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Effect of GGCX on the differentiation function of osteoporosis bone marrow mesenchymal stem cells through regulating TGFβ/smad signaling pathway

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Abstract. – OBJECTIVE: Osteoporosis (OP) has a high incidence and can be found in multiple age groups. The bone marrow mesenchymal stem cells (BMSCs) have the potential for self-renewal and multi-directional differentiation, which are often used for investigating the differentiation function of osteoporosis bone marrow mesenchymal stem cells. γ-glutamyl carboxylase (GGCX) is a carboxylase-related carboxylase and was observed to be abnormally expressed in osteoarthritis. However, the role and related mechanisms of GGCX in OP have not been fully elucidated. This work aimed to evaluate the effect of GGCX on the differentiation function of BMSCs.

PATIENTS AND METHODS: Sprague-Dawley rats were randomly divided into the OP group prepared by ovariectomy and sham group. GGCX expression was tested by enzyme-linked immunosorbent assay (ELISA). BMSCs were isolated from OP rats and transfected with pcDNA-GGCX plasmids. BMSC proliferation was detected by tetrazolium salt colorimetry (MTT) assay. The osteogenic and adipogenic differentiation of BMSCs was analyzed by alizarin red staining and oil red O staining. The ALP activity was determined by alkaline phosphatase (ALP) activity colorimetric assay. Real time-PCR was used to test the expressions of osteogenesis-related genes RUNX2 and OPN mRNA. Western blot was adopted to assess the TGFβ/smad signaling pathway activity.

RESULTS: GGCX expression was significantly decreased in the serum of OP rats compared with the sham group ($p < 0.05$). The transfection of pcDNA-GGCX plasmid significantly promoted BMSC cell proliferation, increased calcified nodule formation, inhibited adipogenic differentiation, enhanced ALP activity, elevated RUNX2, and OPN mRNA expressions, and upregulated TGFβ1, Smad2, and Smad7 expressions ($p < 0.05$).

CONCLUSIONS: GGCX secretion is reduced in osteoporosis. GGCX can regulate osteoporosis via promoting the TGFβ/smad signaling pathway, facilitating BMSCs osteogenic differentiation, and inhibiting BMSCs adipogenic differentiation.

Key Words:
Osteoporosis, GGCX, BMSC, TGFβ/smad signaling pathway, Differentiation.

Introduction

As a common disease in orthopedics, osteoporosis (OP) is induced by a large number of factors, including age, hormone secretion levels, glucocorticoids, and other drugs[1,2]. The typical characteristics of OP are decreased bone mass and degeneration of bone tissue microstructure. The decrease of bone mass leads to the reduction of bone mineral density (BMD), which is a precursor to OP[3]. The incidence rate of OP is increasing year by year. Before 2008, the incidence rate was about 15%, whereas in recent years it has risen to about 30%, which is closely related to the global aging society[4]. OP affects about 200 million people around the world, which brings huge medical costs to global health care[5]. The drug treatment of OP includes bisphosphonates, estrogens, and selective estrogen receptor modulators, whose main

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purpose is to prevent bone resorption and promote bone remodeling[6,7]. The application of the dietary supplements, including calcium and vitamin D (VD), is also used as a basic treatment. However, since OP requires lifelong treatment, there are potential side effects such as gastrointestinal symptoms and venous thrombosis, and even the risk of tumorigenesis[8,9]. The clinical symptoms of OP are not evident, and it is often diagnosed when fractured. Therefore, this study intends to explore the pathogenesis and molecular mechanism of OP to determine biomarkers and therapeutic targets.

γ-glutamyl carboxylase (GGCX) is a vitamin K (VK)-related carboxylase that activates γ-carboxyglutamic acid (GLA) proteins by adding a carboxyl to the γ position of glutamine[10]. GLA proteins include VK-dependent coagulation factors (coagulation factors II, VII, IX, and X), protein C, protein S, and protein Z, as well as bone component proteins, such as matrix GLA protein (MGP), osteocalcin (OCN), and periostin, and GLA-rich proteins[11,12]. It was found that vitamin K is closely related to osteoarthrosis[13,14]. Recent studies[15,16] confirmed reduced GGCX expression in osteoarthritis, suggesting that GGCX is closely related to bone and joint disease. However, the role and mechanism of GGCX in OP has not been elucidated.

Materials and Methods

Experimental Animals

Twenty healthy female Sprague-Dawley (SD) rats weighted 225 ± 20 g and 8-12 weeks old were purchased from the Experimental Animal Center and fed in SPF environment. Feeding conditions include the constant temperature at 21 ± 1°C, constant relative humidity at 50-70 %, and 12 h daily/night cycle.

Ethics Statement

All experiments were approved by the Ethics Committee of the 2nd Hospital of Shantou. Animal experiments were performed in strict accordance with the experimental design by experienced technicians to minimize animal suffering, and the experimental procedures were performed in strict accordance with Legislation Regarding the Use and Care of Laboratory Animals of China.

Main Reagents and Instruments

The sodium pentobarbital was purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd (Shanghai, China). Western blot related chemical reagents were purchased from Beyotime (Shanghai, China). ECL reagents were purchased from Amersham Biosciences (Shanghai, China). Rabbit anti-mouse TGFβ1, Smad2, and Smad7 monoclonal antibodies, and goat anti-rabbit horseradish peroxidase (HRP) IgG labeled secondary antibody were purchased from Cell Signaling (Boston, MA, USA). The pcDNA-GGCX plasmid was constructed by GenePharma (Shanghai, China). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (Corning, NY, USA). Low-glucose Dulbecco's Modified Eagle's Medium (DMEM) medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) and tetrazolium salt colorimetry (MTT) powder were purchased from Gibco (Carlsbad, CA, USA). Dexamethasone, ascorbic acid, oil red O, β-sodium phosphinate, and collagenase were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ALP active colorimetric quantitative detection kit was purchased from Nanjing Jiancheng Reagent Company (Nanjing, China). Other commonly used reagents were purchased from Sangon Biotechnology (Shanghai, China). The ABI 7700 Fast Quantitative PCR Amplifier was purchased from ABI (New York, NY, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The SW-CJ clean bench was purchased from Suzhou Purification Equipment Factory (Jiangsu, China). The surgical microscopy equipment was purchased from Suzhou Medical Instrument Factory (Jiangsu, China).

Animal Modeling and Grouping

Twenty healthy female SD rats were equally and randomly divided into 2 groups, including the OP group that was prepared by ovariectomy and the sham group. According to Hoffmann et al, the rats were anesthetized by 30 mg/kg sodium pentobarbital intraperitoneal injection. The bilateral ovaries were exposed, and the fallopian tubes and blood vessels were ligated. The ovaries were completely removed, and the uterus was put back to the abdomen. The sham group received the same surgical procedure without removing the ovaries. A conventional 200,000 IU/kg penicillin anti-inflammatory treatment was given after surgery.

Bone Marrow Mesenchymal Stem Cells (BMSCs) Isolation and Culture

The OP group rats were sacrificed by cervical dislocation. After 75% alcohol disinfection, the

subcutaneous adipose tissue of groin was isolated and digested with 0.1% collagenase. BMSCs were cultured in low glucose DMEM medium containing 10% FBS, 100 mg/l streptomycin, 100 U/ml penicillin, and 2 mmol/l glutamine. The 2-4th generation cells in the logarithmic growth phase were used for experiments. The cultured BMSCs were randomly divided into 3 groups, including the OP group that was normally cultured, blank control group, and GGCX group, which were transfected with empty plasmid control and pcDNA-GGCX plasmid, respectively.

pcDNA-GGCX Plasmid Transfection

pcDNA-GGCX and the negative control plasmids were separately added to 200 μL of serum-free DMEM medium and incubated at room temperature for 15 min. The mixed Lipofectamine 2000 was separately mixed and incubated at room temperature for 30 min. The mixture was added to the cells in 6-well plate together with 1.6 mL of serum-free DMEM medium and cultured in a 5% CO_2 incubator at 37°C for 6 hours. The medium was replaced to the osteogenic induction medium, including 10% FBS, low-glucose DMEM medium, 10^{-8} mol/L dexamethasone, 50 mg/L ascorbic acid, and 10 mmol/L β-glyceryl phosphates. Then, the cells were further cultured for other 48 hours.

Real-time PCR

The total RNA was extracted from the BMSCs by TRIzol and reverse transcribed to cDNA. The primers were designed by PrimerPremier 6.0 software and synthesized by Invitrogen (Carlsbad, CA, USA) (Table I). The real time-PCR was performed at 55°C for 1 min, followed by 45 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as an internal reference. The relative expression of mRNA was calculated by the $2^{-\Delta Ct}$ method.

MTT Assay

The BMSCs in logarithmic phase were seeded in 96-well plate at 3000 cells/well and treated as abovementioned for 72 h. After added with 20 μL MTT for 4 h, the plate was added with 150 μL DMSO for 10 min and tested at 570 nm to obtain the absorbance value (A).

ALP Content Measurement

According to the instruction, the cells were collected and centrifuged at 1000 rpm for 10 min. Subsequently, the cells were treated with Triton-X100. The optical density (OD) values were measured at 520 nm.

Oil Red O Staining

The cells were stained by oil red O working dyeing solution for 10 min and observed under the phase-contrast microscope. In the case of lipid titration, isopropanol was added to the dyed oil droplet sample for 10 min. The OD value of the solution was measured by a microplate reader.

Alizarin Red Staining

After fixing with 70% ethanol for 1 h, the cells were washed 3 times with double distilled water and stained by alizarin red solution for 10 min. Then, the purple-red calcium nodules were observed under the microscope. Alizarin red staining was quantitatively analyzed using a microplate reader to detect OD values at 570 nm.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to test the GGCX content in the serum. The OD value of the standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

Western Blot

The cells were lysed on ice and quantified by bicinchoninic acid (BCA) method. The isolated proteins were electrophoresed using 10% SDS-PAGE at 60V for 5 h. The gel was transferred to polyvinylidene difluoride (PVDF) membrane by semi-dry transfer method at 100 mA for 1.5 h. After being blocked by 5% skim milk for 2 h, the membrane was incubated in TGF-β1, Smad2, and Smad7 (1:1000, 1:1000, and 1:2000) primary antibodies at 4°C overnight. After incubation in secondary antibody away from light for 30 min, the membrane was imaged using chemiluminescence reagent for 1 min and analyzed by image processing system software and Quantity one software. The experiment was repeated for four times (n=3).

Statistical Analysis

All data analyses were performed on the Statistical Product and Service Solution 16.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation and were compared by the *t*-test or One-way ANOVA (Tukey post-hoc test). $p < 0.05$ was considered the statistical difference.

Results

Serum GGCX Secretion in OP Rats

The serum levels of GGCX in the sham and OP groups were analyzed by ELISA. The results showed that GGCX secretion in the serum of OP rats was significantly decreased compared with the sham group ($p < 0.05$) (Figure 1).

The Impact of Regulating GGCX on GGCX Expression in BMSCs from OP Rats

The effect of GGCX plasmid transfection on the expression of GGCX in OP rat BMSCs was detected by real time-PCR. It was demonstrated that the transfection of GGCX plasmid significantly upregulated the expression of GGCX in BMSCs from OP rats ($p < 0.05$) (Figure 2).

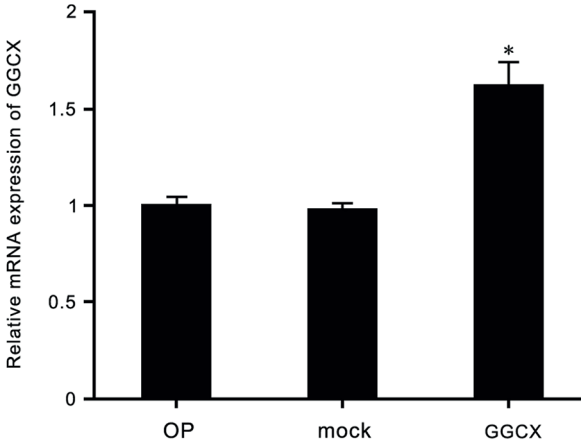

Figure 2. The influence of regulating GGCX on GGCX expression in BMSCs from OP rats. n=10, * $p < 0.05$ *vs.* OP group.

The Influence of Regulating GGCX on BMSCs Proliferation

The effect of the transfection of GGCX plasmid on the proliferation of OP rat BMSCs was detected by MTT assay. It was revealed that the transfection of GGCX plasmid markedly promoted the proliferation of BMSCs in OP microenvironment compared with the OP group ($p < 0.05$) (Figure 3), suggesting that targeting GGCX can regulate the proliferation of BMSC cells in the OP microenvironment.

The Effect of Regulating GGCX on Calcified Nodule Formation in BMSCs From OP Rats

The transfection of the GGCX plasmid apparently increased the formation of calcified nodules in BMSCs in the OP microenvironment compared with the OP group ($p < 0.05$) (Figure 4).

The Impact of Regulating GGCX on the Adipogenic Differentiation Ability of BMSCs from OP Rats

The transfection of GGCX plasmid significantly restrained the adipogenic differentiation ability of BMSCs in the OP microenvironment compared with the OP group ($p < 0.05$) (Figure 5).

The Influence of Regulating GGCX on ALP Activity in BMSCs from OP Rats

ALP activity was analyzed by ALP activity colorimetry. The transfection of GGCX plasmid

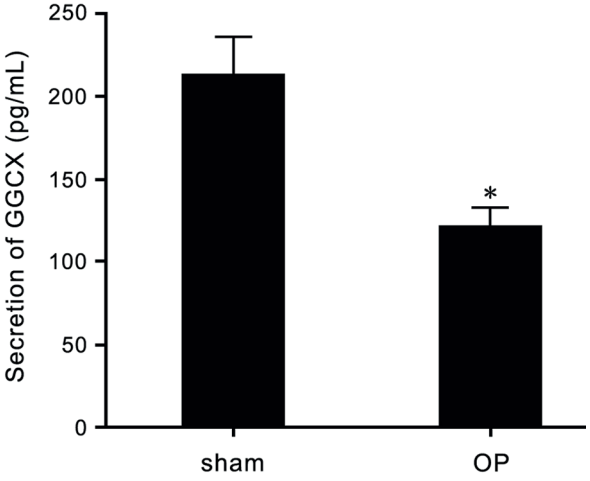

Figure 1. Serum GGCX secretion in OP rats. n=10, * $p < 0.05$ *vs.* sham group.

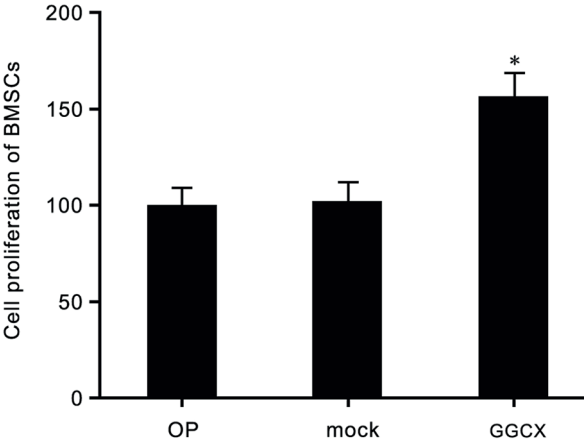

Figure 3. The influence of regulating GGCX on BMSCs proliferation. n=10, * $p < 0.05$ *vs.* OP group.

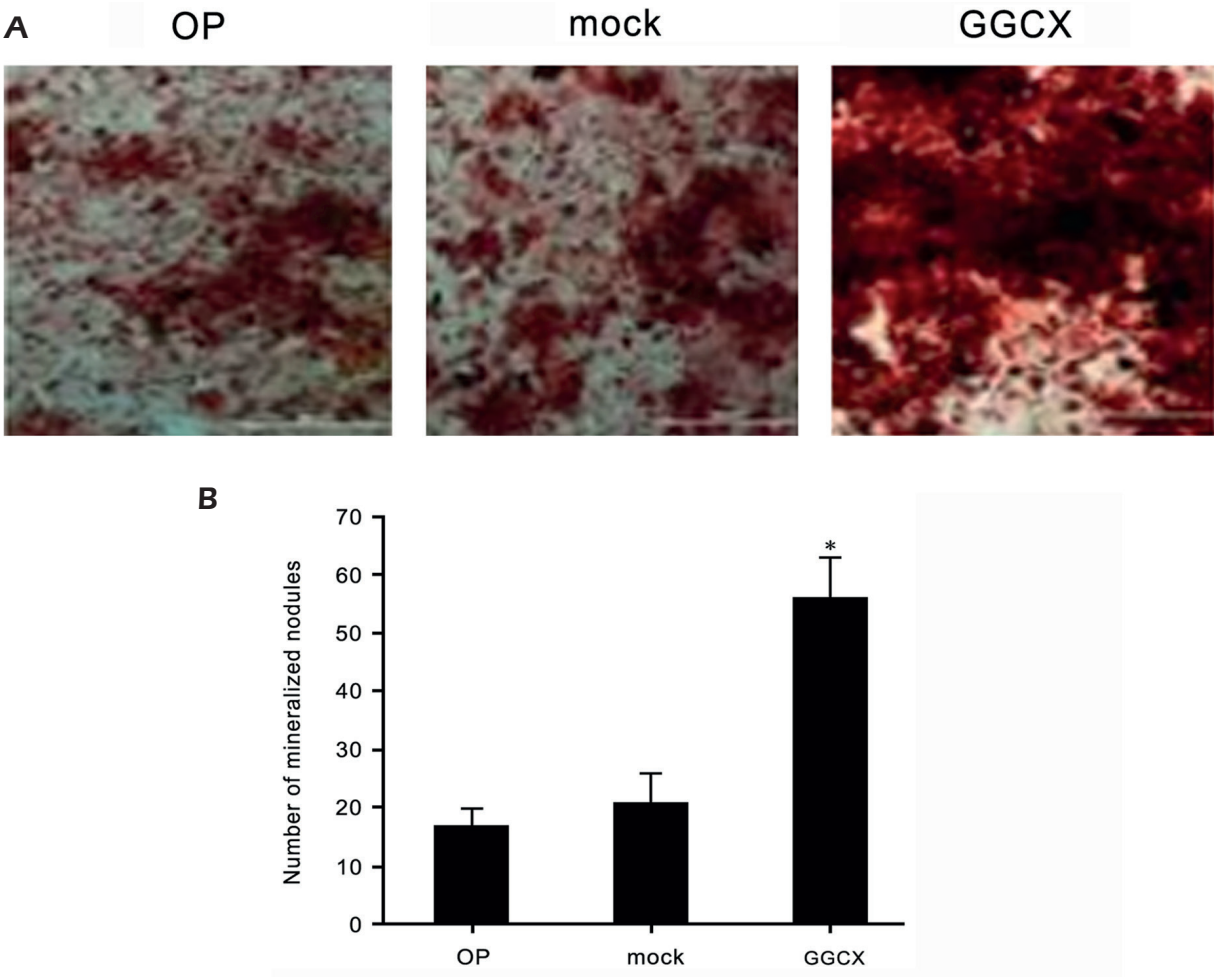

Figure 4. The effect of regulating GGCX on calcified nodule formation in BMSCs from OP rats. ***A,*** Alizarin red staining detection of calcified nodule formation (x 200). ***B,*** Calcified nodule number analysis. n=10, *$p < 0.05$ *vs.* OP group.

significantly enhanced ALP activity in BMSCs under OP microenvironment compared with the OP group ($p < 0.05$) (Figure 6).

The Effect of Regulating GGCX on RUNX2 and OPN Expressions in BMSCs from OP Rats

Transfection of GGCX plasmid markedly promoted osteogenesis in BMSCs in OP microenvironment by upregulating RUNX2 and OPN mRNA expressions ($p < 0.05$) (Figure 7).

The Impact of Regulating GGCX on TGFβ/smad Signaling Pathway in BMSCs from OP Rats

Western blot was performed to evaluate the effect of GGCX on TGFβ/smad signaling pathway. The transfection of GGCX plasmid apparently enhanced the expressions of TGFβ1, Smad2, and Smad7 in BMSCs from OP rats ($p < 0.05$) (Figure 8).

Discussion

The bone marrow stromal system is an important component that constitutes bone marrow and promotes bone formation and differentiation. It is mainly composed of stromal cell lineages, including undifferentiated stromal stem cells and terminally differentiated adipocytes, osteoblasts, hematopoietic support cells, and other cell types. Among them, the bone marrow mesenchymal stem cells (BMSCs) play a key role in maintaining bone balance[18,19]. It was found that the absolute number decreased and the ability of the osteogenic differentiation attenuated in BMSCs during the

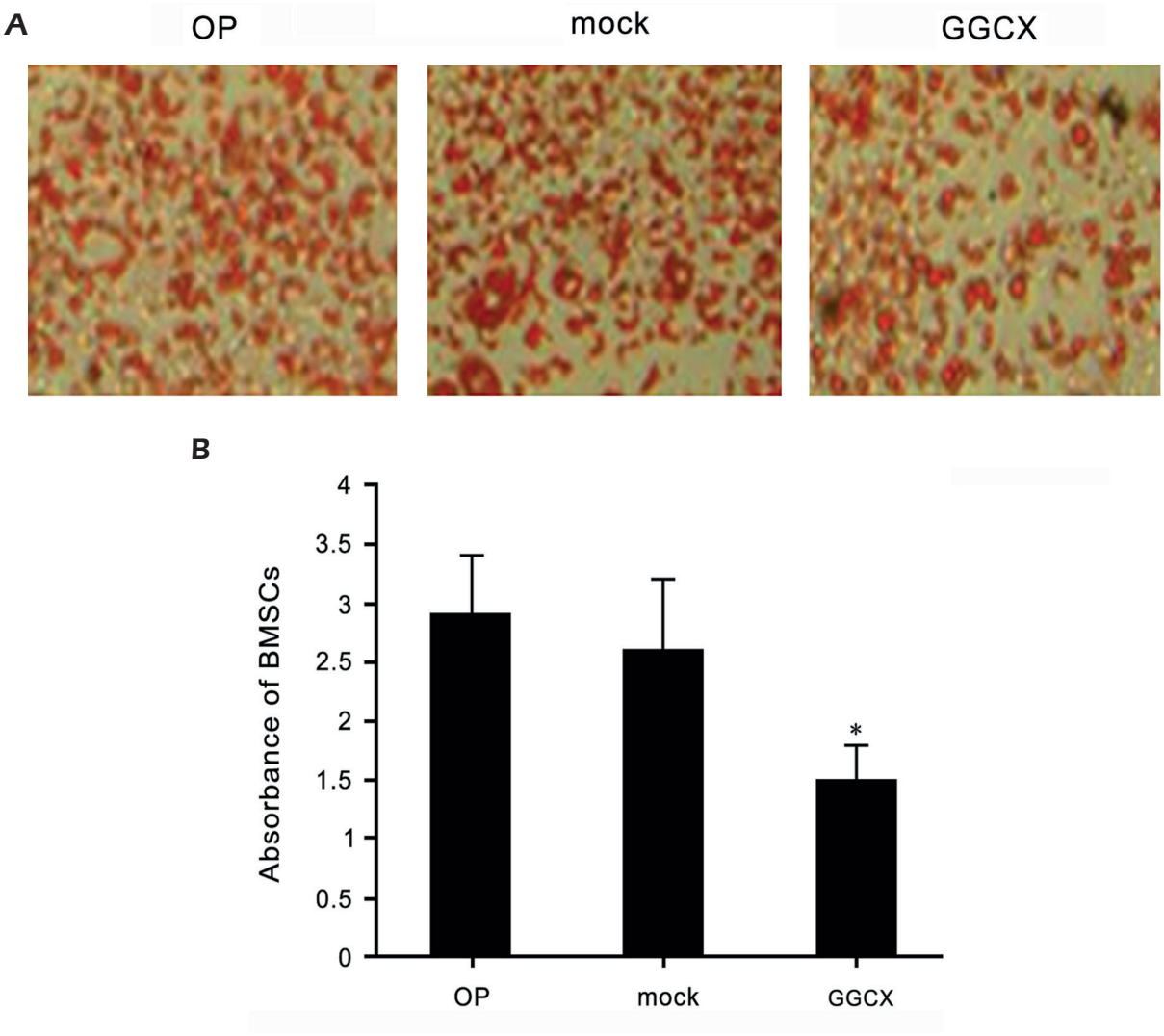

Figure 5. The impact of regulating GGCX on the adipogenic differentiation ability of BMSCs from OP rats. ***A,*** Oil red O staining detection of adipogenic differentiation (x 200). ***B,*** Oil red O staining quantitative analysis. n=10, *$p < 0.05$ *vs.* OP group.

OP process. Therefore, the number and function of BMSCs are an important factor leading to osteoporosis in the elderly[20]. Therefore, it is speculated that the regulation of BMSCs may be one of the key methods for the treatment of OP.

It was observed that VK plays a crucial role in bone development, while GGCX is a vitamin-dependent carboxylase. The loss of the GGCX expression leads to the lack of activity of matrix GLA protein, which cannot bind to calcium ions, resulting in bone-related diseases[11,21]. In this report, we established a rat model of ovariectomized OP and found that GGCX was decreased in the serum of OP rats, suggesting that it may participate in the occurrence and development of OP. Furthermore, OP rat BM-

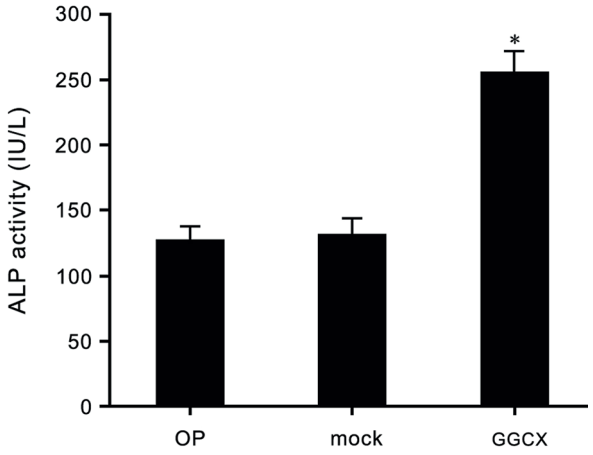

Figure 6. The influence of regulating GGCX on ALP activity in BMSCs from OP rats. n=10, *$p < 0.05$ *vs.* OP group.

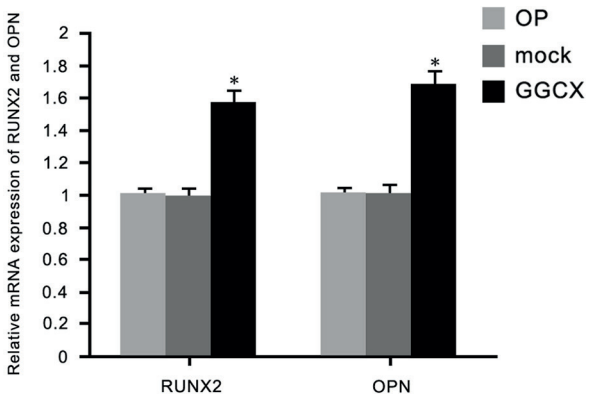

Figure 7. The effect of regulating GGCX on RUNX2 and OPN expressions in BMSCs from OP rats. n=10, $*p < 0.05$ *vs.* OP group.

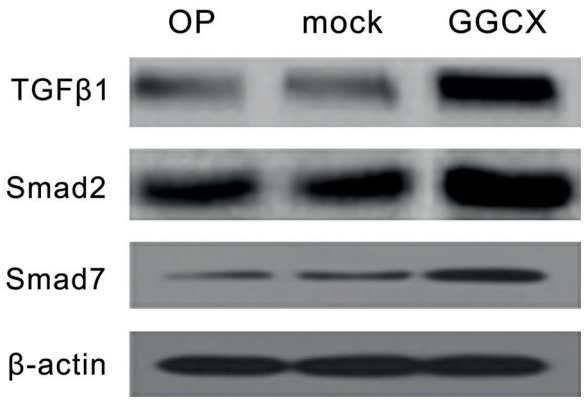

Figure 8. The impact of regulating GGCX on TGFβ/smad signaling pathway in BMSCs from OP rats. n=10.

SCs were isolated and cultured, and the GGCX expression was targeted and transfected. The GGCX plasmid transfection promoted the proliferation of BMSCs, increased the expression of osteogenesis-related genes RUNX2 and OPN mRNA, facilitated osteogenic differentiation, and inhibited adipogenic differentiation. It suggested that the targeting GGCX is beneficial to regulate the differentiation of BMSCs into the osteogenic direction in the OP environment.

Further analysis revealed that the transfection of GGCX plasmid activated the TGFβ/smad signaling pathway. The TGFβ/Smad signaling pathway is involved in a variety of cellular biological processes, including regulation of cell growth, apoptosis, and cell differentiation, thereby maintaining cell homeostasis[22]. TGFβ/Smad signaling pathway plays a key role in osteoblast differentiation. It can participate in the regulation of osteoblast secretion and promote the expression of osteogenesis-related genes RUNX2 and OPN, which is beneficial to the further differentiation and maturation of osteoblasts[23,24]. The present results indicated that the targeting GGCX can promote the differentiation of BMSCs into the osteogenic direction by regulating TGFβ/Smad signaling pathway. It is proposed to further analyze the role and related mechanism of targeting GGCX in clinical OP patients.

Conclusions

GGCX secretion is reduced in osteoporosis. GGCX can regulate osteoporosis via promoting the TGFβ/smad signaling pathway, facilitating BMSCs osteogenic differentiation, and inhibiting BMSCs adipogenic differentiation.

Conflict of Interests

The Authors declare that they have no conflict of interests

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