Long non-coding RNA growth arrest-specific transcript 5 regulates rheumatoid arthritis by targeting homeodomain-interacting protein kinase 2

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Abstract

Objective

It has been proved that fibroblast-like synoviocytes (FSs) play a critical role in the course of rheumatoid arthritis (RA), is a systemic autoimmune disease affecting multiple joints. Until now, no effective treatment has been established. Long non-coding RNA Growth Arrest-Specific Transcript 5 (GAS5) has been identified as a tumour-suppressor lncRNA in various cancers. However, the expression, biological role and clinical significance of GAS5 in RA is completely unknown. In this study, we test the hypothesis that GAS5 might inhibit proliferation and inflammatory response of FSs in RA.

Methods

The expression of GAS5 was examined in synovial tissues from RA patients and normal individuals.

Results

The expression of GAS5 was significantly reduced in RA synovial tissues and RA FSs, whereas the expression of homeodomain-interacting protein kinase 2 (HIPK2) was increased, indicating that it plays a critical role in inflammation and autoimmune diseases. We found that overexpression of GAS5 decreased the level of HIPK2, TNF-α and IL-6.

Conclusion

The methylation-specific PCR results suggested that the GAS5 gene promoter was significantly methylated in RA synovial tissues and RA FSs. More importantly, treatment with methylation inhibitor 5-aza-2-deoxycytidine (5-azadC) inhibited hypermethylation of GAS5 promoter and expression of HIPK2. These results indicated that GAS5 regulates RA via potentially targeting HIPK2. Therefore, this study may provide a potential therapeutic target for RA.

Key words

rheumatoid arthritis, lncRNA GAS5, fibroblast-like synoviocytes, homeodomain-interacting protein kinase 2, DNA methylation

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Introduction

Rheumatoid arthritis (RA) is one of the most commonly systemic autoimmune disease characterised by synovial inflammation in the musculoskeletal joints resulting in cartilage degradation and finally bone destruction with consequent disability (1, 2). RA has affected approximately 1% of the population worldwide, which put huge economic pressures on normal life (3, 4). Treatment options include glucocorticoids, monoclonal antibodies, methotrexate, and other pharmacological agents such as targeting inflammatory mechanisms and the monoclonal antibodies include TNF-α (5), granulocyte-macrophage colony-stimulating factor (6) and so on. Despite all these standard therapies, many RA patients fail to respond (7, 8). Therefore, it is emergency to identify the mechanism of RA occurrence and development and effective therapeutic approaches for RA (9). During the occurrence and the progression of RA, fibroblast-like synoviocytes (FSs) proliferate and secrete large amounts of synovial fluid and cytokines such as integrins, VCAM-1 and Cadherin-11 (10-12) and play a key role in initiating and perpetuating inflammation in RA development (13, 14).

Long non-coding RNAs (lncRNAs) cannot be translated into proteins and is a transcription product of more than 200 nucleotides with a length of 200 nt to ~100 kb (15, 16). lncRNAs is well known as the transcriptional noise (17, 18). Because of their widespread presence, lncRNAs have been reported to participate in various biological processes and perform an increasingly pivotal role in human diseases (19, 20). Studies have shown that lncRNAs participate in liver cancer, osteosarcoma, neurodegenerative disease, cardiovascular disease and other diseases (21). Increasing evidence has also shown that lncRNAs play an important role in RA progression. For example, lncRNA MEG3 was found downregulated in synovial tissues and FSs in RA mice and enforced MEG3 inhibited the expression of IL-6 and TNF-α and cell proliferation of FSs (22). LncRNA Growth Arrest-Specific Transcript 5 (GAS5) was previously shown to downregulate in many cancers such as breast cancer (23, 24) and prostate cancer (25, 26), which has been identified as a tumour-suppressor lncRNA. However, the biological role and clinical function of GAS5 in RA is still unknown. Therefore, in this study, we investigated the expression of GAS5 in RA synovial tissues and RA FSs. We found that GAS5 regulates RA via targeting homeodomain-interacting protein kinase 2 (HIPK2) which has been proved to play a key role in inflammation and autoimmune diseases. Our findings will provide new insights into the molecular function of GAS5 in RA and may hopefully provide a potential therapeutic target for RA.

Materials and methods

Study subjects

In this study, 20 RA patients' samples and 20 trauma patients' samples were collected. The 20 RA patients underwent surgical resection or joint replacement surgery at the Second Affiliated Hospital of Harbin Medical University. The mean age of 20 RA patients are 52±3.2 years old and there were 10 males and 10 females. The mean age of the control group, 20 trauma patients, is 53±3.5 years old. The work described was carried out in accordance with The Code of Ethics of the World Medical Association and the eligible patients were diagnostically confirmed in accordance with the American College of Rheumatology 1987 revised criteria for RA (27).

Cell culture and identification

For FSs culture, the extracted synovial tissues were cut into paste and digested with 0.15% type II collagenase (Thermo, USA). After digestion, the collected cells were cultured with high-glucose DMEM medium (Hyclone, Logan, UT) and supplemented with 15% FBS (Aidenbach, Germany) for 7 days. Washed the cells with PBS (Gibco, USA) to remove the non-adherent or dead cells and kept the cells in high-glucose DMEM medium. When reached 70–80% confluence, the cells were subcultured into a ratio of 1: 2. After three passages, the FSs were used for further experiments. To characterise the cytological phenotypes of

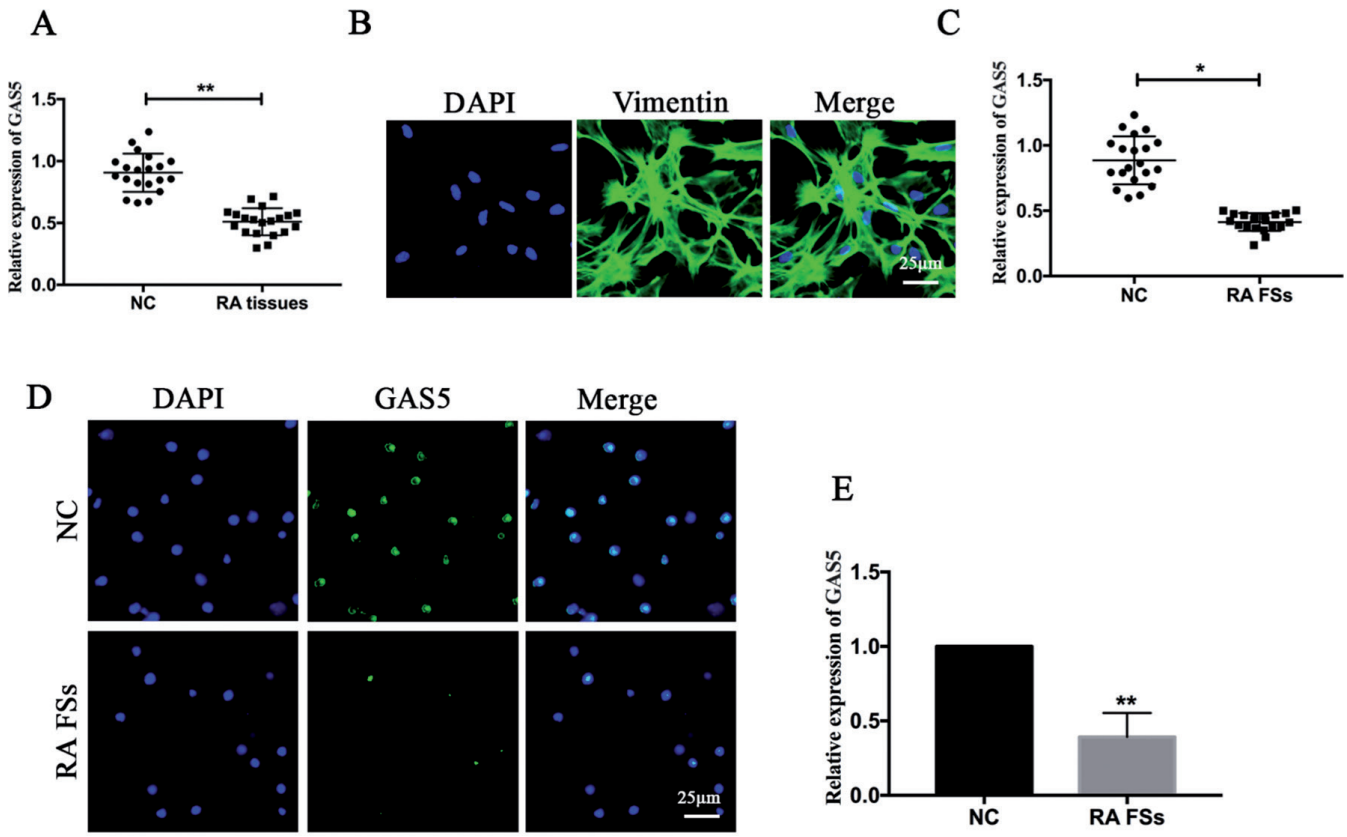

Fig. 1. GAS5 expression is reduced in RA synovial tissues and RA FSs.
A: The expression of GAS5 in RA synovial tissues determined by RT-qPCR. ***p*<0.01, NC: the control group, n=20; the RA group: rheumatoid arthritis tissues, n=20.
B: The FSs were identified by vimentin in the immunofluorescence assay.
C: The expression of GAS5 in RA FSs determined by RT-qPCR. **p*<0.05, NC: the control FSs group, n=20; the RA group: rheumatoid arthritis FSs, n=20.
D: The expression of GAS5 in RA FSs measured by FISH.
E: Relative expression of GAS5 in (**D**).
***p*<0.01, NC: the control FSs group, n=20; the RA group: rheumatoid arthritis FSs, n=20. The experiments were independently repeated 3 times.

synovial cultures, the FSs were identified by vimentin staining.

Cell transfection

The sequence of GAS5 was using human liver cDNA (Invitrogen) and cloned into pCDNA3.1 vector (pGAS5). HIPK2-siRNA and its control were acquired from Santa Cruz Biotechnology. FSs were seeded into a six-well plate and grown to 60–80% confluency, then transfected plasmids or its control plasmids using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols.

Immunofluorescence staining

1×105 FSs cells were seeded on the 3.5 cm cell plate. After 24h, cells were fixed with 4% paraformaldehyde (Boster, China) for 20 min, and subsequently permeabilised using 0.5% Triton X-100 (Boster, China) for 30 minutes, then blocked by 5% BSA for 1h. Primary antibody was applied overnight at 4°C in 1% bovine serum albumin buffer. Secondary antibodies were applied after wash with PBS. After 1h, all cells were counterstained with DAPI (Boster, China) for 5 min. The slices were photographed by a fluorescence microscope.

ELISA assay

Protein in the cultured supernatant were quantified by the TNF-α ELISA KIT and IL-6 ELISA Kit (Boster, China) according to the manufacturer's instructions.

Total RNA extraction and quantitative real-time PCR analysis (qRT-PCR)

Total RNA was isolated using TRIZOL reagent (Invitrogen) from synovial tissues and FSs. According to the manufacturer's protocol. qRT-PCR was employed to determine the mRNA expression of GAS5, HIPK2, interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α). qRT-PCR was performed using SYBRGreen qPCR Master Mix (TAKARA, Japan). The primer sequences are as follows. The mRNA levels of β-actin acted as an internal control.

GAS5 Forward: 5'- CTTCTGGGCTCAAGTGATCCT-3',
GAS5 Reverse: 5'- TTGTGCCATGAGACTCC ATCAG-3'.
HIPK2 Forward: 5'- AATTTGTGCCCGACCTGATC -3',
HIPK2 Reverse: 5'- ACTGAGTAGCCAGCGTGCTT -3'.
TNF-α Forward: 5'- ACTCCCAGAAAAGCAAGCAA-3',
TNF-α Reverse: 5'- CAGTTCCACATCTCGGATCA-3'.
IL-6 Forward: 5'- GAGCCCACCAGGAACGAAAGTC-3',

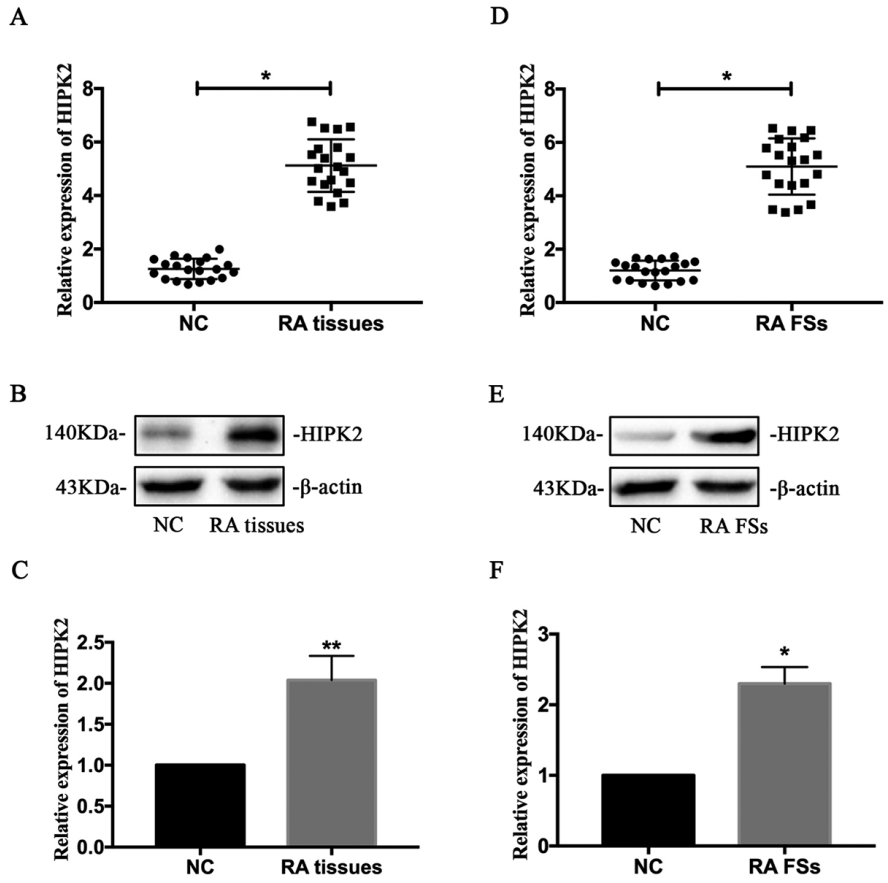

Fig. 2. The expression of HIPK2 in RA synovial tissues and RA FSs.
A: The expression of HIPK2 in RA synovial tissues determined by RT-qPCR. *p<0.05, NC: the control group, n=20; the RA group: rheumatoid arthritis tissues, n=20.
B: Western blot showed that the expression of HIPK2 in RA synovial tissues and control group.
C: Relative expression of HIPK2 in (**B**), * p<0.05. n=3.
D: The expression of HIPK2 in RA FSs determined by RT-qPCR. ** p<0.01, NC: the control FSs group, n=20; the RA group: rheumatoid arthritis FSs, n=20.
E: Western blot showed that the expression of HIPK2 in RA FSs and control group.
F: Relative expression of HIPK2 in (E), * p<0.05. n=3. The experiments were independently repeated 3 times.

IL-6 Reverse: 5'- TGTTGTGGGTGGTATCCTCTGTGAA-3'.
β-actin Forward: 5'- CCCATCTATGAGGGTTACGC-3',
β-actin Reverse: 5'- TTTAATGTCACGCACGATTTC-3'.

Western blot analysis
Total cell or tissue extracts were made using cell lysis buffer followed by immunoblotting with anti-HIPK2 (CST, USA), TNF-α (Abcam, USA), IL-6 (Abcam, USA) and anti-β-actin (Santa Cruz Biotechnology, CA). Cells were lysed in ice-cold RIPA buffer with protease inhibitors (Roche Applied Science) on ice for 30 min. Centrifugate for 20 minutes, 13500 rpm at 4°C. The supernatant was transferred to a new Eppendorf tube and the protein concentration measured using a BCA protein assay kit (Applygen, China). 30 ug of cell lysates was resolved with 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Then, the membranes blocked with 5% skim milk for 2 hr at room temperature (RT). After blocking, the membranes were incubated with primary antibody at 4°C overnight. After washing and incubating, the membranes with secondary antibodies at RT for 1hr. The protein bands were visualised using ECL reagent (HaiGene, China) and analysed using ImageJ 1.61 software.

RNA fluorescence in situ hybridisation (FISH)
FISH was performed according to the manufacturer's protocols. Briefly, FSs were seeded and fixed with 4% paraformaldehyde. After 48 hr, permeabilised FSs with 1% Triton X-100 for 20 minutes and incubated cells with 2xSSC for 40 min at 37°C. Then, washed the slides and rehydrated in series alcohol, subsequently added to each slide, and hybridised for 24hr at 37°C. Finally, the sections were counterstained with DAPI for 5 minutes to stain the cell nucleus.

MTT assay
FSs were seeded in 96-well culture plates at a density of 10000 cells per well. When 60–80% confluence was reached, FSs were transfected with the required oligonucleotide. After transfection for 24 hr, 48 hr, or 72 hr, the cells were stained with 20 μl of MTT (5 mg/ml), (Sigma, USA) for 4 h at 37°C. The cell medium was carefully aspirated and 150 μl of dimethyl sulfoxide added to each well. Then, absorbance was measured at 490 nm.

BrdU treatment and immunostaining
For BrdU assay, FSs were treated with 3 μM BrdU (abcam, USA) for 2 hours and stained with anti-BrdU antibody (abcam, USA) staining. For immunostaining, the FS cells were fixed with 4% paraformaldehyde for 20 min at RT. Then, the cells were blocked with 5% BSA for 1hr. Finally, the cells were washed three times with PBS and incubated with anti-BrdU antibody overnight at 4°C. After that, 80 μl DAPI was added to the cells to stain the nuclear.

Treatment with 5-azadC
The FSs were seeded with a density of $2x10^5$ cells/ml into a six-well plate. After 12 hr, 5-azadC was applied to the cells at a concentration of 1.5 μM and incubated for another 48 hr at 37°C.

Methylation-specific PCR (MSP)
The methylation level of GAS5 promoter was examined by MSP. The genomic DNA and amplified CpG sites of GAS5 promoter region were extracted and the methylated and unmethylated sequences were as follows:
GAS5-M forward: 5´-CTTTTCGAGGTAGGAGTCGACTCC-3´;

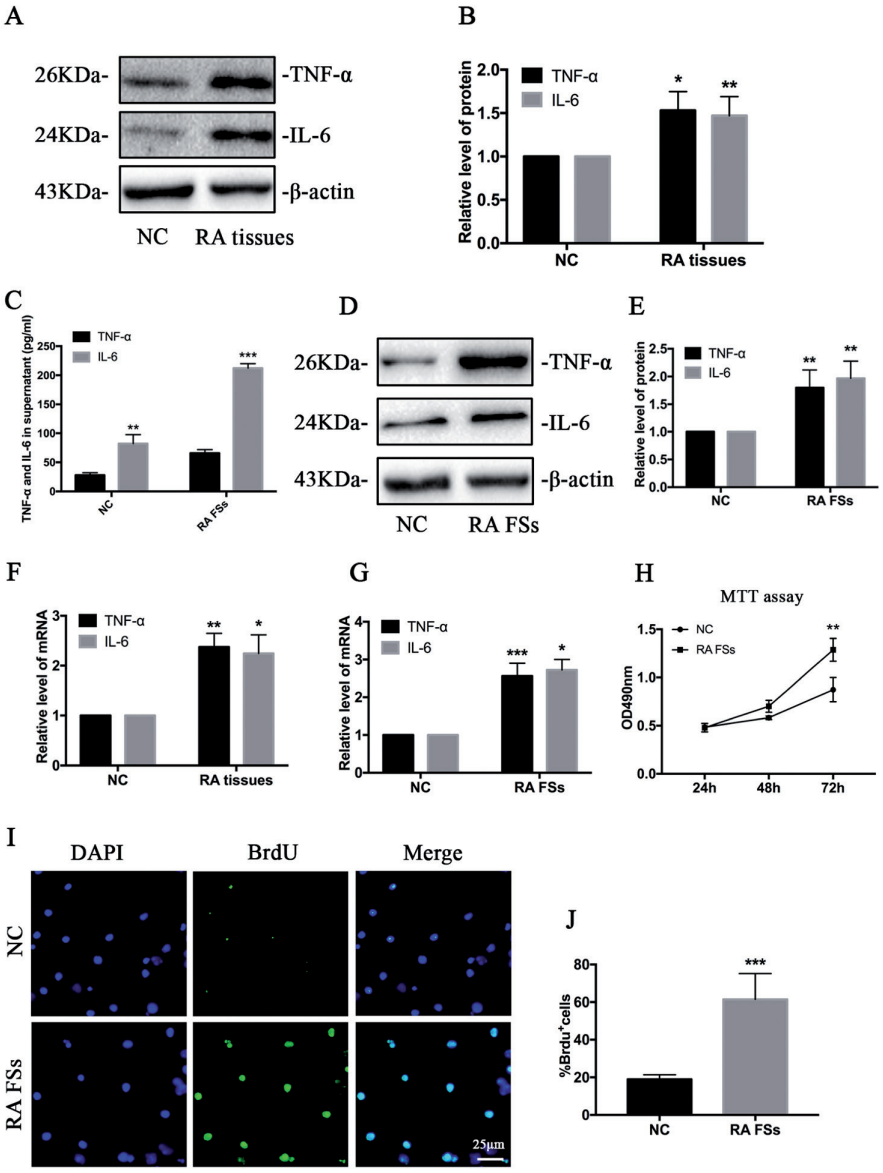

Fig. 3. The level of inflammatory cytokines and the proliferation of FSs in RA.
A: Western blots showed the protein expression of TNF-α and IL-6 in RA synovial tissues and control group. Representative images are shown.
B: Relative protein levels of TNF-α and IL-6 in RA synovial tissues. The results are presented as mean ± SD, and. **p*<0.05, ** *p*<0.01 *versus* the control group.
C: ELISA assay of TNF-α and IL-6 levels in cultured supernatant of RA FSs cells. The results are presented as mean ± SD, and. **p*<0.05, ** *p*<0.01 *versus* the control group.
D: Western blots showed the protein expression of TNF-α and IL-6 in RA synovial tissues. Representative images are shown.
E: Relative protein levels of TNF-α and IL-6 in RA FSs and control FSs. The results are presented as mean ± SD, and. ** *p*<0.01 *versus* the control FSs.
F: qRT-PCR data showed the mRNA expression of TNF-α and IL-6 in RA synovial tissues and control group. The results are presented as mean ± SD, and. **p*<0.05, ** *p*<0.01 *versus* the control group.
G: qRT-PCR data showed the mRNA expression of TNF-α and IL-6 in RA FSs and control FSs. The results are presented as mean ± SD, and. **p*<0.05, *** *p*<0.005 *versus* the control FSs.
H: The MTT assay showed the cell proliferation of RA FSs and control FSs. ** *p*<0.01.
I: The BrdU assay showed the cell proliferation of RA FSs and control FSs.
J: The percent of BrdU positive ($BrdU^+$) cells in RA FSs and control FSs. The results are presented as mean ± SD, *** *p*<0.005.
The experiments were independently repeated 3 times.

GAS5-M reverse: 5´-TTTTTCGAGGTAGGAGTCGATTTT-3´;
GAS5-U forward: 5´-CTTTTTGAGGTAGGAGTTGACTCC-3´;
GAS5-U reverse: 5´-TTTTTTGAGGTAGGAGTTGATTTT-3´.

Statistical analysis

Data are presented as mean ± standard deviation (SD), and the experiment was repeated at least three times. Statistical differences were assessed by one-way analysis of variance (ANOVA). or two-way ANOVA. Statistical differences were considered when **p*<0.05.

Results

GAS5 is involved in RA

To examine the expression of GAS5 in RA synovial tissues. qRT-PCR was used to examine the expression of GAS5 between the RA synovial tissues and control synovial tissues. The results demonstrated that the expression of GAS5 was significantly lower in the RA synovial tissues than in the control synovial tissues (Fig. 1A). FSs were identified by the morphology and vimentin staining (Fig. 1B). Comparison of the expression of GAS5 between the FSs isolated from RA patients and control individuals was examined via qRT-PCR. The results showed that the expression of GAS5 was significantly decreased in the RA FSs (Fig. 1C). Furthermore, fluorescence *in situ* hybridisation was used to detect the expression of GAS5 and the results showed that the expression of GAS5 in RA FSs was decreased compared with control FSs (Fig. 1D-E). These data indicated that GAS5 has a lower expression in RA synovial tissues and RA FSs.

The expression of HIPK2 was upregulated in RA synovial tissues and RA FSs

To identify the expression of HIPK2 in RA synovial tissues and control synovial tissues, qRT-PCR and western blot were performed. The qRT-PCR results showed that the mRNA level of HIPK2 was increased in the RA synovial tissues compared with the control synovial tissues (Fig. 2A). Western blotting results showed the expression of HIPK2 protein was increased in RA synovial

tissues compared with the control synovial tissues (Fig. 2B, C). Comparison of the expression of HIPK2 between the RA FSs and control FSs was also examined by qRT-PCR and western blot. The mRNA level of HIPK2 in RA FSs was increased compared with control FSs (Fig. 2D). The protein level of HIPK2 in RA FSs showed the same pattern (Fig. 2E, F). These data indicated that the expression of HIPK2 was upregulated both in RA synovial tissues and RA FSs.

Downregulated GAS5 and upregulated HIPK2 was accompanied by high expression of inflammatory cytokine and proliferation of FSs

Western blot, qRT-PCR, MTT assay, and BrdU assay were performed to explore whether GAS5 exerted effects on the secretion of cytokines and proliferation in FSs isolated from RA patients. The western blot results showed that the expression of TNF-α and IL-6 was higher in both RA synovial tissues and FSs compared with the control group (Fig. 3A-E). The ELISA assay results showed that the level of TNF-α and IL-6 were higher in the cultured supernatant of RA FSs compared with the control group (Fig. 3C). The qRT-PCR results showed that the mRNA levels of TNF-α and IL-6 were higher in both RA synovial tissues and FSs compared with the control group (Fig. 3F, G). Furthermore, the MTT and BrdU assay suggested that the cell proliferation of RA FSs was higher than that of the control group (Fig. 3H-J). These results showed that the decreased expression of GAS5 and increased expression of HIPK2 were accompanied by high levels of inflammatory cytokine and cell proliferation of FSs.

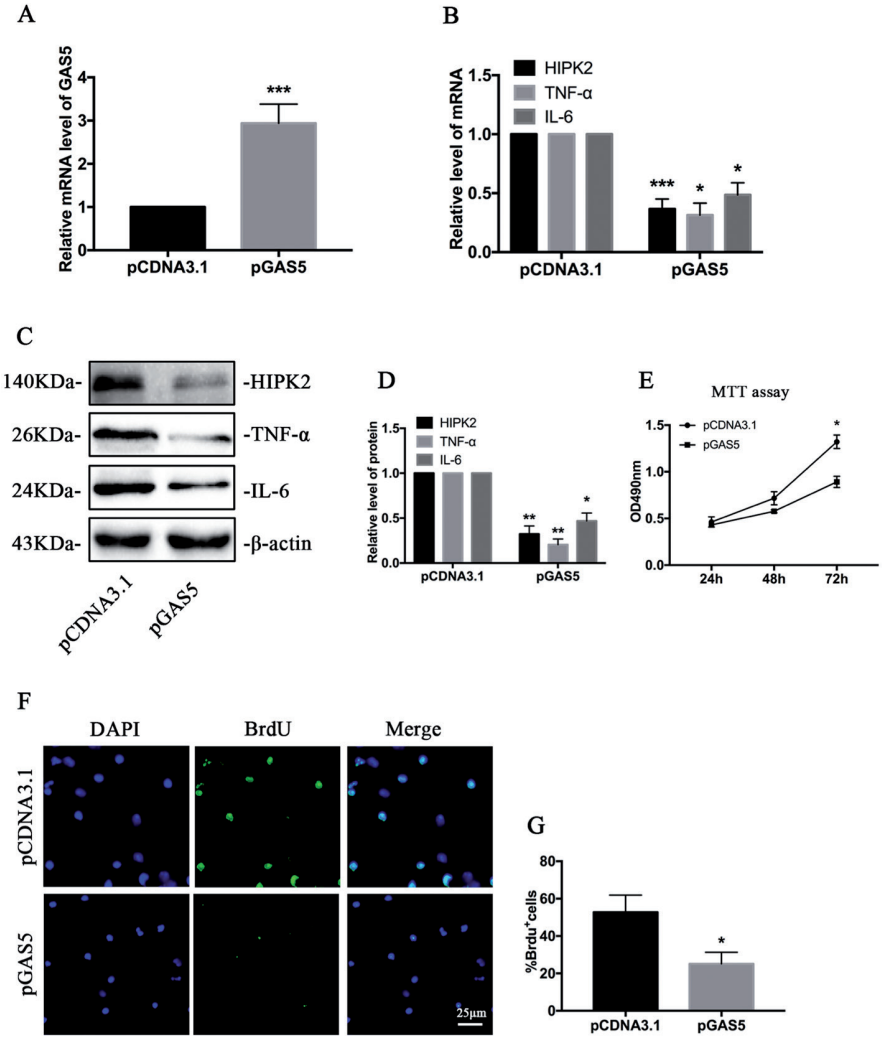

Fig. 4. GAS5 was overexpressed in FSs by transfected with pGAS5.
A: qRT-PCR data identified the mRNA level of GAS5 after transfection. The results are presented as mean ± SD, and *** $p<0.005$.
B: qRT-PCR data showed the mRNA expression of HIPK2, TNF-α, and IL-6 in RA FSs after transfected with pGAS5 or pCDNA3.1. The data are presented as mean ± SD, *$p<0.05$, *** $p<0.005$.
C: Western blot showed the protein expression of HIPK2, TNF-α, and IL-6 in RA FSs after transfected with pGAS5 or pCDNA3.1. Representative images are shown.
D: Relative protein levels of HIPK2, TNF-α and IL-6 in RA FSs after transfected with pGAS5 or pCDNA3.1. The results are presented as mean ± SD, and. *$p<0.05$, ** $p<0.01$.
E: The MTT assay showed the cell proliferation of RA FSs after transfected with pGAS5 or pCDNA3.1. *$p<0.05$.
F: The BrdU assay showed the cell proliferation of RA FSs after transfected with pGAS5 or pCDNA3.1.
G: The percent of BrdU positive ($BrdU^+$) cells in RA FSs after transfected with pGAS5 or pCDNA3.1. The results are presented as mean ± SD, *$p<0.05$. The experiments were independently repeated 3 times.

Enforced expression of GAS5 decreased the expression of HIPK2, inflammatory cytokine and cell proliferation of FSs

To investigate the potential influence of GAS5 in HIPK2 expression, GAS5 overexpression was enforced in RA FSs by transfected with pGAS5 or its control plasmid pCDNA3.1. The transfection efficiency was identified via qRT-PCR (Fig. 4A). Firstly, the qRt-PCR data showed that the mRNA level of HIPK2, TNF-α and IL-6 were decreased in the pGAS5 group compared with the pCDNA3.1 group (Fig. 4B). Western blot results evidenced that the protein expression of HIPK2 and TNF-α and IL-6 were also decreased in the pGAS5 group compared with the pCDNA3.1 group (Fig. 4C, D). The MTT and BrdU assay showed that the cell proliferation of the pGAS5 group was inhibited compared with the pCDNA3.1 group (Fig. 4E- G). The results indicated that GAS5 overexpression decreased HIPK2 expression and inhibited inflammatory cytokine expression and cell proliferation of FSs.

Knockdown of expression of HIPK2 inhibited inflammatory cytokine expression and cell proliferation of FSs

HIPK2 expression was silenced in FSs by transfected with HIPK2-siRNA or

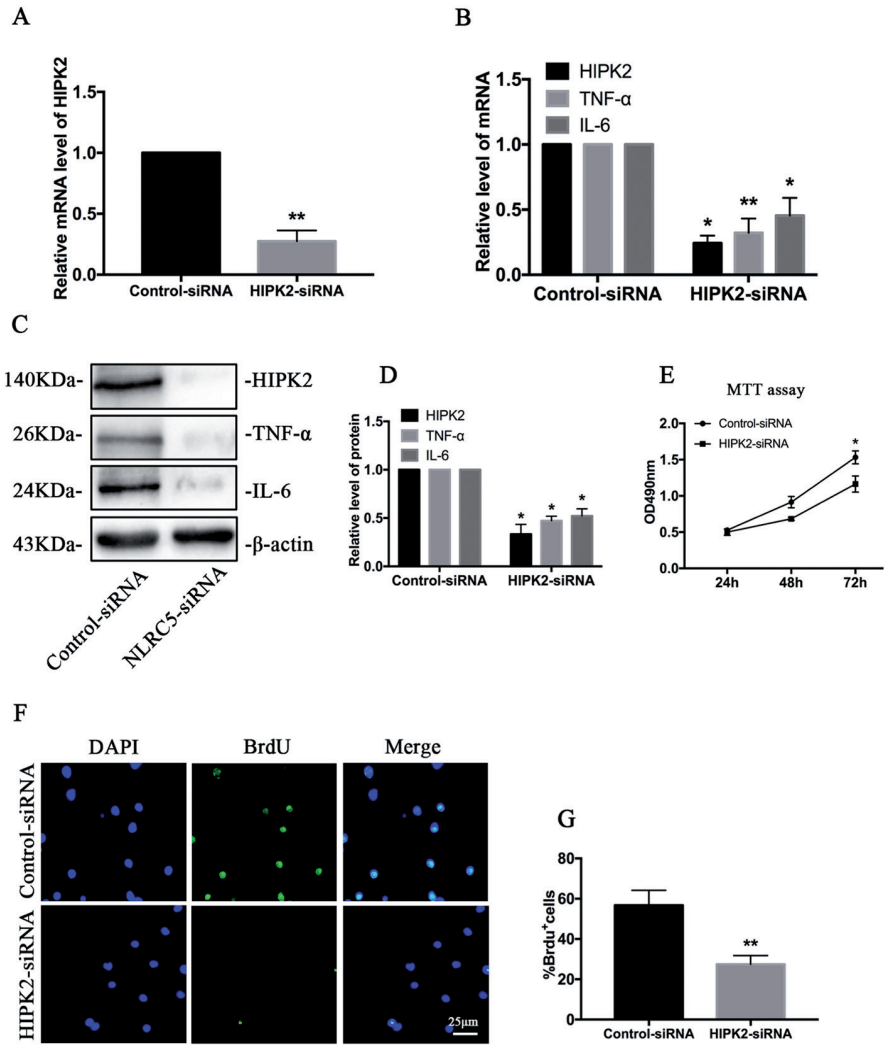

Fig. 5. HIPK2 was knockdown in RA FSs by transfected with HIPK2-siRNA.
A: qRT-PCR data identified the mRNA level of HIPK2 after transfection. The results are presented as mean ± SD, and *p<0.05.
B: qRT-PCR data showed the mRNA expression of HIPK2, TNF-α, and IL-6 in RA FSs after transfected with HIPK2-siRNA or control-siRNA. The data are presented as mean ± SD, *p<0.05, ** p<0.01.
C: Western blot showed the protein expression of HIPK2, TNF-α, and IL-6 in RA FSs after transfected with HIPK2-siRNA or control-siRNA. Representative images are shown.
D: Relative protein levels of TNF-α and IL-6 in RA FSs after transfected with HIPK2-siRNA or control-siRNA. The results are presented as mean ± SD, and. *p<0.05.
E: The MTT assay showed the cell proliferation of RA FSs after transfected with HIPK2-siRNA or control-siRNA. *p<0.05.
F: The BrdU assay showed the cell proliferation of RA FSs after transfected with HIPK2-siRNA or control-siRNA.
G: The percent of BrdU positive ($BrdU^+$) cells in RA FSs after transfected with HIPK2-siRNA or control-siRNA. The results are presented as mean ± SD, ** p<0.01. The experiments were independently repeated 3 times.

its control-siRNA. The transfection efficiency was identified via qRT-PCR (Fig. 5A). Then, qRT-PCR, western blot, MTT assay, and BrdU assay were performed. The qRT-PCR data showed a significant decrease of HIPK2, TNF-α, and IL-6 in the HIPK2-siRNA group compared with the HIPK2-control group (Fig. 5B). Western blot data indicated that the protein level of HIPK2, TNF-α, and IL-6 were also significantly decreased in the HIPK2-siRNA group compared with the HIPK2-control group (Fig. 5C, D). Likewise, the MTT and cell BrdU assay suggested that the cell proliferation ability of the HIPK2-siRNA group was inhibited compared with the HIPK2-control group (Fig. 5E-G). The results suggested that knockdown of the expression of HIPK2 inhibited the expression of inflammatory cytokine and cell proliferation of FSs.

DNA methylation contributed to the downregulation of GAS5

MSP was performed to analyse the contribution of methylation in alteration of GAS5. The results suggested that GAS5 promoter was significantly methylated in RA synovial tissues and RA FSs, whereas the control group had an unmethylated GAS5 promoter region (Fig. 6A-B). The GAS5 promoter methylation level was reduced after transfection with methylation inhibitor 5-azadC (Fig. 6C). qRT-PCR was performed to show whether the methylation inhibitor 5-azadC had a significant influence on the mRNA expression of GAS5 and the results showed that the mRNA level of GAS5 was increased in FSs after treatment with 5-azadC compared with control group (Fig. 6D). Furthermore, the qRT-PCR and western blot results showed that the mRNA level and protein levels of HIPK2 were lower in FSs after stimulation with 5-azadC (Fig. 6E-G). In addition, the western blot data showed that stimulation with 5-azadC inhibited the expression of TNF-α and IL-6 (Fig. 7A-B) and the mRNA levels of TNF-α and IL-6 were also decreased after stimulation with 5-azadC (Fig. 7C). Importantly, MTT assay and BrdU analysis illustrated that stimulation with 5-azadC could significantly inhibit cell proliferation of FSs (Fig. 7D-F). These results suggested that DNA methylation contributed to the downregulation of GAS5.

Discussion

RA is one of the most common systemic autoimmune diseases (28, 29). Studies have proved that smoking, diet, birthweight, socioeconomic status and location of residence are dangerous risk factors associated with RA (30, 31). Given the disability and huge economic pressures on RA patients and family, it is important to identify the mechanism of RA development and establish new strategies for RA treatment. Previous studies indicated that lncRNAs dysregulation may affect epigenetic information and be considered to play a significant role in the patho-

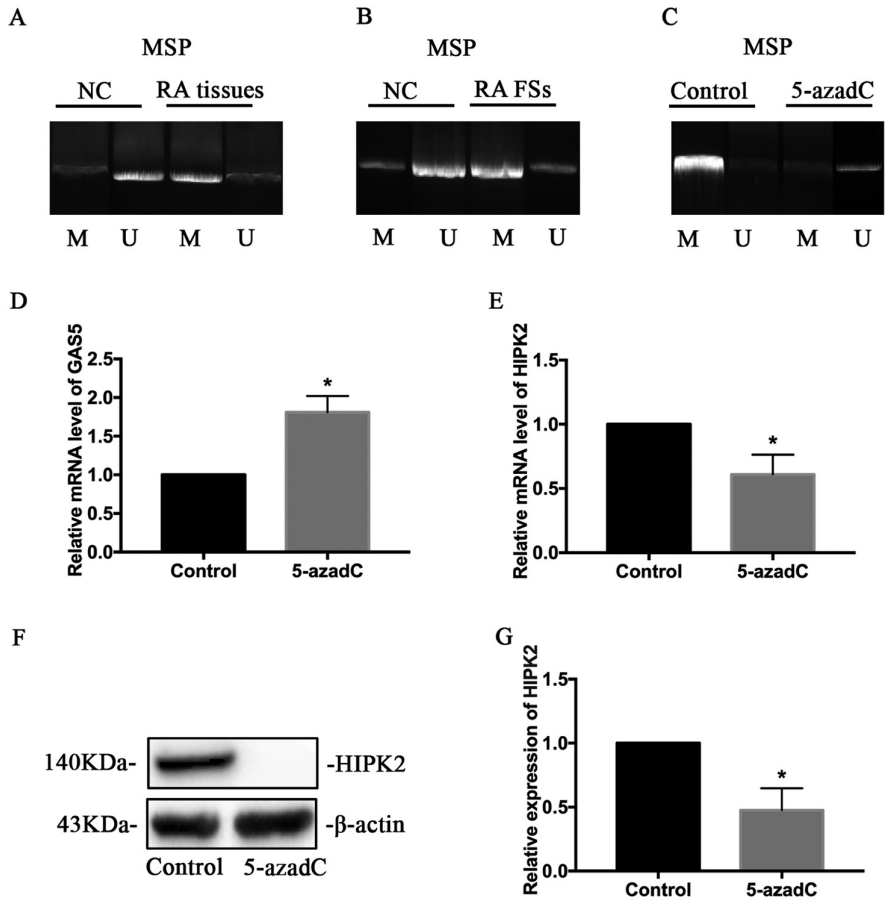

Fig. 6. Detection of GAS5 methylation in RA FSs by MSP.
A: MSP showed the methylation levels of GAS5 in synovial tissues of control and RA patients, M referred to PCR products of methylated alleles and U referred PCR products of unmethylated alleles, respectively.
B: MSP showed the methylation levels of GAS5 in FSs of control and RA patients.
C: Treatment with 5-azadC for 48 hr, the methylation levels of GAS5 in FSs of the control and RA group.
D: qRT-PCR showed the mRNA expression of GAS5 in RA FSs after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, *p<0.05.
E: qRT-PCR showed the mRNA expression of HIPK2 in RA FSs after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, *p<0.05.
F: Western blot showed the expression of HIPK2 in RA FSs after treatment with 5-azadC for 48 hr. Representative images are shown.
G: Relative protein levels of HIPK2 after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, and. *p<0.05. The experiments were independently repeated 3 times.

genesis of RA. Therefore, identification and investigation of RA-associated lncRNAs clinical significance and functions may provide a missing piece of the pathogenesis of RA (32, 33).

GAS5 in humans is a ~650 bases long ncRNA and identified as potential tumour suppressor genes (23, 24). For example, low expression of GAS5 is an adverse prognostic factor to survival in breast cancer and GAS5 overexpression attributed to cancer cell growth arrest (25, 26). However, the relationship between expression of GAS5 and RA progression remains unclear. So, we hypothesised that GAS5 affects the proliferation of FSs and developed this research. qRT-PCR results showed that the mRNA level of GAS5 was decreased in RA synovial tissues and FSs, indicating a potential function of GAS5 in RA development. To further explore the function of GAS5 in RA, GAS5 was overexpressed to observe the effect on inflammatory factors TNF-α and IL-6 expression and the proliferation ability of FSs. We transfected FSs with pGAS5 or pCDNA3.1 for 48 hr and the data showed that transfection of pGAS5 significantly decreased inflammatory factors expression and inhibited cell proliferation ability of FSs, indicating that GAS5 inhibits RA development through the potential inhibition of inflammatory factors and cell proliferation ability.

HIPK2 is one of the members of the homeodomain-interacting protein kinase family and is able to interact with transcription factors. HIPK2 can regulate many biological processes such as tumourigenesis (34), cell proliferation (35), inflammation (36), neural development (37), tissue fibrosis (34), DNA damage response (38) and cell apoptosis (39). Additionally, down-regulated HIPK2 has been reported to impair the pro-apoptosis ability and participate in drug resistance. In 2017, Li Y *et al*. (40) demonstrated that HIPK2 may play a role in RA pathogenesis using the whole-exome sequencing technique, indicating a close relationship between HIPK2 and RA. In this study, the protein and mRNA level of HIPK2 was observed to be increased in RA synovial tissues and FSs. Therefore, we suspected that GAS5 may participate in the development of RA by regulating HIPK2. To explore whether GAS5 regulates the expression of HIPK2 during the development of RA, we detected the effect of GAS5 overexpression on HIPK2 expression. We transfected pGAS5 into FSs for 48 hr, and the results showed that transient transfection of pGAS5 decreased HIPK2 expression. Overall, these data suggested that GAS5 targeting HIPK2 participates in the development and progression of RA.

DNA methylation has been shown to affect many biological processes and genome dynamics related to human disease (41-44). We speculated that the loss of GAS5 in RA synovial tissues and FSs might have a relationship with promoter hypermethylation. In our study, we found that GAS5 promoted hypermethylation in RA synovial tissues and FSs. Furthermore, the methylation inhibitor 5-azadC remarkably decreased the hypermethylation of the GAS5 and the expression of HIPK2, which indicated that GAS5 potentially regulates the expression of HIPK2.

In conclusion, our research showed that overexpression of GAS5 and silencing of HIPK2 decreased FSs proliferation ability and inflammatory reaction. The

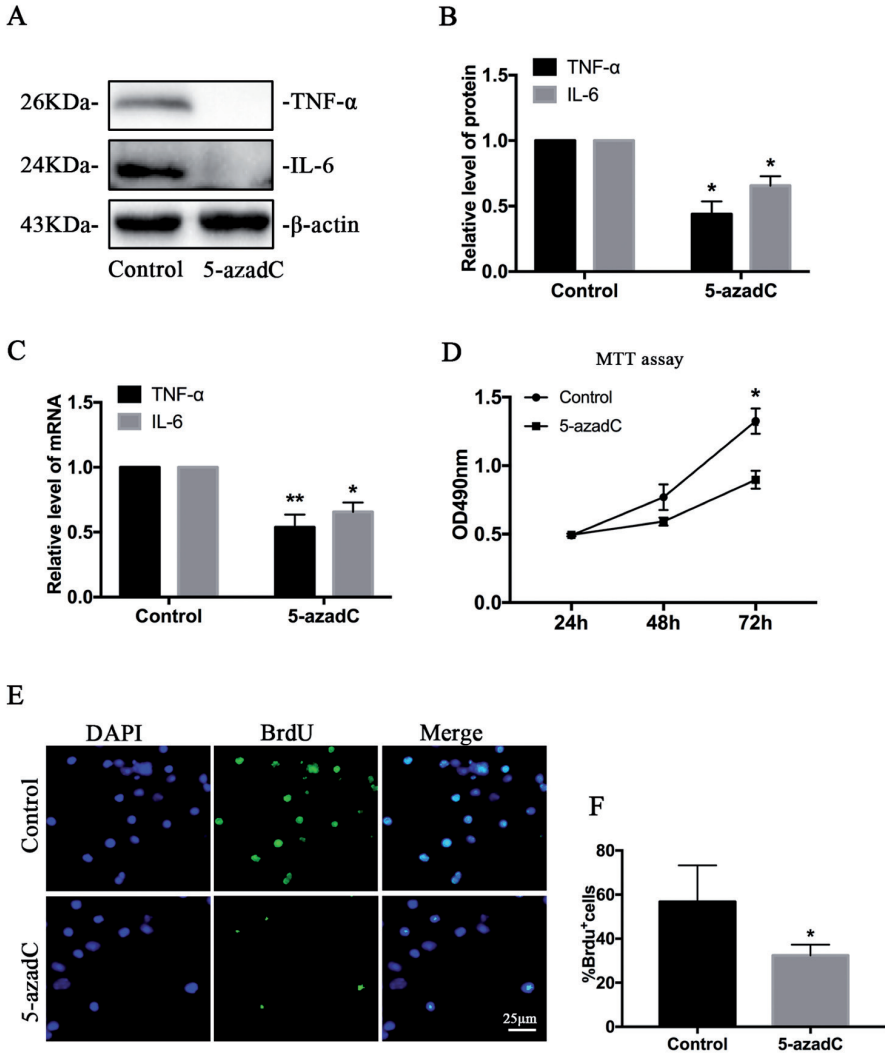

Fig. 7. The expression of inflammatory cytokine and FSs proliferation after treatment with methylation inhibitor 5-azadC.

A: The protein expression of TNF-α and IL-6 in FSs after treatment with 5-azadC and the representative images are shown.

B: Relative protein levels of TNF-α and IL-6 after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, and. *p<0.05, ** p<0.01.

C: qRT-PCR showed the mRNA expression of TNF-α and IL-6 in FSs after treatment with %-azadC. The results are presented as mean ± SD. *p<0.05, ** p<0.01.

D: The MTT assay illustrated that stimulated with 5-azadC could significantly inhibit cell proliferation of FSs. The results are presented as mean ± SD. *p< 0.05.

E: The BrdU assay illustrated the proliferation of FSs after treatment with 5-azadC.

F: The percent of BrdU positive ($BrdU^+$) cells in FSs after treatment with 5-azadC. The results are presented as mean ± SD, *p<0.05. The experiments were independently repeated 3 times.

loss of GAS5 we observed in RA synovial tissues and FSs were mainly caused by the hypermethylation of the GAS5 gene promoter. Methylation inhibitor 5-azadC could offset the hypermethylation of GAS5 and decrease HIPK2 expression. These results indicated GAS5 might regulate the expression of HIPK2 during the development and progression of RA. These results give us the novel insight that GAS5 regulates RA by potentially targeting HIPK2. Our findings provide new insights into the molecular function of GAS5 in RA and hopefully may provide a potential therapeutic target for RA.

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