European Review for Medical and Pharmacological Sciences 2018; 22: 7156-7163

MOTS-c improves osteoporosis by promoting osteogenic differentiation of bone marrow mesenchymal stem cells via TGF-β/Smad pathway

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Abstract. – OBJECTIVE: To explore whether MOTS-c could improve osteoporosis by promoting osteogenic differentiation of rat bone mesenchymal stem cells (BMSCs) via transforming growth factor-β (TGF-β)/Smad pathway.

MATERIALS AND METHODS: Rat BMSCs were isolated and cultured, followed by osteogenic and lipid differentiation. CCK-8 (cell counting kit-8) assay was performed to detect the highest treatment dose of MOTS-c that did not affect cell proliferation. Expressions of osteogenesis-related genes (ALP, Bglap, and Runx2) were detected by qRT-PCR (quantitative Real-Time Polymerase Chain Reaction) and Western blot, respectively. Alizarin red staining and alkaline phosphatase (ALP) cytochemical staining were carried out to evaluate the effect of MOTS-c on BMSCs osteogenesis. TGF-β/Smad pathway-related genes (TGF-β1, TGF-β2, and Smad7) in BMSCs treated with MOTS-c were detected. Finally, TGF-β1 was knocked down to investigate the regulatory effect of MOTS-c on BMSCs osteogenesis.

RESULTS: BMSCs exhibited an elongated morphology and was identified with a high purity by flow cytometry. After osteogenic differentiation, alizarin red staining and ALP staining were all positive. MOTS-c treatment could remarkably stimulate the formation of calcified nodules in BMSCs. Besides, TGF-β/Smad pathway-related genes were significantly upregulated after BMSCs were treated with MOTS-c. Promoted osteogenesis by MOTS-c treatment was reversed by the TGF-β1 knockdown.

CONCLUSIONS: MOTS-c promotes cell differentiation of BMSCs to osteoblasts via TGF-β/Smad pathway.

Key Words:
MOTS-c, Bone marrow mesenchymal stem cell, Osteogenesis, TGF-β/Smad pathway, Osteoporosis.

Introduction

Osteoporosis is a systemic bone disease characterized by low bone mass, destruction of bone microstructure, increased bone fragility, and high fracture risk. Osteoporosis, as a degenerative geriatric disease, gradually damages the bone quality of the elderly. Therefore, osteoporosis has become a global health problem that endangers life quality of affected population[1].

Bone mesenchymal stem cells (BMSCs) are stem cells expressed in the bone marrow stroma[2,3]. In recent years, osteogenic differentiation of BMSCs has been well recognized due to the increased incidence of osteoporosis. The application prospect of BMSCs is very broad. Researches[4,5] have shown that osteogenic differentiation of BMSCs is a series of well-ordered and precisely regulated process. Multiple signaling pathways have been confirmed to participate in the osteogenic differentiation of BMSC, among which, transforming growth factor-β (TGF-β)/Smad exerts a major role[6].

Mitochondrial open reading frame of the 12S rRNA-c (MOTS-c) is a newly identified polypeptide, which is encoded by the open reading frame of mitochondrial 12S rRNA. Scholars[7,8] have shown that MOTS-c can prevent age-dependent insulin resistance induced by high-fat diet, as well as diet-induced obesity activated by AMPK. Previous investigations[9,10] have demonstrated that AMPK can stimulate the proliferation, differentiation and mineralization of osteoblasts, which exerts an important role in the cellular functions of osteoblasts. The role of MOTS-c in osteoblast formation, however, has not been reported yet.

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This work aims to explore whether MOTS-c could improve osteoporosis by promoting osteogenic differentiation of rat BMSCs via TGF-β/Smad pathway.

Materials and Methods

Isolation and Culture of BMSCs

4-week old Sprague Dawley rats weighing 80-100 g (Jilin University, Changchun, China) were executed with dislocation of the cervical vertebra. The femur and tibia were collected under aseptic condition. The marrow cavity was washed with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA). After centrifugation at 1000 r/min for 5 min, BMSCs were re-suspended in DMEM containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C. BMSCs were then seeded in 6-well plates at a density of 1×10^6/L, followed by cell passage with 0.25% trypsin when the confluence was up to 80-90%. This study was approved by the Animal Ethics Committee of Jilin University Animal Center.

Osteogenic differentiation was induced by DMEM containing 10 mmol/L sodium β-glycerophosphate and 50 μg/mL Vitamin C.

Lipid differentiation was induced by DMEM containing 0.5 mmol/L LIBMX, 5 mg/L insulin and 100 mg/L indomethacin.

BMSCs Identification

Third-passage BMSCs were digested with trypsin, followed by centrifugation at 1000 rpm for 3 min. The supernatant was discarded, and the cells were washed 2-3 times with phosphate-buffered saline (PBS). CD44 and CD45 antibodies were diluted with PBS and added to the cells. After incubation for 30 min, the cell suspension was centrifuged at 1000 rpm for 3 min, and the supernatant was discarded. Subsequently, the cell suspension was transferred into a special detection tube, followed by the detection of cell surface antigen using flow cytometry.

RNA Extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

The TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA). After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes. Primers used in this study were as the follows: ALP, F: 5'-AAGGCTTCTTCTTGCTGGTG-3', R: 5'- GCCTTACCCTCATGATGTCC-3'; Bglap, F: 5'-AGCAAAGGTGCAGCCTTTGT-3', R: 5'-GCGCCTGGTCTCTTCACT-3'; Runx2, F: 5'-ACTTCCTGTGCTCCGTGCTG-3', R: 5'-TCGTTGAACCTGGCTACTTGG-3'; PPARG, F: 5'-CAAACTGCCTCTCTGGGAAG-3', R: 5'-TCCTGGTTTCACCTCTAGCC-3'; CEBPA, F: 5'-AAGCCAAGAAGTCGGTGGACAAGA-3', R: 5'-GGTCATTGTCACTGGTCAACTCCA-3'; KLF5, F: 5'-ACCAGACGGCAGTAATGGAC-3', R: 5'-GACTTGGCATGGTGTACGTG-3'; GAPDH, F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'.

Cell Transfection

Third-passage BMSCs in good growth condition were selected for cell transfection according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 6 h later. The interference sequences were as the follows: TGF-β1 siRNA, 5'-GCTAGCATGGAGGCGGCGG-3'; Negative control, 5'-TTCTCCGAACGTGTCACGT-3'.

Cell Counting Kit-8 (CCK-8) Assay

BMSCs treated with different doses of MOTS-c were seeded in the 96-well plates. After culturing for 24 h, serum-free DMEM was replaced. 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added into each well. Absorbance values at the wavelength of 450 nm were detected by the microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated for 5 times.

Alizarin Red Staining

BMSCs were treated with or without MOTS-c for osteogenic differentiation, respectively. After 14 days, cells were washed with PBS twice, fixed with 4% paraformaldehyde for 15 min, and stained with 1% alizarin red staining for 5 min. Calcified nodules were observed and captured using an inverted microscope.

Oil Red Staining

After lipid differentiation of BMSCs for 14 days, cells were washed with PBS twice, fixed with 4% paraformaldehyde for 15 min and stained with oil red solution. Lipid particles were observed and captured using an inverted microscope.

ALP Cytochemical Staining

BMSCs were treated with or without MOTS-c for osteogenic differentiation, respectively. After 14 days, ALP cytochemical staining was performed according to the instructions of ALP assay kit (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, incubation solution was added on the slides in the 6-well plate and incubated for 15 min at 37°C. ALP cytochemical staining was performed for 5 min, followed by washing with running water. Images were observed and captured using an inverted microscope.

Western Blot

The total protein of BMSCs was extracted by the RIPA (radioimmunoprecipitation assay) lysate (Yeasen, Shanghai, China). The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by a SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) under denaturing conditions and then transferred to PVDF (polyvinylidene difluoride) membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. After washing 3 times with TBST (Tris-buffered saline-Tween; Yeasen, Shanghai, China), membranes were incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 16.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The t-test was used for comparing differences between the two groups. $p<0.05$ was considered statistically significant ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

Results

Phenotype and Multi-Lineage Differentiation of BMSCs

We observed an elongated morphology of BMSCs on the first day of cell culture (Figure 1A). Third-passage BMSCs were selected to identify the surface antigens by flow cytometry. The data showed positive-antigen CD44 (99.42%) and negative-antigen CD45 (0.02%), which were consistent with the phenotype characteristics of BMSCs (Figure 1B). Calcified nodules are the significant hallmark of osteoblast maturation. Our experiment found that calcified modules were formed in BMSCs after osteogenic differentiation for 14 days (Figure 1C), indicating the osteogenic capacity of BMSCs. Expression levels of osteogenesis-related genes, including ALP, Runx2, and Bglap at different time points of osteogenic differentiation were detected. The results demonstrated that mRNA levels of ALP, Runx2, and Bglap were increased in a time-dependent manner (Figure 1D). Furthermore, lipid differentiation of BMSCs was found. Multiple lipid particles were observed in oil red staining after lipid differentiation of BMSCs for 14 days (Figure 1E). Lipid differentiation-related genes, including PPARG, CEBPA, and KLF5, were upregulated in a time-dependent manner (Figure 1F).

MOTS-c Promoted Osteogenic Differentiation of BMSCs

CCK-8 assay demonstrated that 1.0 μM of MOTS-c was the highest dose that did not affect cell proliferation (Figure 2A), which was selected for the following experiments. After osteogenic differentiation of BMSCs treated with 1.0 μM MOTS-c for 7 days, protein expressions of ALP, Bglap, and Runx2 were remarkably increased than those of negative controls (Figure 2B). The mRNA levels of ALP, Bglap, and Runx2 were also increased (Figure 2C-2E). ALP cytochemical staining illustrated stained cytoplasm of BMSCs after MOTS-c treatment and large particles, indicating increased ALP activity (Figure 2F). A great number of calcified nodules was observed compared with that of negative controls (Figure 2G). These data demonstrated that MOTS-c could remarkably promote osteogenic differentiation of BMSCs.

MOTS-c Increased Expressions of TGF-β/Smad Pathway-Related Genes in the Osteogenic Differentiation of BMSCs

It is reported that TGF-β/Smad pathway is the vital pathway in the differentiation process of osteoblasts. In the present study, mRNA levels of TGF-β1, TGF-β2, and Smad7 in BMSCs treated with 1.0 μM MOTS-c for 7 days were remar-

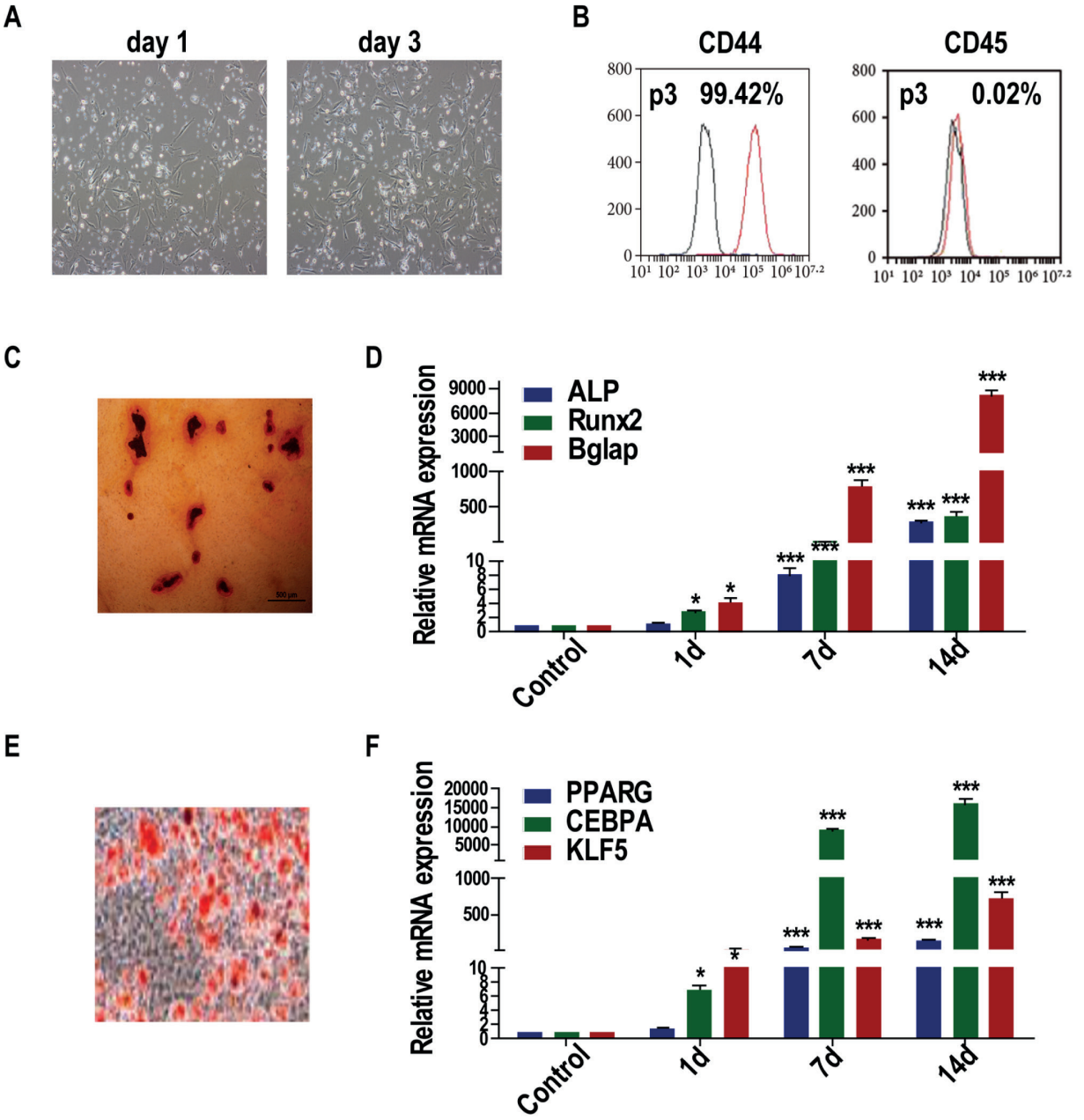

Figure 1. Phenotype and multi-lineage differentiation of BMSCs. ***A,*** Primary BMSCs exhibited elongated morphology after cell culture for 1 and 3 days, respectively. ***B,*** Surface antigens of BMSCs were identified by flow cytometry, including positive-antigen CD44 and negative-antigen CD45. ***C,*** Calcified nodules were formed after osteogenic induction for 14 days. ***D,*** The mRNA levels of osteogenesis-related genes were detected by qRT-PCR after osteogenic induction at different time points. ***E,*** Lipid particles were observed after lipid induction of BMSCs for 14 days. ***F,*** The mRNA levels of lipid induction-related genes were detected by qRT-PCR after lipid induction at different time points.

kably increased compared with those of controls (Figure 3A-3C). Western blot also showed the increased protein expressions of TGF-β1, TGF-β2, and Smad7 (Figure 3D). Therefore, MOTS-c was confirmed to promote osteogenic differentiation of BMSCs via TGF-β/Smad pathway.

MOTS-c Promoted Osteogenic Differentiation of BMSCs via TGF-β/Smad Pathway

BMSCs were further assigned into three groups, namely control group, MOTS-c treatment group, and MOTS-c treatment with TGF-β1

knockdown group. Expression levels of osteogenesis-related genes were detected after osteogenic differentiation for 7 days. The mRNA levels of ALP, Bglap, and Runx2 in MOTS-c treatment group were higher than those of control group, which were partially reversed by TGF-β1 knockdown (Figure 4A-4C). Similar findings were obtained after detecting protein levels of ALP, Bglap, and Runx2 in three groups (Figure 4D), suggesting that TGF-β/Smad pathway is activated in the osteogenic differentiation of BMSCs.

Discussion

Osteoporosis is a multi-factor and age-related metabolic disease[11,12]. The exact mechanism of its occurrence and progression, however, has not

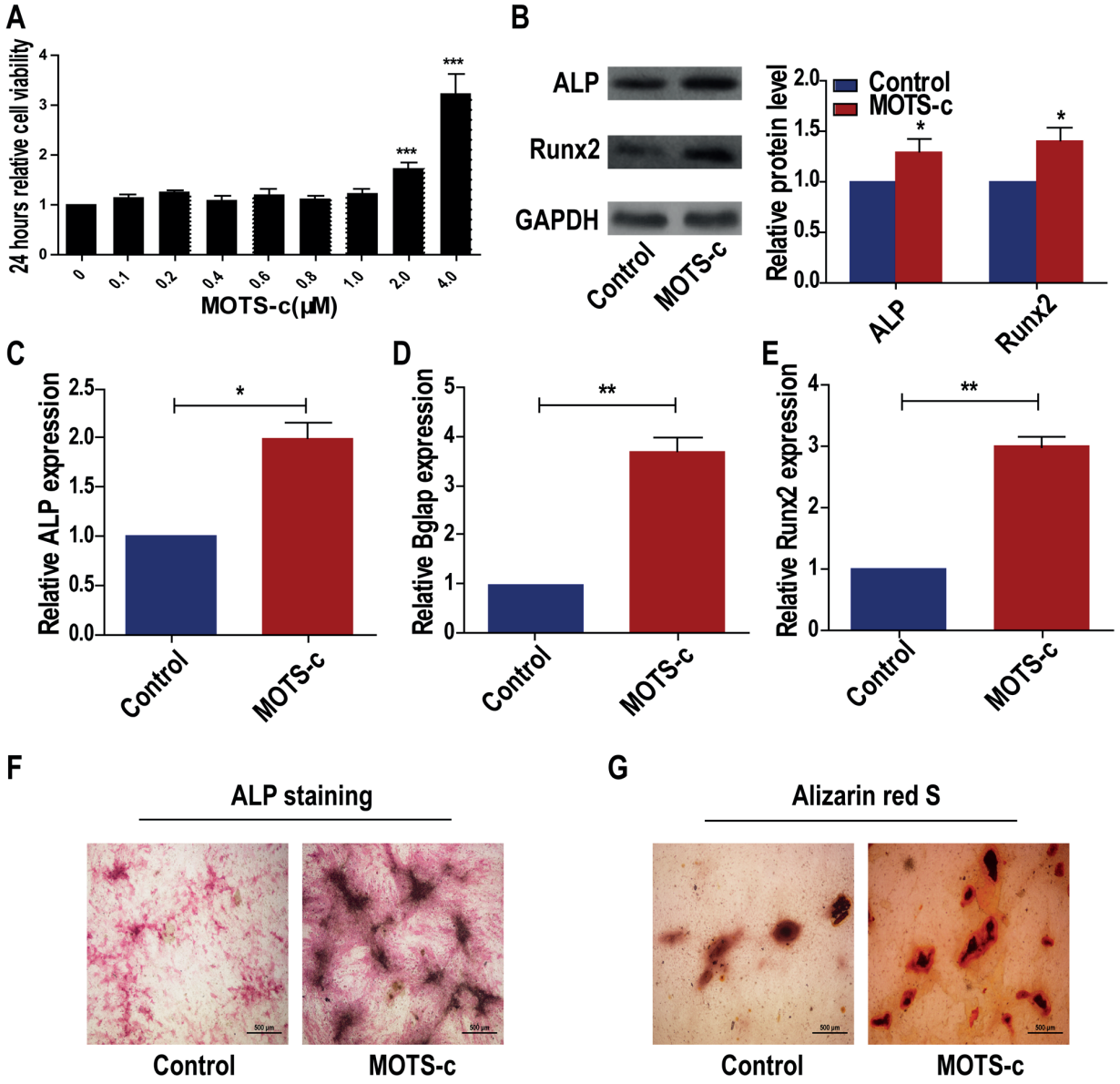

Figure 2. MOTS-c promoted osteogenic differentiation of BMSCs. ***A,*** Cell viability was detected by CCK-8 after BMSCs were treated with different doses of MOTS-c. ***B,*** Protein expressions of ALP and Runx2 in BMSCs treated with MOTS-c after osteogenic differentiation for 7 days. ***C-E,*** Expression levels of ALP (***C***), Bglap (***D***) and Runx2 (***E***) in BMSCs treated with MOTS-c after osteogenic differentiation for 7 days. ***F,*** Oil red staining of ALP in BMSCs treated with MOTS-c after osteogenic differentiation for 14 days. ***G,*** Calcified nodules in BMSCs treated with MOTS-c after osteogenic differentiation for 14 days.

been fully elucidated. BMSCs, as the progenitor cells of osteoblasts and adipocytes, exert an essential role in the physiological and pathophysiological process of bone formation. In this work, BMSCs were derived from the bone marrow of rat femur. Specific morphology of BMSCs was observed and surface antigens of BMSCs were upregulated after *in vitro* culture, indicating the high purity of isolated BMSCs. Under different inductions, two kinds of mesenchymal cells (osteoblasts and adipocytes) were differentiated, further indicating that the adherent cells used in this experiment were BMSCs.

It has been reported that MOTS-c can prevent ovariectomy-induced osteoporosis in mice via AMPK pathway[8]. However, the role of MOTS-c in regulating osteogenic differentiation of BMSCs remains unclear. In this study, BMSCs were treated with different doses of MOTS-c and 1.0 μM was selected as the optimal treatment concentration. Reports have confirmed that ALP, Bglap, and Runx2 are important genes for osteogenic differentiation. Our results found that MOTS-c can upregulate expression levels of ALP, Bglap, and Runx2. Moreover, MOTS-c could promote the formation of mineralized nodules, indicating the osteogenic capacity of BMSCs regulated by MOTS-c.

TGF-β/Smad pathway exerts an important role in the balance of bone formation and resorption[13]. TGF-β1 is one of the members of TGF-β family. It not only promotes the autocrine or paracrine secretion of immune cells, but also stimulates the differentiation of BMSCs into osteoblasts. The specific role of TGF-β1 in bone remodeling has been well recognized[14,15]. This investigation indicated that MOTS-c upregulates TGF-β/Smad pathway-rela-

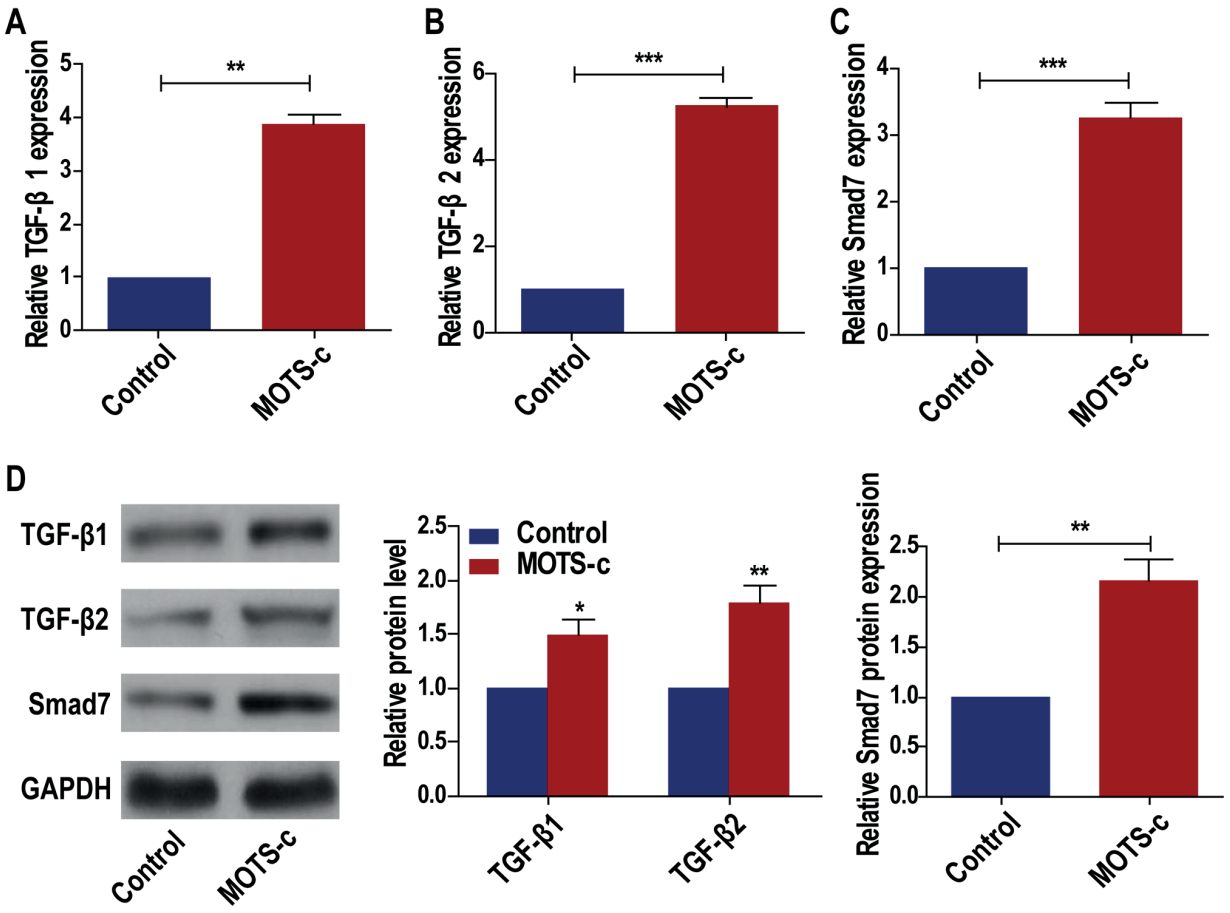

Figure 3. MOTS-c increased expressions of TGF-β/Smad pathway-related genes in the osteogenic differentiation of BMSCs. ***A-C,*** The mRNA levels of TGF-β1 (***A***), TGF-β2 (***B***) and Smad7 (***C***) in BMSCs treated with MOTS-c after osteogenic differentiation for 7 days. ***D,*** The protein levels of TGF-β1, TGF-β2 and Smad7 in BMSCs treated with MOTS-c after osteogenic differentiation for 7 days.

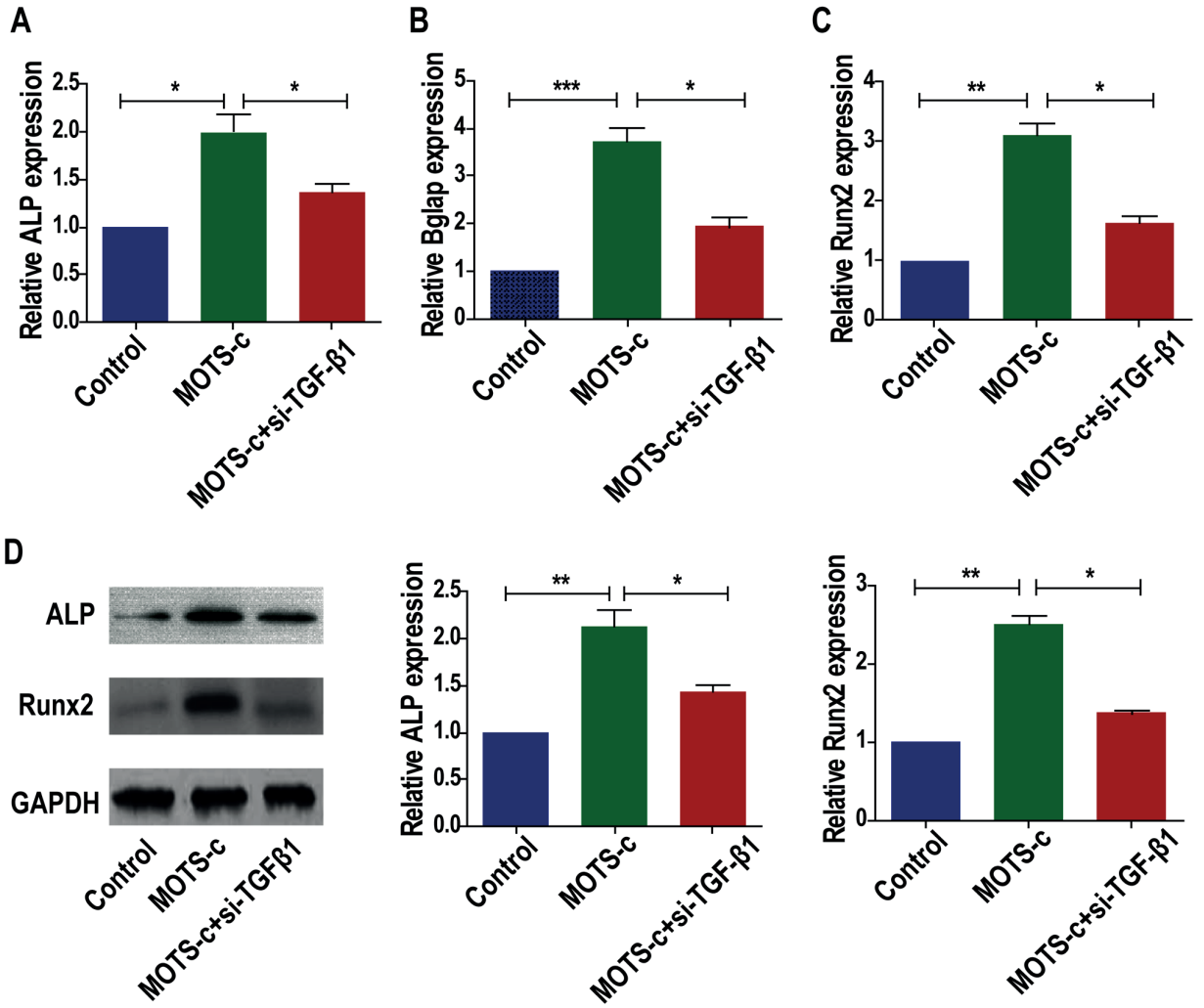

Figure 4. MOTS-c promoted osteogenic differentiation of BMSCs via TGF-β/Smad pathway. ***A-C,*** The mRNA levels of ALP (***A***), Bglap (***B***) and Runx2 (***C***) in control group, MOTS-c treatment group and MOTS-c treatment with TGF-β1 knockdown group. ***D,*** Protein levels of ALP and Runx2 in control group, MOTS-c treatment group and MOTS-c treatment with TGF-β1 knockdown group.

ted genes during the osteogenic differentiation of BMSCs. To further prove that MOTS-c mediates osteogenic differentiation of BMSCs through TGF-β/Smad pathway, small interference sequence of TGF-β1 was constructed. Knockdown of TGF-β1 remarkably inhibited osteogenic differentiation of BMSCs by MOTS-c treatment, suggesting that MOTS-c promotes cell differentiation of BMSCs to osteoblasts via TGF-β/Smad pathway.

Conclusions

We found that MOTS-c promoted cell differentiation of BMSCs to osteoblasts via TGF-β/Smad pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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