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LncRNA GAS5 induces chondrocyte apoptosis by down-regulating miR-137

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Abstract. – OBJECTIVE: Long non-coding RNA (lncRNA) participates in the pathogenesis of human knee osteoarthritis (KOA). Growth arrest specificity 5 (GAS5) is a member lncRNA, but its role in pathological regulation of KOA is still unknown. This study aims to explore the mechanism of GAS5 in KOA on chondrocyte apoptosis and other pathological processes.

PATIENTS AND METHODS: The serum and cartilage tissues were collected from 35 patients with KOA and 30 patients with traumatic amputation admitted to our hospital from April 2016 to April 2020. The expressions of GAS5 and miR-137 were detected and analyzed. Chondrocytes were extracted from cartilage tissues of KOA patients, and the genes were regulated by transfection. Then, the cells were detected, including apoptosis, apoptosis-related proteins (caspase-3, Bax/Bcl-2), and proliferation. The targeting relationship between GAS5 and miR-137 was verified.

RESULTS: GAS5 was up-regulated in serum and cartilage tissues of KOA patients, and down-regulation of GAS5 could inhibit the apoptosis process of chondrocytes and promote proliferation. MiR-137 was down-regulated in samples of KOA patients and was negatively regulated by GAS5. GAS5 induced apoptosis of chondrocytes and inhibited its proliferation through targeted down-regulating miR-137.

CONCLUSIONS: GAS5 is up-regulated in KOA serum, cartilage tissues and cells, and can induce chondrocyte apoptosis through down-regulating miR-137.

Key Words:
Knee osteoarthritis, GAS5, MiR-137, Chondrocytes, Apoptosis.

Introduction

Knee osteoarthritis (KOA) is a common musculoskeletal disorder. It can result in disability, with a lifetime risk of 45%[1,2]. The incidence risk of KOA in women is significantly higher than that of men (43.8% *vs.* 21.1%). The disease is usually hidden, with only 4.4% of males and 19.2% of females showing explicit symptoms[3]. The risk of KOA may be related with the polymorphism of IL-17A and IL-17F genes. In addition, obesity and sedentariness are also risk factors for KOA[4,5]. At present, there is no effective treatment option for non-joint replacement. The key point of the treatment is to identify early non-dominant KOA. The pathological mechanism from a molecular perspective is the basis for individualized KOA treatment[6]. Therefore, we focused on the molecular mechanism of KOA, hoping to provide available solutions for its treatment.

The pathological process of KOA involves cartilage destruction or degradation. The microscopic principle may be related to excessive induction of chondrocyte apoptosis. Maintenance of cartilage homeostasis is crucial to repair joint function[7,8]. Previous studies[9-11] have reported the effect of lncRNA on the progression of KOA diseases. LncRNA ZFAS1 can inhibit the apoptosis of chondrocytes and other molecular processes via regulating Wnt3a signaling pathway. LncRNA ROR can regulate the apoptosis and autophagy of chondrocytes by influencing HIF1α and p53[12,13]. Growth arrest specificity 5 (GAS5), as a member of lncRNA, also participates in the pathological regulation of OA. It can control the behavior of chondrocytes by regulating miR-34a-Bcl-2 pathway[14]. LncRNA-miRNA integrated network often participates in the molecular regulation process of KOA. As reported by Li et al[15], lncRNA CIR-miR-27 molecular network can regulate the degradation of extracellular matrix of chondrocytes in KOA. In the research of Bian et al[16], it was found that miR-137 has a targeted relationship with GAS5 in melanoma and is remarkably down-regulated in KOA patients. It can also be used in pathological processes such as chondrocyte proliferation and inflammation[17]. Therefore, we predicted that GAS5-miR-137 pathway has influence on the changes of molecular mechanism of chondrocytes in KOA.

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At present, there is little research on the mechanism of GAS5-miR-137 apoptosis in OA chondrocytes. We hereby carry out experiments to verify our hypothesis, which is of great significance for OA patients.

Patients and Methods

Human KOA Sample

Fasting venous blood and cartilage tissues during operation (total knee arthroplasty) were collected from KOA patients. There were 14 males and 21 females, aged 30-75 years, with an average age of 61.9±4.8 years. The corresponding control samples were from amputees with trauma at the same time, and these patients had no KOA or rheumatoid arthritis (RA). The collection time of samples started from April 2016 to April 2020. Venous blood samples were centrifuged at 1500 xg at 4°C for 10 min, then the serum was extracted and refrigerated at -70°C. Tissue samples were also prepared at -70°C after freezing in liquid nitrogen. All subjects have signed the informed consent, and this research was supported and approved by the Ethics Committee of our hospital.

Chondrocyte Culture and Transfection

As mentioned before[18], chondrocytes from cartilage tissue of subjects were extracted and cultured in Dulbecco's Modified Eagle's Medium (DMEM) comprising 10% fetal bovine serum (FBS) (Yaji Biological Technology Co., Ltd., Shanghai, China, PM150220B), and the environmental parameters were set to 37°C and 5% CO_2.

Next, chondrocytes were transfected according to the instructions of Lipofectamine™ 2000 kit (BioMag Scientific Inc., Wuxi, China, 11668019). Two days later, cells were collected for further analysis. Transfectants in this study included: GAS5 inhibitory vector (si-GAS5), GAS5 over-expression vector (GAS5), empty vector (vehicle), miR-137 mimic (miR-137), miR-137 inhibitor (Anti-miR-137), and negative control (miR-NC). They were all purchased from Guangzhou Huiyuanyuan Pharmaceutical Technology Co., Ltd.

Quantitative Real-Time PCR

β-Actin was used as internal reference of mRNA, and U6 was used as internal reference of miRNA. TRIzol reagent (Simgen, Hangzhou, China, 5301100) was first utilized to extract the corresponding total RNA from serum, cartilage tissues and chondrocytes, and then the total RNA was reverse transcribed into cDNA and amplified by cDNA first strand synthesis kit (Baiaolaibo Technology Co., Ltd., Beijing, Chia, WE0132-EOK) and SYBR Premix Ex Taq ™ kit (Biocreative Co., Ltd., Beijing, China, RR820A). All primers were synthesized by Shanghai Daixuan Biotechnology Co., Ltd. The primer sequences were as follows: GAS5: forward sequence: 5'-TCTGAGCAGGAATGGCAGTGT-3', reverse sequence: 5'-CATCCTCCTTTGCCACAGAAC-3'. miR-137: forward sequence: 5'-GTGACGGGTATTCTTGGGT-3', reverse sequence: 5'-GACTACGCGTATTCTTAAGCAA-3'. β-Actin: forward sequence: 5'-GCCGGGACCTGACTGACTAC-3', reverse sequence: 5'-TTCTCCTTAATGTCACGCACGAT-3'. U6: forward sequence: 5'-CGCTTCGGCAGCACATATAC-3', reverse sequence: 5'-TTCACGAATTTGCGTGTCAT-3'. Finally, $2^{-\Delta\Delta CT}$ was utilized to determine the relative expression.

CCK-8 in Determining Cell Proliferation

Cell proliferation was determined by Cell Counting Kit-8 (CCK-8) (Zhen Shanghai and Shanghai Industrial Co., Ltd., Shanghai, China, HZ-CCK8-Gu). The collected chondrocytes were inoculated into 96-well plates. Ten mL CCK-8 solution was put into each well at different incubation time points (0 h, 24 h, 48 h, 72 h), and incubated with cells for 2 h. Finally, the absorbance value was measured at 450 mm wavelength using NE90002 microplate reader (Yihui Biological Technology Co., Ltd., Shanghai, China).

Flow Cytometry in Determining Cell Apoptosis

The cells were digested with trypsin (Guang Rui Biological Technology Co., Ltd., Shanghai, China, X4380) and washed with phosphate-buffered saline (PBS). Then, 500 µL binding buffer containing fluorescein isothiocyanate (5 µL) and propidium iodide (5 µL) (Jinsui Biotechnology Co., Ltd., Shanghai, China, J31405, J81767) was added and evenly mixed, the mixture reacted for 10 min. Finally, the cell apoptosis rate was measured within 1 hour using an ACEA NovoCyte flow cytometry (Bidake Biotechnology Co., Ltd., Changzhou, China, 1026).

Western Blot Analysis

Proteins were isolated from chondrocytes with Radio-Immunoprecipitation Assay buffer (Halling Biological Technology Co., Ltd., Shanghai, China, HLIT0050), and bicinchoninic acid (BCA) kit (Jingke Chemical Technology Co., Ltd., Shanghai,

China, JK-201) was applied to measure the concentration. Cell lysates were separated by 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (Ruichu Biotechnology Co., Ltd., Shanghai, China, R07016), transferred to polyvinylidene fluoride membrane (Shifeng Biotechnology Co., Ltd., Shanghai, China, A3805), and then sealed for 2 h with blocking solution (Yiyan Biological Technology Co., Ltd., Shanghai, China, EY-24283). After that, the membrane was incubated with primary antibody including caspase-3, Bcl-2 Associated X (Bax), B-cell lymphoma-2 (Bcl-2) and β-Actin at 4°C overnight. All antibodies were purchased from Shanghai Zhenyu Biotechnology Co., Ltd. Then, the sample was incubated with horseradish peroxidase labeled goat anti-rabbit secondary antibody (AmyJet Scientific Inc., Wuhan, China, A1204-1) for 2 h. Finally, the protein band was visualized by electrochemical luminescence color development kit (Wine Da Industrial Co., Ltd., Shanghai, China, RPN2232), and then measured by MicroChemi 4.2 system (Dongsheng Innovation Biotechnology Co., Ltd., Beijing, China).

Dual-Luciferase Reporter

We explored the targeting relationship between GAS5 and miR-137 by constructing wild type (Wt) and mutant type (Mut) of GAS5. The GAS5 fragments above were co-transfected with miR-137 and miR-NC. After 48 hours of cell lysis, Dual-Luciferase reporter (Cosmo Biotechnology Co., Ltd., Tianjin, China) was applied to detect luciferase activity in cells.

RNA Co-Immunoprecipitation

The experiment was conducted using EZMagna RIP kit (Taize Jiaye Technology Development Co., Ltd., Beijing, China, 17-701). Cells were lysed, incubated with protein A magnetic beads and primary antibody for 1 hour, and then immunoprecipitated at 4°C for 8 hours. Last, RNA was purified, and the expression levels of GAS5 and miR-137 were detected.

Statistical Analysis

All the data were expressed as mean±standard deviation. Data processing and picture generation were all performed by GraphPad 6 software. And all experiments were independently conducted at least 3 times. Independent sample *t*-test, one-way ANOVA, LSD-t test, repeated measurement ANOVA, Bonferroni test and other methods were used to evaluate the differences of data between groups, and $p<0.05$ was considered statistically significant. The diagnostic efficacy of GAS5 and miR-137 in serum for KOA was also evaluated through the receiver operating characteristic (ROC) curve of the subjects, and Pearson correlation coefficient was applied to evaluate the correlation between GAS5 and miR-137 expression in serum and tissues.

Results

GAS5 Is Up-Regulated in KOA

To verify whether GAS5 has abnormal imbalance in KOA serum and cartilage tissues, we detected the relative expression of GAS5 in KOA serum and cartilage tissues. We found that the expression of GAS5 in serum and cartilage of KOA patients was significantly higher than that of traumatic amputees ($p<0.001$), suggesting that GAS5 may be involved in pathological changes of KOA. We did correlation analysis of GAS5 in serum and cartilage tissues and found that GAS5 expression in two different lesion sites showed a significantly positive correlation ($r=0.692$, $p<0.001$), indicating that GAS5 expression in serum might become a biological indicator for noninvasive screening of KOA. We also drew ROC curve for further analysis. In our results, the area under the curve (AUC) of serum GAS5 screening KOA was up to 0.860, indicating that GAS5 was expected to be an auxiliary indicator for the recognition of KOA (Figure 1).

GAS5 Has the Ability to Inhibit Chondrocyte Apoptosis and Promote Proliferation

Chondrocytes were isolated from the cartilage tissues of the subjects, and *in vitro* experiments were conducted to explore the molecular mechanism of GAS5. There was abnormally high expression of GAS5 in chondrocytes of OA patients. Then, we transfected the chondrocytes with GAS5 over-expression and inhibition vectors to realize up-regulation and down-regulation. In the cell function test, up-regulation of GAS5 considerably induces chondrocyte apoptosis, the mechanism of which might be related to the increase of apoptosis factors caspase-3, Bax/Bcl-2 levels and the inhibition of cell proliferation. However, we obtained contrary results in down-regulation of GAS5, the chondrocyte apoptosis was inhibited and the proliferation was promoted. The above results had significant differences ($p<0.05$). The decrease of caspase-3 and Bax/Bcl-2 levels can inhibit cell apoptosis and had an protective effect in KOA[19,20]. Based on the above results, we believed that GAS5

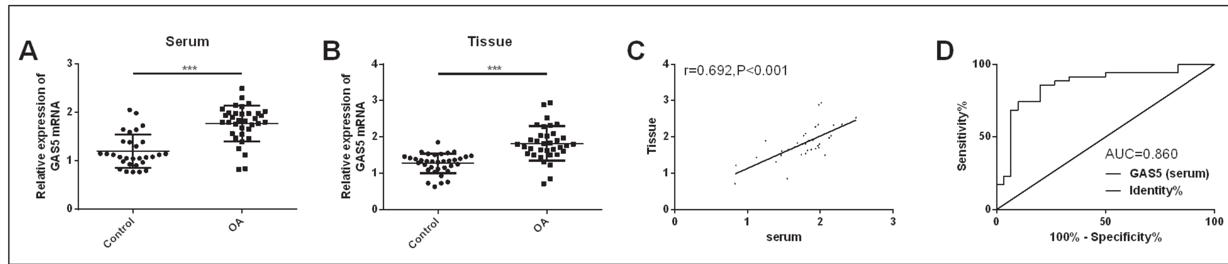

Figure 1. Expression and diagnostic value of GAS5 in KOA. **A**, The expression of GAS5 in KOA serum was higher than that in traumatic amputees. **B**, The expression of GAS5 in KOA cartilage tissues was higher than that in traumatic amputees. **C**, The expression of GAS5 in KOA serum was remarkably positively correlated with cartilage tissues (r=0.692, p<0.001). **D**, The diagnostic performance (AUC) of GAS5 in KOA serum reached 0.860. Notes: Comparison between the two groups, ***p<0.001.

participates in the pathological mechanism of KOA and can regulate the apoptosis and proliferation of chondrocytes. The development of GAS5 biological inhibitors may have important therapeutic effects on improving the progression of KOA diseases (Figure 2).

MiR-137 Is Down-Regulated in KOA

The expression level of miR-137 in KOA was also analyzed. Both the expression levels in serum and cartilage tissues of KOA patients were low (p<0.001). The expression levels of the two lesion sites were remarkably positively correlated (r=0.673, p<0.001), while miR-137 and GAS5 in cartilage tissues were remarkably negatively correlated (r=-0.610, p<0.001). In addition, the AUC of serum in diagnosing KOA reached 0.854. The above results indicate that miR-137 may play a certain role in the disease process of KOA and has a potential connection with GAS5. Its expression level in serum may be helpful for monitoring KOA conditions, and as a serological diagnostic indicator, it may be helpful for early identification of KOA (Figure 3).

GAS5, as a Molecular Sponge of MiR-137, Negatively Regulates its Transcription

Through biological analysis, we further explored the regulatory mechanism of GAS5. We predicted in Starbase that there were targeted binding sites between GAS5 and miR-137, which might be the downstream effector of GAS5. We also found that the expression of miR-137 changed inversely with the change of GAS5. After the up-regulation of GAS5, the expression of miR-137 was notably down-regulated. According to Dual-Luciferase reporter and

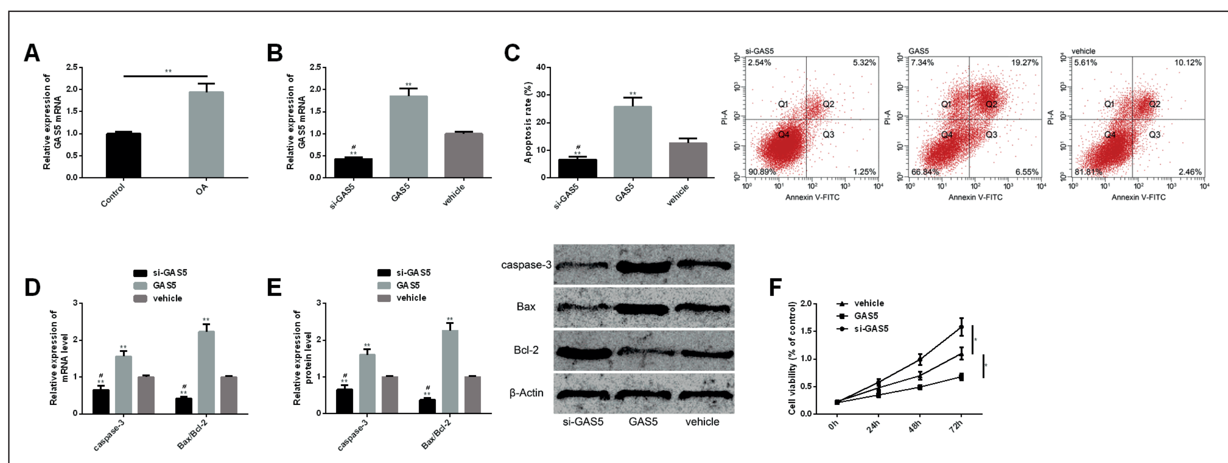

Figure 2. Effect of GAS5 on biological function of chondrocytes. **A**, The expression of GAS5 in chondrocytes was remarkably higher. **B**, Transfection efficiency of GAS5. **C**, Effect of GAS5 on chondrocyte apoptosis, as well as its cell flow picture. **D**, The effect of GAS5 on transcription level of chondrocyte apoptosis-related factors. **E**, The effect of GAS5 on the level of chondrocyte apoptosis-related factor protein, as well as its protein profiling. **F**, The effect of GAS5 on chondrocyte proliferation. Notes: Comparison between the two groups or comparison with vehicle, **p<0.01. Comparison with GAS5, $^{\#}p$<0.05.

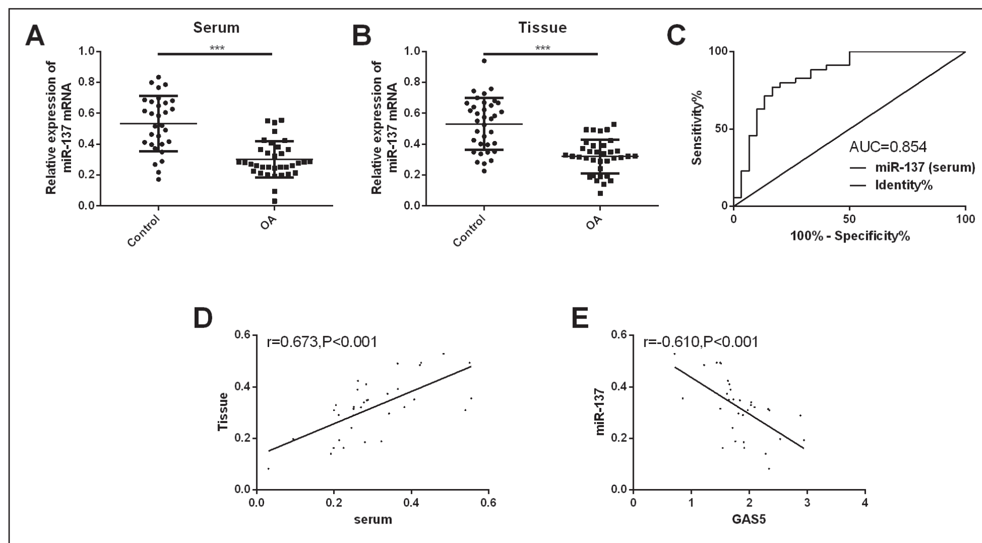

Figure 3. Expression and diagnostic value of miR-137 in KOA. **A**, Expression of miR-137 in KOA serum. **B**, Expression of miR-137 in KOA cartilage tissues. **C**, ROC curve of serum miR-137 for KOA diagnosis. **D**, Correlation between miR-137 in serum and miR-137 in cartilage tissues. **E**, Correlation between miR-137 and GAS5 in cartilage tissues. Notes: Comparison between the two groups, ***p<0.001.

RNA co-immunoprecipitation analysis, it was found that miR-137 significantly down-regulated GAS5-Wt but had no remarkable effect on GAS5-Mut. In addition, the expression level of GAS5 in miR-137 complex containing Ago2 was notably increased. All results showed significant difference ($p < 0.05$). It is also suggested that GAS5 may negatively regulate its expression via sponging miR-137 (Figure 4).

GAS5 Down-Regulate MiR-137 to Induce Chondrocyte Apoptosis and Inhibits Proliferation Level

In view of the interaction between GAS5 and miR-137, we tried to verify whether GAS5 mediated the regulation of miR-137 on chondrocyte biological behavior. On the basis of GAS5, we transfected miR-137, Anti-miR-137 and miR-NC for double intervention. The results showed that the miR-137 expression in chondrocytes co-expressed by GAS5, Anti-miR-137 and miR-NC was notably increased. Moreover, miR-137 expression in chondrocytes co-expressed by GAS5+Anti-miR-137 was notably higher than that of GAS5+miR-NC, while the miR-137 expression in chondrocytes co-expressed by GAS5+miR-137 had no remarkable change, suggesting that GAS5 could offset the increase of miR-137 expression by transfection with miR-137, while the co-action with Anti-miR-137 could enhance the expression

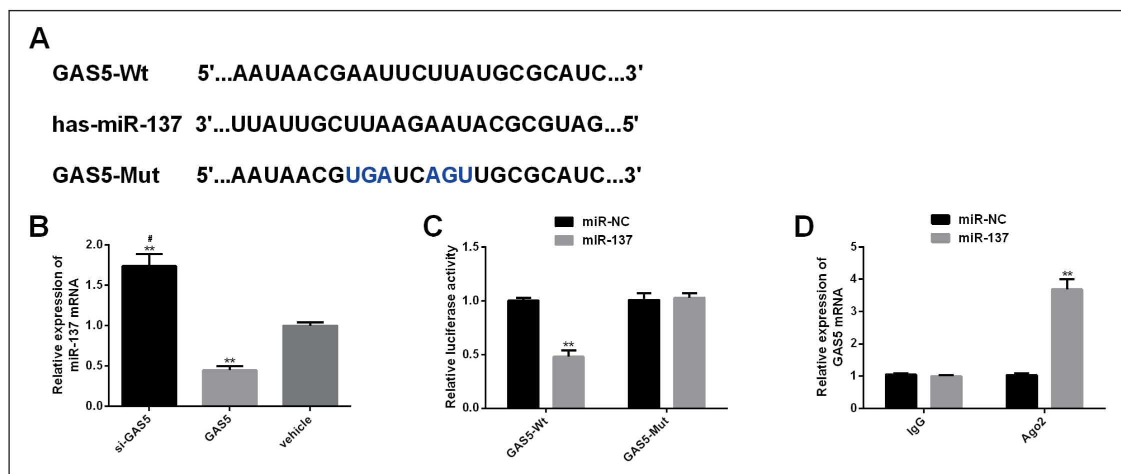

Figure 4. Targeted relationship between GAS5 and miR-137. **A**, Binding sites of GAS5 and miR-137. **B**, Up-regulation or down-regulation of GAS5 on miR-137 expression. **C**, Dual luciferase report assay **D**, RNA co-immunoprecipitation test. Notes: Comparison with vehicle/miR-NC, **p<0.01. Comparison with GAS5, $^{\#}p$<0.05.

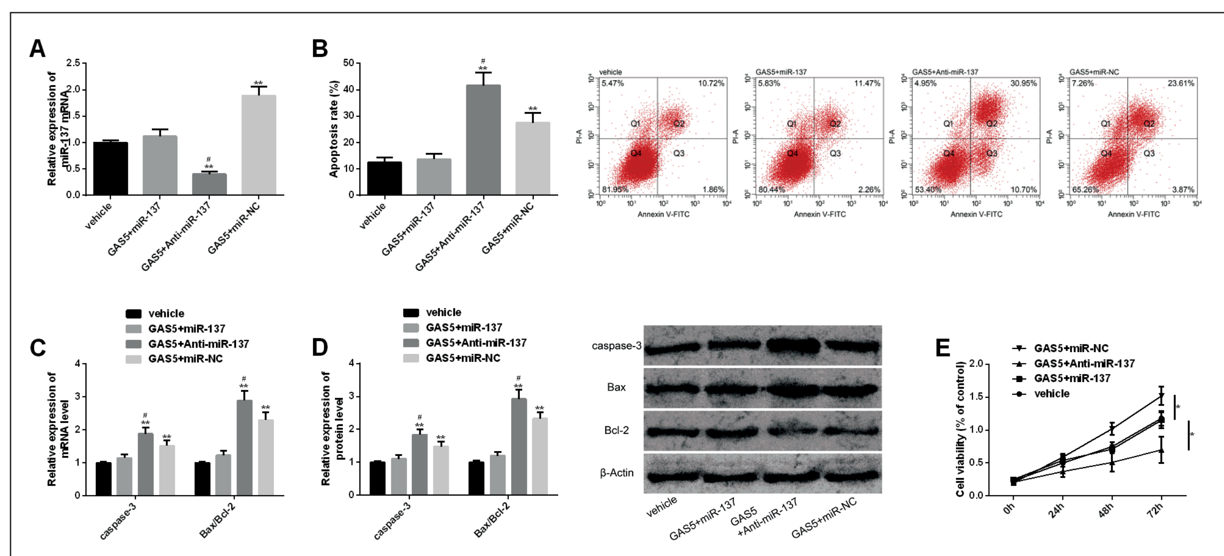

Figure 5. GAS5 mediates the effect of miR-137 on chondrocyte function. **A**, Co-expression of GAS5 and miR-137. **B**, GAS5 mediated the effect of miR-137 on chondrocyte apoptosis, as well was its cell flow picture. **C**, GAS5 mediated the effect of miR-137 on the transcription level of chondrocyte apoptosis factor. **D**, GAS5 mediated miR-137 effect on chondrocyte apoptosis factor protein level, as well as its protein profiling. **E**, GAS5 mediated the effect of miR-137 on chondrocyte proliferation. Notes: Comparison with vehicle or between groups, $^{**}p<0.05$. Comparison with GAS5, $^{\#}p<0.05$.

of miR-137. In cell function analysis, chondrocytes co-expressed by GAS5+Anti-miR-137 also showed higher apoptotic level, higher caspase-3 and Bax/Bcl-2 levels, and remarkably blocked cell proliferation. This suggested that Anti-miR-137 could further induce GAS5 to promote apoptosis and inhibit cell proliferation (Figure 5).

Discussion

As a progressive disease that gradually weakens people's athletic ability, KOA has various negative effects on 9 million American adults, and this trend is getting younger, bringing heavy pressure to society and economy[21,22]. Because of lncRNA's outstanding performance in KOA process, its targeted therapy research in KOA has become a hot topic in the current search for therapy strategies[23,24]. Here, we mainly focused on the role of GAS5 in KOA, which is of great significance to reduce the patient's condition and social pressure.

We separately verified the maladjustment of GAS5 and its potential target gene miR-137 in KOA and found that GAS5 was remarkably up-regulated in both serum and cartilage tissues of KOA patients. In addition to the up-regulation of GAS5 in KOA, we have also witnessed its similar disorders in Klinefelter's syndrome, atherosclerosis, myocardial ischemia-reperfusion injury and other diseases[14,25-27]. As a serum indicator, GAS5 has great efficiency in screening KOA, and we are optimistic about the diagnostic efficacy of GAS5. After down-regulating GAS5, we found that GAS5 significantly suppressed chondrocyte apoptosis and promoted cell proliferation. The mechanism of action might be related to low levels of caspase-3 and Bax/Bcl-2. The development of GAS5 inhibitors for targeted delivery of chondrocytes from KOA patients had certain effect on the treatment of KOA. The biological efficacy of GAS5 in OA is controversial to some extent. In the research of Li et al[28], GAS5 is down-regulated in chondrocytes ATDC5 under the intervention of lipopolysaccharide, and its over-expression is beneficial to relieve inflammatory injury of cells. Similar to our results, Ji et al[14] pointed out that silencing GAS5 expression in KOA can remarkably improve the proliferation ability and inhibit apoptosis of chondrocytes, thus exerting therapeutic effect.

Correspondingly, the biological function of miR-137 in KOA also showed us the favorable side of the development of miR-137 expression promoter for the reversal of KOA process. Its mechanism of action is mediated by GAS5, and they have an interactive relationship. The serological screening efficiency of miR-137 for KOA was comparable to GAS5, indicating that they may become sensitive and minimally invasive indicators for KOA diagno-

sis. miR-137 not only has great performance in KOA application, but also has certain therapeutic effect in RA and spinal cord injury. According to Du et al[29], its expression in RA fibroblast-like synovial cells is down-regulated. Up-regulation of its expression can target ADAMTS-5, significantly block malignant performance of cells and suppress inflammatory state. In the report of Gao et al[30], it has a protective effect on the apoptosis and inflammatory damage of astrocytes in mice by targeted inhibition of MK2.

Although our research stops here, it is also a new beginning. It is still unknown whether GAS5-miR-137 axis has regulatory effect on other pathological processes such as autophagy and inflammatory reaction of KOA, whether it mediates a certain pathway to play a role, whether it can reflect the disease severity of KOA, and whether it has significant correlation with pathological parameters of OA patients. Besides, further exploration of downstream targets of miR-137 is also conducive to understanding the role of molecular regulatory networks in disease regulation in KOA. All the above questions are helpful for us to further explore the potential clinical application of GAS5-miR-137 axis, and we plan to gradually solve the above questions in the future.

Conclusions

To sum up, we found for the first time that GAS5 induces chondrocyte apoptosis through down-regulation of miR-137. GAS5-miR-137 molecular pathway may be a major breakthrough in KOA treatment, and targeted negative modulators of GAS5 or miR-137 may provide a new treatment system for OA patients.

Conflict of Interests

The authors declare that they have no conflict of interest.

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