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Effect of LGR4 on synovial cells and inflammatory factors in rats with traumatic osteoarthritis

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Abstract. – OBJECTIVE: Traumatic arthritis is one of the most common diseases in orthopedics. LGR4 is involved in bone formation and bone development. However, the role of LGR4 in synovial cells of rats with traumatic osteoarthritis has not been reported.

MATERIALS AND METHODS: Sprague Dawley (SD) rats were randomly divided into the control group and model group. The Real Time-Polymerase Chain Reaction (RT-PCR), Western blot, and Enzyme-Linked Immunosorbent Assay (ELISA) were used to analyze the expression of LGR4 in synovial tissue and synovial fluid. Synovial cells were isolated and cultured, followed by transfection of LGR4-pcDNA3.1 plasmid into cells. Cell proliferation was analyzed by MTT and EdU assay, and the Caspase-3 activity was assessed using the Caspase-3 activity kit. The secretion of the inflammatory factors interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) was detected by ELISA. NF-κB signaling pathway changes were evaluated by the Western blot.

RESULTS: In the model group, LGR4 mRNA expression in synovial tissue was significantly decreased, and the secretion of LGR4 in the synovial fluid was significantly decreased compared with the control group ($p<0.05$). LGR4 protein expression in the synovial membrane in the model group tissue was reduced. The transfection of LGR4-pcDNA3.1 plasmid into synovial cells promoted the LGR4 expression, inhibited the proliferation of synoviocytes, increased the Caspase-3 activity, the secretion of IL-1, TNF-α, and IL-6, as well as the decreased expression of NF-κB with a statistical significance, compared with the control group ($p<0.05$).

CONCLUSIONS: LGR4 expression is reduced in the rat model of traumatic osteoarthritis. The upregulation of LGR4 expression can inhibit the secretion of the inflammatory factors and inhibit the proliferation of the synovial cells by regulating NF-κB signaling pathway, which may alleviate the development of the joint inflammation.

Key Words:

LGR4, Traumatic osteoarthritis, Synovial cells, Inflammatory factors, NF-κB.

Introduction

Osteoarthritis (OA), a common disease in orthopedics, is mainly a joint degenerative disease and is clinically a frequently occurring disease. Patients often have limited activity due to joint inflammation, which gradually develops, and can be disabling in severe cases, leading to a decrease in the quality of life of patients and severely threatening human health[1,2]. OA is more common in middle-aged and elderly people, indeed, the incidence rate increases with age, and nearly half of the elderly over 60 years old are affected by OA[3]. The global stepping into an aging society has led to an increase in OA patients with years. OA occurs in the most important and widely used joints, of which the knee joint is the most common, seriously affecting the lower limb movement of patients[4]. The cause of OA is primary or secondary, and secondary osteoarthritis is more common with the trauma, being the common factor[5]. Traumatic arthritis is one of the most common diseases in orthopedics, and can be found in people of all ages, mainly in middle-aged populations[6]. Traumatic arthritis causes the degeneration of articular cartilage, which leads to bone hyperplasia and ossification[7]. Clinically, traumatic arthritis is characterized by different changes of subchondral bone and cartilage destruction, resulting in clinical chronic pain and tenderness, joint stiffness, deformity, joint swelling, total joint abnormal joint pain, and movement disorders[8]. Although there are various treatments for traumatic arthritis, there is still a lack of effective treatment. Therefore, finding and

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elucidating the key molecular targets for the treatment of traumatic arthritis has currently become one of the hot spots[9,10].

G protein-coupled receptors are a family of proteins in the human body. They can bind and regulate the G proteins and participate in the intracellular signal transduction, regulate cell proliferation, differentiation, apoptosis, and other processes, thereby regulating various biological processes of the body[11,12]. LGR4 is located on the long arm of chromosome 11 and belongs to the G protein-coupled receptor rhodopsin family. It is also a member of the glycoprotein hormone receptor subfamily, also known as GPR48, containing an N-terminal extracellular domain 7 crosses functional domains such as membrane domains, and C-terminal cytoplasmic regions[13,14]. LGR4 is involved in the regulation of several physiological functions and participates in tissue growth, differentiation, and movement[15]. Luo et al[16] have shown that LGR4 is involved in bone formation, remodeling, and organ development. However, the role of LGR4 in rat synovial cells with traumatic osteoarthritis has not been reported.

Materials and Methods

Animals

Healthy Sprague Dawley (SD) rats, male, 8 weeks old, specific-pathogen-free (SPF) grade, with body weight of 250 ± 20 g, were purchased from the Experimental Animal Center of this unit, and fed by SPF Animal Experiment Center. The feeding conditions included the temperature at 21 ± 1ºC and relative humidity 50-70%, ensuring a 12/day cycle every 12 hours.

Ethics Statement

All research subjects have signed informed consents. This study has been approved by the Ethical Committee of The 80th Army Hospital of the Chinese People's Liberation Army.

Reagents and Equipment

TRIzol reagent and type I collagenase were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LGR4-pcDNA3.1 plasmid was designed and synthesized by Shanghai Gene Pharmaceutical Co. Ltd (Shanghai, China). The RNA extraction kit, RT-PCR primer, reverse transcription (RT) kit, and real time-PCR reagent were purchased from Axygen Scientific Inc. (Union City, CA, USA). The polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Washington, NY, USA), Western blot related chemical reagent was purchased from Biyuntian Biotechnology Co., Ltd. (Nantong, China), enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). The rabbit anti-mouse NF-κB monoclonal antibody, rabbit anti-mouse LGR4 antibody, goat anti-rabbit Horseradish peroxidase (HRP)-labeled IgG secondary antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium, fetal bovine serum (FBS), and cyan chain double-antibody were purchased from HyClone (San Angelo, TX, USA). The Dimethyl sulfoxide (DMSO) and tetrazolium salt colorimetry (MTT) powder were purchased from Gibco (Grand Island, NY, USA), while the trypsin digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). The EdU kit was purchased from Nanjing Kaiji Biotechnology Development Co. Ltd (Nanjing, China). The Caspase-3 activity assay kit was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). ABI7900 HT real time-PCR was purchased from ABI (Waltham, MA, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The CK2 fluorescence microscope was purchased from Olympus Corporation (Shinjuku, Tokyo, Japan).

Preparation of Rat Traumatic Osteoarthritis Model

Healthy male SD rats were randomly divided into 2 groups (n=10 in each group). The rats in model group were used to prepare a rat model of traumatic osteoarthritis by excising the medial meniscus. The rats were anesthetized with sodium pentobarbital, and the conventional knee joint was sterilized and prepared for skin followed by confirmation of its entrance in the joint cavity to establish the lateral entrance at the intercondylar fossa. Then, the low-temperature plasma knife was used to cut the medial meniscus at the middle 1/3 junction, causing meniscus defect, hemostasis, and stitching the wound layer by layer. The post-operative routine consisted of intramuscular injection of 3.2 million U/streptomycin antibiotics, once a day for 7 days.

Isolation and Culture of Synovial Cells

The synovial tissue was isolated under aseptic conditions followed by removal of fat, connective tissue, and subsequent digestion with 4 mg/ml type I collagenase at 37°C for 2 h. The cell suspension was collected, filtered through a 70 μm cell strainer, and centrifuged at 2000 rpm for 6 min. The isolated cells were cultured in 10% FBS, 90% high glucose DMEM/F12 medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin) at 37°C in a 5% CO_2 incubator. The cells in the logarithmic growth phase were used for experiments. The cells were randomly divided into 3 groups: the control group, NC group (transfected with pcDNA3.1 empty plasmid), and LGR4 group (transfected with LGR4-pcDNA3.1 plasmid). The LGR4-pcDNA3.1 plasmid sequence was 5'-AUGGAGCGAGAUAUG-3'. The pcDNA3.1 empty plasmid and the LGR4-pcDNA3.1 plasmid liposome were separately added to 200 μL of serum-free DMEM medium, mixed thoroughly, and incubated at room temperature for 15 min. The mixed Lipofectamine 2000 was separately mixed and incubated for 30 min at room temperature followed by removal of the serum, rinsing with Phosphate-Buffered Saline (PBS), and subsequent addition of 1.6 ml serum-free DMEM medium. The cells were cultured in a 5% CO_2 incubator at 37°C for 6 hours followed by changing the medium to the serum-containing DMEM medium and further cultured for 48 hours for experimental research.

Real Time-PCR Analysis of LGR4 Expression

The synovial specimens were isolated, and the tissue blocks were placed in a laboratory containing liquid nitrogen for grinding, and an appropriate amount of lysate was added to collect the synovial cells of each group. The total RNA was extracted using the TRIzol reagent and reversely transcripted into cDNA using the kit (Axygen Scientific Inc., Union City, CA, USA). The primers were designed according to each gene sequence by PrimerPremier6.0, and were synthesized by Shanghai Yingjun Biotechnology Co. Ltd. (Guangzhou, China) (Table I). GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out using the $2^{-\Delta Ct}$ method.

MTT Assay Analysis of Proliferation of Synovial Cells

The synovial cells in the logarithmic growth phase were collected and inoculated into the 96-well culture plate supplemented with DMEM/F12 medium containing 10% fetal bovine serum at a cell number of 5×10^3, and the supernatant was discarded after 24 hours of culture, and randomly divided into the groups as mentioned above. After 48 hours of cell treatment, 20 μL sterile MTT was added into the wells. After 4 hours of continuous culture, the supernatant was completely removed, 150 μL/well of DMSO was added, and the shaker was shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (Absorbance, A) value was measured at a wavelength of 570 nm, and the proliferation rate of each group was calculated. The experiment was repeated three times.

Caspase-3 Activity Analysis

The changes in the Caspase-3 activity were examined according to the kit instructions. The trypsin digested cells were centrifuged at 600 g at 4°C for 5 min followed by discarding the supernatant and addition of cell lysis buffer into the cell lysate, which was placed on ice for 15 min. After that, the cells were centrifuged at 20000 g for 5 min at 4°C followed by addition of 2 mM Ac-DEVD-pNA. After that, the OD value at 405 nm was measured to reflect the Caspase-3 activity changes.

EdU Assay

The cell proliferation was measured using the EdU assay kit. According to the kit instructions, the cells were incubated with 10 μM EdU solution for 4 h, fixed in 4% paraformaldehyde, permeated with 0.5% Triton X-100, and reacted with the kit reaction mixture for 30 min at room temperature,

Table I. Primer sequences for real-time PCR.

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACTCTGTCAGTGG	TAATCGAATGTACGTGGT
LGR4	CAGGACTCCTCACTCTAAG	GCTTGACTGACGAGTGA

followed by staining with Hoechst 33342 cell nucleus and observation under a fluorescence microscope. The percentage of cell proliferation: number of cells stained with EdU / number of cells stained with Hoechst 33342 × 100%.

Western Blot Analysis of NF-κB Protein Expression

We extracted the total protein from tissues and cells: we added lysate, lysed the cells on ice for 15-30 min, disrupted the cells by 5 s × 4 times, centrifugated at 4°C at 10 000 × g for 15 min, and transferred the supernatant to the new Eppendorf (EP) tube. The BCA method was used to quantify the protein, which was stored at -20°C for the Western blot analysis. The isolated protein was separated on a 10% SDS-PAGE, and transferred to a PVDF membrane by a semi-dry transfer method at 100 mA for 1.5 h and blocked with 5% skim milk powder for 2 h. NF-κB (1:1000) and β-actin (1:2000) monoclonal antibody were incubated with the membrane at 4°C overnight. After washing with PBST, the corresponding secondary antibodies were added and incubated for 30 min at room temperature under dark. After PBST washing, the chemiluminescence was added for the visualization of the protein band. X-film and strip density measurements were separately scanned using the protein image processing system software and the Quantity one software. The experiment was repeated four times (n=4).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) and analyzed by the Statistical Product and Service Solution SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, USA). The mean values of the two groups were compared using the Student's *t*-test test and the differences between the groups were analyzed by the analysis of variance (ANOVA) with the Tukey post-hoc test. $p<0.05$ indicates the statistical difference.

Results

The Expression of LGR4 in Synovial Tissue of Rats with Traumatic Osteoarthritis

The expression of LGR4 mRNA and the protein was detected by Real Time-PCR and Western blot, respectively. Compared with the control group, the expression of LGR4 mRNA in the synovial tissues was significantly decreased in the traumatic osteoarthritis model group ($p<0.05$). Consistently, the expression of LGR4 protein in the synovial tissues of rats was also significantly reduced in the model of traumatic osteoarthritis (Figure 1).

The Secretion of LGR4 in Synovial Fluid of Rats with Traumatic Osteoarthritis

The expression of LGR4 in the synovial fluid was analyzed by ELISA. Compared with the con-

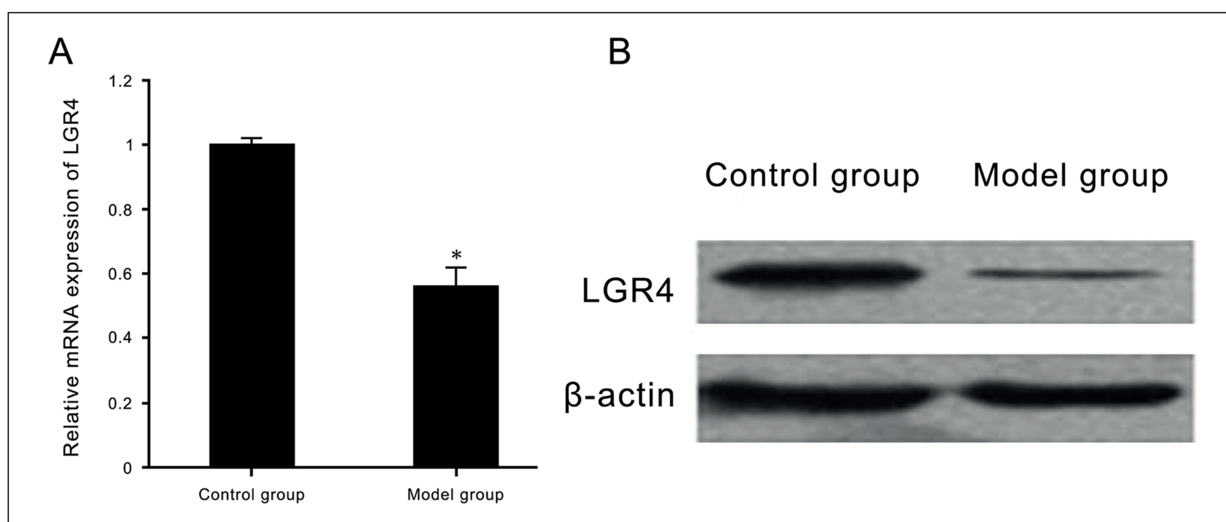

Figure 1. Expression of LGR4 in synovial tissue of rats with traumatic osteoarthritis. ***A,*** Real Time-PCR was used to detect the expression of LGR4 mRNA in synovial tissue of rats with traumatic osteoarthritis, compared with the control group, *$p<0.05$. ***B,*** Western blot analysis of LGR4 protein in rats with traumatic osteoarthritis expression in the membrane tissue.

trol group, the secretion of LGR4 in the synovial fluid of rats was significantly reduced in the model group ($p<0.05$) (Figure 2).

Effects of LGR4 on the Expression of LGR4 in Synovial Cells

The pcDNA3.1 empty plasmid and LGR4-pcDNA3.1 plasmid were transfected into the synovial cells of osteoarthritis rats, and the expression of LGR4 was analyzed. The results showed that the transfection of LGR4-pcDNA3.1 plasmid significantly upregulated the expression of LGR4 mRNA and protein in the synovial cells of osteoarthritis rats. Compared with the control group, the difference was statistically significant ($p < 0.05$) (Figure 3).

Effect of LGR4 on the Proliferation of Synovial Cells

MTT and EdU proliferation assays were performed to analyze the effect of LGR4 on the proliferation of the synovial cells in rats with osteoarthritis. The results showed that the transfection of LGR4-pcDNA3.1 plasmid significantly inhibited the proliferation of the rat synovial cells compared with the control group ($p<0.05$) (Figure 4).

Effect of LGR4 on Caspase-3 Activity in Synovial Cells

The transfection of LGR4-pcDNA3.1 plasmid significantly promoted the activity of Caspase-3 in rat synovial cells. Compared with the control group, the difference was statistically significant ($p<0.05$) (Figure 5).

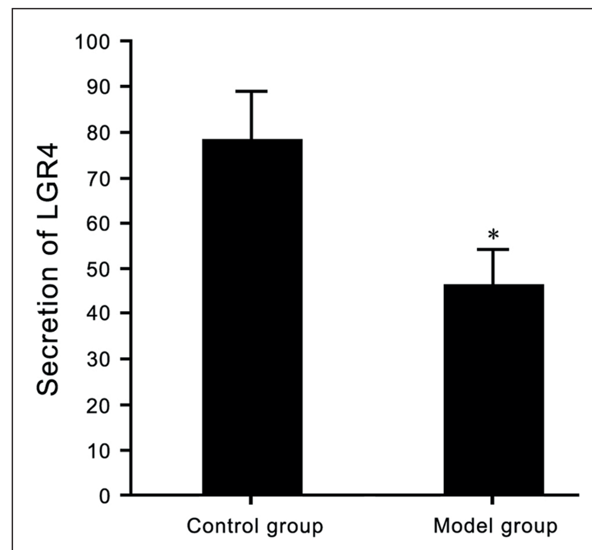

Figure 2. Secretion of LGR4 in synovial fluid of rats with traumatic osteoarthritis. Compared with the control group, *$p<0.05$.

Effects of LGR4 on Inflammatory Factors of Synovial Cells

ELISA was used to analyze the inflammatory factors IL-1, TNF-α, and IL-6 in the synovial cells of osteoarthritis rats after transfection of LGR4-pcDNA3.1 plasmid. The results showed

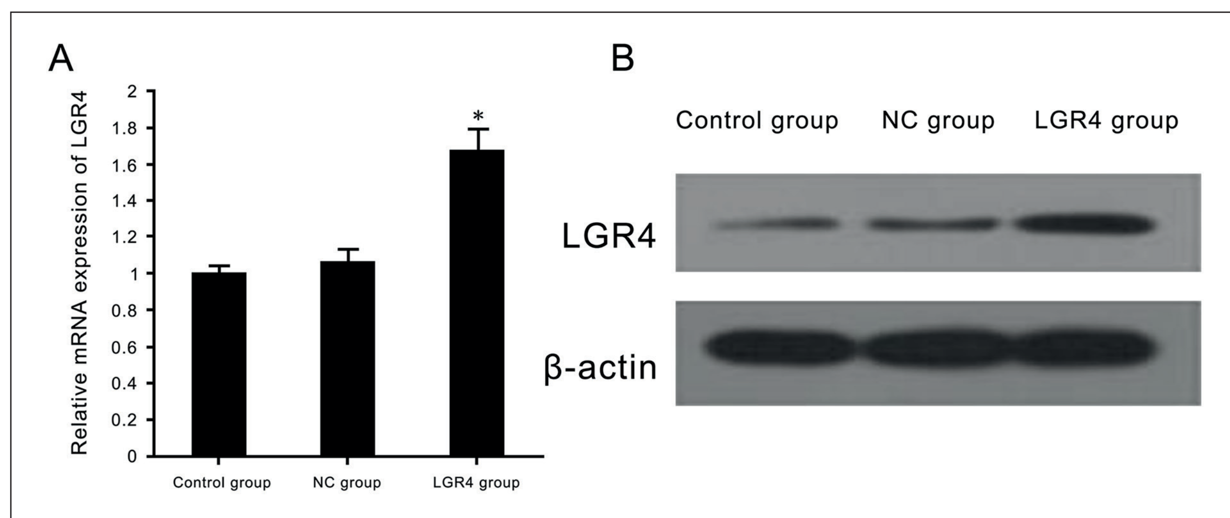

Figure 3. Effect of LGR4 on the expression of LGR4 in the synovial cells of rats with osteoarthritis. ***A,*** Real Time-PCR was used to detect the expression of LGR4 mRNA in the synovial cells of osteoarthritis rats. Compared with the control group, *$p<0.05$. ***B,*** Western blot analysis of LGR4 protein in the synovial cells of osteoarthritic rats expression.

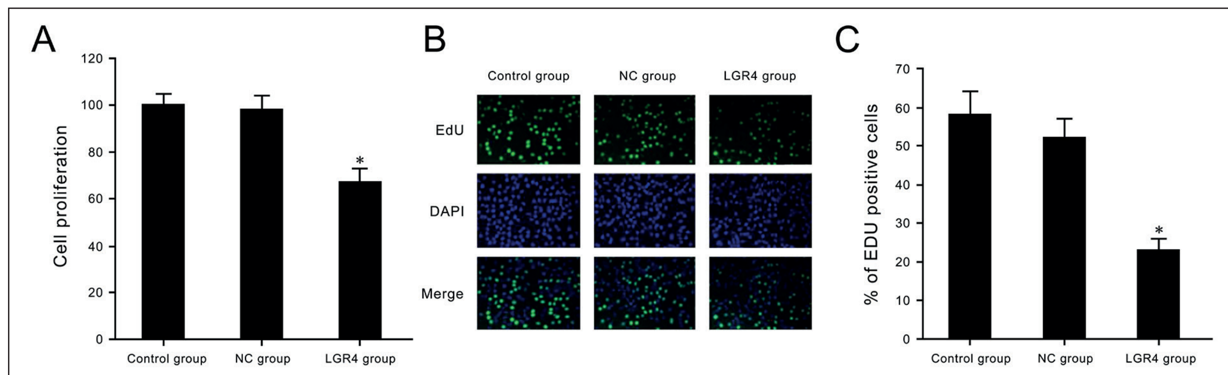

Figure 4. Effect of LGR4 on the proliferation of the synovial cells in rats with osteoarthritis. ***A,*** MTT assay was used to detect the effect of LGR4 on the proliferation of the synovial cells in rats with osteoarthritis. ***B,*** EdU proliferation assay was used to upregulate the effect of LGR4 on the proliferation of the synovial cells in osteoarthritis rats. ***C,*** The statistical analysis of EdU proliferation test. Compared with control group, *$p<0.05$.

that the upregulation of LGR4 expression in the synovial cells of osteoarthritis rats significantly inhibited the secretion of the inflammatory factors IL-1, TNF-α, and IL-6 compared with the control group ($p<0.05$) (Figure 6).

Effects of LGR4 on NF-κB Signaling Pathway in Synovial Cells

The Western blot analysis showed that the transfection of LGR4-pcDNA3.1 plasmid significantly inhibited the NF-κB expression (Figure 7).

Discussion

As traumatic osteoarthritis caused by joint trauma belongs to secondary osteoarthritis, the speed of the development and degree of trauma is closely related. Patients may have degenerative changes of the articular cartilage, progressive joint pain, and limited activity, similar to osteoarthritis[17,18]. However, the regulatory mechanisms involved in the traumatic osteoarthritis are not fully understood. The synovial cells play an important role in the progression of joint inflammation, which provides nutrients and oxygen to

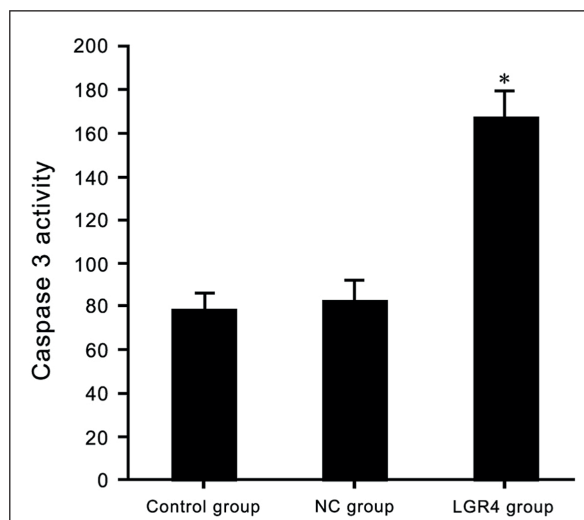

Figure 5. Effect of LGR4 on Caspase-3 activity in the synovial cells of osteoarthritis rats. Compared with control group, *$p<0.05$.

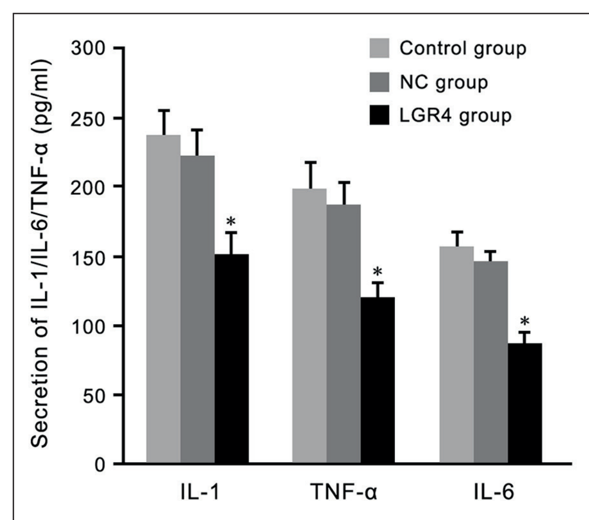

Figure 6. Effect of LGR4 on the inflammatory factors in the synovial cells of rats with osteoarthritis. Compared with control group, *$p<0.05$.

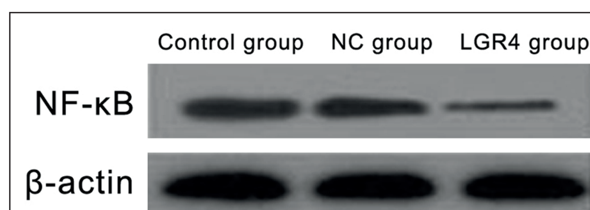

Figure 7. Effect of LGR4 on NF-κB signaling pathway in the synovial cells of rats with osteoarthritis.

articular chondrocytes. Therefore, the abnormal synovial tissue lesions lead to abnormal synovial fluid components, which alter the microenvironment of the joints and promote the development of osteoarthritis[19,20]. LGR4 has been shown to be involved in the development of bone and blood, and is associated with inflammatory response factors[16]. This study found that LGR4 mRNA and the protein expression in the synovial tissue of rats was significantly reduced in the rat model of the traumatic osteoarthritis, with a decreased secretion in the synovial fluid, suggesting that LGR4 might be involved in the progression of traumatic osteoarthritis.

In this study, the mechanism of LGR4 in traumatic osteoarthritis was further elucidated by transfecting LGR4 plasmid into the synovial cells of traumatic osteoarthritis. The results confirmed that the transfection of LGR4-pcDNA3.1 plasmid significantly promoted the LGR4 expression, inhibited the proliferation of synoviocytes, increased the activity of Caspase-3, and decreased the secretion of the inflammatory factors IL-1, TNF-α, and IL- 6. The synovial cell proliferation can lead to severe hypoxia in the joint cavity, increase synovial inflammation, and accelerate the cartilage cell hyperplasia in the microenvironment of synovitis, leading to rapid exacerbation of osteoarthritis[21,22]. In the present study, we observed that the overexpression of LGR4 in the synovial cells of traumatic osteoarthritis can inhibit synoviocytes proliferation, promote apoptosis, thereby alleviating inflammation, reducing the secretion of the inflammatory factors in the joint cavity, and ameliorating the progression of the joint inflammation. The NF-κB signaling pathway is a key cellular transcriptional regulator that maintains its inhibitory state under normal physiological conditions. However, under the stimulation of the external factors, NF-κB signaling pathway can be activated by upregulating the expression of the inflammatory factors. Then, the cytokines such as IL-1, TNF-α, IL-6 and tumor necrosis factor participate in the inflammatory response[23]. This study showed that the overexpression of LGR4 in the synovial cells of traumatic osteoarthritis inhibited the proliferation of synovial cells and inhibited the progression of traumatic arthritis by decreasing the expression of NF-κB. In further studies, an in-depth analysis of the expression and possible mechanisms of LGR4 in clinical patients is required to provide a reference for further elucidation of the role of LGR4 in traumatic osteoarthritis.

Conclusions

We demonstrated that the expression of LGR4 in synovial tissue and synovial fluid is reduced in the rat model of traumatic osteoarthritis. The upregulation of LGR4 expression in the synovial cells of rats with traumatic osteoarthritis can inhibit the secretion of the inflammatory factors by regulating NF-κB signaling pathway, and promote the proliferation of synovial cells, which may alleviate the development of the joint inflammation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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