to upregulate the expression of SATB2 [37]. Hence, it was found that nearly 75% of super-enhancer RNAs (seRNAs) show the peaks of m6A. In addition, the level of m6A methylation in seRNAs was decreased in Mettl3 KO models [38]. Furthermore, pre-miR-320 methylation in the nucleus results in the down-regulation of miR-320 expression in the cytoplasm, contributing to the osteogenic potential of METTL3 [19]. Additionally, the down-regulation of miR-320 rescued the bone mass loss shown in METTL3 +/- knockout [23]. In this study, we treated BMSCs cells with CUR alone or in the combination with BMSC-derived exosomes. BMSC-derived exosomes significantly enhanced the role of CUR in promoting the expression of METTL3 and RUNX2 mRNA and in suppressing the expression of miR-320 in BMSCs cells.
The bioinformatics studies showed that the 3'-UTR of RUNX2 contains four miR-320 binding sites. The interaction of RUNX2 3'-UTR and miR-320 is also specific, as mutations in the 3'-UTR of RUNX2 blocked the binding between miR-320 and RUNX2 3'-UTR [39]. In this study, we performed luciferase assay to explore the inhibitory role of miR-320 in the expression of RUNX2. The luciferase activity of wild type RUNX2 was significantly repressed by miR-320 in BMSCs and MG63 cells.

RUNX2 belongs to the family of RUNT related transcription factors that are essential in the course of skeletal development during embryogenesis. RUNX2 is also recognized for its oncogenic functions. In fact, numerous studies revealed that the dysregulation of RUNX2 leads to tumor progression as well as tumor invasion [40]. Additionally, RUNX2 was found to be involved in bone development as well as hypertrophic differentiation of chondrocytes [41, 42]. Past studies showed that the adipocytic differentiation of MSCs was inhibited by RUNX2 [43]. It was also shown that the differentiation of MSCs into osteoblasts is the main pathway for bone formation [39]. Runx2 can activate and regulate osteogenesis via many different signaling pathways, such as the TGF-β1, BMP, Wnt, Hedgehog, as well as NELL-1 signaling pathways [26, 27]. The mice carrying the homozygous Runx2 −/− mutations lack differentiated osteoblasts upon birth [44, 45]. While Runx2 does not act as a crucial regulator in the differentiation of adipocytes, its role in enhancing osteogenesis might affect the differentiation of adipocyte lineages [46]. In addition, we treated BMSCs cells with METTL3 siRNA and checked the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs cells. The expression of METTL3, RUNX2, BGLAP and LAP was notably suppressed by METTL3 siRNA, while the expression of miR-320 was apparently enhanced by METTL3 siRNA in BMSCs cells.

However, our study is limited by the lack of clinical validation. Although a conclusion that exosomes derived from CUR-treated BMSCs could promote the therapeutic effect of CUR, future clinical validation should be conducted.

**Conclusion**

In conclusion, our study demonstrated that BMSC-derived exosomes could modulate the signaling pathway of METTL3/miRNA-320/RUNX2 to attenuate osteoporosis by promoting
osteogenic differentiation of BMSCs. Moreover, exosomes derived from CUR-treated BMSCs promoted the therapeutic effect of CUR.

Conflict of interest

None

Author Contributions Statement

Y.H.Z and C.L.Z wrote the main manuscript text and N.G.W and C.L.Z prepared figures 1-7. All authors reviewed the manuscript

Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure legends

Fig.1

Characterization of exosomes isolated from bone marrow stem cells.

A: The primary bone marrow stem cells showed a long spindle shape.

B: Representative images of BMSC-derived exosomes observed under an electron microscope.

Fig.2

CUR treatment strengthened the therapeutic effect of BMSC-derived exosomes on osteoporosis mice (* P-value < 0.05 vs. SHAM group; ** P-value < 0.05 vs. OSTEOPOROSIS group; # P-value < 0.05 vs. OSTEOPOROSIS + CUR group).

A: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Bone volume/Tissue volume ratio in osteoporosis mice.

B: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Bone mineral density in osteoporosis mice.
C: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Trabecular number in osteoporosis mice.

D: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Trabecular thickness in osteoporosis mice.

E: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Trabecular separation in osteoporosis mice.

F: Representative micro-CT images of mice groups.

**Fig.3**

CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3, miR-320 and RUNX2 in osteoporosis mice (* P-value < 0.05 vs. Sham group; ** P-value < 0.05 vs. Osteoporosis group; # P-value < 0.05 vs. Osteoporosis + EXO group).

**Fig.4**

Immunohistochemistry analysis showed that CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3 protein in osteoporosis mice.

**Fig.5**

A: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3 mRNA in osteoporosis mice.

B: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3 protein in osteoporosis mice.

C: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of miR-320 in osteoporosis mice.

D: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of RUNX2 mRNA in osteoporosis mice.

E: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of RUNX2 protein in osteoporosis mice.
Exosomes derived from CUR-treated BMSCs strengthened the ability of CUR in altering the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs cells (* P-value < 0.05 vs. NC group; ** P-value < 0.05 vs. CUR group).

A: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of METTL3 mRNA in BMSCs cells.

B: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of METTL3 protein in BMSCs cells.

C: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in suppressing the expression of miR-320 in BMSCs cells.

D: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of RUNX2 mRNA in BMSCs cells.

E: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of RUNX2 protein in BMSCs cells.

F: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of BGLAP mRNA in BMSCs cells.

G: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of LAP mRNA in BMSCs cells.

Fig.6

METTL3 siRNA transfection altered the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs cells (* P-value < 0.05 vs. NC siRNA group).

A: The expression of METTL3 mRNA was suppressed by METTL3 siRNA in BMSCs cells.

B: The expression of METTL3 protein was suppressed by METTL3 siRNA in BMSCs cells.

C: The expression of miR-320 was increased by METTL3 siRNA in BMSCs cells.

D: The expression of RUNX2 mRNA was suppressed by METTL3 siRNA in BMSCs cells.
E: The expression of RUNX2 protein was suppressed by METTL3 siRNA in BMSCs cells.

F: The expression of BGLAP mRNA was suppressed by METTL3 siRNA in BMSCs cells.

G: The expression of LAP mRNA was suppressed by METTL3 siRNA in BMSCs cells.

**Fig.7**

MiR-320 suppressed the expression of RUNX2 through binding to its 3’ UTR.

A: Sequence analysis indicated that miR-320 could potentially bind to the 3’ UTR of RUNX2.

B: The luciferase activity of wild type RUNX2 was remarkably suppressed by miR-320 in BMSCs cells (* P-value < 0.05 vs. control group).

C: The luciferase activity of wild type RUNX2 was remarkably suppressed by miR-320 in MG63 cells (* P-value < 0.05 vs. control group).

D: The expression of RUNX2 mRNA was repressed by miR-320 precursors in a dose dependent manner in BMSCs cells (* P-value < 0.05 vs. scramble group).

E: The expression of RUNX2 mRNA was repressed by miR-320 precursors in a dose dependent manner in MG63 cells (* P-value < 0.05 vs. scramble group).

**Fig.8**

Schematic illustration of the study.

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Characterization of exosomes isolated from bone marrow stem cells.
A: The primary bone marrow stem cells showed a long spindle shape.
B: Representative images of BMSC-derived exosomes observed under an electron microscope.
CUR treatment strengthened the therapeutic effect of BMSC-derived exosomes on osteoporosis mice (* P-value < 0.05 vs. SHAM group; ** P-value < 0.05 vs. OSTEOPOROSIS group; # P-value < 0.05 vs. OSTEOPOROSIS + CUR group).

A: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Bone volume/Tissue volume ratio in osteoporosis mice.
B: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Bone mineral density in osteoporosis mice.
C: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Trabecular number in osteoporosis mice.
D: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Trabecular thickness in osteoporosis mice.
E: CUR treatment enhanced the capability of BMSC-derived exosomes in restoring the Trabecular separation in osteoporosis mice.
F: Representative micro-CT images of mice groups.
CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3, miR-320 and RUNX2 in osteoporosis mice (* P-value < 0.05 vs. Sham group; ** P-value < 0.05 vs. Osteoporosis group; # P-value < 0.05 vs. Osteoporosis + EXO group).

A: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3 mRNA in osteoporosis mice.

B: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3 protein in osteoporosis mice.

C: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of miR-320 in osteoporosis mice.

D: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of RUNX2 mRNA in osteoporosis mice.

E: CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of RUNX2 protein in osteoporosis mice.
Immunohistochemistry analysis showed that CUR treatment enhanced the capability of BMSC-derived exosomes in maintaining the expression of METTL3 protein in osteoporosis mice.
Exosomes derived from CUR-treated BMSCs strengthened the ability of CUR in altering the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs cells (* P-value < 0.05 vs. NC group; ** P-value < 0.05 vs. CUR group).

A: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of METTL3 mRNA in BMSCs cells.

B: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of METTL3 protein in BMSCs cells.

C: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in suppressing the expression of miR-320 in BMSCs cells.

D: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of RUNX2 mRNA in BMSCs cells.

E: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of RUNX2 protein in BMSCs cells.

F: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of BGLAP mRNA in BMSCs cells.

G: Exosomes derived from CUR-treated BMSCs strengthened the effect of CUR in promoting the expression of LAP mRNA in BMSCs cells.
METTL3 siRNA transfection altered the expression of METTL3, miR-320, RUNX2, BGLAP and LAP in BMSCs cells (* P-value < 0.05 vs. NC siRNA group).

A: The expression of METTL3 mRNA was suppressed by METTL3 siRNA in BMSCs cells.
B: The expression of METTL3 protein was suppressed by METTL3 siRNA in BMSCs cells.
C: The expression of miR-320 was increased by METTL3 siRNA in BMSCs cells.
D: The expression of RUNX2 mRNA was suppressed by METTL3 siRNA in BMSCs cells.
E: The expression of RUNX2 protein was suppressed by METTL3 siRNA in BMSCs cells.
F: The expression of BGLAP mRNA was suppressed by METTL3 siRNA in BMSCs cells.
G: The expression of LAP mRNA was suppressed by METTL3 siRNA in BMSCs cells.
MiR-320 suppressed the expression of RUNX2 through binding to its 3’ UTR.
A: Sequence analysis indicated that miR-320 could potentially bind to the 3’ UTR of RUNX2.
B: The luciferase activity of wild type RUNX2 was remarkably suppressed by miR-320 in BMSCs cells (* P-value < 0.05 vs. control group).
C: The luciferase activity of wild type RUNX2 was remarkably suppressed by miR-320 in MG63 cells (* P-value < 0.05 vs. control group).
D: The expression of RUNX2 mRNA was repressed by miR-320 precursors in a dose dependent manner in BMSCs cells (* P-value < 0.05 vs. scramble group).
E: The expression of RUNX2 mRNA was repressed by miR-320 precursors in a dose dependent manner in MG63 cells (* P-value < 0.05 vs. scramble group).
Schematic illustration of the study.

Curcumin+exo

(+)

METTL3

miR-320

RUNX3

Osteogenic differentiation

Osteopocosis

Schematic illustration of the study.