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Downregulation of GNAS inhibits osteogenesis of bone marrow mesenchymal stem cells and promotes osteoporosis through the Wnt pathway

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Abstract. – OBJECTIVE: This study aims to explore the role of GNAS in accelerating the progression of osteoporosis by inhibiting osteogenesis of BMSCs by the Wnt pathway.

PATIENTS AND METHODS: GNAS levels in OP tissues and BMSCs undergoing osteogenesis for different time points were detected. Regulatory effects of GNAS on osteogenesis-related gene expressions, ALP activity, capability of mineralization, and activation of the Wnt pathway in BMSCs were assessed through a series of functional experiments. At last, rescue experiments were performed to further verify the significance of the Wnt pathway during GNAS-mediated osteogenesis development.

RESULTS: GNAS was downregulated in OP tissues relative to normal bone tissues. With the prolongation of osteogenesis, GNAS level gradually increased in BMSCs. Knockdown of GNAS downregulated expression levels of ALP and RUNX2, and attenuated ALP activity and capability of mineralization in BMSCs. GNAS was able to activate the Wnt pathway in BMSCs. Notably, overexpression of Wnt3a could reverse the regulatory effects of GNAS on osteogenesis-related gene expressions, ALP activity, and capability of mineralization in BMSCs.

CONCLUSIONS: Downregulation of GNAS suppresses osteogenesis of BMSCs through the Wnt pathway, thus aggravating the progression of osteoporosis.

Key Words:
Osteoporosis, BMSCs, GNAS, Wnt pathway, Osteogenesis.

Introduction

The incidence of osteoporosis (OP) is on the rise due to the aging population. OP patients are prone to develop fractures, which severely affect life quality of the elderly[1,2]. The imbalance between bone absorption and bone formation contributes to the etiology of OP. Currently, preventive and therapeutic drugs for OP are developed based on inhibition of bone absorption. Although the bone remodeling process is blocked, the loss of bone volume and multiple adverse events are unavoidable. It is of significance to develop effective approaches to accelerate bone formation, thereafter, preventing the incidence of OP[3].

Mesenchymal stem cells (MSCs) are extensively distributed in various tissues and organs, showing self-renewal and multi-differentiation potentials. Bone marrow derived MSCs (BMSCs) are well concerned nowadays because of their critical roles in regulating bone metabolism and relevant signaling pathways[4]. Key events and factors influencing differentiation potential of BMSCs are the research focuses[5].

Guanine nucleotide binding protein subunit α (GNAS) is a key component of the cell membrane receptor pathway, which is able to enhance the second messenger cyclic AMP by activating adenylate cyclase and in turn, affects life activities[6]. It is reported that GNAS in human and mice could encode multiple protein splicing. Base mutations of GNAS are closely linked to the occurrence of McCune-Albright syndrome (manifested as multiple bone dysplasia, non-prominent skin brown pigmentation, and precocious puberty) and pseudo-hypoparathyroidism[7]. An et al[8] have shown the promotive function of GNAS in the osteogenesis of BMSCs.

The Wnt pathway is a vital signaling in mediat-

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ing bone metabolism. It may be utilized as a therapeutic target for OP and bone repair due to the crucial role in osteogenesis of BMSCs[9-11]. Yu et al[10] discovered that Wnt4 inhibits TGFI3-induced activation of the NF-κB pathway in macrophages and osteoclast precursor cells, and cell differentiation of BMSCs into osteoblasts through the Wnt pathway. In this paper, we aim to elucidate the role of GNAS in influencing BMSCs differentiation and the involvement of the Wnt pathway.

Patients and Methods

BMSCs Culture and Transfection

Human bone marrow was extracted, diluted in phosphate-buffered saline (PBS), and centrifuged at 1800 r/min for 10 min. The precipitant was diluted in the isodose lymphocyte separation solution, and centrifuged at 3000 r/min for 30 min. The medium layer was yellowish brown cell layer, which was collected, washed with PBS, and centrifuged at 1000 r/min for 5 min, for a total of three times. After adjustment of cell density to 2×10^6/mL, cell suspension was inoculated and cultured for 24 h. Non-adherent cells were discarded by replacing the medium and PBS washing on the other day. Subsequently, medium was replaced every two days. This study was approved by the ethics committee of Taizhou Municipal Hospital. For transfection, 100 pmoL vector and 5 μl of Lipofectamine2000 were respectively diluted in 250 μl of serum-free Opti-MEM. The mixture was applied in BMSCs for 6-8 h.

Osteogenesis

The fourth-generation BMSCs were cultured overnight at 70-80% confluence. Osteogenic induction medium was applied: Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) + 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) + 1% penicillin-streptomycin + 10 mmol/L β-glycerophosphate + 50 μg/ml ascorbic acid. Fresh medium was replaced every 2 days. Osteogenic induction was conducted for different time points in BMSCs.

ALP Activity Determination

After osteogenesis in BMSCs, cells were washed with pre-cold PBS for three times and lysed in pre-cold 1% Triton X-100 on ice for 30 min. Cell lysate was subjected to ALP activity determination, and the value was normalized to that of total protein concentration.

Alizarin Red Staining (ARS)

BMSCs were collected, resuspended in low-glucose DMEM, and cultured overnight. On the next day, complete DMEM was replaced. Until the formation of visible mineralized nodules, cells were incubated in 95% ethanol for 10 min, followed by incubation with 0.1% ARS-Tris-HCL solution (pH4.3) at 37#C for 30 min. Nodules were observed and captured using an inverted microscope.

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

Total RNA of BMSCs was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) method. The cDNA was obtained through reverse transcription of RNA, and the SYBR Green method was used for PCR detection. The primer sequences were listed as follows: GNAS, F: 5′-ACATCACCGTGGCACCCAGACCTCCCTC-3′, R: 5′-ATCTTTTTGTTGGCCTCACG-3′; RUNX2, F: 5′-GACGTGCCCAGGCGTATTTC-3′, R: 5′-AAGGTGGCTGGGTAGTGCATTC-3′; ALP, F: 5′-GGAATACGAATGAGAAGGCC-3′, R: 5′-CAGTTCAGTGCGGTTCCAGACATAG-3′; GAPDH, F: 5′-TTCTTTTGCGTCGCCAGCCGA-3′, R: 5′-GTCACCACCCGCCCAATACGA-3′.

Western Blot

The total protein from BMSCs was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and loaded for electrophoresis. Protein sample was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), they were blocked in 5% skim milk for 2 h, incubated with primary antibodies (Cell Signaling Technology; Danvers, MA, USA) at 4°C overnight. The membranes were then washed with Tris-Buffered Saline with Tween-20 (TBST) and followed by the incubation of secondary antibody at room temperature for 1 h. Bands were exposed by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA) and analyzed by Image Software.

Statistical Analysis

Statistical Product and Service Solution 22.0 (SPSS IBM Corp., Armonk, NY USA) was used for all statistical analysis. Data were expressed as mean ± SD. The *t*-test was used for analyzing

differences between the two groups. $p<0.05$ indicated the significant difference.

Results

GNAS Was Downregulated in OP

QRT-PCR data revealed a lower abundance of GNAS in OP tissues than that of normal bone tissues (Figure 1A). Similarly, protein level of GNAS was identically downregulated in three tested cases of OP tissues compared to that of normal ones (Figure 1B). Downregulated GNAS may be related to the progression of OP.

Knockdown of GNAS Alleviated Osteogenesis of BMSCs

Dynamical levels of GNAS in BMSCs undergoing osteogenesis for different time points were determined. It is shown that GNAS level time-dependently increased with the prolongation of osteogenesis in BMSCs from day 3 to day 10 (Figure 2A). Subsequently, sh-GNAS was constructed and its transfection efficacy was tested (Figure 2B, 2C). In BMSCs undergoing osteogenesis, transfection of sh-GNAS markedly downregulated mRNA levels of ALP and RUNX2 (Figure 2D), as well as protein level of RUNX2 (Figure 2E). Moreover, ALP activity (Figure 2F) and capability of mineralization (Figure 2G) were markedly declined after silence of GNAS in BMSCs.

Overexpression of GNAS Accelerated Osteogenesis of BMSCs

To further ascertain the biological function of GNAS in influencing osteogenesis of BMSCs, LV-GNAS was constructed and transfection of it remarkably upregulated GNAS level (Figure 3A, 3B). Relative levels of ALP and RUNX2 were markedly upregulated in BMSCs overexpressing GNAS under osteogenic induction (Figure 3C, 3E). In addition, overexpression of GNAS greatly enhanced ALP activity (Figure 3D) and capability of mineralization (Figure 3F) in BMSCs.

GNAS Regulated the Wnt Pathway

Western blot was conducted to investigate the regulatory effects of GNAS on the Wnt pathway. As data revealed, transfection of sh-GNAS markedly downregulated protein levels of Wnt3a and β-catenin in BMSCs (Figure 4A). Conversely, overexpression of GNAS yielded the opposite trends on protein levels of Wnt3a and β-catenin (Figure 4B).

GNAS Regulated Osteogenesis of BMSCs Through the Wnt Pathway

Of note, in BMSCs co-transfected with sh-GNAS and pcDNA-Wnt3a, mRNA levels of ALP and RUNX2, as well as protein levels of Wnt3a, β-catenin, and RUNX2 were much higher compared with those transfected with only sh-GNAS (Figure 5A, 5B). Furthermore, declined ALP

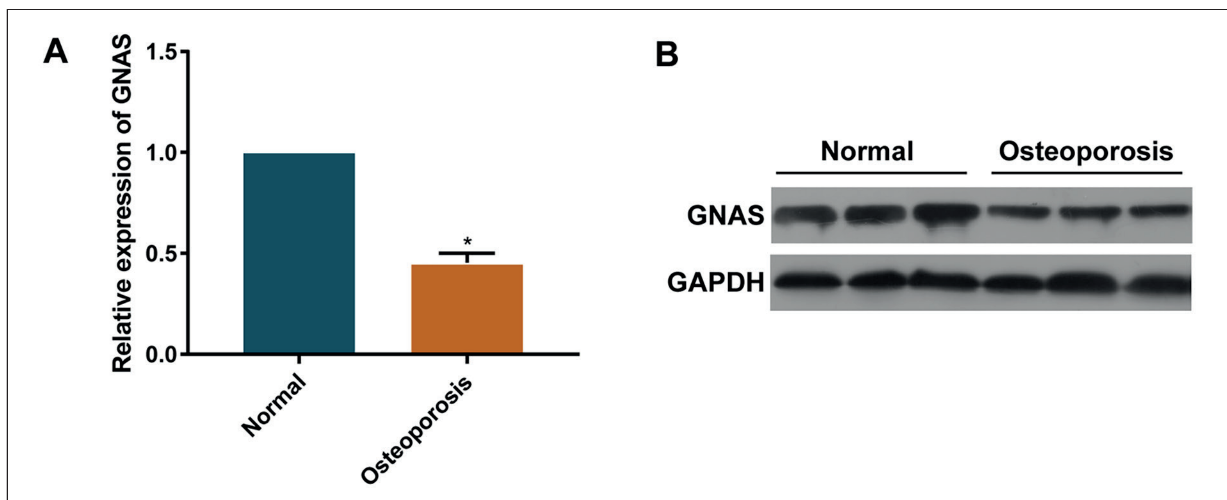

Figure 1. GNAS was downregulated in OP. **A, B,** The mRNA (**A**) and protein levels of GNAS (**B**) in OP tissues and normal bone tissues.

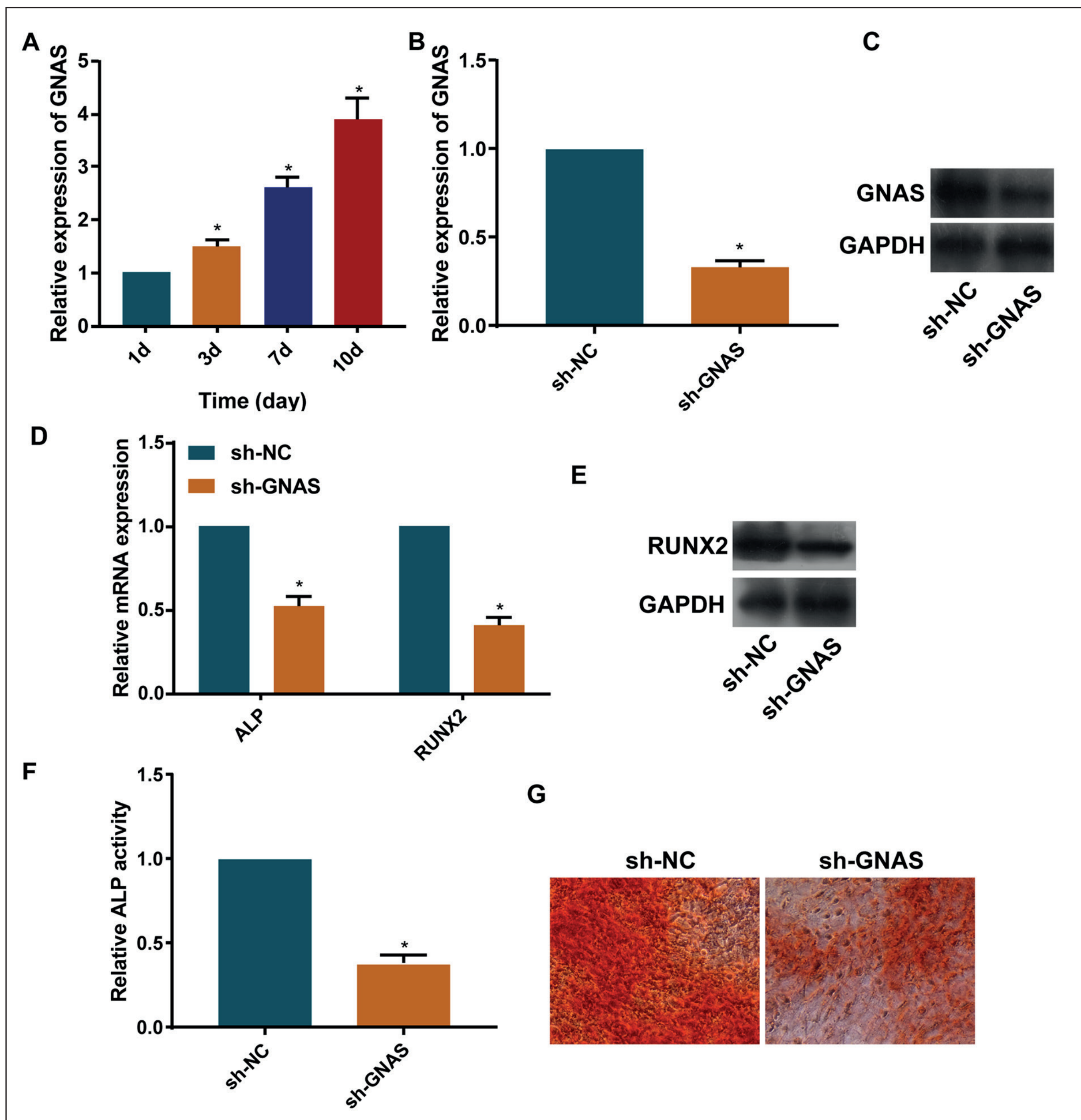

Figure 2. Knockdown of GNAS alleviated osteogenesis of BMSCs. **A,** GNAS level in BMSCs undergoing osteogenesis for day 1, 3, 7 and 10. **B, C,** mRNA (**B**) and protein level of GNAS (**C**) in BMSCs transfected with sh-NC or sh-GNAS. **D,** mRNA levels of ALP and RUNX2 in BMSCs transfected with sh-NC or sh-GNAS. **E,** Protein level of RUNX2 in BMSCs transfected with sh-NC or sh-GNAS. **F,** ALP activity in BMSCs transfected with sh-NC or sh-GNAS. **G,** Capability of mineralization in BMSCs transfected with sh-NC or sh-GNAS (magnification 100×).

activity (Figure 5C) and capability of mineralization (Figure 5D) in BMSCs with GNAS knockdown were partially reversed by overexpression of Wnt3a. It is further confirmed that the Wnt pathway was responsible for GNAS-mediated osteogenesis of BMSCs.

Discussion

OP is a systematic metabolic bone disease associated with aging. With the acceleration of aging, OP has become a critical public health problem globally[12]. Primary OP generally affects middle-aged and

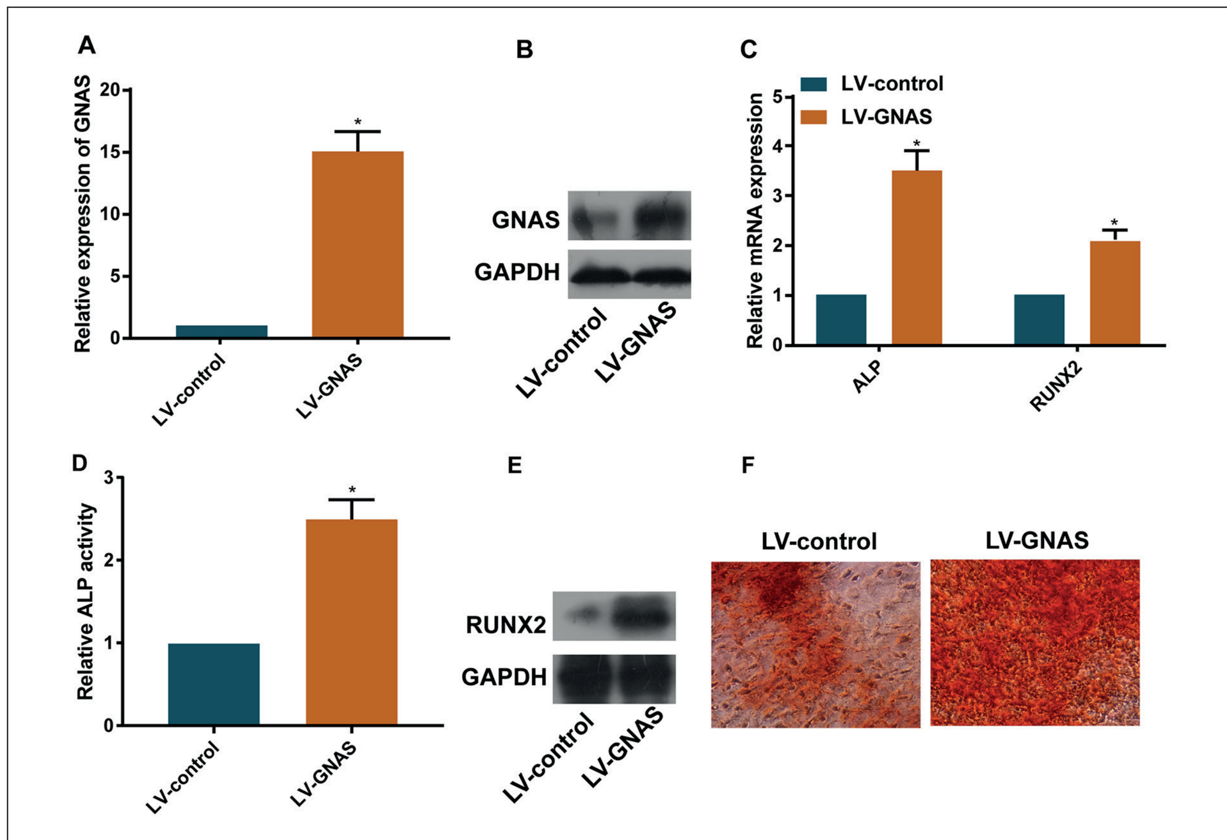

Figure 3. Overexpression of GNAS accelerated osteogenesis of BMSCs. **A, B,** mRNA (**A**) and protein level of GNAS (**B**) in BMSCs transfected with LV-control or LV-GNAS. **C,** mRNA levels of ALP and RUNX2 in BMSCs transfected with LV-control or LV-GNAS. **D,** ALP activity in BMSCs transfected with LV-control or LV-GNAS. **E,** Protein level of RUNX2 in BMSCs transfected with LV-control or LV-GNAS. **F,** Capability of mineralization in BMSCs transfected with LV-control or LV-GNAS (magnification 100×).

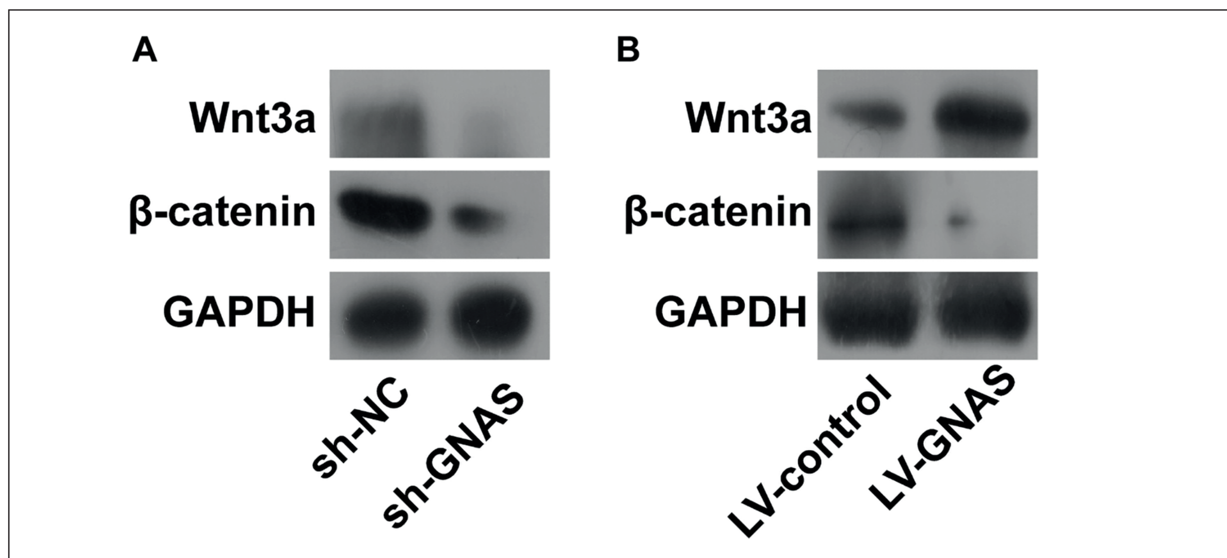

Figure 4. GNAS regulated the Wnt pathway. **A,** Protein levels of Wnt3a and β-catenin in BMSCs transfected with sh-NC or sh-GNAS. **B,** Protein levels of Wnt3a and β-catenin in BMSCs transfected with LV-control or LV-GNAS.

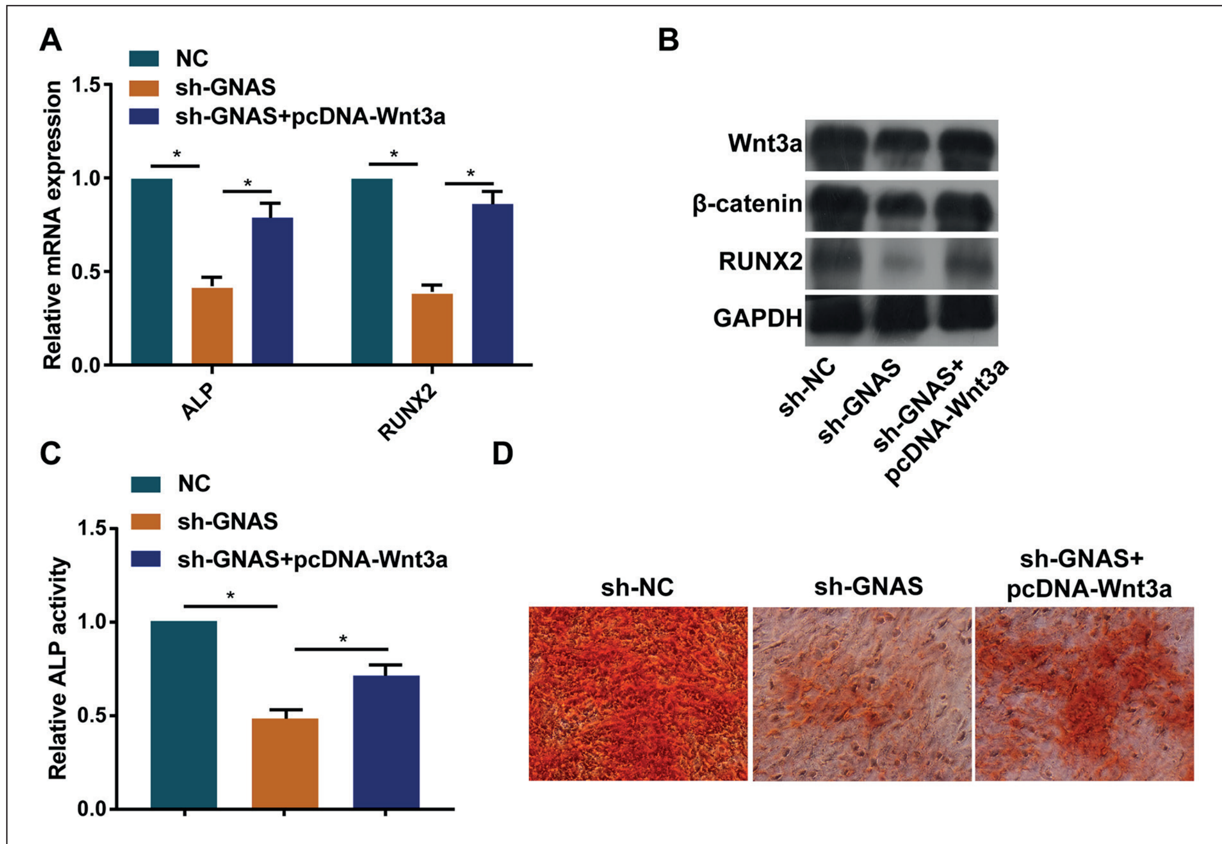

Figure 5. GNAS regulated osteogenesis of BMSCs through the Wnt pathway. BMSCs were transfected with NC, sh-GNAS or sh-GNAS+pcDNA-Wnt3a. **A,** mRNA levels of ALP and RUNX2. **B,** Protein levels of Wnt3a, β-catenin, and RUNX2. **C,** ALP activity. **D,** Capability of mineralization (magnification 100×).

post-menopausal women, with the clinical manifestations of bone volume decline and microstructural changes of bone tissues. Degenerations of osteoblast function and quantity are of significance in the occurrence and progression of OP[13]. Under a certain induction, BMSCs can be differentiated into osteoblasts, and further utilized as a vital approach for OP prevention and treatment[14,15].

GNAS locates on chromosome 20q 13.32 and consists of 13 exons. It is a complex imprinted expression gene encoding multiple gene products. The GSα protein is a heterotrimeric G protein subunit. In addition, a variety of transcripts and proteins are encoded by different promoters and variable splicing methods[16]. GNAS deficiency exerts a key role in ectopic osteoblast differentiation[17,18]. In our experiment, GNAS was lowly expressed in OP tissues, and responsible for osteogenesis of BMSCs.

The name Wnt is a portmanteau created from the name Wingless and the name Int-1[19]. The Wnt pathway accurately regulates a variety of cellular behaviors and tumor phenotypes[20]. Bone metabolism is dynamically mediated by the Wnt/β-catenin pathway[21]. Blockade of the Wnt pathway triggers the phosphorylation of β-catenin by interacting with axin, APC, and GSK3β in a ubiquitin/proteasome pathway. Conversely, the activated Wnt pathway induces the binding between Wnt proteins (Wnt3a, Wnt7b) with Frizzled family receptor and LRP5/6, thus transporting the biological signal to the Dishevelled protein inside the cell. As a result, accumulated cytoplasmic β-catenin is translocated into the nucleus, where interacting with TCF/LEF to further regulate bone metabolism-related genes[22,23]. Gaur et al[24] demonstrated that the Wnt pathway triggers osteoblast differentiation and accelerates bone fracture healing by upregulating RUNX2. In our study, silence of GNAS downregulated mRNA levels of ALP and RUNX2, protein levels of Wnt3a, β-catenin, and RUNX2, ALP activity, and calcification ability of BMSCs. The above findings were partially reversed after overexpression of Wnt3a.

To sum up, GNAS was identified in our research to promote osteogenesis of BMSCs through the Wnt pathway, thus alleviating the progression of OP. Our findings provide novel directions in clinical prevention and treatment of OP.

Conclusions

The downregulation of GNAS suppresses osteogenesis of BMSCs through the Wnt pathway, thus aggravating the progression of osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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