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MOTS-c improves osteoporosis by promoting the synthesis of type I collagen in osteoblasts via TGF-β/SMAD signaling pathway

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Abstract. – OBJECTIVE: To investigate whether MOTS-c can regulate the synthesis of type I collagen in osteoblasts by regulating TGF-β/SMAD pathway, thereby improving osteoporosis.

MATERIALS AND METHODS: Viability of hFOB1.19 cells treated with MOTS-c was detected by CCK-8 assay. The mRNA and protein levels of TGF-β, SMAD7, COL1A1 and COL1A2 in hFOB1.19 cells were detected by quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blot, respectively. We then changed expressions of TGF-β and SMAD7 by plasmids transfection to detect levels of COL1A1 and COL1A2 in hFOB1.19 cells by qRT-PCR and Western blot, respectively.

RESULTS: Cell viability was significantly increased after treatment of 1.0 μM MOTS-c for 24 h or 0.5 μM MOTS-c for 48 h in a time-dependent manner. The mRNA and protein expressions of TGF-β, SMAD7, COL1A1 and COL1A2 in hFOB1.19 cells were dependent on the concentration of MOTS-c. In addition, MOTS-c increased the expressions of COL1A1 and COL1A2, which were partially reversed by knockdown of TGF-β or SMAD7.

CONCLUSIONS: MOTS-c could promote osteoblasts to synthesize type I collagen via TGF-β/SMAD pathway.

Key Words:
MOTS-c, TGF-β/SMAD pathway, Osteoblasts, Type I collagen, Osteoporosis.

Introduction

Osteoporosis is a kind of systemic skeletal disease that is characterized by bone mass decrease and bone structural damage, which results in increased brittleness of bone and fracture risk. It is a complex disease caused by multi-system, multi-link, multi-level and multi-factor interaction[1,2]. According to a survey conducted in the United States, osteoporosis is presented in 13% to 18% of people over 50 years old, and bone mass loss is presented in 37% to 50% of the population[3]. Therefore, it is necessary to conduct in-depth research on osteoporosis. There is a coupled dynamic balance between bone formation and bone resorption, which is the basis for maintaining the stability of the bone morphology. Bone homeostasis would be destroyed once the dynamic balance of bone formation and resorption coupling is broken[4]. The imbalance between the formation and absorption of bone inorganic minerals and organic matrices, especially Type I collagen, is particularly critical in the above process. Type I collagen is made of two α1 chains and one α2 chain, which accounts for about 80-90% of the bone organic matter. Functionally, type I collagen is the main component of the extracellular matrix of bone[5]. Various causes may be responsible for osteoporosis, such as the reduced synthesis and reluctant decomposition of type I collagen, alteration in morphology and stability of type I collagen, and decreased deposition of calcium, phosphorus, and other minerals in collagen scaffolds. Effects of different cytokines are varied during the synthesis of type I collagen procollagen by osteoblasts, of which the transforming growth factor TGF-β is considered to be the most direct cytokine affecting the synthesis and metabolism of type I collagen[6]. TGF-β is widely distributed in cells and exerts crucial role in chemotactic cytokines, stimulation of cell proliferation, differentiation, migration, and inhibition of immune surveillance[7]. The SMAD protein family is classical transduction molecules in cytoplasmic TGF-β pathway[8]. It is reported that abnormal transduction of TGF-β/SMAD pathway inhibits

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the bone formation and accelerates the bone destruction, thus leading to pathological bone metabolism[9]. MOTS-c is a polypeptide with 16 amino acids encoded by the open reading frame of mitochondrial 12S r RNA[10]. Lee et al[10] reported that MOTS-c significantly improves obesity and insulin resistance induced by age and diet, as well as fatty liver caused by high-fat diet. MOTS-c treatment has presented effective improvements in the bone density, bone tissue volume ratio, number of trabecular bone and trabecular thickness[11]. Therefore, MOTS-c may become a very promising drug for the treatment of osteoporosis. AMPK (serine/threonine kinase) is also proved to participate in bone metabolism. Activated AMPK promotes osteoblast differentiation and inhibits osteoclast differentiation[12]. At present, few studies have reported the effect of TGF-β/SMAD signaling pathway on stimulating osteoblasts to secrete type I collagen. This study aims to investigate the role of MOTS-c in the synthesis of type I collagen in osteoblasts, which provides new suggestions for discovering the pathogenesis and treatment of osteoporosis.

Materials and Methods

Cell Culture

The hFOB1.19 cell line (ATCC, Manassas, VA, USA) was cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA) and 100 U/ml penicillin and 100 μg/mL streptomycin (Beyotime, Shanghai, China) in a 5% CO_2 incubator at 37°C. Cells with good growth and proliferation condition were selected for further experiments.

Cell Counting Kit-8 (CCK-8) Assay

Cells in logarithmic growth phase were digested with trypsin (Beyotime, Shanghai, China) and inoculated in 96-well plates at a density of 3×10^3 cells/well. The medium was replaced with fresh Roswell Park Memorial Institute-1640 (RPMI-1640) containing different concentrations of MOTS-c (Sigma-Aldrich, St. Louis, MO, USA) 24 h later. After culturing for 24 h and 48 h, respectively, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) were added to each well and the optical density (OD) was measured at the wavelength of 450 nm.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

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, including TGF-β, SMAD7, COL1A1 and COL1A2. The primer sequences were: TGF-β (F: 5'-CACCATCCATGACATGAACC-3', R: 5'-TCATGTTGGACAACTGCTCC-3'), SMAD7 (F: 5'-CGAGTCCTTTCCTCTCTC-3', R: 5'-GGCTCAATGAGCATGCTCAC-3'), COL1A1 (F: 5'-ACCAGCATCACCCTTAGCAC-3', R: 5'-CTGGTCCTGATGGCAAAACT-3'), COL1A2 (F: 5'-GGCTGCTCCAAAAAGACAAATGA-3', R: 5'-AGACAGGGCCAACCTCCACA-3').

Western Blot

The total protein extracted from the treated hFOB1.19 cells was detected for its concentration by the bicinchoninic acid (BCA, Abcam, Cambridge, MA, USA) method. Protein sample was then separated by electrophoresis at 100 V for 1 h. Subsequently, the protein sample in the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Merck, Millipore, Billerica, MA, USA) was transferred to the polyvinylidene difluoride (PVDF) membrane (Merck, Millipore, Billerica, MA, USA) at 80 V for 2 h. These protein-loaded membranes were blocked with 5% skim milk at 37°C for 1 h and then incubated with primary antibodies (Abcam, Cambridge, MA, USA) overnight at 4°C. After being washed with tris-buffered saline-tween (TBST, Yeasen, Shanghai, China) for at least three times, the membranes were incubated with specific secondary antibody (Beyotime, Shanghai, China) at room temperature for 2 h. The protein blot on the membrane was exposed by the electrochemiluminescence (ECL, Thermo Fisher Scientific, Waltham, MA, USA) method.

Cell Transfection

Cells in logarithmic growth phase were digested and centrifuged 24 h prior to transfection for preparing single cell suspension. The density of hFOB1.19 cells was adjusted to 3×10^4/mL with the complete medium. For cell transfection, hFOB1.19 cells were transfected with MOTS-c+TGF-β inhibitor or MOTS-c+SMAD7 inhibitor (Invitrogen, Carlsbad, CA, USA) according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) were used to perform statistical analysis. Measurement data were expressed as mean±standard deviation ($\bar{x}$±s). Differences between two groups were compared using independent sample *t*-test analysis. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

MOTS-c Promoted the Proliferation of hFOB1.19 Cells

Viability of hFOB1.19 cells treated with 0.5 μM or 1.0 μM MOTS-c was detected by CCK-8 assay. The results showed that the cell viability of those treated with MOTS-c was remarkably higher than that of the negative controls (Figure 1A). In addition, the viability of hFOB1.19 cells treated with 1.0 μM MOTS-c showed a time-dependent elevation (Figure 1B). These results indicated that MOTS-c could affect the proliferation of hFOB1.19 cells.

MOTS-c Increased the Transcription of TGF-β, SMAD7, COL1A1 and COL1A2 in hFOB1.19 Cells

We first detected mRNA levels of TGF-β, SMAD7, COL1A1 and COL1A2 in hFOB1.19 cells that were treated with different concentrations of MOTS-c. The results showed that the mRNA expressions of these genes were increased in a dose-dependent manner (Figure 2 A). After hFOB1.19 cells were treated with 1.0 μM MOTS-c for 1 and 3 days, TGF-β, SMAD7, COL1A1, and COL1A2 genes were significantly up-regulated compared to those of the control group (Figure 2B). These results suggested that MOTS-c promotes the expressions of TGF-β, SMAD7, COL1A1 and COL1A2, contributing to the synthesis of type I collagen-related genes in osteoblasts.

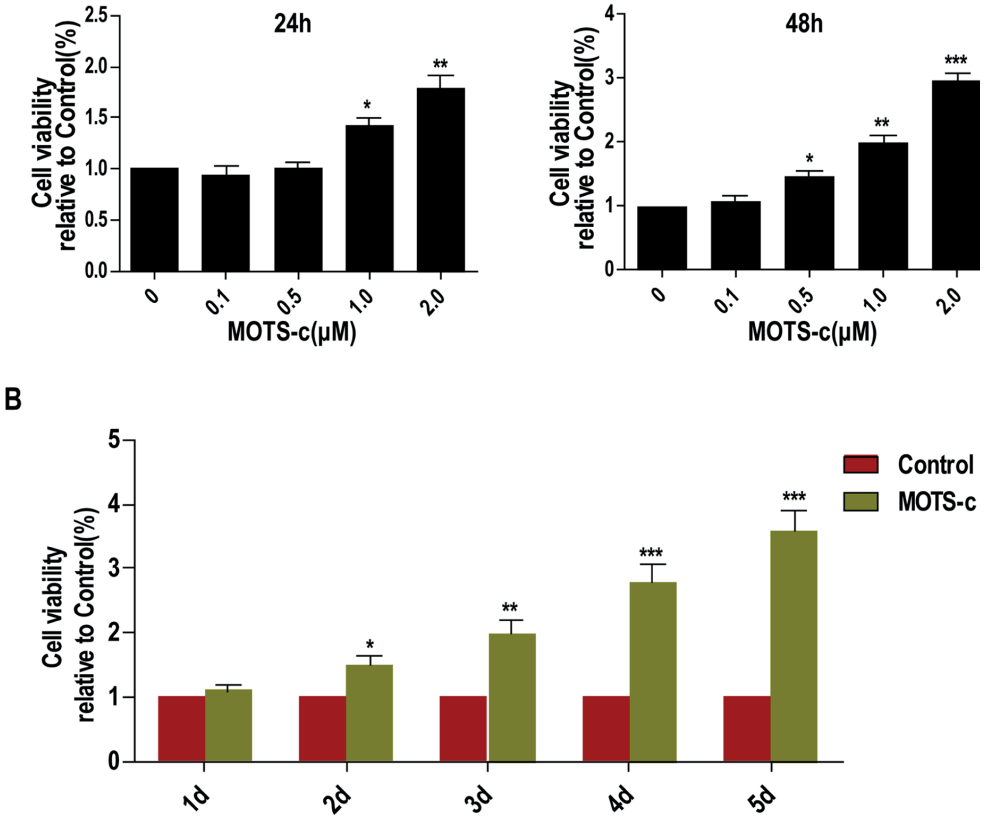

Figure 1. MOTS-c promoted the proliferation of hFOB1.19 cells. ***A,*** The hFOB1.19 cells were treated with different concentrations of MOTS-c and then cultured for 24 h and 48 h, respectively. Viability of treated cells was measured by CCK-8 assay. ***B,*** Cell viability was gradually increased after treatment with 1.0 μM MOTS-c.

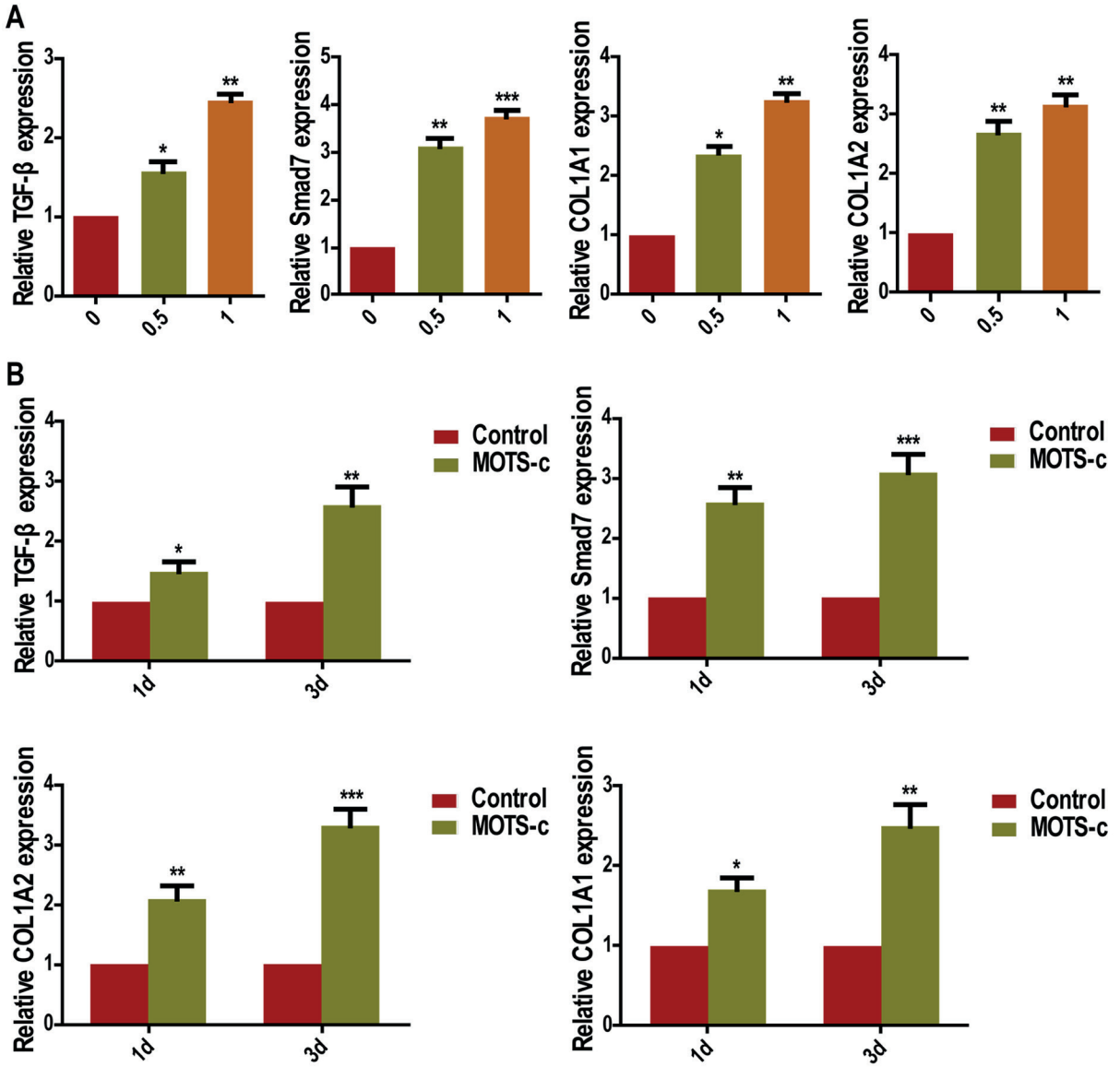

Figure 2. MOTS-c increased the transcription of TGF-β, SMAD7, and Type I Collagen-related genes in hFOB1.19 Cells. ***A,*** The hFOB1.19 cells were treated with different concentrations of MOTS-c and qRT-PCR was used to detect the expressions of TGF-β, SMAD7, COL1A1 and COL1A2. ***B,*** The hFOB1.19 cells were treated with 1.0 μM MOTS-c and the expressions of TGF-β, SMAD7, COL1A1 and COL1A2 in cells cultured for 1 day and 3 days were detected by qRT-PCR, respectively.

MOTS-c Enhanced the Synthesis of TGF-β, SMAD7, COL1A1 and COL1A2 in hFOB1.19 Cells

After treatment of hFOB1.19 cells with different concentrations of MOTS-c, the protein expressions of TGF-β, SMAD7, COL1A1 and COL1A2 were determined by Western blot. Our results indicated that the protein expressions of these genes were increased in a dose-dependent manner (Figure 3A). The protein expressions of TGF-β, SMAD7, COL1A1 and COL1A2 in hFOB1.19 cells were also increased after treatment with 1.0 μM MOTS-c in a time-dependent manner (Figure 3B). The above evidence further showed that MOTS-c not only promotes the protein synthesis of TGF-β and SMAD7, but also the synthesis of type I collagen in osteoblasts.

MOTS-c Contributed to the Synthesis of Type I Collagen in hFOB1.19 Cells Via TGF-β/SMAD Pathway

According to the different treatments in hFOB1.19 cells, cells were divided into the control group, MOTS-c group and the MOTS-c+TGF-β inhibitor

group. After cell culture for 24 h, the expressions of COL1A1 and COL1A2 in MOTS-c group were remarkably elevated compared to those of the control group, which were partially reversed by TGF-β inhibitor (Figure 4A-4B). Similarly, hFOB1.19 cells were divided into the control group, MOTS-c group and MOTS-c+SMAD7 inhibitor group based on different treatments. The data showed thatCOL1A1 and COL1A2 were remarkably upregulated in MOTS-c group compared to those of control group, which were partially decreased by smad7 inhibitor (Figure 4A-4B). The above results indicated that MOTS-c promotes the synthesis of type I collagen in osteoblasts *via* TGF-β/SMAD pathway.

Discussion

Osteoporosis is a metabolic bone lesion characterized by the reduction of bone tissue per unit volume. Bone metabolism is highly converted in human primary osteoporosis, which is manifested as stronger bone resorption than bone formation[13]. Osteoporosis and fracture, especially hip fracture, are the leading causes of disability and death in the elderly[14,15]. Bone remodeling is mediated by the balance of bone resorption and formation[16]. Among the bone metabolism regulators, TGF-β family proteins are greatly involved in the entire process of osteogenic differentiation[17]. TGF-β cytokine is a multi-functional protein and closely related to cell proliferation, differentiation, apoptosis and angiogenesis. Non-parenchymal cells release TGF-β when the body is damaged. TGF-β binds to corresponding receptors on the cell surface, which in turn activates the SMAD pathway[18,19]. SMAD7 is an inhibitory protein in the TGF-β/SMAD pathway, which negatively regulates the phosphorylation of smad2/3 protein and thereby inhibiting TGF-β pathway[20]. Researches[21] have

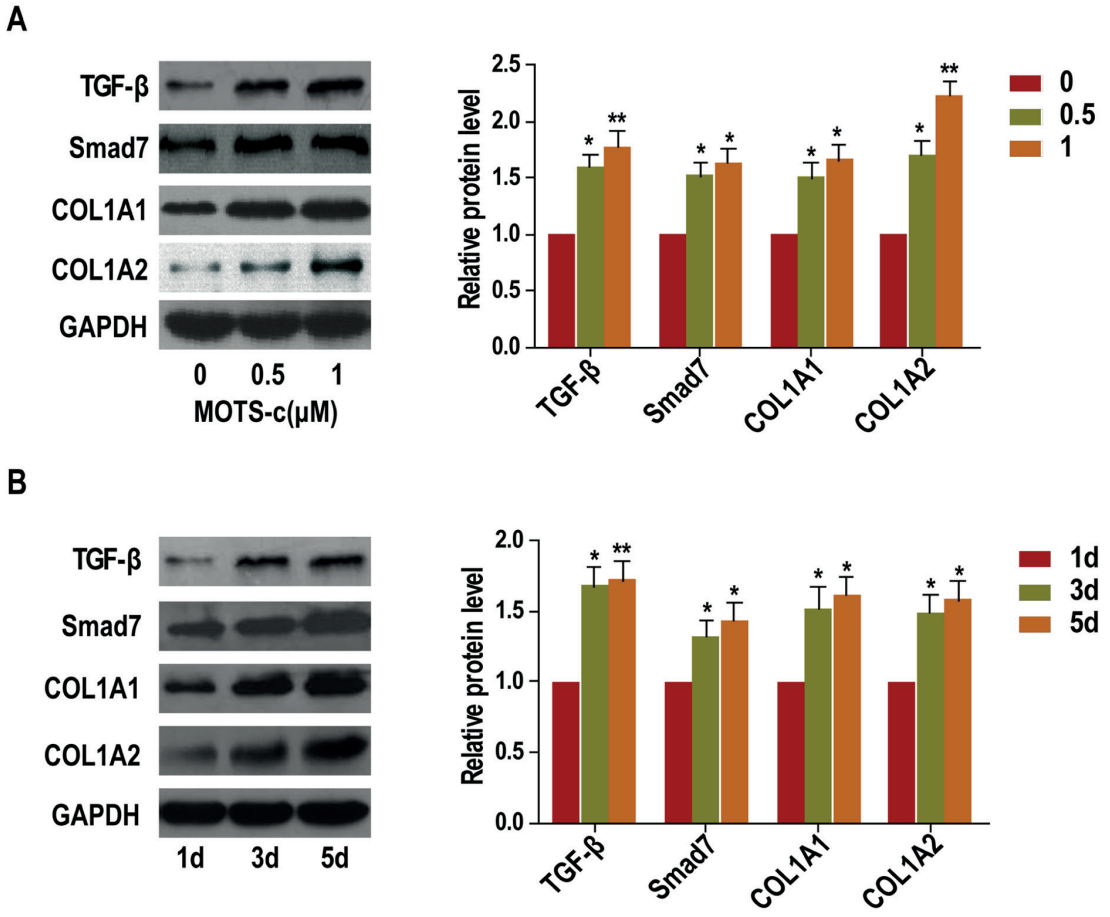

Figure 3. MOTS-c enhanced the synthesis of TGF-β, SMAD7 and Type I Collagen-related genes in hFOB1.19 cells. ***A,*** The hFOB1.19 cells were treated with different concentrations of MOTS-c and Western blot was used to detect the protein expressions of TGF-β, SMAD7, COL1A1 and COL1A2. ***B,*** The hFOB1.19 cells were treated with 1.0 μM MOTS-c and the protein expressions of TGF-β, SMAD7, COL1A1 and COL1A2 in cells cultured for 1 day and 3 days were detected by Western blot, respectively.

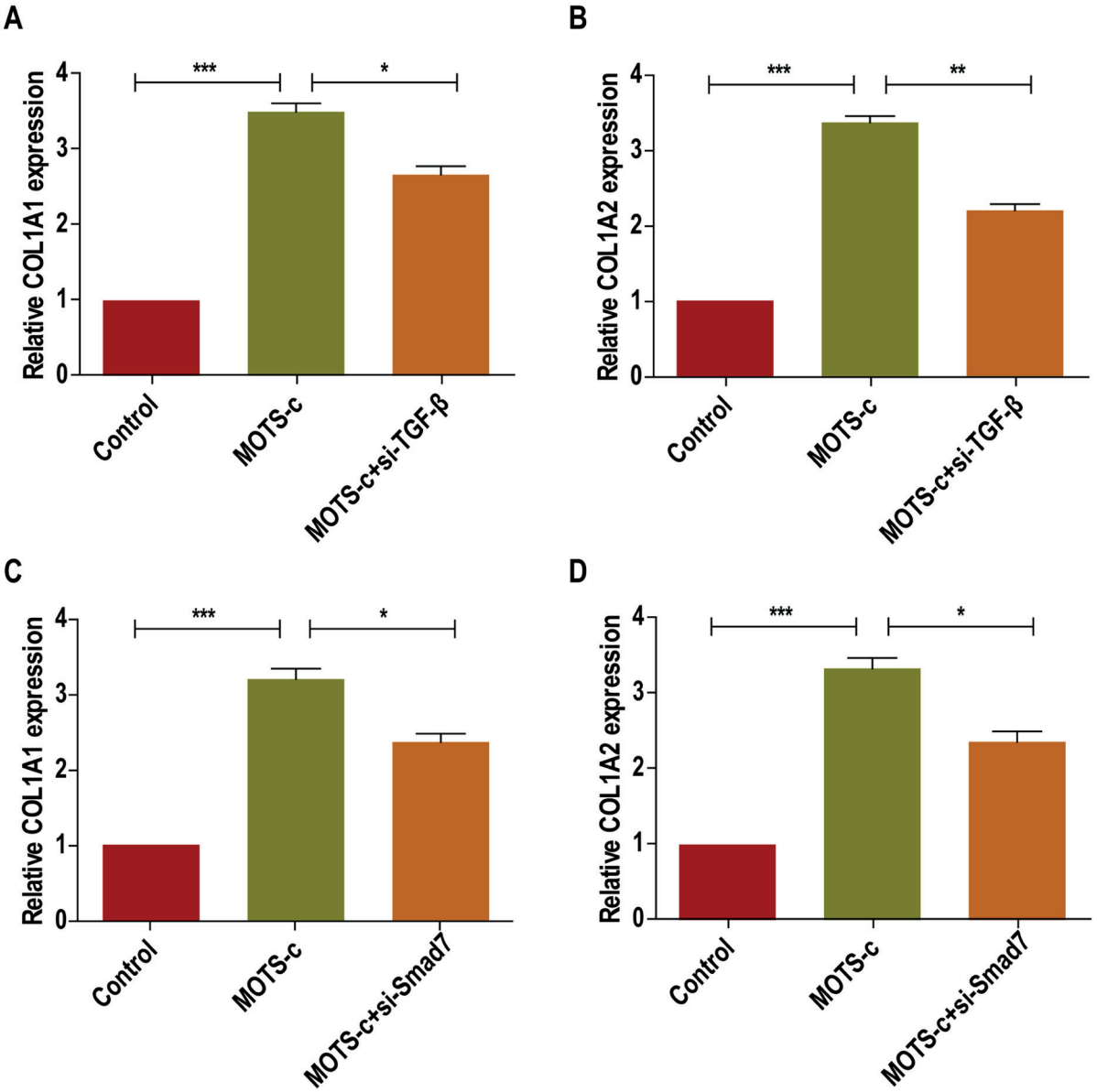

Figure 4. MOTS-c contributed to the synthesis of type I collagen in hFOB1.19 cells via TGF-β/SMAD pathway. ***A-B,*** The hFOB1.19 cells were divided into the control group, MOTS-c group and MOTS-c+TGF-β inhibitor group. The expressions of COL1A1 and COL1A2 were detected by qRT-PCR. ***C-D,*** The hFOB1.19 cells were divided into the control group, MOTS-c group and MOTS-c+SMAD7 inhibitor group. The expressions of COL1A1 and COL1A2 were detected by qRT-PCR.

shown that TGF-β/SMAD pathway mediates the deposition of extracellular matrix (ECM), there by participating in renal tubulointerstitial fibrosis and development of diabetic nephropathy. In addition, high concentration and long-term stimulation of TGF-β1 induce osteogenic differentiation of bone marrow mesenchymal stem cells (MSCs) *in vitro*[22]. TGF-β pathway-related genes exert anti-osteoporosis effect by regulating the function of bone deposits and osteoclasts. TGF-β also affects the bone formation by promoting the proliferation and differentiation of osteoblasts, as well as the synthesis of extracellular matrix[23]. In this work, we found that MOTS-c improves osteoporosis by promoting expressions of genes that are related to the synthesis of type I collagen in osteoblasts *via* TGF-β/SMAD pathway. Lee et al[24] found that MOTS-c could also affect osteoporosis through AMPK pathway, which was similar to that of our findings. In summary, our study first demonstrated that MOTS-c could promote the synthesis of type I collagen in osteoblasts *via* TGF-β/SMAD pathway,

which provides theoretical basis and new research directions for further exploring the mechanism of osteoporosis formation.

Conclusions

We displayed that MOTS-c could promote osteoblasts to synthesize type I collagen *via* TGF-β/SMAD pathway.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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