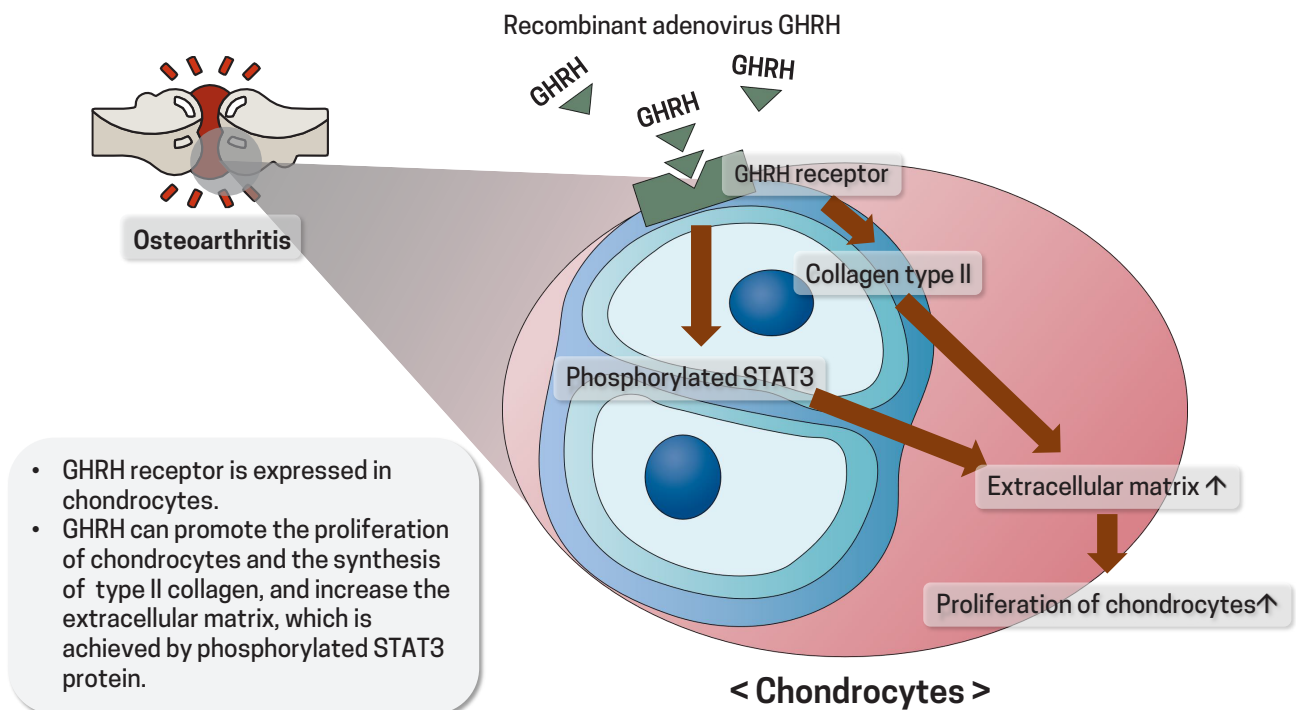


Effect of growth hormone releasing hormone on chondrocytes of osteoarthritis

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Background/Aims: To evaluate the effect and possible mechanism of growth hormone releasing hormone (GHRH) on chondrocytes of osteoarthritis (OA).

Methods: Articular chondrocytes were cultured and the expression of GHRH receptor in chondrocytes was detected. Then recombinant adenovirus GHRH (Ad-GHRH) was transfected to one group of chondrocytes. The expression of collagen type II, matrix metalloproteinase 13 (MMP-13) and signal transducer and activator of transcription 3 (STAT3) in each experimental group was determined by Western blot.

Results: The GHRH receptor was expressed in chondrocytes, and this provided a basis for further study of the role of GHRH in chondrocytes. Cell proliferation of the Ad-GHRH group was significantly higher than that of the OA group by CCK-8 assay. Compared with the OA-group, the protein expression of MMP-13 was decreased in the Ad-GHRH group. Compared with the OA-group, the protein expression of collagen type II, phosphorylated STAT3 (P-STAT3) were increased in the Ad-GHRH group.

Conclusions: Our results show that the GHRH receptor is expressed in chondrocytes. GHRH can promote the proliferation of chondrocytes and the synthesis of type II collagen, and increase the extracellular matrix, which is achieved by phosphorylated STAT3 protein.

Keywords: Osteoarthritis; Chondrocytes; Growth hormone releasing hormone; Matrix metalloproteinase 13; Collagen type II

INTRODUCTION

Osteoarthritis (OA) is a type of joint disease that results from breakdown of joint cartilage and underlying bone. The most common symptoms are joint pain and stiffness. Usually the symptoms develop over years and eventually lead to joint malformation and dysfunction [1]. OA is the most common form of arthritis. In China, about 100 million people are affected. Among those over 55 years of age, about 80% of people are affected [2]. OA can affect work and normal daily activities. Because of joint deformity and dysfunction, it seriously diminishes patient health and quality of life and poses a substantial economic burden for both families and society [3]. OA has become a medical and social issue of concern.

The pathogenesis of OA is still unclear. The most basic pathological changes are degeneration and disappearance of cartilage, which result in cartilage matrix degradation, chondrocytes death and joint integrity damage [4]. A variety of factors can regulate the synthesis and catabolism of extracellular matrix (ECM), and participate in the degradation of OA cartilage [5]. The chondrocytes is the only cell type in mature cartilage tissue and secretes ECM. Therefore, the survival of chondrocytes is very important for the maintenance of ECM. Damage of chondrocytes function and survival will inevitably compromise the structure and cause dysfunction of articular cartilage, which has an important

influence on the pathogenesis of OA [6].

Growth hormone releasing hormone (GHRH) is a peptide hormone secreted by the hypothalamus. Its main function is to regulate the release of pituitary growth hormone. However, in recent years a large number of studies have shown that GHRH and its specific receptor are expressed not only in the pituitary gland but also in many extra pituitary tissues, such as uterus, ovary, oviduct and testicle, placenta, cerebral cortex, pituitary, kidney, prostate, liver, lung and gastrointestinal tract [7,8]. Although the physiological significance of ectopic GHRH production has not been clarified, studies suggest that. GHRH is involved in a variety of cell proliferation, aging and other physiological and pathological processes. GHRH agonists affect peripheral tissues by direct receptor binding and stimulating cell proliferation. However, there are no published reports describing the effect of GHRH on articular chondrocytes of OA.

To evaluate the effect of GHRH on chondrocytes, we first cultured the articular chondrocytes and detected the expression of GHRH receptor. Then recombinant adenovirus GHRH (Ad-GHRH) was transfected to chondrocytes to observe the expression of collagen type II, matrix metalloproteinase 13 (MMP-13) and signal transducer and activator of transcription 3 (STAT-3). The effect and possible mechanism of GHRH on OA was determined.

METHODS

Animals

All animal care and experimental procedures were approved and conducted in accordance with the Institutional Animal Care and Use Committee of Jinzhou Medical University (2018014) and conformed to the Guide for the Care and Use of Laboratory Animal published by the U.S. National Institutes of Health.

Forty-eight male wistar rats (200 g; 12 weeks old) were obtained from the animal center of Jinzhou Medical University (Jinzhou, China; License number: SYXK 2014-0002). The maintenance and care of the experimental rats were in accordance with international relevant laws and regulations. The animals were maintained at the laboratory Animal Center for 1 week before the experiment. They were housed at humidity 50% \pm 5%, room temperature 23°C \pm 1°C with a 12-hour light cycle and were given food and water.

Anterior cruciate ligament transection model

The rats were randomly divided into control group, OA group and Ad-GHRH group. The rats were anesthetized (intraperitoneal injection of 10% chloral hydrate 4 mL/kg). A para-patellar skin incision was made on the medial side of the left knee joint, and thereafter on the medial side of the patellar tendon. Then the patella was dislocated laterally to provide access to the joint space. The anterior cruciate ligament was cut by ophthalmic scissors. A positive anterior drawer test validated complete transection of the ligament. The joint was then irrigated with 0.9% sodium chloride injection. The patellar ligament and skin were sutured. Control group underwent the same surgical procedure without cutting the anterior cruciate ligament. Each animal received an injection of penicillin 100,000 U daily for 3 days after surgery.

Cell culture

The rats were sacrificed and the limbs were sterilized with alcohol. The thigh bone was separated with aseptic scissors. Fascia, muscle and connective tissue were removed as much as possible. Cartilage tissue was removed from the joints and cut into 1 mm³ pieces. The tissue was washed for two times with phosphate buffer saline and 0.25% pancreatin was added. Tissue was digested for 15 to 20 minutes in a 37°C rocking bed, followed by addition of 0.2% collagenase for 30 to 60 minutes. Culture medium was added

to terminate digestion. The digested solution was filtered through 200- μ m nylon mesh and centrifuged at 12,000 \times g for 5 minutes. The cells were cultured with DMEM in an atmosphere of 5% CO₂ at 37°C. The chondrocytes of Ad-GHRH group derived from cartilage of the OA group of rats.

Expression of GHRH receptor, GHRH, MMP-13, collagen type II and STAT3 protein

Chondrocytes were lysed with protease and phosphatase inhibitors and centrifuged at 12,000 \times g at 4°C for 5 minutes. The supernatant was collected and mixed with loading buffer. Samples were resolved by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane with 80 V for 1 hour. Protein was electrically transferred onto a nitrocellulose membrane (60 V, 3 hours). After transfer, the membrane was blocked with 5% non-fat dry milk for 2 hours and incubated overnight with primary antibodies (Abcam Technology, Cambridge, MA, USA). Subsequently, the membrane was incubated with secondary antibodies at room temperature for 2 hours. Bands were visualized with the EC3 Imaging System (UVP LLC, Upland, CA, USA). A quantitative analysis of grey intensity was carried out with Image J software (National Institutes of Health, Bethesda, MD, USA).

Inoculated GHRH recombinant adenovirus expression vector

GHRH recombinant adenovirus expression vector was structured by the GENE Company (Shanghai, China). Chondrocytes were cultured in 96-well plates and grown to approximately 90% confluence. The growth medium was then removed, and 0.2 mL fresh medium containing adenovirus was added. The plates were incubated at 37°C for 4 hours to allow the virus to infect the cells. Then, the cells were removed. The culture medium containing the virus was extracted, and replaced with fresh culture medium. After incubation under these conditions for 96 hours, cells were assayed as indicated in the text.

Proliferation of cartilage cells

The cell counting kit-8 (CCK-8) assay was used to detect the proliferation of chondrocytes. Chondrocytes were suspended by trypsin digestion. The number of cells was 1×10^6 /mL. The cells were seeded into 96-well plates at a density of 1×10^5 cells per hole. Cells were cultured for 24 hours, then 10

μL CCK-8 reagent was added and the cells were incubated at 37°C for 3 hours. Absorbance of 450 nm was measured by an enzyme scale.

Statistical analysis

Experimental data were presented as means with standard deviations (SD) and analyzed with SPSS version 19.0 (IBM Co., Armonk, NY, USA). Intergroup data were compared by analysis of variance and $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS

Existence of GHRH receptors on chondrocytes

We used pituitary tissue of the rat brain as a positive control and skeletal muscle as a negative control. The expression of GHRH receptors was detected by Western blot. It can be

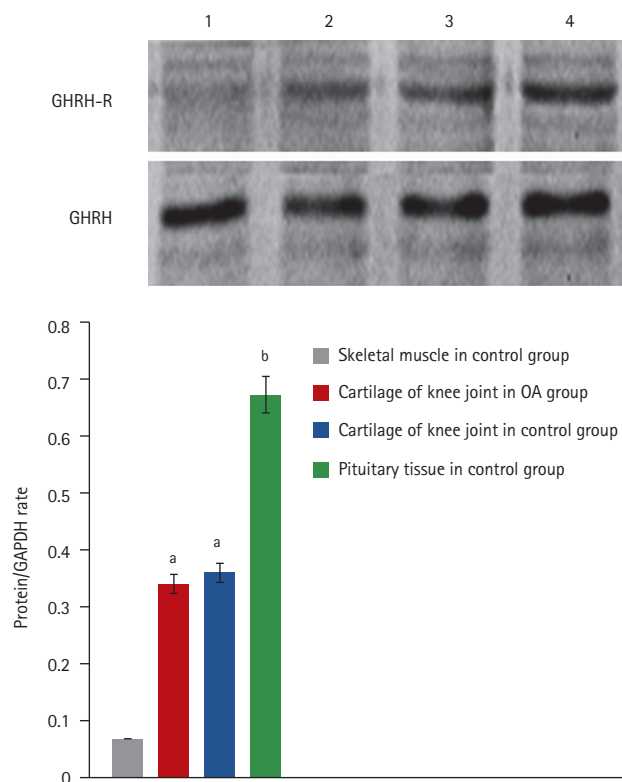


Figure 1. Western blot analysis. Lane 1, skeletal muscle in control group; line 2, cartilage of knee joint in osteoarthritis (OA) group; line 3, cartilage of knee joint in control group; line 4, pituitary tissue in control group. Integrated density values of growth hormone releasing hormone (GHRH) receptor (GHRH-R) are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ^a $p < 0.05$ vs. skeletal muscle in control group, ^b $p < 0.01$ vs. skeletal muscle in control group.

seen that the level of GHRH receptor protein is high in the pituitary gland and cartilage of rat knee joint. These results indicate that GHRH receptors are expressed on chondrocytes (Fig. 1).

Level of GHRH in chondrocytes

Western blot was used to detect the level of GHRH in chondrocytes. There was no significant difference in the level of GHRH between the OA group and the control group, which was significantly lower than that of the Ad-GHRH group (Fig. 2).

GHRH promotes the proliferation of cartilage cells

The proliferation of the chondrocytes was studied by CCK-8 assay. The results showed that the cell proliferation in the OA group was significantly lower than that of the control

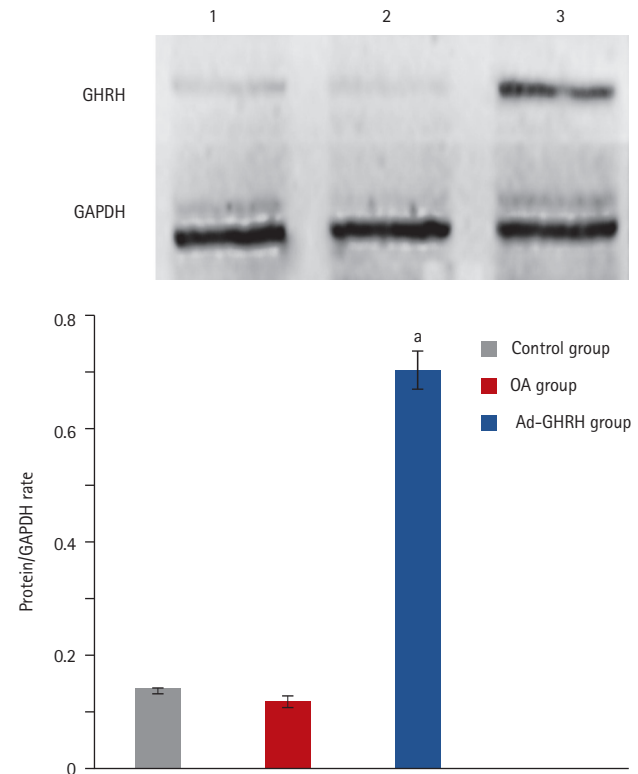


Figure 2. Western blot analysis. Lane 1, control group; line 2, osteoarthritis (OA)-group; line 3, adenovirus growth hormone releasing hormone (Ad-GHRH) group. Integrated density values of growth hormone releasing hormone (GHRH) are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ^a $p < 0.01$ vs. OA group.

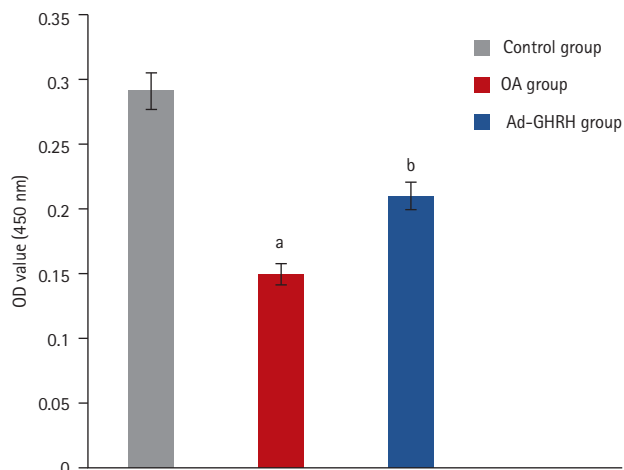


Figure 3. Cell viability of chondrocytes was measured using CCK-8 kit after treatments. Each bar is mean ± SD of at least three independent experiments. OD, optical density; CCK-8, cell counting kit-8; OA, osteoarthritis; Ad-GHRH, adenovirus growth hormone releasing hormone. ^a $p < 0.05$ vs. control group, ^b $p < 0.01$ vs. OA group.

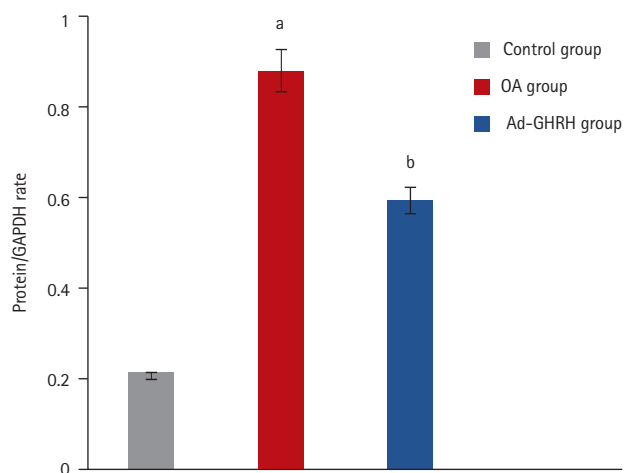
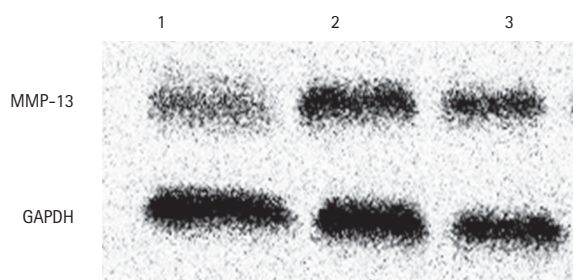


Figure 4. Western blot analysis. Lane 1, control group; line 2, osteoarthritis (OA)-group; line 3, adenovirus growth hormone releasing hormone (Ad-GHRH) group. Integrated density values of matrix metalloproteinase 13 (MMP-13) are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ^a $p < 0.05$ vs. control group, ^b $p < 0.01$ vs. OA group.

group, and the cell proliferation of the Ad-GHRH group was significantly higher than that of the OA group. The difference was statistically significant (Fig. 3).

Expression of MMP-13 and collagen type II

To evaluate the effect of GHRH on chondrocytes and extracellular matrix, we determined the expression of MMP-13 and type II collagen by Western blot analysis. The results showed that compared with the OA-group, the protein expression of MMP-13 was decreased in the Ad-GHRH group (Fig. 4). Compared with the OA-group, the protein expression of type II collagen was increased in the Ad-GHRH group (Fig. 5).

Expression of STAT3

We further determined the expression of STAT3 and phosphorylated STAT3 (P-STAT3). Western blot analysis showed that the expression of P-STAT3 in the Ad-GHRH group was significantly higher than in the OA group, and the difference was statistically significant (Fig. 6).

DISCUSSION

GHRH was first demonstrated in 1961. The 44-amino-acid forms of GHRH were first isolated and characterized from human pancreatic tumors that caused acromegaly. The action of GHRH is initiated by binding to the GHRH receptor. In recent years, many studies have shown that the GHRH receptor is expressed in a variety of non-hypothalamic tissues. To our knowledge, our study is the first to investigate whether chondrocytes express the GHRH receptor. We detected the expression of GHRH receptor in chondrocytes by Western blot. GHRH receptor is highly expressed in the pituitary gland and is not expressed in muscle tissue. Therefore, we used the pituitary tissue in the brain as the positive control and skeletal muscle as the negative control. Results showed that the GHRH receptor is expressed in chondrocytes, and this provided a basis for further study of the role of GHRH in chondrocytes.

The physiological significance of GHRH ectopic production is still unclear. In recent years, GHRH was shown to stimulate many kinds of cell proliferation, especially in some human tumor cell lines, such as stomach, lung, ovarian, and breast. Studies have shown that GHRH can activate the mitogen-activated protein kinase signaling pathway in

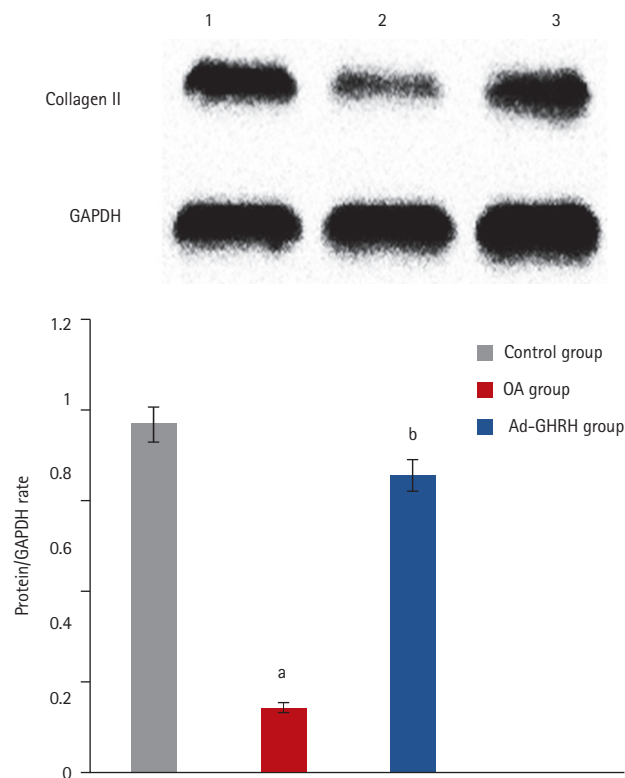


Figure 5. Western blot analysis. Lane 1, control group; line 2, osteoarthritis (OA)-group; line 3, adenovirus growth hormone releasing hormone (Ad-GHRH) group. Integrated density values of collagen type II are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ^a $p < 0.05$ vs. control group, ^b $p < 0.01$ vs. OA group.

MDA-MB-231 human breast cancer cells [9]. Munoz-Moreno et al. [10] showed that the GHRH antagonist inhibits the growth of the tumor by inhibiting the protein kinase B (Akt) and extracellular signal-regulated kinases (ERKs) signaling pathway in prostate cancer cells. Their study also showed that the GHRH receptor is expressed in cardiac myocytes. GHRH activates the ERK1/2 and phosphoinositide 3-kinases (PI3Ks)/AKT signaling pathway through its receptor, which improves the anti-apoptosis ability of cardiac myocytes and reduces the formation of cardiac scar tissue after myocardial infarction [11]. Ludwig et al. [12] found that the GHRH agonist JI-36 can promote the survival and metabolism of beta islet cells and inhibit the apoptosis of beta cells, and that the GHRH antagonist can reverse the effect. Dioufa et al. [13] reported that the GHRH agonist JI-38 promotes skin wound healing by regulating the proliferation of fibroblasts. In China, Ma et al. [14] studied the effect of the GHRH agonist JI-34 on bone marrow mesenchymal stem cells (MSC).

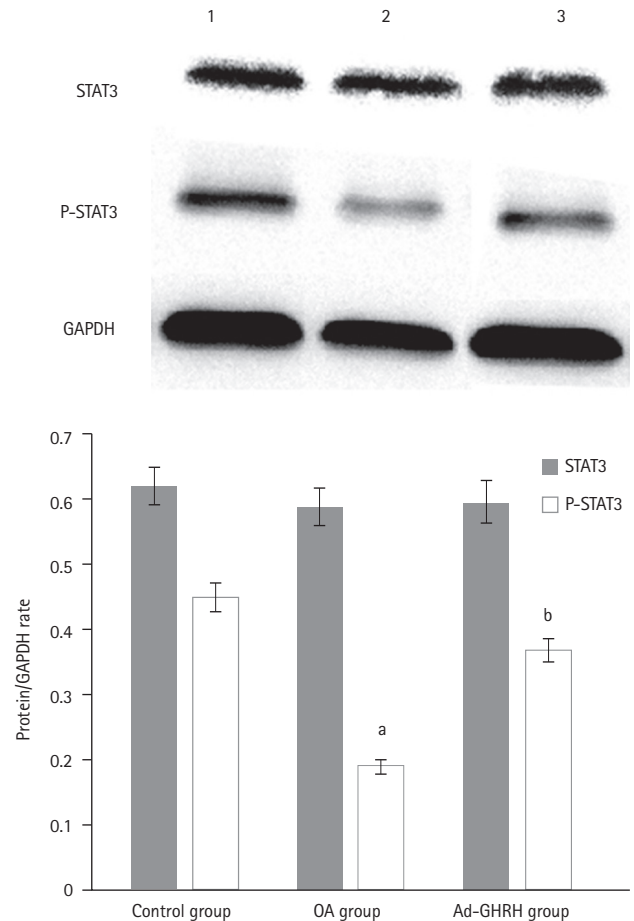


Figure 6. Western blot analysis. Lane 1, control group; line 2, osteoarthritis (OA)-group; line 3, adenovirus growth hormone releasing hormone (Ad-GHRH) group. Integrated density values of signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 (P-STAT3) are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ^a $p < 0.05$ vs. control group, ^b $p < 0.01$ vs. OA group.

Their results showed that after the pretreatment of MSC, JI-34 could increase the proliferation rate of MSC, enhance the anti-apoptosis ability of MSC in hypoxic and ischemic conditions, and enhance the ability of MSC to migrate and promote angiogenesis. These studies suggest that GHRH is involved in a variety of cell proliferation, aging and other physiological and pathological processes.

The role of GHRH in chondrocytes in osteoarthritis is not clear. The number of chondrocytes is decreased in osteoarthritis. The major component of the articular cartilage matrix is collagen type II. It represents 90% to 95% of total collagen content and forms the fibrils that give cartilage its tensile strength. Type II collagen is specifically expressed in car-

tilage and OA essentially involves catabolism and anabolism of articular cartilage. Collagen type II is recognized as the most important biomarker for OA [15]. MMP-13 is a major collagen type II degradation enzyme involved in the pathogenesis of OA [16]. We detected proliferation of chondrocytes and the protein expression of the collagen type II and MMP-13. Our results showed that compared with the OA-group, the protein expression of MMP-13 was decreased and the protein expression of collagen type II was increased in the Ad-GHRH group. These findings indicate that GHRH can promote the proliferation of chondrocytes and the synthesis of matrix protein of extracellular matrix, inhibit the degradation of matrix protein degrading enzyme and delay the development of OA cartilage lesions.

To identify the specific mechanisms of GHRH for chondrocytes and the related signal pathway involved, we verified the common signaling pathways such as Janus kinase (JAK)-STAT. The JAK-STAT signaling pathway is a chain of interactions between proteins in a cell and is involved in processes such as the metabolism of cellular proliferation, differentiation and survival. This pathway communicates information from chemical signals outside of a cell to the cell nucleus, resulting in the activation of genes through a process called transcription. Many studies have shown that the JAK-STAT signaling pathway plays an important role in the repair of articular cartilage [17]. Studies have shown that GHRH can activate the JAK2/STAT3 pathway. Xia et al. [18] have found that intracellular P-STAT3 increased significantly after treatment of JI-34 in mesenchymal stromal cells (MSCs). In our study, the results showed that compared with the OA group, the protein expression of P-STAT3 was increased in the Ad-GHRH group. Our findings indicate that GHRH can act on chondrocytes by binding to the GHRH receptor on the surface of chondrocytes and triggering the downstream STAT3 signaling pathway.

Collectively, our results demonstrate, for the first time, that the GHRH receptor is expressed in chondrocytes. Our findings further suggest that GHRH may have effects on chondrocytes that promote the proliferation of chondrocytes and the synthesis of collagen type II, and increase the extracellular matrix, which is achieved by phosphorylated STAT3 protein. At present, GHRH and its corresponding agonists are also used in the study of the treatment of endocrine system diseases such as congenital growth hormone deficiency, as well as in the study of other tissues that pro-

mote cell proliferation. The results of this study contribute to our knowledge of the effect and mechanism of GHRH on chondrocytes and provide a new target for the treatment of OA.

However, this study has several limitations. It is important to determine the role of GHRH in OA cartilage more directly after stimulation with exogenous GHRH and/or knockdown of GHRH receptors in cartilage cells. Unfortunately, these were not been determine in this study because that there was no suitable recombinant GHRH protein and our funds were limited. We will study these issues in our future research.

KEY MESSAGE

1. That the growth hormone releasing hormone (GHRH) receptor is expressed in cartilage cells.
2. GHRH can promote the proliferation of cartilage cells and the synthesis of type II collagen, and increase the extracellular matrix, which is achieved by phosphorylated signal transducer and activator of transcription 3 protein.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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