LGR5 enhances the osteoblastic differentiation of MC3T3-E1 cells through the Wnt/β-catenin pathway

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Abstract. Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is a Wnt-associated gene that contributes to cell proliferation and self-renewal in various organs. LGR5 is expressed in Ewing sarcoma, and LGR5-overexpressing mesenchymal stem cells promote fracture healing. However, the effects of LGR5 on osteoblastic differentiation remain unclear. The aim of the present study was to explore the function of LGR5 in osteoblastic differentiation. LGR5 was overexpressed or knocked down in the MC3T3-E1 pre-osteoblastic cell line via lentiviral transfection and its function in osteoblastic differentiation was investigated. The mRNA expression levels of the osteoblast differentiation markers alkaline phosphatase (ALP), osteocalcin and collagen type I a1 were determined, and ALP and Alizarin red staining were performed. In addition, the effects of LGR5 modulation on β-catenin and the expression of target genes in the Wnt pathway were investigated. The results revealed that the overexpression of LGR5 promoted osteoblastic differentiation. This was associated with enhancement of the stability of β-catenin and its levels in the cell nucleus, which enabled it to activate Wnt signaling. By contrast, the inhibition of LGR5 decreased the osteogenic capacity of MC3T3-E1 cells. These results indicate that LGR5 is a positive regulator of osteoblastic differentiation, whose effects are mediated through the Wnt/β-catenin signaling pathway. This suggests suggesting that the regulation of LGR5/Wnt/β-catenin signaling has potential as a therapy for osteoporosis.

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Introduction

Bone homeostasis is maintained by the balance between the formation of bone by osteoblasts and the resorption of bone by osteoclasts (1). Osteoporosis is a systemic skeletal disease that results from the disruption of bone homeostasis with excessive bone resorption and/or reduced bone formation (2). Treatments for osteoporosis include antiresorptive agents to inhibit bone resorption and anabolic agents to promote bone formation (3). Although medications for osteoporosis have been developed and used successfully in recent years, most of them are antiresorptive drugs. Antiresorptive drugs including bisphosphonates, estrogen and receptor activator of NF-κB ligand inhibitors, prevent the loss of bone rather than restore it (4,5). In addition, parathyroid hormone, the only US Food and Drug Administration-approved anabolic agent, has limitations of high cost and invasive modes of administration (6). Therefore, it is necessary to explore the mechanisms of osteoblastic differentiation to facilitate the search for new anabolic agents for the treatment of osteoporosis.

Leucine-rich repeat-containing G-protein coupled receptors (LGRs) belong to the G-protein-coupled receptor family, which transmit extracellular signals into the cytoplasm (7). LGR5 is one of the group B LGR proteins (LGR4-6), which recognize R-spondin (Rspo) proteins to activate Wnt signaling (8,9). LGR5 is considered a stem cell marker, and plays an important role in normal development and cancer. It is involved in the self-renewal and stem cell development of tissues including hair follicles, the stomach, small intestine and colon (10-12). The genetic deletion of LGR5 in mice results in 100% neonatal lethality (13). Also, LGR5 has been shown to promote tumor growth and progression in colorectal carcinoma (14), basal cell carcinoma (15), glioblastoma (16) and neuroblastoma (17). Since its close homologs LGR4 and LGR6 have been reported to participate in bone formation (18,19), the role of LGR5 in bone remodeling has also become a topic of interest. Furthermore, LGR5 is upregulated in Ewing sarcoma, a malignant bone tumor, and promotes tumor progression through Wnt/β-catenin signaling (20). A recent study revealed that mesenchymal stem cells overexpressing LGR5 promote the healing of fractures through Wnt/ERK signaling pathways (21). All these previous findings suggest a potential role of LGR5 in bone remodeling. However, the effects of LGR5

on osteoblastic differentiation and the underlying mechanism remain unclear. Thus the present study aimed to explore the function of LGR5 in osteoblastic differentiation using the MC3T3-E1 pre-osteoblastic cell line.

Materials and methods

Cell culture. The MC3T3-E1 murine pre-osteoblastic cell line and C2C12 myoblastic cell line were obtained from the American Type Culture Collection. MC3T3-E1 cells were cultured in α-minimum essential medium (HyClone; Cytiva) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The medium was supplemented with 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. C2C12 cells were cultured in high-glucose DMEM culture medium (HyClone; Cytiva) containing 10% FBS and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. The culture medium was replaced thrice each week. After the cells had grown to 70% confluence, the medium was changed to osteogenic media containing 4 mM β-glycerophosphate and 25 μg/ml ascorbic acid for the induction of osteoblastic differentiation. Dickkopfs-1 (Dkk-1; PeproTech, Inc.) was used as a Wnt inhibitor at a concentration of 100 ng/ml and Wnt-3a (PeproTech, Inc.) was used as a Wnt activator at a concentration of 20 ng/ml. Rspo-2 (PeproTech, Inc.) was added for osteogenic induction at a concentration of 100 ng/ml. The duration of treatment is specified in each respective assay section.

Lentiviral transfection of MC3T3-E1 cells. The lentivirus-based LGR5 overexpression vector based on green fluorescent protein (GFP)-PURO and short hairpin RNA (shRNA)-GFP-PURO were purchased from Shanghai GeneChem Co., Ltd.. A lentivirus-based LGR5 overexpression vector (lv-LGR5) was designed using the primer sequences of murine LGR5 (GenBank number, NM_010195.2) cDNA as follows: Sense, 5'-CTTCTCGAGCTACTTCGGGCACCA TGGAC-3' and antisense, 5'-GCGGGTACCTTAGAGACA TGGGACAAATG-3'. The sequences of the shRNA targeting LGR5 (sh-LGR5) were as follows: Sense, 5'-GCAACAACA UCAGGUCAAUTT-3' and antisense, 5'-AUUGACCUGAUG UUGUUGCTT-3'. A scrambled shRNA (TTCTCCGAACGT GTCACGTAA) with no complementary sequences in the murine genome, was used as a negative control. MC3T3-E1 cells were seeded in 6-well plates ($1.5x10^4$ cells/cm^2). When the cells had grown to 70% confluence, the experimental lentivirus or negative control lentivirus at a multiplicity of infection of 20 in 1 ml was used to transfect the cells at 37˚C for 24 h. Post-transfection, the transfection medium was discarded and replaced by selective medium including 2 μg/ml puromycin (Sigma-Aldrich; Merck KGaA). After 9 days, the third passage of stable clones was collected in the following experiments. The transfection efficiency was evaluated based on the percentage of GFP-positive cells, and the overexpression or knockdown of LGR5 was verified using reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis.

Immunofluorescence (IF). MC3T3-E1 and C2C12 cells were seeded on coverslips in 24-well plates ($2x10^4$ cells/cm^2). After washing with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.5% Triton X-100 for another 20 min at room temperature. The cells were then incubated with primary antibody against LGR5 (cat. no. ab273092; 1:100) at 4˚C overnight after blocking with 5% bovine serum albumin (2 h at room temperature). The next day, cells were incubated with fluorescent-labeled secondary antibody (cat. no. A0408; 1:200; Beyotime Institute of Biotechnology) for 1 h at room temperature and treated with 4,6-diamidino-2-phenylindole (cat. no. C1002; 1:1,000; Beyotime Institute of Biotechnology) for 15 min for nuclear staining at room temperature. The samples were observed under a confocal microscope.

Cell viability and apoptosis assay. For the assessment of cell viability, transfected cells seeded in 96-well plates ($2x10^4$ cells/cm^2) were treated with 20 μl/well 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; 5 mg/ml). The supernatant was removed after incubation at 37˚C for 2 h. The formazan product in each well was dissolved by dimethyl sulfoxide with gentle shaking for 5 min. The absorbance was then detected using a microplate reader (Bio-Rad Laboratories, Inc.) at 570 nm. Cell viability rates were expressed as fold changes relative to that of the control group.

Cell apoptosis was detected using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, transfected cells were lysed within ice-cold lysis buffer for 30 min and then pelleted by centrifugation for 10 min (200 x g at 4˚C). Mouse monoclonal antibodies against histones (biotin-labeled) and DNA (peroxidase-labeled) from the kit were used to bind nucleosomes for 2 h at 15-25˚C, and the antibody-nucleosome complexes were then bound to the microplate by streptavidin. After washing the immobilized antibody-histone complexes, the samples were incubated with 2,2'-azino-di(3-ethylbenzthiazoline-sulfonate). The apoptosis was colorimetrically determined at 405 nm based on the amount of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates, which was determined using the quantitative sandwich enzyme immunoassay principle. The results were expressed as the ratio of absorbance of the treated (apoptotic) sample to that of the control group.

RT-qPCR analysis. Total cellular RNA was collected using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 7 or 14 days after osteogenic induction according to the manufacturer's instructions. Cells treated with Dkk-1, Wnt-3a and Rspo-2 were collected at 7 days. The RNA was used to generate cDNA using a PrimeScript™ RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was then performed using SYBR® Premix Ex Taq™ (Takara Bio, Inc.) and an ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were used as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and primer annealing and extension at 60˚C for 30 sec and final extension for 1 min at 72˚C. Each sample was separately examined in triplicate. The expression values of the target genes were quantified by the $2^{-\Delta\Delta Cq}$ method (22) with normalization to β-actin. The qPCR primers used were as follows: β-actin forward, 5'-TGACAGGATGCAGAAGGAGA-3' and reverse, 5'-CGC TCAGGAGGAGCAATG-3'; LGR5 forward, 5'-TCTTCA

CCTCCTACCTGGACCT-3' and reverse 5'-GGCGTAGTC TGCTATGTGGTGT-3'; alkaline phosphatase (ALP) forward, 5'-TCGGGACTGGTACTCGGATAAC-3' and reverse, 5'-GTT CAGTGCGGTTCCAGACATAG-3'; osterix (OSX) forward, 5'-GGAGGCACAAAGAAGCCATACGC-3' and reverse 5'-TGCAGGAGAGAGGAGTCCATTG-3'; runt related transcription factor 2 (RUNX2) forward, 5'-GACGAGGCAAGA GTTTCACC-3' and reverse, 5'-GGACCGTCCACTGTCACT TT-3'; collagen type I α1 (COL-1a1) forward, 5'-AAGAAG CACGTCTGGTTGGAG-3' and reverse, 5'-GGTCCATGT AGGCTACGCTGTT-3'; osteocalcin (OCN) forward, 5'-CAA GCAGGGAGGCAATAAGG-3' and reverse, 5'-CGTCAC AAGCAGGGTTAAGC-3'; lymphoid enhancer-binding factor 1 (Lef1) forward, 5'-CGCTGATCAATGCCCCAACTTTCC GGAGGA-3' and reverse, 5'-CCGCTCGAGTCAGATGTA GGCAGCTGTCATTCTG-3'; T-cell factor 1 (Tcf1) forward, 5'-TGCTGTCTATATCCGCAGGAAG-3' and reverse, 5'-CGA TCTCTCTGGATTTTATTCTCT-3'; β-catenin forward, 5'-ACGCTGCTCATCCCACTAAT-3' and reverse, 5'-AGT TCCGCGTCATCCTGATA-3'.

Western blot analysis. Cell lysates were collected from cells on days 0, 7 and 14 of osteogenic induction using ice-cold RIPA lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS with protease inhibitor cocktail (Roche Diagnostics)] at 7 or 14 days after osteogenic induction, and total proteins were extracted using a Protein Extraction kit (Beyotime Institute of Biotechnology). Nuclear and cytoplasmic proteins were extracted using a Nucleoprotein Extraction kit (Sangon Biotech Co., Ltd.) according to the manufacturer's instructions. Protein content was quantified using a Bicinchoninic Acid Protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Equal amounts of proteins (40 μg/lane) were loaded onto gels for 8-12% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk diluted in Tris-buffered saline containing 0.05% (*v/v*) Tween-20 (TBST) for 2 h at room temperature, and then incubated with primary antibodies at 4˚C overnight. After washing thrice with TBST, the membranes were incubated with HRP-conjugated secondary antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at room temperature. The expression of target proteins was visualized by the reaction of HRP with a chemiluminescent substrate (EMD Millipore). GAPDH was used as the control, with the exception of nuclear protein, where histone H3 was used. The primary antibodies used in this experiment were as follows: LGR5 (1:1,000; cat. no. ab273092; Abcam), β-catenin (cat. no. 9562; 1:1,000; Cell Signaling Technology, Inc.), phosphorylated (inactive) β-catenin (cat. no. 9566; 1:1,000; Cell Signaling Technology, Inc.), GAPDH (cat. no. 8884; 1:2,000, Cell Signaling Technology, Inc.) and histone H3 (cat. no. 4499; 1:5,000; Cell Signaling Technology, Inc.).

ALP staining and Alizarin red staining. ALP and Alizarin red staining were performed on MC3T3-E1 cells cultured in osteogenic media for 7 and 14 days, respectively. For ALP staining, cells fixed with 4% paraformaldehyde (4˚C for 30 min) were treated with an ALP substrate mixture from an ALP staining kit (Sigma-Aldrich; Merck KGaA) in darkness (at room temperature for 30 min). After rinsing the cells three times with PBS, they were observed under a light microscope (Leica Microsystems, Inc.). For Alizarin red staining, cells fixed in ice-cold 70% ethanol (4˚C for 20 min) were stained with 3% Alizarin red S solution (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. The cells were washed with double-distilled water three times, and images of mineralized nodules were obtained under a light microscope (Leica Microsystems, Inc.).

TOPflash dual-luciferase reporter assays. A TOPflash Wnt/b-catenin activity assay was performed to detect the activation of canonical Wnt signaling. MC3T3-E1 cells seeded in six-well plates ($2x10^5$ cells/well) were transfected with plasmids containing TOPflash/FOPflash firefly luciferase (500 ng/well; BioVector NTCC, Inc.) and pRL-SV40-renilla luciferase reporter (20 ng/well; (BioVector NTCC, Inc.) for 24 h. The medium was then replaced with osteogenic culture medium containing control lentivirus, lv-LGR5 with or without Wnt-3a or Dkk-1, or Wnt-3a with or without Dkk-1 for another 48 h. The luciferase assay was performed using a Dual-Luciferase Assay kit (Promega Corporation) according to the manufacturer's protocol. The luciferase activity was presented as the ratio of TOPflash/FOPflash, with *Renilla* luciferase plasmids as the internal control.

Statistical analysis. All independent experiments were performed at least three times and the data are presented as the mean ± SD. Statistical differences were determined by the Student's t-test, or one-way ANOVA followed by a post hoc Fisher's least significant difference test or Dunnett's test. Statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression level of LGR5 increases during the osteoblastic differentiation of MC3T3-E1 cells. To explore whether LGR5 was expressed in MC3T3-E1 cells, IF analysis was first conducted. The results verified the expression of LGR5 in MC3T3-E1 cells, and demonstrated that LGR5 was mainly distributed in the cytoplasm and membrane (Fig. 1A). The MC3T3-E1 cells were then induced to undergo osteoblastic differentiation with osteogenic media for 7 or 14 days. Successful induction was confirmed by the increased mRNA expression levels of the osteoblast differentiation markers ALP, OCN and COL-1a1, as well as the transcription factors RUNX2 and OSX (Fig. 1B). Osteoblastic differentiation was also confirmed by the results of ALP staining at day 7 and Alizarin red staining at day 14 during induction (Fig. 1C). The expression of LGR5 during osteoblastic differentiation was also detected by RT-qPCR and western blotting. The mRNA and protein levels of LGR5 increased during differentiation (Fig. 1D and E), suggesting a potential role of LGR5 in the osteoblastic differentiation of MC3T3-E1 cells.

Effects of LGR5 regulation on the viability and apoptosis of MC3T3-E1 cells. To investigate the effects of LGR5

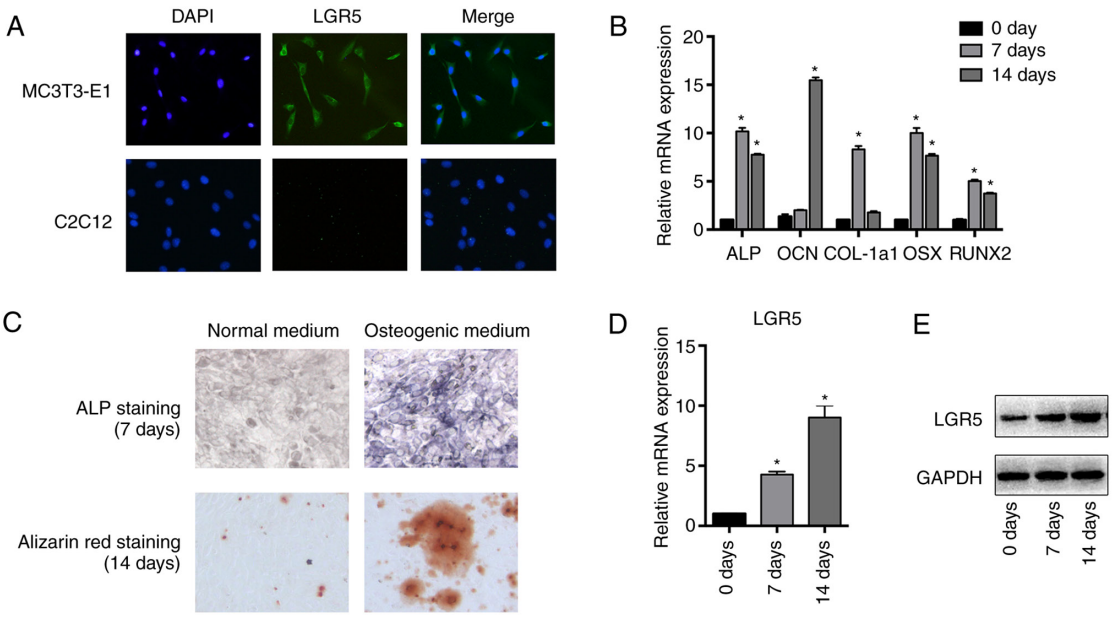

Figure 1. Expression of LGR5 increases during the osteoblastic differentiation of MC3T3-E1 cells. (A) Immunofluorescence analysis of LGR5 protein expression in MC3T3-E1 cells. C2C12 mouse myoblast cells were used as control cells (magnification, x100). (B) MC3T3-E1 cells were induced for 7 or 14 days in osteogenic media, and the mRNA levels of the osteoblast differentiation markers ALP, COL-a1, OCN, OSX and RUNX2 were detected by reverse transcription-quantitative PCR analysis. (C) ALP and Alizarin red staining after osteogenic induction for 7 or 14 days, respectively (magnification, x100). (D) mRNA and (E) protein expression of LGR5 after osteogenic induction for 7 or 14 days. *P<0.05 vs. 0 day. LGR5, leucine-rich repeat-containing G-protein coupled receptor; ALP, alkaline phosphatase; OCN, osteocalcin; COL-1a1, collagen type I α1; OSX, osterix; Runx2, runt related transcription factor 2.

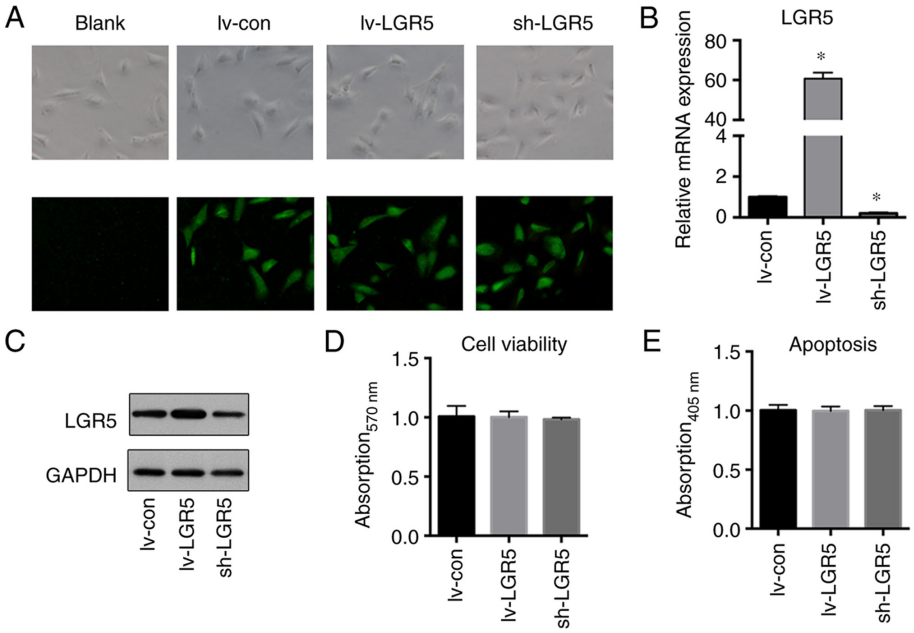

Figure 2. Effects of LGR5 regulation on the viability and apoptosis of MC3T3-E1 cells. (A) Immunofluorescence analysis demonstrated the presence of GFP-positive cells, indicating successful transfection by the lentiviruses after 3 days (magnification, x100). (B) Reverse transcription-quantitative PCR and (C) western blotting verified the successful overexpression or knockdown of the LGR5 gene in MC3T3-E1 cells compared with the control. (D) Cell viability of the transfected cells as evaluated by MTT assay. (E) Cell apoptosis results evaluated using a Cell Death Detection ELISA assay. *P<0.05 vs. lv-con. lv-LGR5, lentivirus-based LGR5 overexpression vector; sh-LGR5, lentivirus-based short hairpin RNA vector targeting LGR5; lv-con, Lentivirus-based negative shRNA control; LGR5, leucine-rich repeat-containing G-protein coupled receptor.

regulation on cell viability and apoptosis, LGR5 was overexpressed or knocked down in MC3T3-E1 cells via lentiviral transfection. Fluorescence microscopy confirmed high efficiencies of lentivirus transfection with a large proportion of GFP-positive cells (Fig. 2A). The successful overexpression or knockdown of LGR5 gene was then verified by RT-qPCR and western blotting (Fig. 2B and C). Neither overexpression nor knockdown of the LGR5 gene significantly affected the cell viability or apoptosis of MC3T3-E1 cells (Fig. 2D and E).

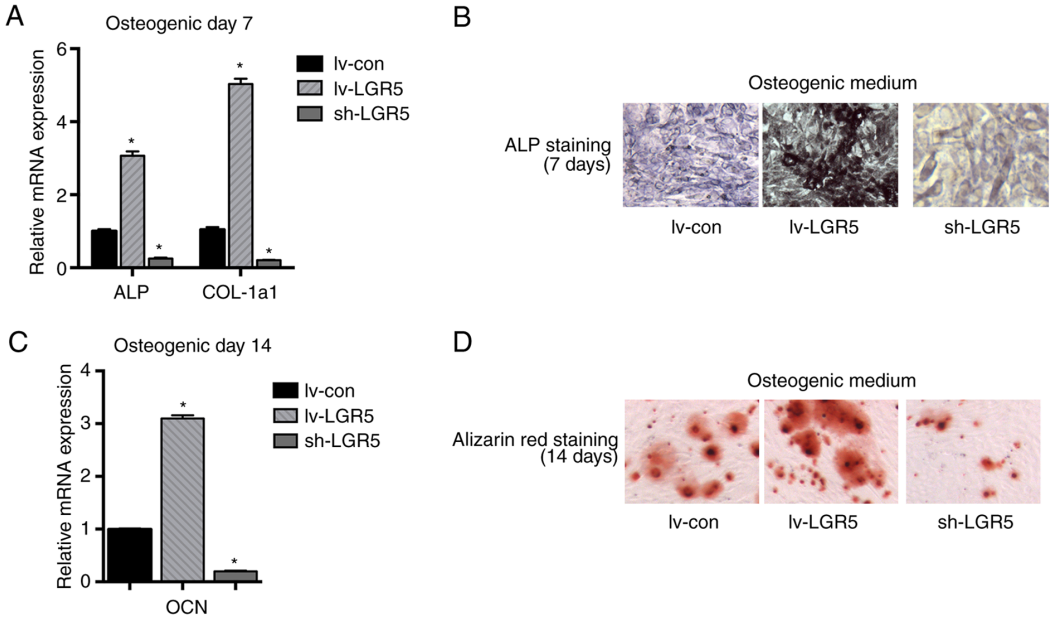

Figure 3. LGR5 promotes the osteogenic differentiation of MC3T3-E1 cells. (A) mRNA levels of the osteoblast differentiation markers ALP and COL-1a1 were detected in MC3T3-E1 cells after osteogenic induction for 7 days. (B) ALP staining of MC3T3-E1 cells after osteogenic induction for 7 days (magnification, x100). (C) mRNA levels of the osteoblast differentiation marker OCN after osteogenic induction for 14 days. (D) Alizarin red staining of MC3T3-E1 cells after osteogenic induction for 14 days (magnification, x100). *P<0.05 vs. lv-con. lv-LGR5, lentivirus-based LGR5 overexpression vector; sh-LGR5, lentivirus-based short hairpin RNA vector targeting LGR5, lv-con, Lentivirus-based negative shRNA control; LGR5, leucine-rich repeat-containing G-protein coupled receptor; ALP, alkaline phosphatase; OCN, osteocalcin; COL-1a1, collagen type I α1.

LGR5 enhances the osteoblastic differentiation of MC3T3-E1 cells. To explore the role of LGR5 in osteoblastic differentiation, the transfected MC3T3-E1 cells were cultured in osteogenic media for 7 or 14 days. After 7 days, LGR5 overexpression significantly increased the mRNA levels of ALP and COL-1a1, which are known as early- and middle-stage osteogenic differentiation marker genes. The knockdown of LGR5 significantly inhibited the expression of these mRNAs (Fig. 3A). Concurrently, ALP staining also suggested that LGR5 played a positive role in the osteoblastic differentiation of the MC3T3-E1 cells (Fig. 3B). Following 14 days of induction, the mRNA level of OCN, the late-stage osteogenic gene, was significantly upregulated by LGR5 overexpression (Fig. 3C). Furthermore, Alizarin red staining after 14 days also revealed that LGR5 enhanced the mineralization of MC3T3-E1 cells at the late stage of osteoblastic differentiation (Fig. 3D). These results together suggest that LGR5 promoted the osteoblastic differentiation of MC3T3-E1 cells.

LGR5 activates the Wnt signaling pathway by stabilizing β-catenin. Since LGR5 is a facultative Wnt receptor component mediating the activation of Wnt signaling (9), and Wnt signaling plays a pivotal role in osteoblast differentiation (23), whether LGR5 promoted osteoblastic differentiation through Wnt/β-catenin signaling in MC3T3-E1 cells was explored. Neither the overexpression nor the knockdown of LGR5 affected the mRNA level of β-catenin (Fig. 4A). The protein level of β-catenin in transfected MC3T3-E1 cells was then assessed by western blotting. LGR5 overexpression increased the protein level of β-catenin in the total cell lysates (Fig. 4B), but reduced the level of phosphorylated β-catenin in the cytoplasm (Fig. 4C). In addition, the level of intranuclear β-catenin was substantially increased by LGR5 overexpression (Fig. 4D). These results indicate that LGR5 activated Wnt/β-catenin signaling in the cells by increasing the cytoplasmic stabilization and nuclear accumulation of β-catenin. Furthermore, LGR5 upregulated the mRNA levels of Lef1 and Tcf1, two target genes of the Wnt pathway (Fig. 4E). The TOPflash dual luciferase activity assay also confirmed the activation of Wnt/β-catenin signaling in MC3T3-E1 cells with LGR5 overexpression (Fig. 4F).

The Wnt inhibitor Dkk-1 was then added to the cells to further verify that the potentiating effects of LGR5 on osteoblastic differentiation were dependent on the Wnt/β-catenin pathway. The results indicated that LGR5-enhanced osteoblastic differentiation was significantly abolished by Dkk-1 treatment (Fig. 4F and G). Thus, it was concluded that LGR5 promoted the osteoblastic differentiation of MC3T3-E1 cells through activation of the Wnt/β-catenin pathway. Since LGR5 has been reported to be a target gene of Wnt (9,24-26), Wnt-3a was added as a Wnt activator, to explore whether LGR5 is downstream of Wnt/β-catenin signaling. Neither Wnt-3a nor Dkk-1 affected the expression level of LGR5 (Fig. 4H), suggesting that LGR5 was not the downstream mediator of Wnt/β-catenin signaling in MC3T3-E1 cells. In addition, Wnt-3a significantly promoted the mRNA levels of the osteogenic differentiation markers ALP, OCN and COL-1a1, while LGR5 knockdown had no discernible impact on Wnt-3a-induced osteogenesis (Fig. 4I).

The Rspo family of proteins (Rspo1-4) are agonists of the canonical Wnt/β-catenin signaling pathway (27). Among the four proteins, Rspo-2 has the highest affinity for LGR5 (23). Rspo-2 has been identified to be a pivotal protein in embryonic development (28), tumor growth (29) and osteoblastogenesis (30). In the present study, the effects of Rspo-2 on cells with LGR5 overexpression or knockdown were explored. The results showed that the knockdown of LGR5 markedly

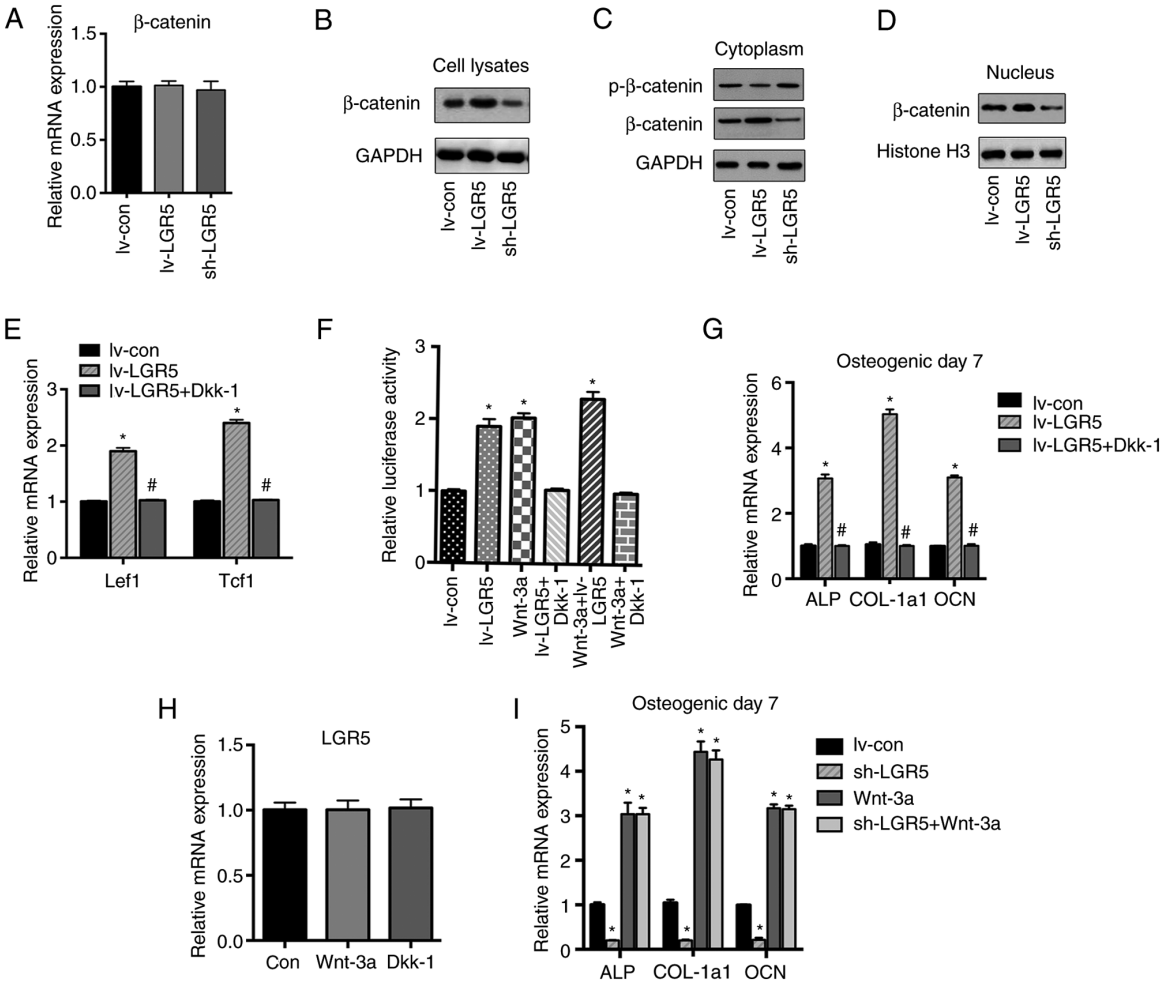

Figure 4. LGR5 activates the Wnt signaling by stabilizing β-catenin. (A) The mRNA level of β-catenin was detected in cells transfected with lentiviruses after osteogenic induction for 7 days. (B) The β-catenin protein level in total cell lysates was assessed by western blot analysis after the osteogenic induction of MC3T3-E1 cells for 7 days. (C) p-β-catenin (inactive) and β-catenin protein levels in the cytoplasm of MC3T3-E1 cells after osteogenic induction for 7 days. (D) β-catenin protein level in the nucleus of MC3T3-E1 cells after osteogenic induction for 7 days. (E) Reverse transcription-quantitative PCR analysis of the Wnt target genes Lef1 and Tcf1 after osteogenic induction for 7 days. (F) TOPflash dual-luciferase activity assay of MC3T3-E1 cells treated with lentivirus transfection, Dkk-1 and/or Wnt-3a after induction for 2 days. (G) The mRNA levels of osteogenic marker genes ALP, COL-a1 and OCN in cells treated with LGR5 overexpression alone or with Dkk-1 after osteogenic induction for 7 days. (H) The mRNA level of LGR5 in cells treated with Dkk-1 or Wnt-3a after osteogenic induction for 7 days. (I) The mRNA levels of osteogenic marker genes in cells treated with lentivirus transfection and/or Wnt-3a after osteogenic induction for 7 days. *P<0.05 vs. lv-con. #P<0.05 vs. lv-LGR5. lv-LGR5, lentivirus-based LGR5 overexpression vector; sh-LGR5, lentivirus-based short hairpin RNA vector targeting LGR5; lv-con, lentivirus-based negative shRNA control; con, untreated control; LGR5, leucine-rich repeat-containing G-protein coupled receptor; p-, phospho-; Lef1, lymphoid enhancer-binding protein 1; Tcf1, T-cell factor 1; ALP, alkaline phosphatase; COL-1a1, collagen type I α1; OCN, osteocalcin; Dkk-1, Dickkopfs-1.

inhibited the Wnt/β-catenin signaling (Fig. 5A and B) and osteogenic differentiation (Fig. 5C and D) induced by Rspo-2. These results indicate that LGR5 acted as a key receptor for R-spo-2 in the promotion of osteogenesis.

Discussion

Wnt signaling is widely known to play a pivotal role in bone remodeling and development, particularly its major branch, the canonical (Wnt/β-catenin) pathway (23,31). Wnt/β-catenin signaling is initiated through the binding of Wnt to the frizzled receptor and low-density lipoprotein receptor-related protein (LRP)5/6 coreceptors (23). After this, the β-catenin 'destruction complex' which comprises adenomatous polyposis coli, glycogen synthase kinase 3 and the scaffolding protein Axin, is inactivated to inhibit β-catenin phosphorylation and proteasomal degradation. Consequently, the amount of β-catenin that translocates into the nucleus is increased, and target genes including Lef1 and Tcf1 are activated (23,32). Activation of this pathway promotes osteoblastic differentiation and bone formation (23,31) (Fig. 6).

LGR5, also known as G-protein-coupled receptor 49, is a marker of matured stem cells and is essential for the normal embryonic development of various organs and tissues (33,34). LGR5 drives the self-renewal of stem cells in the stomach (11), small intestine, colon (12), hair follicles (10) and mammary glands (35). Similarly, LGR5 is upregulated in the colorectal cancer, basal cell carcinoma and glioblastoma cell lines, and promotes the initiation and proliferation of carcinomas (14-16). The underlying mechanisms of LGR5 involve the promotion of cancer stem cell proliferation and self-renewal via the potentiation of canonical Wnt/β-catenin signaling (36).

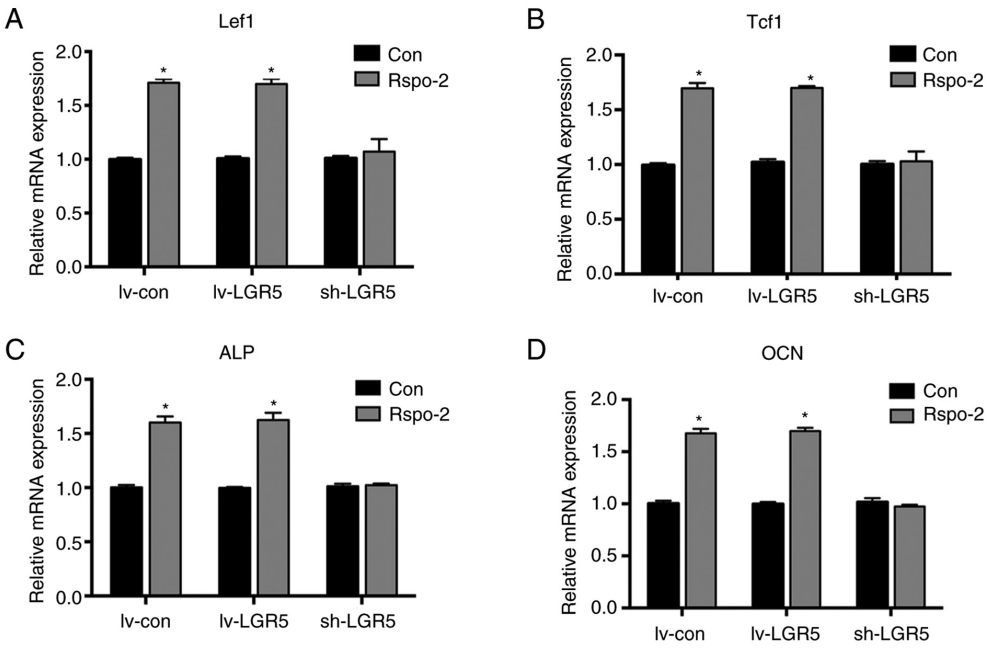

Figure 5. Effects of Rspo-2 on cells with modulated levels of LGR5. Reverse transcription-quantitative PCR analysis of the Wnt target genes (A) Lef1 and (B) Tcf1 after osteogenic induction for 7 days. The mRNA levels of osteogenic marker genes (C) ALP and (D) OCN in cells with or without Rspo-2 treatment after osteogenic induction for 7 days. *P<0.05 vs. Con. Con, untreated control; lv-LGR5, lentivirus-based LGR5 overexpression vector; sh-LGR5, lentivirus-based short hairpin RNA vector targeting LGR5; lv-con, lentivirus-based negative shRNA control; LGR5, leucine-rich repeat-containing G-protein coupled receptor; Lef1, lymphoid enhancer-binding protein 1; Tcf1, T-cell factor 1; ALP, alkaline phosphatase; OCN, osteocalcin; Rspo-2, R-spondin-2.

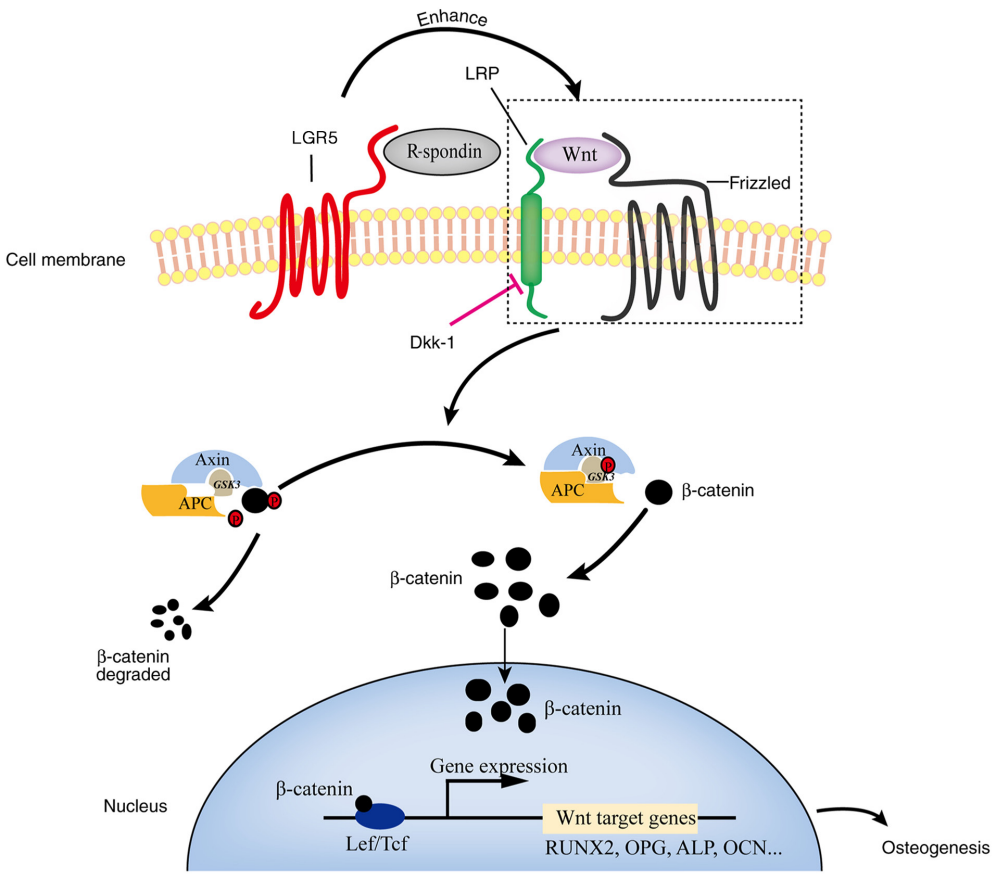

Figure 6. Schematic diagram of the molecular mechanism by which LGR5 regulates osteogenic differentiation through Wnt/β-catenin signaling. Once activated by its ligand R-spondin, LGR5 recruits the frizzled/LRP Wnt receptor complex, thereby inhibiting β-catenin degradation and increasing the nuclear accumulation of β-catenin. Inside the cell nucleus, β-catenin binds to Tcf/Lef transcription factors and then induces the expression of the downstream target genes (such as RUNX2, OPG, ALP and OCN), thus enhancing osteogenesis in osteoblasts. LGR5, leucine-rich repeat-containing G-protein coupled receptor LRP, low density lipoprotein receptor-related protein; Dkk-1, Dickkopfs-1; APC, adenomatous polyposis coli; GSK3, glycogen synthase kinase 3; Lef, lymphoid enhancer-binding factor; Tcf, T cell-factor; Runx2, runt related transcription factor 2; OPG, osteoprotegerin; ALP, alkaline phosphatase; OCN, osteocalcin.

LGR5 is a type B LGR protein, along with the closely related receptors LGR4 and LGR6. Both LGR4 and LGR6 have been reported to play positive roles in bone formation. LGR4 promotes bone formation via Wnt/β-catenin signaling and inhibits bone resorption by suppressing RANK signaling (18,37), and LGR6 promotes osteoblastic differentiation in MC3T3-E1 cells through Wnt/β-catenin signaling (19). Considering its homology with LGR4 and LGR6, we hypothesized LGR5 may also play a critical role in osteoblastic differentiation. In addition, recent studies have shown that LGR5 is upregulated in bone-associated Ewing sarcoma and promotes tumorigenesis through Wnt/β-catenin signaling (20). Bone marrow stem cells with LGR5 overexpression have been demonstrated to have greater potential for the promotion of fracture healing (21). In the present study, MC3T3-E1 cells that overexpressed LGR5 exhibited enhanced differentiation potential, as verified by the expression of osteogenic marker genes, as well as ALP and Alizarin red staining. Since LGR5 and its family members LGR4 and LGR6 are known as receptors of the Rspo family, which activate Wnt/β-catenin signaling by complexing with frizzled/LRP receptors (9,24), whether the potentiating effects of LGR5 on osteogenesis were mediated through Wnt/β-catenin signaling were then explored. The results demonstrated that LGR5 overexpression did not alter the transcriptional level of β-catenin but significantly elevated the protein level of β-catenin in total cells. Furthermore, western blotting showed that LGR5 reduced β-catenin phosphorylation levels in the cytoplasm, and increased the accumulation of β-catenin in the nucleus, indicating that the degradation of β-catenin in the cytoplasm was decreased. These results suggest that LGR5 overexpression reinforced the Wnt/β-catenin signaling pathway by increasing the cytoplasmic stabilization and nuclear accumulation of β-catenin. As a consequence, the expression of osteoblastic differentiation-associated genes was triggered and osteogenesis was enhanced. In addition, the results indicate that the Wnt signaling antagonist Dkk-1 blocked the interaction of Wnt ligand with frizzled and LRP receptors, thereby abrogating the potentiating effects of LGR5 on osteoblastic differentiation. LGR5 knockdown antagonized the activation of Wnt/β-catenin and osteogenesis induced by Rspo-2, while LGR5 knockdown did not affect the osteogenesis of MC3T3-E1 cells induced by Wnt-3a, a potent Wnt/β-catenin activator. These results together demonstrate that LGR5 acted as the Rspo receptor. Previous studies have reported that LGR5 recruits the LRP-frizzled receptor complex, and then binds to Wnt ligands (9,24). Overall, the activation of canonical Wnt signaling enhances osteogenic gene expression and promotes osteoblastic differentiation (Fig. 6).

In summary, through LGR5 gene regulation in MC3T3-E1 cells, the present study revealed the potentiating effects of LGR5 on osteoblastic differentiation. The study demonstrated that LGR5 promotes osteoblastic differentiation through Wnt/β-catenin signaling at the cellular level. Therefore, the regulation of LGR5/Wnt/β-catenin signaling may offer promise as a potential therapy for osteoporosis and other bone loss conditions. However, the role of LGR5 in animals requires further study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Authors' contributions

WY performed the majority of experiments and drafted the manuscript. WY and CRX confirm the authenticity of all the raw data. CRX, FCC and PC assisted with the experiments. CRX and LY analyzed the data and drafted the manuscript. LY and XYP conceived the study, supervised the experiments and edited the manuscript. All authors read and approved the final manuscript.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Jiang M, Wang T, Yan X, Liu Z, Yan Y, Yang K, Qi J, Zhou H, Qian N, Zhou Q, *et al*: A novel rhein derivative modulates bone formation and resorption and ameliorates estrogen-dependent bone loss. J Bone Miner Res 34: 361-374, 2019.
2. Yadav VK, Balaji S, Suresh PS, Liu XS, Lu X, Li Z, Guo XE, Mann JJ, Balapure AK, Gershon MD, *et al*: Pharmacological inhibition of gut-derived serotonin synthesis is a potential bone anabolic treatment for osteoporosis. Nat Med 16: 308-312, 2010.
3. Black DM and Rosen CJ: Clinical practice. Postmenopausal osteoporosis. N Engl J Med 374: 254-262, 2016.
4. Canalis E: Management of endocrine disease: Novel anabolic treatments for osteoporosis. Eur J Endocrinol 178: R33-R44, 2018.
5. Riggs BL and Hartmann LC: Selective estrogen-receptor modulators-mechanisms of action and application to clinical practice. N Engl J Med 348: 618-629, 2003.
6. Leder BZ: Parathyroid hormone and parathyroid hormone-related protein analogs in osteoporosis therapy. Curr Osteoporos Rep 15: 110-119, 2017.
7. Luo J, Zhou W, Zhou X, Li D, Weng J, Yi Z, Cho SG, Li C, Yi T, Wu X, *et al*: Regulation of bone formation and remodeling by G-protein-coupled receptor 48. Development 136: 2747-2756, 2009.
8. Xu JG, Huang C, Yang Z, Jin M, Fu P, Zhang N, Luo J, Li D, Liu M, Zhou Y and Zhu Y: Crystal structure of LGR4-Rspo1 complex: Insights into the divergent mechanisms of ligand recognition by leucine-rich repeat G-protein-coupled receptors (LGRs). J Biol Chem 290: 2455-2465, 2015.

9. de Lau W, Barker N, Low TY, Koo BK, Li VS, Teunissen H, Kujala P, Haegebarth A, Peters PJ, van de Wetering M, *et al*: Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 476: 293-297, 2011.
10. Jaks V, Barker N, Kasper M, van Es JH, Snippert HJ, Clevers H and Toftgård R: Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet 40: 1291-1299, 2008.
11. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, *et al*: Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell 6: 25-36, 2010.
12. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ and Clevers H: Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449: 1003-1007, 2007.
13. Morita H, Mazerbourg S, Bouley DM, Luo CW, Kawamura K, Kuwabara Y, Baribault H, Tian H and Hsueh AJ: Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension. Mol Cell Biol 24: 9736-9743, 2004.
14. Kemper K, Prasetyanti PR, De Lau W, Rodermond H, Clevers H and Medema JP: Monoclonal antibodies against Lgr5 identify human colorectal cancer stem cells. Stem Cells 30: 2378-2386, 2012.
15. Tanese K, Fukuma M, Yamada T, Mori T, Yoshikawa T, Watanabe W, Ishiko A, Amagai M, Nishikawa T and Sakamoto M: G-protein-coupled receptor GPR49 is up-regulated in basal cell carcinoma and promotes cell proliferation and tumor formation. Am J Pathol 173: 835-843, 2008.
16. Nakata S, Campos B, Bageritz J, Bermejo JL, Becker N, Engel F, Acker T, Momma S, Herold-Mende C, Lichter P, *et al*: LGR5 is a marker of poor prognosis in glioblastoma and is required for survival of brain cancer stem-like cells. Brain Pathol 23: 60-72, 2013.
17. Vieira GC, Chockalingam S, Melegh Z, Greenhough A, Malik S, Szemes M, Park JH, Kaidi A, Zhou L, Catchpoole D, *et al*: LGR5 regulates pro-survival MEK/ERK and proliferative Wnt/beta-catenin signalling in neuroblastoma. Oncotarget 6: 40053-40067, 2015.
18. Zhu C, Zheng XF, Yang YH, Li B, Wang YR, Jiang SD and Jiang LS: LGR4 acts as a key receptor for R-spondin 2 to promote osteogenesis through Wnt signaling pathway. Cell Signal 28: 989-1000, 2016.
19. Liu SL, Zhou YM, Tang DB, Zhou N, Zheng WW, Tang ZH, Duan CW, Zheng L and Chen J: LGR6 promotes osteogenesis by activating the Wnt/β-catenin signaling pathway. Biochem Biophys Res Commun 519: 1-7, 2019.
20. Scannell CA, Pedersen EA, Mosher JT, Krook MA, Nicholls LA, Wilky BA, Loeb DM and Lawlor ER: LGR5 is expressed by ewing sarcoma and potentiates Wnt/β-catenin signaling. Front Oncol 3: 81, 2013.
21. Lin W, Xu L, Pan Q, Lin S, Feng L, Wang B, Chen S, Li Y, Wang H, Li Y, *et al*: Lgr5-overexpressing mesenchymal stem cells augment fracture healing through regulation of Wnt/ERK signaling pathways and mitochondrial dynamics. FASEB J 33: 8565-8577, 2019.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Krishnan V, Bryant HU and Macdougald OA: Regulation of bone mass by Wnt signaling. J Clin Invest 116: 1202-1209, 2006.
24. Carmon KS, Gong X, Lin Q, Thomas A and Liu Q: R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc Natl Acad Sci USA 108: 11452-11457, 2011.
25. Glinka A, Dolde C, Kirsch N, Huang YL, Kazanskaya O, Ingelfinger D, Boutros M, Cruciat CM and Niehrs C: LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling. EMBO Rep 12: 1055-1061, 2011.
26. Gong X, Carmon KS, Lin Q, Thomas A, Yi J and Liu Q: LGR6 is a high affinity receptor of R-spondins and potentially functions as a tumor suppressor. PLoS One 7: e37137, 2012.
27. Yoon JK and Lee JS: Cellular signaling and biological functions of R-spondins. Cell Signal 24: 369-377, 2012.
28. Yamada W, Nagao K, Horikoshi K, Fujikura A, Ikeda E, Inagaki Y, Kakitani M, Tomizuka K, Miyazaki H, Suda T and Takubo K: Craniofacial malformation in R-spondin2 knockout mice. Biochem Biophys Res Commun 381: 453-458, 2009.
29. Zhang H, Han X, Wei B, Fang J, Hou X, Lan T and Wei H: RSPO2 enhances cell invasion and migration via the WNT/β-catenin pathway in human gastric cancer. J Cell Biochem 120: 5813-5824, 2019.
30. Friedman MS, Oyserman SM and Hankenson KD: Wnt11 promotes osteoblast maturation and mineralization through R-spondin 2. J Biol Chem 284: 14117-14125, 2009.
31. Leucht P, Minear S, Ten Berge D, Nusse R and Helms JA: Translating insights from development into regenerative medicine: The function of Wnts in bone biology. Semin Cell Dev Biol 19: 434-443, 2008.
32. Baron R and Kneissel M: WNT signaling in bone homeostasis and disease: From human mutations to treatments. Nat Med 19: 179-192, 2013.
33. Barker N and Clevers H: Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology 138: 1681-1696, 2010.
34. Liu D, He XC, Qian P, Barker N, Trainor PA, Clevers H, Liu H and Li L: Leucine-rich repeat-containing G-protein-coupled receptor 5 marks short-term hematopoietic stem and progenitor cells during mouse embryonic development. J Biol Chem 289: 23809-23816, 2014.
35. Plaks V, Brenot A, Lawson DA, Linnemann JR, Van Kappel EC, Wong KC, de Sauvage F, Klein OD and Werb Z: Lgr5-expressing cells are sufficient and necessary for postnatal mammary gland organogenesis. Cell Rep 3: 70-78, 2013.
36. Xu L, Lin W, Wen L and Li G: Lgr5 in cancer biology: Functional identification of Lgr5 in cancer progression and potential opportunities for novel therapy. Stem Cell Res Ther 10: 219, 2019.
37. Luo J, Yang Z, Ma Y, Yue Z, Lin H, Qu G, Huang J, Dai W, Li C, Zheng C, *et al*: LGR4 is a receptor for RANKL and negatively regulates osteoclast differentiation and bone resorption. Nat Med 22: 539-546, 2016.