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Down-regulation of *LGR6* promotes bone fracture recovery using bone marrow stromal cells

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ABSTRACT

Objective: The Leucine-rich repeat-containing G-protein coupled receptor 6 (*LGR6*) is a well-known marker of stem cells. In present study, we aimed to further explore the effects of *LGR6* on promoting osteogenic differentiation of bone marrow stromal cells (BMSCs) and bone healing.

Methods: Flow cytometry assay was used to determine the expression of BMSCs surface markers, and western blot was performed to detect the *LGR6* protein expression. The osteogenic differentiation of BMSCs was qualified using ALP and ARS staining. Protein expression of osteogenic genes (ALP, Collagen I, Runx2 and OCN) were evaluated using western blot. *In vivo*, BMSCs transfected with sh-*LGR6* or *LGR6* cDNA were injected into the fracture site to establish rat fracture healing model. X-ray system and hematoxylin-eosin (HE) staining were conducted to observe the fracture recovery. Biomechanical test was performed to detect the changes of maximum load, elastic modules and bone mineral density.

Results: In BMSCS, CD90 and CD44 were positively expressed, while CD11b was negatively expressed. Expression level of *LGR6* gradually decreased with the osteogenic differentiation of BMSCs. The osteogenic genes expression level during the osteogenic differentiation significantly increased with the down-regulation of *LGR6*. *In vivo,* 8 weeks after injection, rats treated with *LGR6* knocked-down BMSCs showed increased number of fibroblasts. Maximum load, elastic modulus and the bone mineral density were enhanced with the knocking-down of *LGR6*.

Conclusion: Inhibition of *LGR6* promoted the osteogenic differentiation of BMSCs *in vitro*. Moreover, transplantation of *LGR6*-knockout BMSCs in rat models contributes to a better recovery after the fracture.

1. Introduction

Bone is a vital regulated organ in locomotion, supporting organ and maintaining mineral homeostasis and structural robustness [1]. Due to the metabolic disorder, trauma, and cancer resection *etc.*, bone defects such as atrophy and delayed bone regeneration often occurred [2]. Clinical treatments for compromised bone healing require multiple procedures and they are complex and costly [3]. It has been reported that over 5% of bone fractures fail to heal or demonstrate a delay in healing, leading to increased health cost and morbidity [4]. Therefore, effective strategies to improve bone healing are in great demand.

In recent years, scientists have paid more attention on mesenchymal stem cells (MSCs). Implantation of MSCs is considered to be an therapeutic strategy to improve bone healing as MSCs can proliferate and differentiate into multiple cells, including bone, cartilage, adipose, and muscle cells [4]. Bone marrow stromal cells (BMSCs), containing a small number of MSCs, is an increasingly important source of cells for cell therapy and tissue repair [5,6]. Through differentiating into selected cell types and secrete paracrine factor, BMSCs act as strong candidates for regulation of angiogenesis at the beginning of bone

Abbreviations: BMSCs, bone marrow stromal cells; MSCs, mesenchymal stem cells; *LGR6*, leucine-rich repeat-containing G-protein coupled receptor 6; FBS, fetal bovine serum; PE, phycoerythrin; OIM, osteogenic induction medium; ALP, alkaline phosphatas; ARS, Alizarin Red S; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; OCN, osteocalcin; TBST, Tris Buffered Saline Tween; SD, Sprague-Dawley; BMD, bone marrow density; EDTA, Ethylenediaminetetraacetic Acid

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formation [7]. Previous study by Nishida et al. revealed that BMSCs protected and repaired damaged neurons through producing growth factors [8]. Another study by Wang et al. indicated that BMSCs induced bone formation when implanted with biodegradable scaffolding, suggesting a promising prospect for clinical treatments of bone damage [5].

Leucine-rich repeat-containing G-protein coupled receptor 6 (*LGR6*) belongs to leucine-rich repeat-containing subgroup of the G protein-coupled 7-transmembrane protein superfamily [9]. It is categorized into type B of *LGR* protein family, functioning in organogenesis and homeostasis in various tissues [10]. Along with the other two type B *LGR*s, *i.e. LGR4* and *LGR5*, *LGR6* is frequently expressed in distinct types of adult stem cells [11]. Specifically, *LGR6* marked a group of stem cells in taste buds, lung and skin [12,13]. The study of *LGR6* in stem cells thus becomes a hit. Evidences that confirm the significance of *LGR6* have been accumulating. For instance, Lehoczky and Tabin found that type B *LGR*s marked in nail stem cells and could be a contributor to digit tip regeneration [14]. In addition, *LGR6* promoted wound healing, angiogenesis, and nascent hair follicle development in damaged epithelial tissues [15]. However, not enough studies have been focused on the mechanism of *LGR6* in BMSCs or its role in bone damage recovery.

In the present study, we used a microarray to explore the differentially expressed genes between fibroblasts and BMSCs. The role of *LGR6* during the osteogenic differentiation were evaluated *in vivo* and *vitro*. After osteogenic induction, BMSCs with lower expression of *LGR6* were found to highly express Collagen I, Runx2 (runt-related transcription factor 2), OCN (osteocalcin), ALP (alkaline phosphatas) and had a deeper mineralization, indicating that down-regulation of *LGR6* promotes the osteogenic differentiation of BMSCs *in vitro*. Moreover, transplantation of *LGR6*-knockout BMSCs facilitated the healing of bone fracture *in vivo*, providing novel clinical therapy possibilities for bone healing.

2. Materials and methods

2.1. BMSCs isolation and culture

Marrow samples used for BMSCs isolation were obtained from 11 patients undergoing hip surgeries (Female = 6, Male = 5, Age = 7–25) at the First Hospital of Lanzhou University. All patients were informed the consent and the present study was approved by the ethical committee of the First Hospital of Lanzhou University. BMSCs isolation was performed as previous reported [7,8]. In brief, bone marrow obtained from patients under sterile conditions was filtered with a cell strainer (Thermo Fisher Scientific, Waltham, MA, USA). Then the marrow was centrifuging at 400 × *g* for 10 min. The supernatant was discarded and the remaining cells were re-suspended in α-MEM (Thermo Fisher Scientific) consists of 10% FBS (fetal bovine serum) and 1% antibiotics (streptomycin and penicillin, GIBCO BRL, Grand Island, NY, USA), and cultured in 175 cm^2 culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. After 24 h culturing, the cultures were washed with PBS (phosphate buffered saline) to remove non-adherent cells. The medium was changed every 2–3 day. Cells were passaged when reaching approximately 80% confluence and the cells at passage 5 were used for the experiments.

2.2. Fibroblast cells

Two fibroblast cells (CRL2352 and CRL2429) from ATCC (American Type Culture Collection, VA, USA) were used to verified the microarray analysis results. The two cells were cultured in ATCC-formulated Iscove's Modified Dulbecco's Medium with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. BMSCs identification

Passage 5 cells were harvested and plated into 24-well plates at a density of 1×10^5 cells/mL. After the overnight incubation, the cells were fixed in 4% paraformaldehyde (Shanghai Suran Chemicals Co. Ltd., Shanghai, China), blocked with confining liquid and washed with PBS. The phycoerythrin (PE)-conjugated primary antibodies against CD44, CD90 and CD11b (20 μL/test, BD Biosciences Systems & Reagents Inc., San Jose, California, USA) were added to cells. Then the BMSCs were detected using a flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Isotype control antibodies were used as negative control here.

2.4. Osteogenic differentiation

To induce osteogenic differentiation, the culture medium was switched to the osteogenic induction medium (OIM): DMEM containing 0.1 μM dexamethasone, 50 μM 1-ascorbate-2-phosphate and 10 mM β-glycerophosphate. ALP and ARS (Alizarin Red S) staining were performed on day 0, 7 and 14, respectively. Expression of osteogenic genes (ALP, Collagen I, Runx2 and OCN) was quantitated by western blot at the same time.

2.5. ALP and ARS staining

The osteogenic differentiation of BMSCs was evaluated by ALP and ARS staining. Cells were seeded into 6-well plates and washed with PBS for following staining. The ALP activity was evaluated by ALP staining kit (Solarbio) and the mineralized deposits were evaluated by the ARS staining solution (Solarbio). The images were observed under microscope with the magnification × 200. The results were quantitated according to the intensity of the staining.

2.6. Western blot

The bicinchoninic acid (BCA) protein concentration assay kit (Beyotime, Shanghai, China) was applied to quantify the proteins expression. After the addition of loading buffer, the proteins (100 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen). The membranes were incubated with 5% skim milk at room temperature for 2 h. Primary antibodies against ALP, Collagen I, Runx2, OCN and GAPDH (Abcam Inc., Cambridge, MA, USA) were added into the membranes for an overnight incubation. After being washed with Tris Buffered Saline Tween (TBST) for 5 min, the membranes were treated with secondary antibodies (Anti-Rabbit IgG, Abcam) and incubated for 2 h. Immunoreactivity was detected using an enhanced chemiluminescence (Merck Millipore, Billerica, MA, USA). GAPDH was used as control.

2.7. Cell transfection

The expression of *LGR6* in BMSCs was down-regulated or over-expressed by transfecting *pcDNA/LGR6* shRNA or pcDNA 3.1*/LGR6* cDNA using Lipofectamine 2000 (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Cells were divided into the following four groups: blank group, cells added with Lipofectamine 2000; vector group, cells transfected with pcDNA 3.1; inhibit group, cells transfected with *LGR6* shRNA (Sangon Biotech, Shanghai, China); overexpress group, cells transfected with *LGR6* cDNA (Sangon Biotech).

2.8. qrt-PCR

Total RNA was extracted by TRIzol® kit (Invitrogen) and reverse-transcribed into cDNA using Reverse Transcription Kit (Takara, Dalian, China). PCR was performed using Maxima SYBR Green qPCR Master