Sialoglycoprotein from *Gadous morhua* eggs improve high bone turnover activity via down-regulating BMP-2/Smads and Wnt/ β -catenin signal pathways

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Abstract The effect of sialoglycoprotein isolated from *Gadous morhua* eggs (*Gm*-SGP) on ovariectomized (OVX) induced osteoporosis, which is characterized by high bone turnover activity was investigated. Results revealed that *Gm*-SGP significantly increased bone mineral density, enhanced bone biomechanical properties and repaired the microstructure of the trabecular bone. Also, the treatment with *Gm*-SGP remarkably decreased biochemical marker contents or activities, such as serum BALP, PICP, BMP-2, TrACP, Cath-K, urine Ca and P, leading to the reduction in

bone turnover. The elevation in the rate of bone formative process contributed in the increase of bone turnover. Both BMP-2/Smads and Wnt/ β -catenin signaling pathways played an important role in osteogenesis. *Gm*-SGP suppressed the key factors expression in these two pathways such as BMP-2, Smad1, Smad4, Lrp-5b, Runx2, Osx, ALP, Col1, OCN and β -catenin. These findings might provide some theoretical basis for the application of *Gm*-SGP as a potential anti-osteoporotic drug or as functional food.

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Graphical Abstract

Keywords *Gadous morhua* eggs · Sialoglycoprotein · Bone turnover · BMP-2/Smads · Wnt/ β -catenin

Introduction

Osteoporosis is the most prevalent metabolic bone disease that results from an increase in bone loss relative to bone formation, leading to low bone mass and deteriorative bone microstructure (Chung et al., 2014). Osteoporosis is considered to be an important global health issue, secondary to coronary heart disease by the World Health Organization (WHO) (Leboime et al., 2010). Nowadays, more than 200 million people are suffering from osteoporosis all over the world (Wirries et al., 2013). Among these people, more than 33% of women over 50 years of age suffer from postmenopausal osteoporosis, which results from dramatic estrogen levels decline after menopause (Riggs et al., 2002). Postmenopausal osteoporosis is closely associated with an excessive increase in the rates of bone formation and bone resorption (excessive high bone turnover activity), leading to accelerated bone loss (Pacifi, 1996). Osteoporosis-induced fracture is the main clinical hazard for osteoporosis patients which can result in disability and even death (Genant et al., 2017). The worldwide hip fracture incidence in women is expected to increase by 310% in 2050 (Herrero and Pico, 2016). This will lead to a heavy economic burden and an increased cost in health care resources (Keen, 2003).

The current strategy for treating postmenopausal osteoporosis major by hormone replacement therapy (HRP), selective estrogen receptor modulators, bisphosphonates and calcitonin (Brewer et al., 2011). Although these drugs have an effect on increasing bone mass, their prolonged use can also induce many adverse reactions such as breast cancer, stroke, cardiovascular disease and many others (Rossouw et al., 2002). Therefore, alternatives to this treatment of osteoporosis with fewer adverse reactions and that would also be more suitable for a long-term use are urgently needed.

Recently, many studies have focused on the bioactive substance extracted from natural products that could be more suitable for the treatment of osteoporosis (Liu et al., 2014). Marine biological resources contain plenty of bioactive substances with novel structure and may have potency for osteoporosis therapy (Xia et al., 2016). *Gadous morhua* is a major marine fishery resource that has not been fully utilized. The eggs of *Gadous morhua* are one of the major byproducts of fish processing, which contain plentiful bioactive substances such as glycoproteins, polyunsaturated fatty acids and lecithin (Wang et al., 2008). Sialoglycoproteins (SGPs) are a new family of glycoproteins that widely exists in fish eggs and that is basically a novel glycoprotein containing high amounts of sialic acid and carbohydrate (Inoue and Inoue, 1997). Recently, our laboratory found that SGP isolated from *Gadous morhua* eggs (Gm-SGP) exhibited an anti-osteoporosis effect with a proliferative response on MC3T3-E1 cells, which play a major role in the bone remodeling process. In addition, Gm-SGP has been shown to enhance fracture healing in osteoporotic mice (Zhan et al., 2017). In the present study, the anti-osteoporotic activity of Gm-SGP was evaluated in vivo by using OVX-induced osteoporosis in rats. The mechanisms involved in the inhibiting effect of Gm-SGP on high bone turnover activity were investigated, which may provide some theoretical basis for the usage of Gm-SGP as a potential oral anti-osteoporotic agent or as functional food.

Materials and methods

Materials and reagents

Fresh eggs of *Gadous morhua* were provided by Shandong Oriental Ocean Group Co., Ltd (Yantai, Shandong, China). The eggs were removed from their envelope and stored at -80°C until use. TRIzol reagent and Maxima SYBR Green qRT-PCR Master Mix were Invitrogen products (Hoffmann-La Roche Ltd, Basel, Switzerland). Moloney

murine leukemia virus reverse transcriptase (M-MLV), random primer, and PageRuler prestained protein ladder were from TaKaRa Bio Inc (Otsu, Shiga, Japan). The primers of bone morphogenetic protein 2 (BMP2), Smad-1, Smad-4, low-density lipoprotein receptor-related protein 5b (Lrp-5b), β -catenin, Runt-related transcription factor 2 (Runx2), Osterix (Osx), and β -actin were synthesized by Sangon Biotech Co. Ltd (Shanghai, China). Rabbit anti-rat BMP-2, Smad-1, p-Smad-1, β -catenin, Runx2, TATA box binding Protein (TBP) and β -actin primary antibodies and goat anti-rabbit secondary antibody IgG-HRP were from Cell Signaling (Beverly, MA, USA).

Preparation of *Gm*-SGP

Unless otherwise stated, all procedures were carried out at 4 °C. Mature *Gadous morhua* eggs (300 g) were homogenized in 3 vol (v/w) of 0.4 M NaCl with a squeezer for 5 min, and the residue was then removed after centrifugation at 4500 r/min for 10 min. The supernatant was dialyzed for 24 h to obtain water-soluble glycoproteins. Then, an equal volume of 90% phenol was added and stirred for 12 h at 4 °C. After centrifugation at 10,000 r/min for 10 min, the supernatant was decanted into dialysis bags with 4000 Da sectional molecular weight, then dialyzed against tap water for 3 days, and finally against distilled water for 1 day to remove the phenol. The crude glycoproteins were then applied to a column (2.6 × 50 cm) of QFF (pre-equilibrated with 0.02 M Tris-HCl buffer, pH = 8.2), eluted with a linear gradient of NaCl (0–1 M) in 0.02 M Tris-HCl buffer (pH = 8.0). The elution profile was monitored by the absorbance at 280 nm for protein, by the resorcinol method for sialic acids, and by the phenol-sulfuric acid method for hexose. Sialic acid-positive fractions were collected and applied to a Sephacryl S-300 (1.6 × 100 cm, equilibrated with distilled water), and eluted with distilled water at 1 ml/min. The profile was monitored according to the above methods. After isolation with Sephacryl S-300, a single overlapping peak of protein, carbohydrates and sialic acid was obtained and lyophilized as *Gm*-SGP.

Chemical analysis revealed that the purity of *Gm*-SGP was 87.46% and that the molecular weight was 7000 Da. *Gm*-SGP contained 18.32% of protein, 65.86% of hexose, and 15.7% of N-acetylneuraminic acid (Neu5Ac).

Animals and experimental design

This study was approved by the Ethical Committee of Experimental Animal Care at the Ocean University of China (certificate no. SYXK20120014). Forty female Wistar rats (200 ± 20 g) were purchased from the Vital River Laboratory Animal Center (Beijing, China; Licensed

ID: SCXK2012-0001). The animals were housed at 23 ± 1 °C with a 12 h light/12 h dark cycle and given food and water ad libitum.

After an acclimatization period of 7 days, rats underwent either bilateral ovariectomy (OVX, n = 32) to establish an osteoporotic model, or bilateral laparotomy (Sham, n = 8) as a sham control group. After 30 days, rats were then randomly allocated to 5 groups of 8 animals each according to the body weight: Sham control group, OVX control group, ALN group (1 mg of alendronate sodium per kg body weight), *Gm*-SGP-L group (150 mg of *Gm*-SGP per kg body weight), *Gm*-SGP-H group (450 mg of *Gm*-SGP per kg body weight). The sham control group and the OVX control group were treated with physiological saline (7 mL/kg body weight). A volume of 10 mL/kg body weight was intragastrically administered to rats once a day for 90 days. During the experimental period, rat body weight was recorded every 3 days. Five days before rats were euthanized, urine was collected for 24 h to measure Ca and P contents. After the last administration, rats had no access to food but a free access to water for 10 h. Then, blood was collected from the abdominal aorta and serum was separated to quantify bone-turnover-related biochemical markers. Tibiae and proximal femurs were quickly isolated and fixed in 10% neutral formaldehyde in order to measure BMD, bone biomechanical properties, bone Ca content and bone histomorphology. Distal femurs were quickly put into liquid nitrogen and then stored in – 80 °C for qRT-PCR and Western blot analysis.

Measurement of bone metabolic indicators

Serum bone metabolic indicators, such as BALP, PICP, BMP-2, and Cath-K were measured using Enzyme-linked immunosorbent assay (ELISA) kits (R&D Co. Ltd, USA). Serum tartrate-resistant acid phosphatase (TRAP) and urine Ca and P contents were measured using Chemical assay kits (Jiancheng Biotech, Nanjing, China).

Bone mineral density and bone biomechanical analysis

The BMD of the left tibia was estimated using dual-energy X-ray Bone Densitometer (GK99-UNIGAMMAX-RAY-PLUS, L'ACN, Italy). The values are expressed as grams of mineral contents per cm². Bone biomechanical properties of the tibia including ultimate load and stiffness were measured using a YLS-16A bone strength tester (Yiyan, China).

Bone Ca content analysis

Bone Ca content of femurs were measured with ICP-MS using 7300DV ICP-OES (PerkinElmer, USA).

Histomorphometric observation

The proximal femur was fixed in 10% neutral formaldehyde for 48 h, then decalcified in 10% EDTA and finally embedded in paraffin. Samples were sectioned (5- μ m thickness) and stained with hematoxylin and eosin (H&E). Sections with the widest marrow cavity near the growth plate of the metaphysis of the femurs were selected for bone histomorphology analysis using a BH-2 microscope (Olympus, Tokyo, Japan). Histomorphometric analysis was implemented by ImageJ software (National Institutes of Health).

Real-time quantitative polymerase chain reaction (qRT-PCR) assay

The mRNA expression of key related genes in BMP-2/Smads and Wnt/ β -catenin pathways, including BMP-2, Smad-1, Smad-4, Lrp-5b, β -catenin, Runx2, and Osx were measured by qRT-PCR. The sequences of the primers used in this study are shown in Table S1. RNA extraction and amplification were performed as previously described (Hu et al., 2014). Briefly, total RNA from the femur was extracted with TRIzol reagent, and RNA (1 μ g) was reverse transcribed to cDNA using M-MLV. Then, qRT-PCR analysis was performed on the iCycler iQ5 system (Bio-Rad Inc., Hercules, CA, USA). Twenty-five microliters of reaction mixture including 12.5 μ L of Maxima SYBR Green qPCR Master Mix, 0.75 μ L each of forward and reverse primers (10 μ M), 6 μ L of nuclease-free water and 5 μ L of template (samples were diluted to contain comparable cDNA concentrations) was applied for qRT-PCR. The thermal conditions consisted of an initial denaturation at 95 $^{\circ}$ C for 10 min followed by 45 cycles of denaturation at 95 $^{\circ}$ C for 15 s, annealing at 60 $^{\circ}$ C for 10 s, and extension at 72 $^{\circ}$ C for 45 s. The data normalization was achieved using endogenous reference β -actin. The gene expression levels were analyzed by relative quantification using the standard curve method.

Western blot analysis

The protein expression of BMP-2, Smad-1, p-Smad-1, Samd-4, β -catenin and Runx2 were determined by western blot analysis. Femurs were ground into powder with liquid nitrogen. To obtain total protein, 0.1 g of powder was lysed in Western and IP lysis buffer (Applygen, China) and then centrifuged to remove precipitation. Nuclear proteins were

obtained using a nuclear extract kit (Solarbio, Beijing, China). The proteins were separated on 10% SDS-PAGE gel, then transferred onto 0.45 μ m polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% BSA for 2 h, then incubated respectively with BMP-2, Smad1, pSmad1, Samd4, β -catenin and Runx2 primary antibodies overnight at 4 $^{\circ}$ C. Afterwards, the membranes were incubated with horseradish peroxidase (HRP) conjugated IgG secondary antibody. Protein bands were visualized using an ECL stain kit, then quantified with the ImageJ program. The TBP and β -actin were analyzed as internal controls for nuclear protein and total protein, respectively.

Statistical analysis

Results are presented as mean $\pm$ standard deviation. Statistical comparison of means among multiple groups was performed using one-way analyses of variance (ANOVA) and Duncan post hoc tests using the SPSS software (version 17.0; SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered statistically significant.

Results and discussion

Gm-SGP improved the high bone turnover status in OVX rats

Postmenopausal osteoporosis is closely characterized with simultaneous excessive increase in the rates of bone formation and bone resorption, leading to accelerated bone loss (Pacifi, 1996). Increasing studies have suggested that OVX-induced higher bone turnover rates can be reflected by higher bone formation and resorption markers (Ma et al., 2013). Xia et al. reported that Ca-SGP limited osteoporosis by reducing the expression of bone formation markers such as ALP, OCN, PICP, and BMP-2 as well as a bone resorption marker such as TRAP (Xia et al., 2015). As shown in Table 1, serum contents of bone formation markers, such as BALP, PICP, and BMP-2 were significantly increased in the OVX control group ($P < 0.05$ for all markers). Similarly, bone resorption markers, such as TRAP and Cath-K were also clearly enhanced in the OVX control group compared with the Sham control group ($P < 0.05$ for both markers), indicating that a high bone turnover occurred in OVX rats. Also, Ca and P contents in urine were both remarkably enhanced in the OVX control group which further demonstrates the bone loss in OVX rats. After treatment with different doses of *Gm*-SGP, serum contents of bone formation markers, such as BALP, PICP, and BMP-2 were reduced significantly compared to the OVX control group ($P < 0.01$). In comparison, the

Table 1 Effects of *Gm*-SGP on Biomarker of Bone Turnover in Serum and urine

	Sham	OVX	ALN	<i>Gm</i> -SGP-L	<i>Gm</i> -SGP-H
BALP (U/L)	147.00 ± 8.32	205.61 ± 9.18 ^{aa}	166.78 ± 8.02 ^{aa,bb}	185.77 ± 6.30 ^{aa,bb}	175.30 ± 4.70 ^{aa,bb}
PICP (ng/mL)	8.90 ± 0.79	11.41 ± 0.74 ^{aa}	9.05 ± 0.77 ^{bb}	10.31 ± 0.48 ^{aa,b}	9.29 ± 0.67 ^{bb}
BMP-2 (ng/mL)	5.92 ± 0.30	9.68 ± 0.40 ^{aa}	7.25 ± 0.42 ^{aa,bb}	8.08 ± 0.48 ^{aa,bb}	7.47 ± 0.45 ^{aa,bb}
TRAP (U/L)	24.28 ± 4.52	40.33 ± 6.92 ^{aa}	27.70 ± 4.61 ^{bb}	35.14 ± 3.32 ^{aa,b}	32.36 ± 2.88 ^{aa,bb}
Cath-K (pg/ml)	1334.75 ± 64.06	1743.22 ± 70.25 ^{aa}	1529.04 ± 73.00 ^{aa,bb}	1631.72 ± 60.78 ^{aa,bb}	1476.09 ± 60.40 ^{aa,bb}
Urine Ca (mmol/L)	0.48 ± 0.09	1.02 ± 0.18 ^{aa}	0.72 ± 0.14 ^{bb}	0.82 ± 0.21 ^{aa,b}	0.72 ± 0.21 ^{bb}
Urine P (mmol/L)	32.74 ± 9.30	58.76 ± 9.73 ^{aa}	41.33 ± 3.33 ^{bb}	49.13 ± 3.33 ^{aa,b}	47.82 ± 4.48 ^{aa,bb}

Data are presented as mean ± SD (n = 8). Multiple comparisons were done using one-way ANOVA analyses

^a*P* < 0.05; ^{aa}*P* < 0.01 versus the sham control group; ^b*P* < 0.05; ^{bb}*P* < 0.01 versus the OVX control group

levels of bone resorption markers in serum (TRAP and Cath-K) were remarkably decreased (*P* < 0.01). The content in Ca and P in urine was decreased on average by 24.5 and 17.5%, respectively. These results suggest that *Gm*-SGP prominently improved the adverse status of bone turnover.

Gm-SGP enhanced tibial BMD and bone biomechanical property in OVX rats

BMD is a gold standard for the diagnosis of osteoporosis and has been used to reflect bone loss (Cao et al., 2014). As shown in Fig. 1(A), tibial BMD of OVX rats was

Fig. 1 Effects of *Gm*-SGP on BMD, Ca content and biomechanical properties of bones in OVX-induced osteoporotic rats. Right tibias were dissected and their BMD was measured by dual-energy X-ray absorptiometry (A); right femurs were collected to measure bone calcium content by ICP-MS system (B); left femurs were collected

and their ultimate load and stiffness were measured using a three-point bending bone strength tester (C). Data are presented as mean ± SD (n = 8). Multiple comparisons were done using one-way ANOVA analyses. ^a*P* < 0.05, ^{aa}*P* < 0.01 versus the sham control group; ^b*P* < 0.05, ^{bb}*P* < 0.01 versus the OVX control group

significantly decreased and quantitative analysis revealed that the tibial BMD of the OVX group was reduced by 29.7% compared to the Sham control group ($P < 0.01$). However, tibial BMD was recovered in a dose-dependent manner by *Gm*-SGP since quantitative analysis showed that high doses of *Gm*-SGP significantly enhanced tibial BMD (+134%)s when compared with the OVX group [Fig. 1(A)]. ICP-MS analysis [Fig. 1(B)] showed that intragastric administration of *Gm*-SGP clearly increased bone Ca content, which further demonstrates that *Gm*-SGP significantly enhanced BMD and prevented bone loss in OVX rats.

Bone loss and microstructure degeneration induced by estrogen deficiency lead to increased risk of fractures and biomechanical properties is currently the best indicator to predict this risk of fractures (Xia et al., 2015). Ma et al. reported that bone ultimate load property is an indicator for osteoporotic fracture (Ma et al., 2009). Wu et al. reported that enhance the bone stiffness could improve the osteoporosis in OVX rat (Wu et al., 2015). In this study, both ultimate load and tibial stiffness were measured by a three-point bending test to evaluate the bone biomechanical properties. As shown in Fig. 1(C), both ultimate load and tibial stiffness were reduced in the OVX group ($P < 0.01$). Fortunately, compared with the OVX group, high doses of *Gm*-SGP significantly increased ultimate load and stiffness by 25.84% and 20.91%, respectively ($P < 0.01$), indicating that *Gm*-SGP could enhance the bone biomechanical properties in OVX-induced rat.

Gm-SGP restored bone microarchitecture in OVX rats

The changes in bone microarchitecture were examined by H&E staining (Fig. 2). Compared with the OVX control group, the trabecular microarchitecture was thick, serried and the number of trabecular bones was increased after treatment with *Gm*-SGP in OVX rats, these effects being similar in the ALN group. Further verification by quantitative analysis revealed that the trabecular separation (Tb. sp) was significantly increased in OVX rats. In contrast, trabecular area (Tb. Ar) and trabecular thickness (Tb. Th) were clearly decreased in the OVX control group compared to the Sham control group. Treatment with *Gm*-SGP conversely resulted in well-developed trabecular microarchitecture. Morphometry data showed that high doses of *Gm*-SGP reduced Tb. Sp by 36.1% and increased Tb. Ar and Tb. Th by 73.9 and 85.8%, respectively ($P < 0.01$), indicating that *Gm*-SGP prominently restored bone microarchitecture in OVX rats.

Gm-SGP down-regulated key genes expression related to BMP-2/Smads signaling pathway

In this study, we further investigated the molecular mechanisms responsible for the regulation of *Gm*-SGP on high bone turnover activity (i.e., the main characteristic of postmenopausal osteoporosis). The BMP-2/Smads pathway is one of the critical signaling cascades that regulate bone

Fig. 2 Effects of *Gm*-SGP on the bone histomorphology of osteoporotic rats. The proximal femur was fixed in 10% neutral formaldehyde, then decalcified in 10% EDTA for 3 weeks and subsequently embedded in paraffin before being sectioned (5- μ m thickness) and stained with H&E (10 $\times$ magnification). The parameters of bone histomorphometry including trabecular area (Tb. Ar), trabecular

thickness (Tb. Th), and trabecular separation (Tb. Sp) were selected for quantitative analysis. Data are presented as mean $\pm$ SD ($n = 8$). Multiple comparisons were done using one-way ANOVA analyses. ^a $P < 0.05$, ^{aa} $P < 0.01$ versus the sham control group; ^b $P < 0.05$, ^{bb} $P < 0.01$ versus the OVX control group

Fig. 3 Effects of *Gm-SGP* on mRNA and protein expressions of key genes in the BMP-2/Smads signaling pathway. RNA and protein were extracted from distal femurs and the mRNA expression including BMP-2, Smad-1 and Smad-4 were measured by qRT-PCR (A); protein expression of BMP-2 (B), Smad1, p-Smad1 (C) and Smad4

(D) were measured by western blot. Data are presented as mean $\pm$ SD ($n = 8$). Multiple comparisons were done using one-way ANOVA analyses. ^a $P < 0.05$ ^{aa} $P < 0.01$ versus the sham control group; ^b $P < 0.05$, ^{bb} $P < 0.01$ versus the OVX control group

remodeling. BMP-2 induced phosphorylation of type I and type II receptor kinase and then activated type I receptor, transferring the signal to the cytoplasm by phosphorylating the downstream target Smad1/5/8. The latter then interacted with Smad4 and translocated into the nucleus to regulate the target gene (Jian et al., 2015). Yang et al. reported a decrease in BMP-2 and Smad1/5/8, which participated in the canonical BMP-2/Smads signaling pathway inhibiting osteoblast differentiation and high bone turnover rate (Yang et al., 2016). Xia et al. and Wang et al. reported that *Ca-SGP* down-regulates the expression of BMP-2 in mRNA and the secretion levels to improve postmenopausal osteoporosis (Wang et al., 2016; Xia et al., 2016). As shown in Fig. 3(A), treatment with *Gm-SGP* decreased the mRNA levels of BMP-2, Smad-1 and Smad-4 on average by 30.61, 23.30 and 23.15% in OVX rats, respectively

($P < 0.01$). Furthermore, in translation level, protein expression of BMP-2 [Fig. 3(B)], phosphorylated Smad-1 [Fig. 3(C)] and Smad-4 [Fig. 3(D)] were also decreased by *Gm-SGP* in a dose-dependent manner ($P < 0.01$), indicating that the signal transduction in the BMP-2/Smads pathway was inhibited. All these results suggested that *Gm-SGP* can down-regulate the activity of the BMP-2/Smads pathway to inhibit the abnormal high bone turnover rate.

***Gm-SGP* decreased key genes expression related to the Wnt/β-catenin signaling pathway**

Wnt/β-catenin is another key pathway in the regulation of bone formation (Baron and Rawadi, 2007). The canonical Wnt signal is triggered by the interaction of Wnt protein with Frizzled (FZD) receptor and lipoprotein receptor-

Fig. 4 Effects of *Gm-SGP* on mRNA and protein expressions of key genes in the Wnt/ β -catenin signaling pathway. RNA and protein were extracted from distal femurs and the mRNA expression of Lrp-5 and β -catenin were measured by qRT-PCR (A); protein expression of total β -catenin (B) and intracellular β -catenin (C) were measured by

western blot. Data are presented as mean $\pm$ SD ($n = 8$). Multiple comparisons were done using one-way ANOVA analyses. ^a $P < 0.05$, ^{aa} $P < 0.01$ versus the sham control group; ^b $P < 0.05$, ^{bb} $P < 0.01$ versus the OVX control group

related 5/6 protein (LRP 5/6) co-receptor. These results lead to an accumulation of β -catenin in the cytoplasm which then translocates into the nucleus and combines with transcription factors to regulate the transcription of target genes (Day et al., 2005). LRP 5-deficient mice show reduced osteoblast proliferation in OVX mice and maintained the normal bone turnover rate (Babij et al., 2003; Kato et al., 2002). β -catenin gene knock-out mice revealed no bone mass formation and the process of osteoblast differentiation was limited in the early stage (Hu et al., 2005). Wang et al. reported that the Wnt/ β -catenin signaling pathway was overactive in OVX rats, thus contributing to the abnormal high bone turnover rate (Wang et al., 2016). In transcription level, mRNA expression of Lrp-5b and β -catenin were significantly elevated in the OVX control group, demonstrating that the Wnt/ β -catenin

pathway was activated [Fig. 4(A)]. Fortunately, treatment with *Gm-SGP* changed this situation. Figure 4(A) shows that different doses of *Gm-SGP* decreased mRNA expression of Lrp-5b and β -catenin on average by 30.25 and 21.85%, respectively ($P < 0.01$). In the same way, in translation level, *Gm-SGP* clearly down-regulated the protein expression of total β -catenin and intracellular β -catenin which revealed that the accumulation and the intracellular translocation of β -catenin were inhibited by *Gm-SGP* [Fig. 4(B, C)]. All these data suggest that *Gm-SGP* decreased the Wnt/ β -catenin signaling pathway activity to limit OVX-induced osteoporosis.

Fig. 5 Effects of *Gm-SGP* on the transcription of Runx2 and Osx. RNA and protein were extracted from distal femurs and the mRNA expression of Runx2 and Osx were measured by qRT-PCR (A); protein expression of Runx2 was measured by western blot (B). Data

are presented as mean $\pm$ SD (n = 8). Multiple comparisons were done using one-way ANOVA analyses. ^a*P* < 0.05, ^{aa}*P* < 0.01 versus the sham control group; ^b*P* < 0.05, ^{bb}*P* < 0.01 versus the OVX control group

Fig. 6 Effects of *Gm-SGP* on gene expression of osteogenesis markers ALP, Col1, and OCN. RNA was extracted from distal femurs and the mRNA expression of ALP, Col1, and OCN were measured by qRT-PCR. Data are presented as mean $\pm$ SD (n = 8).

Multiple comparisons were done using one-way ANOVA analyses. ^a*P* < 0.05, ^{aa}*P* < 0.01 versus the sham control group; ^b*P* < 0.05, ^{bb}*P* < 0.01 versus the OVX control group

Gm-SGP attenuated the transcriptions of Runx2 and Osterix

Runx-related transcription factor 2 (Runx2) and Osterix (Osx) are essential osteogenic transcription factors of the BMP-2/Smads and Wnt/ β -catenin signaling pathways

(Komori et al., 1997). An increased number of studies has revealed that Runx2 plays an important role in the maturation of osteoblasts (Gerstenfeld et al., 1996). In addition, BMP-2 and Wnt signals transduction is highly dependent on Runx-2 (Lian et al., 2006). In mice with no Runx2 expression, the differentiation of osteoblasts is stopped in

both intramembranous and endochondral skeleton (Nakashima et al., 2002). *Osx* (also known as *Sp7*) is a zinc finger-containing osteoblast-specific transcription factor that plays an essential role in osteoblast proliferation, differentiation and bone formation (Nakashima et al., 2002). Genetic studies have identified *Osx* as a downstream gene of *Runx2* in the osteoblast differentiation signaling pathways (An et al., 2016). As shown in Fig. 5(A), *Gm*-SGP dose-dependently decreased the transcription level of *Runx2* and *Osx*. Indeed, high doses of *Gm*-SGP reduce the mRNA expression of *Runx2* and *Osx* by 47.1 and 37.6% in OVX rats, respectively ($P < 0.01$). In addition, the translational level of *Runx2* was further investigated. As shown in Fig. 5(B), the protein level of *Runx2* was up-regulated in the OVX group and as expected, it was significantly reduced by *Gm*-SGP ($P < 0.01$). All these results further suggest that *Gm*-SGP down-regulated the transcription of *Runx2* and *Osx* to inhibit the activity of the BMP-2/Smads and Wnt/ β -catenin signaling pathways.

***Gm*-SGP decreased the osteogenesis markers expression in OVX rats**

ALP, CoL1, and OCN are important bone phenotypic markers in osteogenesis and the downstream target genes of the BMP-2/Smads and Wnt/ β -catenin signaling pathways (Gerstenfeld et al., 1996). As shown in Fig. 6, *Gm*-SGP decreased their expression levels in a dose-dependent manner. Different doses of *Gm*-SGP reduced the expression of ALP, CoL1, and OCN on average by 42.48, 28.04 and 30.25%, which were significantly up-regulated in the OVX control group ($P < 0.01$). These data further demonstrate that the decrease in the BMP-2/Smads and Wnt/ β -catenin signals by *Gm*-SGP inhibited the abnormal high bone formation rate in vivo.

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Compliance with ethical standards

Conflict interests The authors declare no competing financial interest.

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