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# Overexpression of lncRNA GAS5 attenuates cardiac fibrosis through regulating PTEN/MMP-2 signal pathway in mice

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**Abstract. – OBJECTIVE: The present study aimed at investigating the effect and mechanism of lncRNA Growth Arrest-Specific 5 (GAS5) in cardiac fibrosis induced by isoproterenol (ISO) *in vivo*.**

**MATERIALS AND METHODS: The C57BL/6 mice were injected subcutaneously with ISO to induce cardiac fibrosis and injected intracoronary with lentivirus pcDNA-GAS5. After 3 weeks, cardiac function was detected by echocardiography. The interstitial collagen volume was stained by Masson trichrome. The expression of GAS5 was measured by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The expressions of phosphatase and tensin homologue (PTEN), matrix metalloprotease-2 (MMP-2), α-smooth muscle actin (α-SMA), and collagen I protein were measured by Western blot.**

**RESULTS: Our results indicated that the expression of GAS5 was significantly down-regulated in the fibrotic myocardium. Overexpression of GAS5 after injection with pcDNA-GAS5 could attenuate cardiac fibrosis and improve cardiac function through increasing the expression of PTEN and decreasing the expression of MMP-2, α-SMA, and collagen I.**

**CONCLUSIONS: Overexpression of GAS5 could attenuate cardiac fibrosis induced by ISO. The molecular mechanism was associated with the regulation of PTEN/MMP-2 signaling pathway.**

Key Words
Cardiac fibrosis, Long non-coding RNA, GAS5.

## Introduction

Cardiac fibrosis is an important feature of myocardial structural remodeling and is a common pathological change in the development of various cardiovascular diseases, including atrial fibrillation, myocardial infarction, and valvular heart diseases. The main pathological manifestations of cardiac fibrosis are cardiac fibroblasts proliferation and extracellular matrix (ECM) deposition, thereby leading to myocardial stiffness, cardiac dysfunction, and even malignant arrhythmia and sudden death[1,2]. The pathogenesis of cardiac fibrosis is very complicated, abnormal changes in genes and signaling pathways are closely associated with the progression of cardiac fibrosis.

Long non-coding RNAs (lncRNAs) are a class of molecules greater than 200 nucleotides, which play important roles in various biological processes, including gene expression, transcription, and post-transcriptional regulation[3]. The lncRNA growth arrest-specific 5 (GAS5) is considered as a tumor suppressor in a variety of cancers[4] and is closely associated with the regulation of cardiomyocyte apoptosis[5] and ventricular remodeling[6]. Recently, it has been reported that upregulation of GAS5 could inhibit the activation of cardiac fibroblasts by regulating the phosphatase and tensin homologue (PTEN)/matrix metalloprotease-2 (MMP-2) signaling pathway *in vitro*[7]. However, the regulatory effects of GAS5 and PTEN/MMP-2 signal on cardiac fibrosis *in vivo* have not yet been reported. In the present study, we established a mouse model of cardiac fibrosis induced by isoproterenol (ISO) and investigated the regulate effects of GAS5 and PTEN/MMP-2 signal on cardiac fibrosis.

## Materials and Methods

### Animals and Cardiac Fibrosis Model

Male C57BL/6 mice (8-10 weeks old, weight 20-30 g) were provided by the Animal Experiment Center of Nanjing Medical University. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH. Mice were injected subcu-



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taneously with ISO (5 mg/kg/day for 14 days) to induce cardiac fibrosis. The mice were randomly divided into 4 groups (n=15 in each group): control group (mice were injected subcutaneously with saline), ISO group, ISO + pcDNA-GAS5 group (ISO mice were intracoronary injected with lentivirus pcDNA-GAS5), and ISO + pcDNA vector group (ISO mice were intracoronary injected with empty vector). All mice were housed in a room at 22°C with 12:12 hour light/dark cycles, and fed standard mouse food and water. After 3 weeks, animals were sacrificed and the heart samples were harvested for analysis. This investigation was approved by the Animal Ethics Committee of Lianshui County People's Hospital Animal Center.

### Echocardiography

After 3 weeks, the mice were anesthetized with isoflurane and the cardiac function was detected using a rodent animal ultrasonic instrument (Vevo 2100, VisualSonics, Toronto, Canada). The left ventricular (LV) end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD), LV fractional shortening (FS) were calculated.

### Masson Trichrome Staining

The LV tissue samples were fixed in paraformaldehyde (3.7% in PBS, freshly prepared) for 24 h and then embedded in paraffin. LV sections (4-5 μm) were stained with Masson's trichrome for interstitial fibrosis. The proportion of the total fibrosis area was observed using a microscope (Nikon, Tokyo, Japan) and was calculated by Image J software (NIH, Bethesda, MD, USA), as the blue-stained areas divided by the total LV area.

### Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA in myocardium tissue was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary Deoxyribose Nucleic Acid (cDNA) synthesis was using the reverse transcription (RT) reagent according to kit instructions (TaKaRa Biotechnology, Otsu, Shiga, Japan) and a real-time PCR reaction system was performed by the Eppendorf Mastercycler ep realplex. The primer sequences were as follows: GAS5 (forward) 5'-GGATAACAGAGCGAGCGCAAT-3', (reverse) 5'-CCAGCCAAATGAACAAGCATG-3'. GAPDH (forward) 5'-CTGGAGAAACCTGCCAAGTA-3', (reverse) 5'-TGTTGCTGTAGCCGTATTCA-3'. The relative expression levels of GAS5 mRNA were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta CT}$ method.

### Western Blotting

Western blot analysis was used to determinate the expression of a protein that is extracted from the myocardium. Briefly, gel electrophoresis was performed to separate the different molecular weight proteins and then transferred onto polyvinylidene difluoride (PVDF) membranes. These membranes were incubated with anti-PTEN, anti-MMP-2, anti-α-SMA, anti-collagen I (Cell Signaling Technology, Danvers MA, USA) for overnight at 4°C. After incubating with these primary antibodies, the membranes were washed in Tris-Buffered Saline and Tween 20 (TBST) and then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for another 2 h. Western Blot Detection kit and Image J software (NIH) were used to measure the blot signal and density.

### Statistical Analysis

All results were presented as means ± standard deviations (SD). Differences among different groups were analyzed by one-way analysis of variance (ANOVA), followed by Post-Hoc Test (Least Significant Difference). A value of $p<0.05$ was confirmed to be statistically significant.

## Results

### The Expression of lncRNA GAS5 in the Myocardium

As shown in Figure 1A, the expression of lncRNA GAS5 was decreased in the myocardium of ISO mice compared with the control mice ($p<0.001$). After injection with lentivirus pcDNA-GAS5, the expression of GAS5 was significantly increased in the myocardium ($p<0.001$).

### Overexpression of GAS5 Improved Cardiac Function and Attenuated Cardiac Fibrosis

As shown in Figure 1B and 1C, LVEDD was significantly increased and LVFS were significantly decreased in the ISO group compared with the control group ($p<0.01$; $p<0.001$). Meanwhile, overexpression of GAS5 in myocardium could markedly decrease LVEDD and increase LVFS ($p<0.05$; $p<0.01$).

As shown in Figure 2, the interstitial collagen volume was substantially increased in the ISO group compared with the control group ($p<0.001$). By contrast, overexpression of GAS5 significantly inhibited ISO-induced interstitial fibrosis ($p<0.01$).

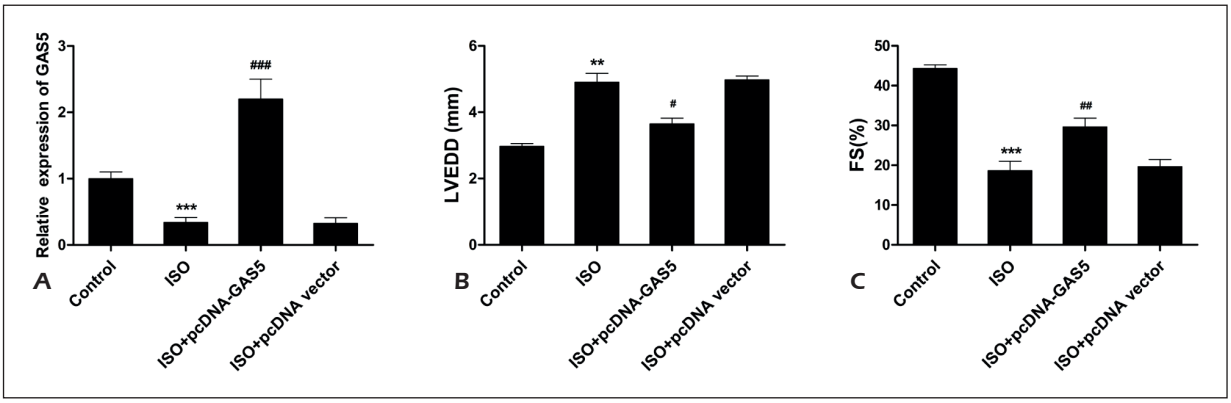


**Figure 1.** The expression of H19 and cardiac function. **A**, The expression of H19 in each group. **B**, The LVEDD in each group. **C**, The LVFS in each group. ***p*<0.01, ****p*<0.001 *versus* control group; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 *versus* ISO group.

### *Overexpression of GAS5 Regulated PTEN/MMP-2 Signaling Pathway*

As shown in Figure 3, the expression of PTEN was significantly decreased, while the expression of MMP-2, α-SMA, and collagen I were significantly increased in the ISO group compared with the control group (*p*<0.05; *p*<0.01; *p*<0.01; *p*<0.001). The overexpression of GAS5 in the myocardium could markedly increase the expression of PTEN and decrease the expression of MMP-2, α-SMA, and collagen I (*p*<0.01; *p*<0.05; *p*<0.01; *p*<0.01).

## Discussion

In this study, we established a mice model of cardiac fibrosis to explore the effect of lncRNA GAS5 on the progression of cardiac fibrosis. Our results indicated that the expression of GAS5 was significantly downregulated in the fibrotic myocardium, which was closely associated with cardiac dysfunction. Furthermore, we found that overexpression of GAS5 could inhibit cardiac fibrosis and improve cardiac function through regulating PTEN/MMP-2 signaling pathway.

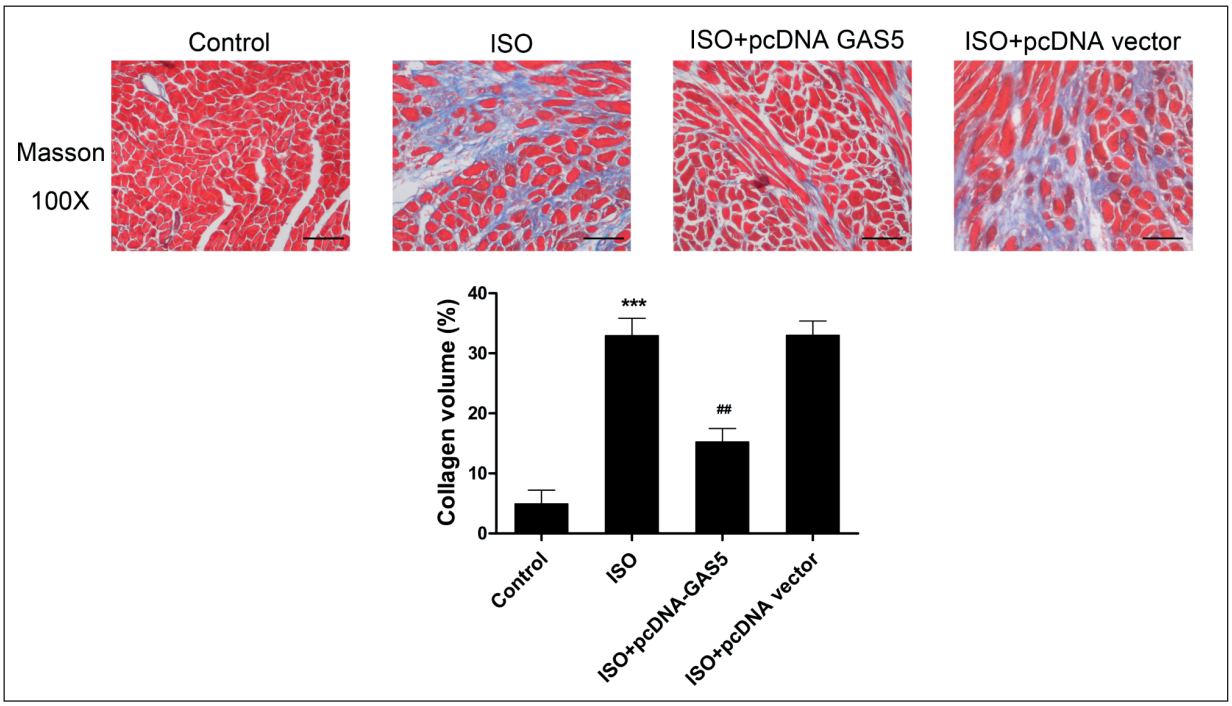


**Figure 2.** Masson trichrome staining and the collagen volume in each group. Bar=50 μm. ****p*<0.001 *versus* control group; ##*p*<0.01 *versus* ISO group.

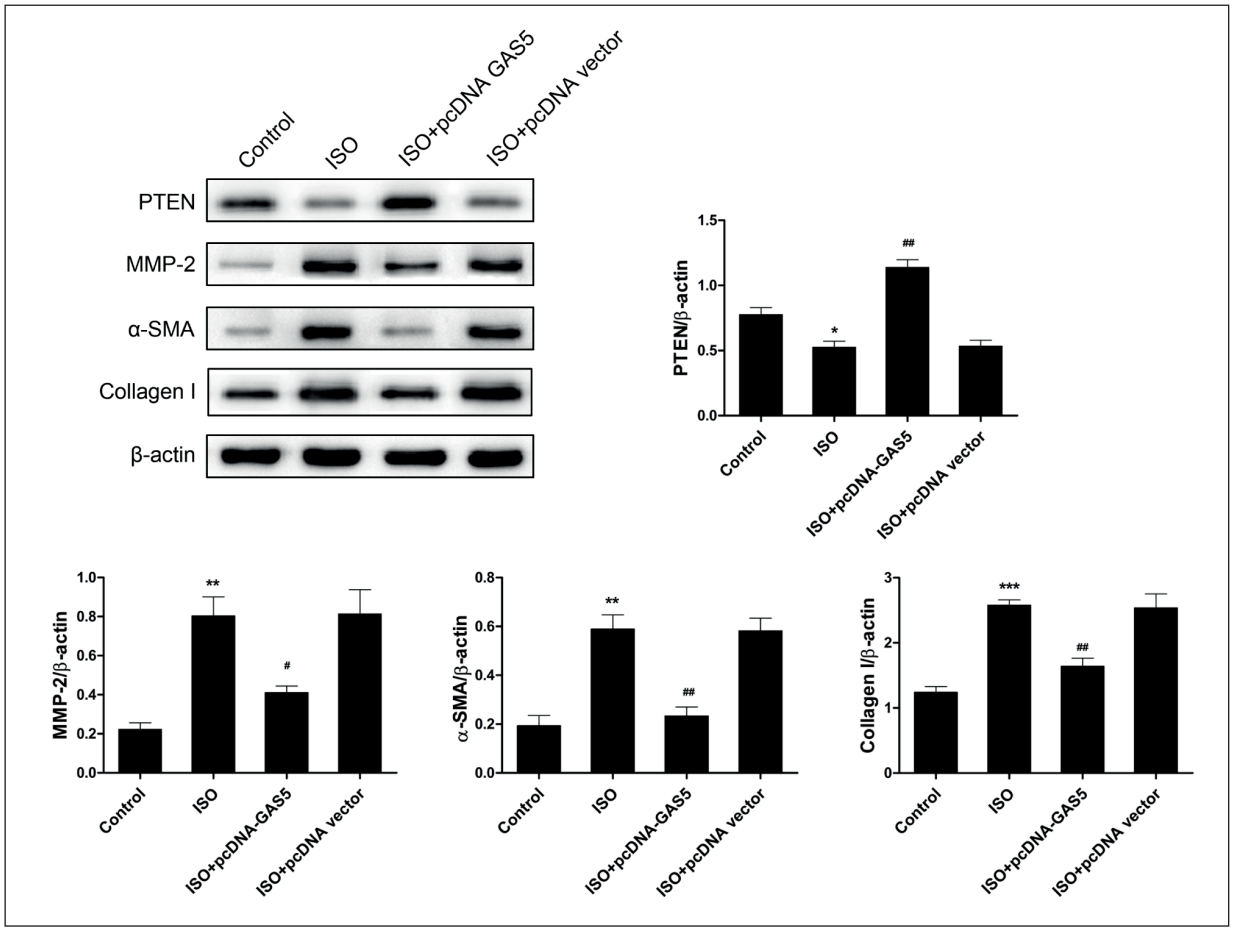


**Figure 3.** The expression of PTEN/MMP-2 signaling pathway in each group. The expressions of PTEN, MMP-2, α-SMA, and collagen I in each group. *$p$<0.05, **$p$<0.01, ***$p$<0.001 *versus* control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 *versus* ISO group.

LncRNA GAS5 plays important roles in various biological processes, including cell proliferation, growth arrest, apoptosis, and tumor metastasis[8-12]. It commonly functions as a competing endogenous RNA by sponging miRNAs and regulating their function. It has been demonstrated that GAS5 is closely associated with the regulation of cardiovascular diseases. Wang et al[6] found that GAS5 could regulate hypertension-induced vascular remodeling. Wei et al[5] reported that upregulation of GAS5 could inhibit cardiac hypertrophy by sponging miR-23a via Wnt/β-catenin signal pathway *in vitro*. Moreover, Tao et al[7] demonstrated that upregulation of GAS5 could inhibit the activation of cardiac fibroblasts by sponging miR-21 *in vitro*. Thus, it implicated that GAS5 may function as a negative regulator of cardiovascular diseases. In this study, our results indicated that GAS5 was downregulated in the fibrotic myocardium. Upregulation of GAS5 after transfecting pcDNA-GAS5 could inhibit cardiac fibrosis and improve cardiac function.

Activation of cardiac fibroblasts, as reflected by the expression of α-SMA, is an important feature during the development of cardiac fibrosis[13]. Collagen I is secreted by activated cardiac fibroblasts and is the most abundant collagen type in the myocardium, constituting approximately 80% of the extracellular matrix[1]. MMP-2 is a gelatinase that can degrade ECM; however, the excessive activation of MMP-2 will promote the deposition of ECM[14]. PTEN was originally identified as a tumor suppressor and plays an important role in controlling tumor cell cycle, differentiation, and apoptosis by regulating MMPs[15,16]. Moreover, Tao et al[7] found that upregulation of GAS5 could inhibit the activation of cardiac fibroblasts by regulating PTEN/MMP-2 signaling pathway *in vitro*. Similarly, in this work, we created a mice model of cardiac fibrosis. We observed that overexpression of GAS5 in the fibrotic myocardium could markedly increase the expression of PTEN and decrease the expression of MMP-2, α-SMA, and collagen I.

## Conclusions

This is the first study to identify that overexpression of GAS5 could attenuate cardiac fibrosis *in vivo*. The molecular mechanism was associated with the regulation of PTEN/MMP-2 signaling pathway. Our results provide new insights into understanding the molecular mechanisms of cardiac fibrosis. Further research is needed to investigate *in vitro*.

### Conflict of Interests

The authors declare that they have no conflict of interest.

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