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 α1 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.

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 (Rsps) 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% CO₂. 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% CO₂. The culture medium was replaced thrice each week. After the cells had grown to 70% confluence, the experimental 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 (shRNA)‑GFP‑PURO were purchased from Shanghai GeneChem Co., Ltd. The lenti-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 (shRNA)‑GFP‑PURO were purchased from Shanghai GeneChem Co., Ltd. The lenti-transfection of MC3T3-E1 cells

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 (shRNA)‑GFP‑PURO were purchased from Shanghai GeneChem Co., Ltd. The lenti-transfection of MC3T3-E1 cells

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^ΔΔCq method (22) with normalization to β-actin. The qPCR primers used were as follows: β-actin forward, 5'-TGACAGGATGCAGAAGGAGA-3' and reverse, 5'-CGCTCACGGAGGAGGAT-3'; LGR5 forward, 5'-CTTTCA
CCTCCTACCTGGACCT-3' and reverse 5'-GGCGTAGTCTTGCTATGTTGTTG-3'; alkaline phosphatase (ALP) forward, 5'-TCGGGACTGTTCTCGGATAAC-3' and reverse, 5'-GTTGAGCTGCTGATGTTCTGTT-3'; osterix (OSX) forward, 5'-GGAGGGCACAAGAGCCCATACGC-3' and reverse 5'-TGCCAGGAGAGGGTCCATTG-3'; runt related transcription factor 2 (RUNX2) forward, 5'-GAGCGAGGCAAGATTTCCAC-3' and reverse, 5'-GGACGGTCTAGCTGTT-3'; osteocalcin (OCN) forward, 5'-CAAGCGAGGAGGAAATAGG-3' and reverse, 5'-CGTCACACGAGGGAACGC-3'; T-cell factor 1 (Tcf1) forward, 5'-GGAGCTGATCAATCGCAGACTTCCGGAGGA-3' and reverse, 5'-CCGCTCGATGCTGATGTTAAGGATGGCAGCTGT-3'; T-cell factor 1 (Tcf1) forward, 5'-GGAGCTGATCAATCGCAGACTTCCGGAGGA-3' and reverse, 5'-CGTCACACGAGGGAACGC-3'; 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) according to the manufacturer's instructions. Equal amounts of proteins (40 µg) 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% 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 level of target proteins was visualized by the reaction of HRP with a chemiluminescent substrate (EMD Millipore). GAPDH was used as the control. 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...
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).
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 (Fig. 4F).

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...
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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 proteosomal 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).

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 interaction of Wnt/β-catenin and osteogenesis induced by Rsps-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 Rsps 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.

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