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Case Report

Effect of DNMT3A Methylated LncRNA GAS5 on Myocardial Fibrosis

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- Myocardial fibrosis
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Abstract

Background: The main pathological manifestations of myocardial fibrosis are the proliferation of myocardial fibroblasts and the mass deposition of collagen fibers in myocardial tissue. It is a common feature of all cardiovascular diseases. Studies have shown that Lnc RNA can regulate myocardial fibrosis by regulating fibroblast proliferation, differentiation and collagen deposition through a variety of mechanisms. This study aims to investigate the expression and biological function of Lnc RNA GAS5 in myocardial fibrosis.

Methods: Animal models were constructed and the pathological changes of rat heart tissue were observed by HE, Masson and Sirius staining. The expressions of caspase1, NLRP3, DNMT3A and Col11 were analyzed by Western blot and q RT-PCR. Pyrodeath of target cells was detected by flow cytometry and Hoechst 33358 staining.After transient transfection of si DNMT3A, the expression of Lnc RNA GAS5 and its effect on pyrodeath of myocardial fibroblasts were recorded

Results: In this study, we identified LncRNA-GAS5 as the key onset of cardiac fibroblast pyroptosis and cardiac fibrosis. Here, we detected ISO-induced cardiac fibrosis models and cardiac fibroblast pyroptosis model. We found that the expression of pyroptosis-related proteins such as caspase 1, NLRP3, and DNMT1 was increased in cardiac fibrosis tissue, while the expression of GAS5 was decreased. The overexpressing of LncRNA GAS5 was shown to increase and inhibit cardiac fibroblast pyroptosis, as well as attenuate caspase 1 and NLRP3 expression in cardiac fibroblast. However, the silencing of GAS5 was also observed; it shows the opposite situation. Furthermore, further studies revealed that treatment of DNMT3A inhibitor led to increased GAS5 expression by reversion of promoter hypermethylation in cardiac fibroblast.

Conclusion: This study showed that DNMT3A methylation of LncRNA GAS5 leads to cardiac fibroblast pyroptosis via affecting NLRP3 axis. Our findings indicate a new regulatory mechanism for cardiac fibroblast pyroptosis, providing a novel therapeutic target for cardiac fibrosis.

INTRODUCTION

Myocardial fibrosis is a common feature of various cardiovascular diseases [1]. In normal heart tissue, cardiomyocytes and Cardiac fibroblasts account for about 90% of the total number of cells, while cardiac fibroblasts alone account for 60% to 70%. Myocardial fibroblasts can synthesize collagen and other Extracellular matrix ECM [2], in which type I collagen (col1) and type III collagen are the main components. Collagenous Content plays an important role in maintaining the normal structure of myocardial tissue and the integrity of cardiac function [3]. Myocardial fibrosis means that under the stimulation of various factors, part of the heart muscle dies, and the compensatory repair damage of the body is excessive, resulting in the proliferation of fiber tissue, which leads to myocardial hypertrophy, gradually forming myocardial fibrosis, and eventually heart failure. The main pathological manifestations of myocardial fibrosis are the proliferation of myocardial fibroblasts and the mass deposition of collagen fibers in myocardial tissue. Therefore, we can think that myocardial fibroblasts are the key to myocardial fibrosis. The death of myocardial fibroblasts and the inflammatory response of myocardial fibroblasts play a key role in cardiac fibrosis [4].

Pyroptosis is a programmed cell death characterized by apoptosis and necrosis, which is related to inflammatory response [5]. Lipopolysaccharide (LPS) is a known substance that can stimulate cells to produce pyrosis [6]. In cardiac fibrosis, Nod-like receptor protein 3 (NLRP3) inflammasome is activated by inflammation, which is subsequently activated by cleaved caspase 1 [7], resulting in cleavage and polymerization of GSDMD, resulting in cell perforation, and thus cell death. New studies have shown that pyrodeath is involved in the pathogenesis of cardiac fibrosis [8]. However, the mechanisms that initiate myocardial fibrosis and cause myocardial fibroblast pyrodeath are still unknown. Therefore, it is necessary to determine the pathological mechanism and effective therapeutic targets of cardiac fibrosis.

Studies have shown that Lnc RNA can regulate myocardial fibrosis by regulating fibroblast proliferation, differentiation and collagen deposition through a variety of mechanisms [9]. Lnc RNA, or Long non-coding RNA, is a functional RNA molecule with a length greater than 200 nucleotides [10]. Lnc RNA plays an important role in many life activities such as epigenetic regulation [11,12], apoptosis regulation and pyroptosis regulation [13], and is widely involved in physiological and pathological processes of the body [14]. Among them, GAS5 is a kind of Lnc RNA related to growth arrest, which plays an important role in the proliferation, apoptosis, invasion and invasion of many malignant tumor cells and is a known tumor suppressor [15]. GAS5 is believed to play an important role in cell apoptosis and apoptosis [16]. Moreover, studies have shown that GAS5 expression in various tumor specimens and fibrosis specimens is significantly lower than that in normal tissues. Our previous study found that Lnc RNA GAS5 could affect the activation and proliferation of myocardial fibroblasts by regulating the expression of micRNA-21.

As one of the most widely studied epigenetic changes, DNA methylation is associated with a variety of biological processes, including fibrosis and pyrosis [17]. DNA methylation is an epigenetic process by which methyl groups are added to DNA [18]. DNA methylation transferase (DNMT3A) has the ability to maintain new DNA methylation [19]. Studies have shown that Lnc RNA can bind to DNMT3A to promote gene expression and abnormal DNA methylation in the process of fibrosis[20]. In recent years, pyrodeath has gradually become a hot topic of research. At present, pyrodeath has been widely reported on the pathogenesis of tumor, while the literature on cardiac diseases, especially myocardial fibrosis, is few. In this study, we found that Lnc RNA GAS5 plays an important role in pyrodeath of cardiac fibroblasts, but the expression and mechanism of GAS5 in pyrodeath and fibrosis of cardiac fibroblasts remain unclear. However, it remains unclear whether Lnc RNA can affect myocardial fibrosis and pyrodeath of myocardial fibroblasts through DNMT3A. The molecular mechanisms involved in this process remain unclear. Therefore, we aimed to investigate whether the NLRP3 signaling pathway is involved in the progression of myocardial fibrosis and hypothesized that DNMT3A of Lnc RNA GAS5 is methylated by modulating the NLRP3 signaling pathway.

MATERIALS AND METHODS

Animal Model

Forty male rats weighing 180~200g were randomly divided into experimental group and control group, with 20 rats in each group. Rats in the experimental group were injected subcutaneously with ISO (isoproterenol), 5 mg/kg for 2 weeks, while rats in the control group were injected with the same amount of normal saline by the same method. After two weeks, all rats were killed under anesthesia and their heart tissue was saved for later use.

HE Staining

After conventional paraffin embedding, the sample was continuously cut into 4 μm, which was waxed with dew and stained with HE. Then look through a microscope and collect the results. Each slice was examined by at least 5 random fields, and semi-quantitative evaluation analysis was performed using the Automatic Photo and Image Analysis System (Image-Pro Plus 6.0, USA).

Masson Staining

The sections were washed with distilled water, lemon red acid red solution for 5-10 min, 2% glacial acetic acid water and 1% phosphomolybdenum. The aqueous solution was decomposed by acid for 3-5 min, dyed with aniline blue solution directly for 5 min, soaked in acetic acid aqueous solution containing 2% glacier, dehydrated conventionally, sealed with neutral resin, observed and photographed under a microscope. Each slice was examined by at least 5 random fields, and semi-quantitative evaluation analysis was performed using the Automatic Photo and Image Analysis System (Image-Pro Plus 6.0, USA).

Sirius Red Staining

The sections were routinely dew axed to water, stained with Sirius red dye solution for 1 h, rinsed with tap water, stained with Mayer hematoxylin nuclei for 8– 10 min, rinsed with tap water, routinely dehydrated, sealed with a neutral resin, and observed and took pictures under the microscope. At least five random fields of each section were examined, and semi quantitative evaluations were analyzed with a Photo and Image Auto analysis System (Image-Pro Plus 6.0, USA).

Immunohistochemical Analysis

Samples were fixed in 4% paraformaldehyde and embedded in paraffin. Samples were cut into 5-μm-thick sections and stained with primary antibodies against caspase 1 (Cell Signaling Technology, MA, USA, 1:200), NLRP3 (Boster Biological Technology, Wuhan, China, 1:200) at 4°C overnight before being washed three times with PBS, and then incubated with the secondary antibody for 1 h at room temperature. Then, the sections were stained with diaminobenzidine. The images were captured by a fluorescence microscope (Nikon 80i, Otawara, Tochigi, Japan). At least five random fields of each section were examined, and semi quantitative evaluations were analyzed with a photo and image auto analysis system (Image-Pro Plus 6.0, USA).

Cell Culture and Treatment

After all the cell extraction instruments were prepared, the 3-5 day-old SD rats were soaked in 75% alcohol for 2 min, the heart tissues were taken, washed twice with PBS, and then put into 1.5ml eppendorf tubes. Cut into the test tube, add 1ml 0.25% trypsin, digest in 37°C water bath for 6-8 min, centrifuge at 1500rpm for 5 min, discard the supernatant. The digestion and centrifugation steps were repeated three times, at which time more cells were obtained. The cells were transferred to DMEM containing 10% fetal bovine serum, and the original medium was

discarded 2 hours later and the new medium was added. ATP was added to the treated cells and LPS+ was added to the untreated cells to stimulate cell drapes. Cells were stimulated with LPS (1 μg/ml) for 5.5 h, and then cultured with ATP (5 ng/ml) for 5h. A group of cells was added to the same amount of PBS and cultured in the same environment as the control group.

qRT-PCR

RNA was extracted from rat hearts and CFs with Trizol reagent (Invitrogen, MA, USA) and was reverse-transcribed into cDNA using the Takara kit according to manufacturer's instructions. Real-time fluorescence quantitative PCR analysis was performed by SYBR Green real-time fluorescence quantitative main mixing method. PrimeScriptTM RT reagent kit (TaKaRa, Japan) was used for reverse transcription of cDNA. The PCR operation follows the instructions for Maxima SYBR Green/ROX qPCR Master Mixture (2X) (Thermo, USA). β-actin was used to normalize mRNA relative expression levels, and 2-ΔΔCT was used to calculate the relative expression levels of target genes.

TUNEL Staining

TUNEL staining Kits (Roche, IN, USA) were used to detect DNA damage. Cells were cultured in 24-well plates. The procedures used depended on the reagent. The nuclei were stained with DAPI. Images were captured by fluorescence microscopy (Nikon 80i). Nuclei labeled with DAPI and TUNEL were considered positive.

Flow Cytometry

Cell apoptosis was determined by cytometry using Annexin V-FITC/propidium iodide (PI) apoptosis detection kits (BD Accuri C6, BD Biosciences, Franklin Lake, NJ) according to the manufacturer's instructions. In brief, the cells were harvested and washed with cold phosphatebuffered saline (PBS). The cells were resuspended and stained using the Annexin V-FITC/PI apoptosis detection kit in accordance with the manufacturer's instructions. The samples were analyzed using Becton-Dickinson flow cytometer.

Hoechst 33258 Staining

After CFs were treated respectively, the medium was removed and washed twice with PBS. Then sufficient Hoechst 33358 staining solution was added to cover cells. After incubation for 5 to 10 min in the dark, cells were washed for three times with PBS. Then, cells were observed and imaged by a fluorescent microscope (Leica DMI-4000 B, Leica, Wetzlar, Germany).

Transfection of siRNA and Plasmid

siRNA targeting GAS5 and DNMT3A mRNA was constructed by Shanghai Gene Pharmaceutical Company, and plasmids overexpressing GAS5 were constructed by Shanghai Sangong Company. GAS5 overexpressed plasmid and empty vector were used to transfect CFs. The sequence is as follows: si-GAS5 (mouse), sense5 '-GUAUAAAGGUACCACAUGUTT-3',anti-sense5'-ACAUGUGGUACCUUUAUA CTT-3'; si-DNMT3A,5'-CCCAGAGUA UGCACCAAUATT-3'(sense) and 5'-UAUUGGUGCAUACUCUGGG TT-3' (anti-sense) were digested and transferred to six-well plates for culture. The nucleotides of si-DNMT3A and si-GAS5 were used at final concentrations of 50 pM and 60 pM, respectively. According to manufacturer's instructions, different doses of siRNA or chaotic ribonucleotides were transfected with RNAimax liposome (Invitrogen, Carlsbad, USA).

Western Blotting

Histamine and cell protein were extracted with strong RIPA lysate, and their protein concentrations were determined by BCA protein assay. Total protein samples were extracted and loaded on 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. 5% skim milk powder incubated at room temperature. After several hours, the membrane was treated with primary antibody caspasase1 (P29466, Affinit, 1:1000); DNMT3A (ab188453, Abcam, 1:1:1000); NLRP3 (bs-10021R, Bioss,1:1000); Type I collagen (ab34710, Abcam, 1:1000); IL-1β at 4°C (ab9722, 1:100000, 1:1000). After three washing with TBS/T-20, the membrane was incubated with horseradish peroxidase labeled Goat anti-Rabbit IgG or Goat anti-mouse IgG (1:10 000) at 37°C for 1-2 hours. The protein was detected using the Tanon imaging system and the signal was observed with an ECL chemiluminescence kit (Thermo Scientific, USA). Beta-actin was used as an internal control for protein volume normalization.

Statistical Analysis

Quantitative data are expressed as mean ± SD. Statistical significance was determined by either the Student's t test for comparison between means or one-way analysis of variance with a post hoc Dunnett's test. If $p < 0.05$, the result was considered statistically significant.

RESULTS

Pathologic and pyrogenic changes of ISO-induced cardiac fibrosis in rats

HE staining results: Cardiac pathological sections of model group (IOS group) and control group (N group) were compared under light microscope after staining. In the model group (IOS group), the myocardial cells were disordered and disordered, and the myocardial fibroblasts were increased and densely distributed. In the control group (group N), myocardial cells were arranged in a regular manner, uniform in size, and there was no significant change in collagen distribution, as shown in Figure 1a ($n = 3$, $P<0.05$, the difference was statistically significant).

Masson staining results: Collagen fibers were blue after Masson staining. In the experimental group (ISO group), the collagen distribution in the intercellular interstitium was significantly increased, with dense arrangement and diverse cell morphology, and there were patches of blue areas in the visual field. In the control group (group N), collagen fibers were less distributed and there were few blue areas in the visual

field. Figure 1b (n=3, P<0.05, the difference was statistically significant).

Sirius red staining results: Collagen fibers will appear yellow or red after staining with Sirius red dye solution. Compared with normal group N, collagen in the ISO group was arranged disordered and increased, and even filled some areas, while in contrast, collagen in the normal group was arranged clearly and in a consistent shape, as shown in Figure 1c (n=3, P<0.05, the difference was statistically significant).

Immunohistochemistry, Western blot and qRT-PCR results

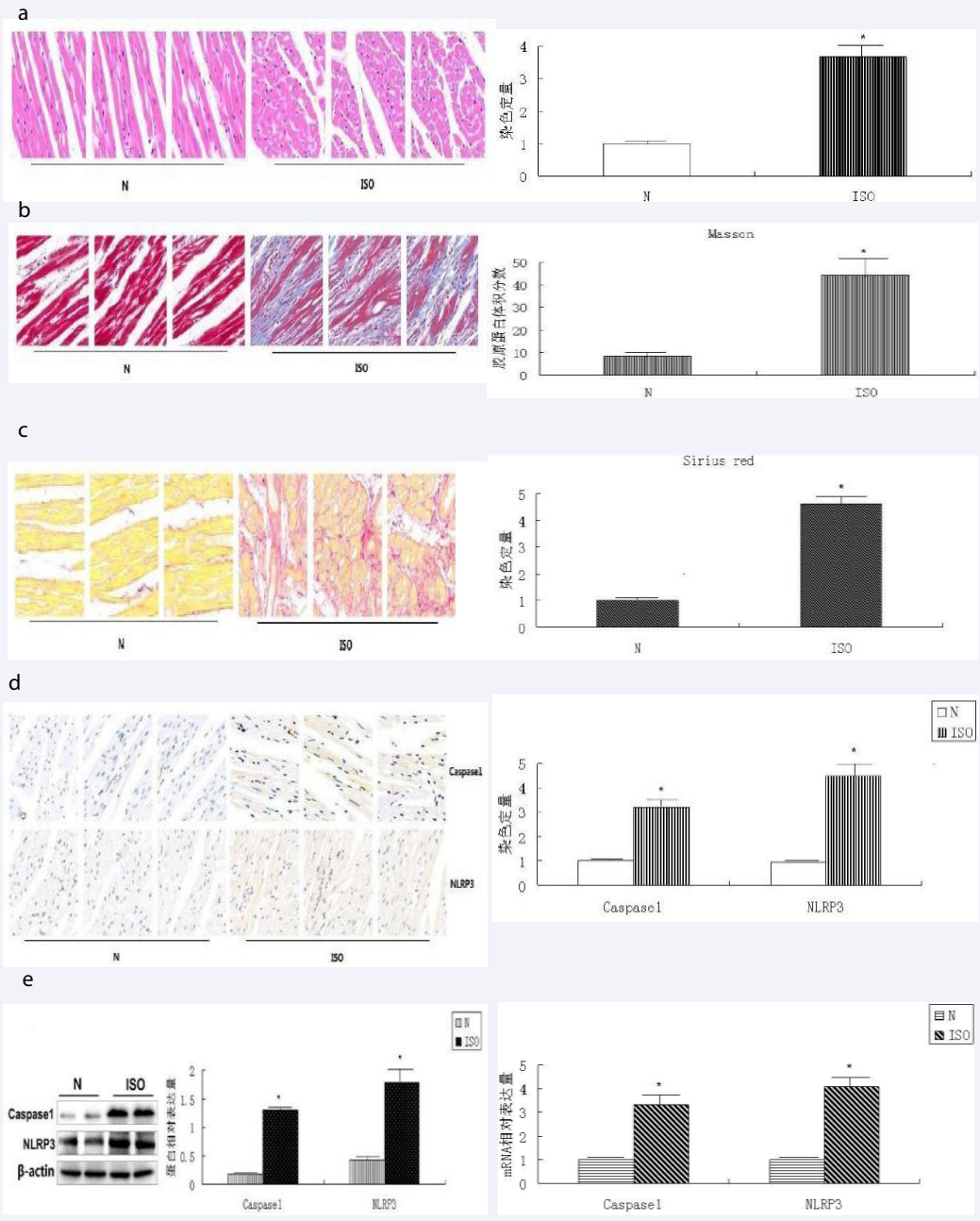

Figure 1 Pathologic and pyrogenic changes of ISO-induced cardiac fibrosis in rats.
A Pathological and pyroptosis changes in ISO-induced cardiac fibrosis. a-A piece of heart tissue was fixed; thin sections were cut and stained with hematoxylin and eosin (H&E). b-Thin sections were cut and stained with Masson's trichrome stain. c-Thin sections were cut and stained with Sirius Red staining. d- NLRP3 and caspase 1 expression in rat cardiac fibrosis tissue was analyzed by Immunohistochemistry. e- NLRP3 and caspase 1 protein expression in rat cardiac fibrosis tissue was analyzed by Western blotting. f- NLRP3 and caspase 1 mRNA expression in rat cardiac fibrosis tissue was analyzed by qRT-PCR. The numbers in the views represent the groups of rats in the experiments. *p < 0.05,

all showed that the expressions of Caspase1 and NLRP3 were significantly increased in the heart tissues of rats treated with ISO, as shown in Figure 1d, Figure 1e and Figure 1f. (n=3, P< 0.05, the difference was statistically significant).

Increased expression of DNMT3A and decreased expression of Lnc RNA GAS5 in ISo-induced myocardial fibrosis tissue and LPS-induced pyroptosis cells

DNMT3A in ISO-induced cardiac fibrosis tissues and LPS-induced pyroptosis of cardiac fibroblasts, qRT-PCR results showed that the expression of Lnc RNA GAS5 was significantly down-regulated in cardiac fibrosis tissues (Figure 2a, P< 0.05, the difference was statistically significant). However, qRT-PCR results showed significantly increased expressions of DNMT3A and Col1A1 in ISO-induced cardiac fibrosis compared to normal cardiac tissue

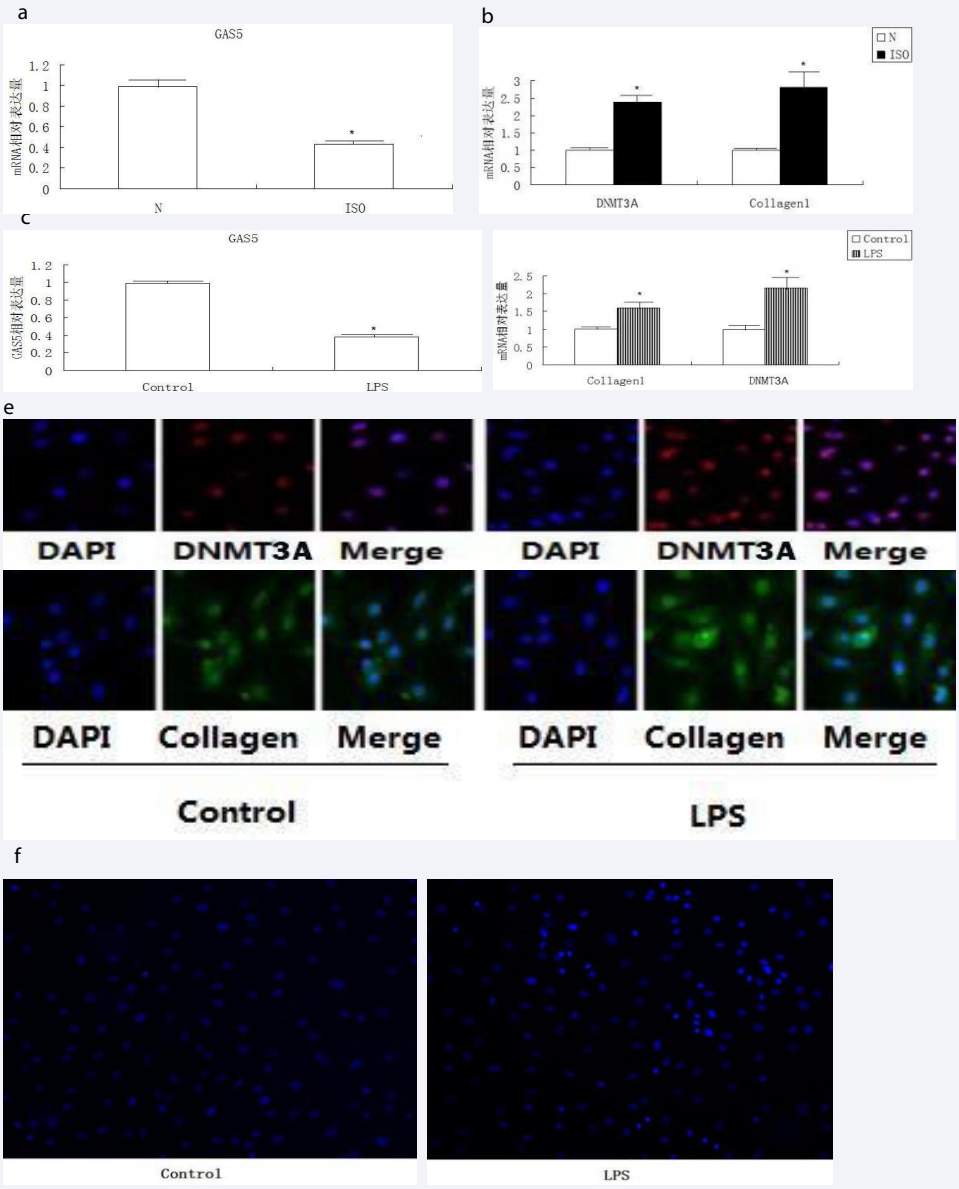

Figure 2 Increased expression of DNMT3A and decreased expression of Lnc RNA GAS5 in ISo-induced myocardial fibrosis tissue and LPS-induced pyroptosis cells.

a. Rat heart tissue RNAwas isolated,qRT-PCR to detect GAS5 expression in ISO-induced myocardial fibrosis model; b.qRT-PCR detection of DNMT3A and Collagen1 expression in ISO-induced myocardial fibrosis model; c.Western blot to detect the expression of DNMT3A, Collagen1 protein in the model of myocardial fibrosis induced by ISO; d.qRT-PCR detection of GAS5 expression in LPS-induced cardiomyocyte fibroblast pyroptosis; e.qRT-PCR detection of DNMT3A, Collagen1 expression in pyroptosis model of myocardial fibroblasts induced by LPS; f.Immunofluorescence detection of Collagen1, DNMT3A protein expression in a model of myocardial fibroblast pyroptosis caused by LPS; g.Hoechest 33258 staining to detect the relative rate of apoptosis in LPS-induced cardiomyocyte fibroblast scorch models.

(Figure 2b, P< 0.05, the difference was statistically significant,). Western blot also showed significantly increased expressions of DNMT3A and Col1A1 proteins (Figure 2c, P< 0.05, the difference was statistically significant). In vitro, we stimulated cardiac fibroblasts with LPS. qRT-PCR results showed significantly increased expressions of DNMT3A and Col11 in LPs-induced cardiac fibroblasts compared with untreated cardiac fibroblasts (Figure 2d, P< 0.05, the difference was statistically significant) Lnc RNA GAS5 expression decreased significantly (Figure 2e, P< 0.05,the difference was statistically significant).

Immunofluorescence assay results showed that protein expressions of DNMT3A and Col11 were significantly increased in LPs-induced myocardial fibroblasts compared with untreated myocardial fibroblasts (Figure 2f, P< 0.05 the difference was statistically significant). In addition, Hoechst 33258 staining showed a significant increase in pyrodeath in LPs-induced myocardial fibroblasts compared to untreated myocardial fibroblasts (Figure 2g, P< 0.05, the difference was statistically significant).

The effect of DNMT3A mediated methylation silencing on the expression of Lnc RNA GAS5 and Lnc RNA GAS5 on the scorch death of myocardial fibroblasts induced by LPS

To investigate the relationship between DNMT3A methylation and GAS5 expression, we transfected si DNMT3A and its negative control (NC) into cardiac fibroblasts, and set up a blank control group. qRT-PCR results showed that GAS5 expression significantly increased in cardiac fibroblasts transfected with si DNMT3A (Figure 3a, P<0.05, the difference was statistically significant), while the expressions of DNMT3A and caspase 1 were significantly decreased in myocardial fibroblasts transfected with si DNMT3A (Figure 3b, P<0.05, the difference was statistically significant).

In order to investigate the effect of GAS5 expression on myocardial fibroblast apoptosis, LPS was used to induce pyrodeath in si GAS5 group, empty group, GAS5 overexpression group (pc-DNA3.1-GAS5) and normal group. qRT-PCR results showed that the expression of GAS5 was significantly increased in cardiac fibroblasts transfected with pc-DNA3.1-GAS5, while the expression level of Caspase1 was significantly decreased (Figure 3c, P<0.05, the difference was statistically significant), the expression of GAS5 in cardiac fibroblasts transfected with si GAS5 was significantly decreased, and the expression of caspase 1 in CFs transfected with si GAS5 was significantly increased (Figure 3d, P<0.05,, the difference was statistically significant). In addition, flow cytometry showed increased apoptosis after si RNA transfection and decreased apoptosis in cardiac fibroblasts transfected with pc-DNA3.1-GAS5 (Figure 3e, P<0.05,Figure 3f, P<0.05, the difference was statistically significant).

DNMT3A silencing of LncRNA GAS5 leads to pyrodeath of myocardial fibroblasts via the NLRP3 signaling pathway

To understand the mechanism by which DNMT3A silencing of Lnc RNA GAS5 leads to pyrodeath of myocardial fibroblasts through the NLRP3 signaling pathway, si DNMT3A was used. Overexpressed GAS5 (pc-DNA3.1-GAS5) and small interfering Rnas (si GAS5) were transfected into CFs. First, we demonstrated that NLRP3 expression was significantly increased in ISO-induced cardiac fibrosis compared with normal myocardial tissue (Figure 4a, Figure 4b, P<0.05, the difference was statistically significant), LPS induced apoptosis of myocardial fibroblasts was significantly increased compared with untreated fibroblasts (Figure 4c, Figure 4d, P<0.05, the difference was statistically significant). The expression of NLRP3 was significantly decreased in CFs transfected with si DNMT3A (Figure 4e, P<0.05, the difference was statistically significant). Moreover, NLRP3 expression was significantly reduced in pc-DNA3.1-GAS5 transfected cardiac fibroblasts (Figure 4f, P<0.05, the difference was statistically significant), while the expression of NLRP3 was significantly decreased in cardiac fibroblasts transfected with si GAS5 (Figure 4g, P<0.05, the difference was statistically significant).

DISCUSSION

In recent years, more and more studies have shown that myocardial fibrosis is closely related to various heart diseases, such as myocardial hypertrophy, cardiac insufficiency and valvular heart disease [22]. Myocardial fibrosis refers to the death of part of the myocardium under the stimulation of various factors, and excessive compensatory repair damage of the body leads to the proliferation of myofibroblasts, the proliferation of collagen fiber tissue, and the disorder of collagen fiber arrangement. Its pathological basis is the increase of the proportion of myocardial interstitium in myocardial tissue [23,24]. Myocardial fibroblasts can be extracellular matrix (ECM), which is mostly collagen fibers, and the increase of collagen synthesis and decrease of collagen degradation are one of the main causes of myocardial fibrosis [25]. It can be seen from the histopathological results of this experiment that the SD rat cardiac fibrosis model is successful. As the main component of non-coding Rnas, most Lnc Rnas have little or no protein-coding ability, yet they play a crucial role in biological processes and cellular metabolism [1,2]. Lnc RNA can regulate myocardial fibrosis by regulating fibroblast proliferation, differentiation, and collagen deposition through various mechanisms. Previous studies have shown that lnc RNA AK014842 and BF607975 are uniformly up-regulated in diabetic cardiomyopathy and diabetic myocardial fibrosis cell models through the establishment of diabetic myocardial fibrosis cell models [26]. The above experiments confirmed that lnc RNA is a key substance in regulating myocardial fibrosis. Lnc RNA GAS5, a kind of Lnc RNA associated with growth arrest, plays an important role in the proliferation, apoptosis, invasion and invasion of many malignant tumor cells, and is a known tumor suppressor. Studies have shown that GAS5 expression in mice, rats, human hepatic fibrosis specimens and activated HSC is significantly decreased, and GAS5 overexpression reduces collagen accumulation in hepatic fibrosis tissues [27]. Previous studies of our group have found that Lnc RNA GAS5 can affect the activation and proliferation of myocardial fibroblasts by regulating the expression of mi R-21, but whether it has other effects on myocardial fibroblasts remains unclear. However,

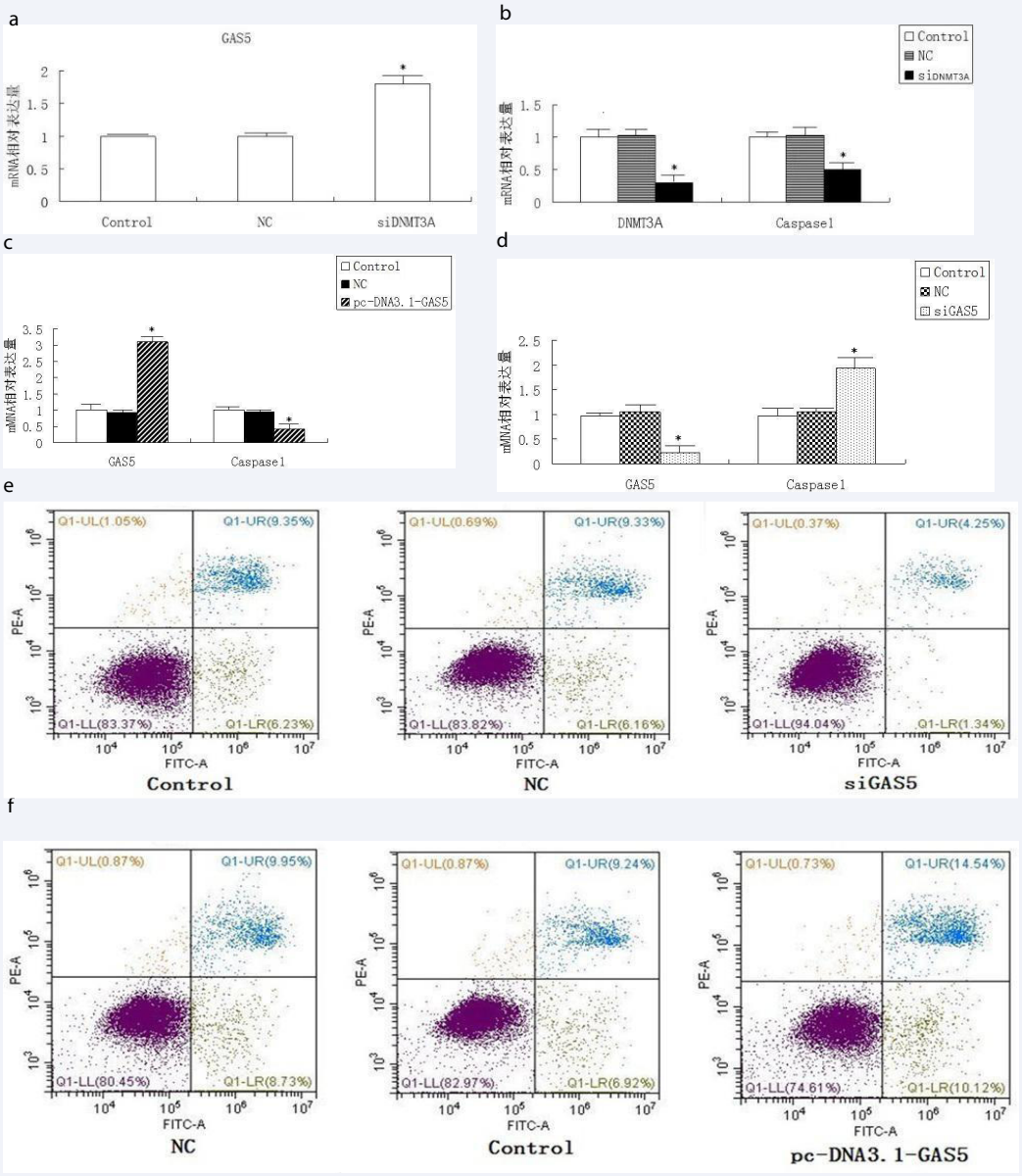

Figure 3 The effect of DNMT3A mediated methylation silencing on the expression of Lnc RNA GAS5 and Lnc RNA GAS5 on the scorch death of myocardial fibroblasts induced by LPS

a. qRT-PCR detection of transient transfection of si DNMT3A on the expression of GAS5 in cardiac fibroblasts; b. Effect of transient transfection si DNMT3A on expression of DNMT3A and Caspase1 in cardiac fibroblasts detected by qRT-PCR; c. Effect of transient transfection pc-DNA3.1-GAS5 on expression of GAS5 and Caspase1 in cardiac fibroblasts detected by qRT-PCR; d. Effect of transient transfection si GAS5 on expression of GAS5 and Caspase1 in cardiac fibroblasts detected by qRT-PCR; e. Flow cytometry detection of transient transfection of si GAS5 to apoptosis in a model of myocardial fibroblast pyroptosis;f.Flow cytometry detection of transient transfection of pc RNA3.1-gas5 to apoptosis in a model of myocardial fibroblast pyroptosis.

relevant studies have suggested that Lnc RNA GAS5 can inhibit the occurrence and development of ovarian cancer by promoting pyrodeath of ovarian cancer cells [28]. Therefore, we speculated that Lnc RNA GAS5 might affect myocardial fibrosis by regulating pyrodeath of myocardial fibroblasts. Pyroptosis, also known as cell inflammatory necrosis, is a programmed cell death that occurs in many cell lines, including endothelial cells (EC), smooth muscle cells (SMC) and cardiomyocytes, and is widely involved in the pathophysiological processes of various diseases [29]. It is important to note that pyrodeath is characterized by necrosis and apoptosis. Although pyroptosis cells maintain an intact plasma membrane until the end of the process, pores with a diameter of 1-2nm form directly in the plasma membrane of pyroptosis cells, leading to cell swelling, which is caused by water entry molecules

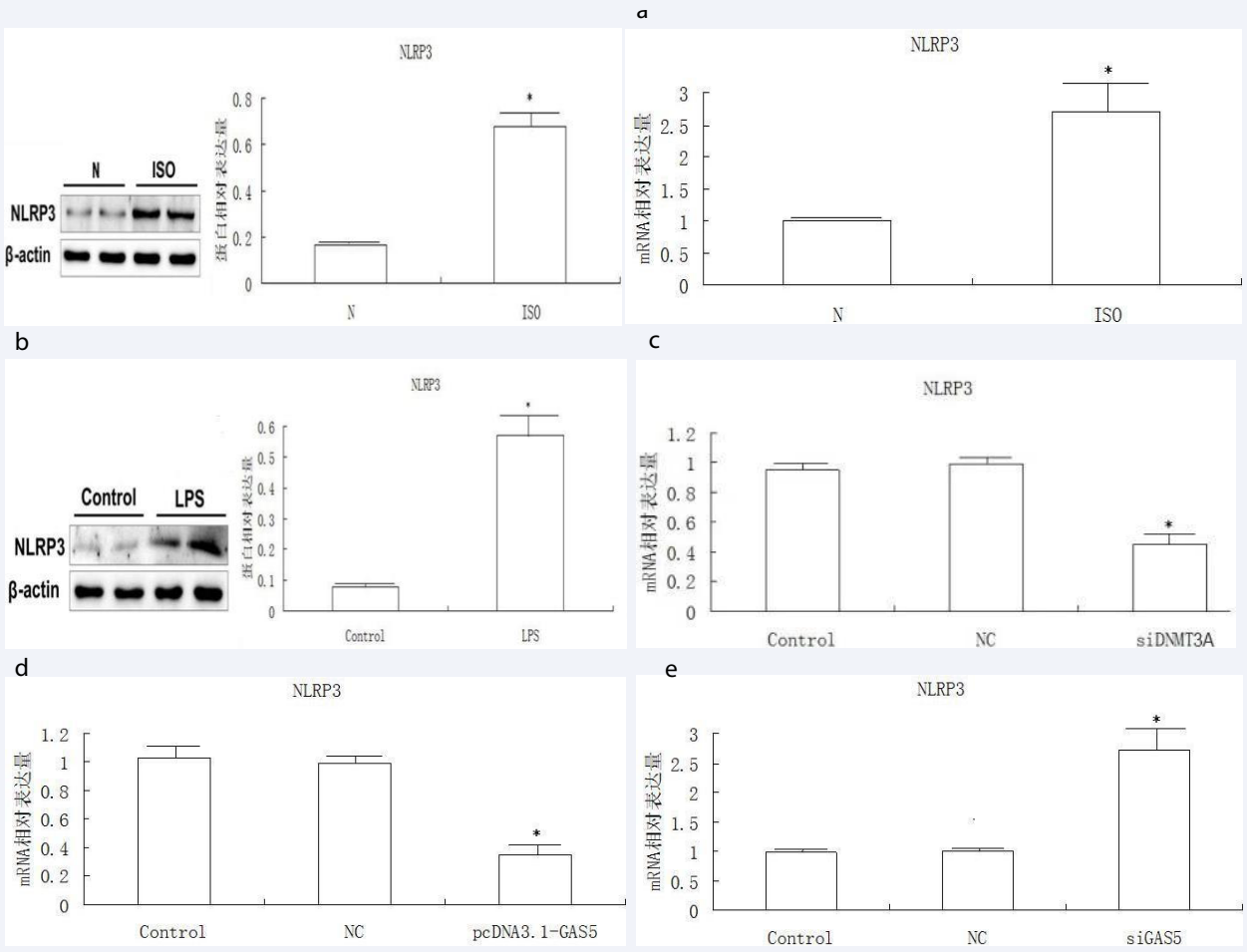

Figure 4 DNMT3A silencing of LncRNA GAS5 leads to pyrodeath of myocardial fibroblasts via the NLRP3 signaling pathway.
a. qRT-PCR detection of NLRP3 expression in ISO-induced myocardial fibrosis model; b.Western blot detection of NLRP3 expression in LPS-induced cardiac fibroblasts model; c. qRT-PCR transient transfection of si DNMTI-induced myocardial fibroblasts pyroptosis model NLRP3 expression. d. qRT-PCR transient transfection of pc DNA3.1-GAS5 myocardial fibroblasts pyroptosis model NLRP3 expression; e. qRT-PCR transient transfection of si GAS5 myocardial fibroblasts pyroptosis model NLRP3 expression

and cell lysis. Through rapid destruction of the plasma membrane, pro-inflammatory cytokines are released into the extracellular environment, causing an inflammatory response similar to necrosis [21]. Therefore, the pro-inflammatory properties of pyrodeath determine its role in a variety of inflammation-related diseases, and in-depth understanding of its molecular mechanisms could help develop new clinical therapies. Pyrodeath occurs more rapidly than apoptosis and is accompanied by the release of a large number of pro-inflammatory cytokines such as IL-1β and IL-18 [30,31]. Epigenetic changes in DNA methylation and Lnc RNA play an important role in many biological processes, including cardiac fibrosis [32,33], but the relationship between Lnc RNA methylation and apoptosis in cardiac fibrosis remains unclear. Therefore, in this study, we aimed to elucidate the underlying mechanism of how Lnc RNA GAS5 and DNA methylation affect myocardial fibroblast apoptosis in cardiac fibrosis. We established a rat myocardial fibrosis model to explore the expressions of GAS5, DNMT3A, NLRP3 and Caspase1 related to scorch death in myocardial tissue during myocardial fibrosis. All the results showed that GAS5 expression decreased, DNMT3A expression increased and pyrodeath increased after the occurrence of cardiac fibrosis. We then established an in vitro model to induce pyrodeath in myocardial fibroblasts using LPS, and detected that GAS5 expression decreased, DNMT3A expression increased, pyrodeath increased, and collagen expression also increased in myocardial fibroblasts. Initially, we found that the expression of Lnc RNA GAS5 was down-regulated in cardiac fibrotic tissue and LPS-induced pyroptosis of myocardial fibroblasts. Overexpression of Lnc RNA GAS5 can reduce myocardial fibroblast pyrodeath by targeting caspase 1, thereby reducing cell death in vitro. Previous studies have shown that many Lnc Rnas are involved in the process of cardiac fibrosis [34]. For example, Lnc RNA GAS5 is down-regulated in cardiac fibrosis and influences proliferation via mi R-21 [35]. As reported in the literature, GAS5 seems to play a role in a variety of diseases, such as inflammatory bowel disease, which shows a significant

reduction in the level of GAS5 in cells [36]. Our results show that the expression of Lnc RNAGAS5 is reduced in cardiac fibrosis and influences pyrodeath of myocardial fibroblasts, thus confirming previously published results. Studies have shown that Lnc RNA can bind to DNMT3A to promote gene expression and abnormal DNA methylation during fibrosis [37]. NLRP3, characterized by apoptosis and pyrodeath, is associated with cell death and inflammatory response [38]. The scorch death of cardiac fibroblasts induced by cardiac fibrosis and LPS was significantly higher than that of normal cardiac tissue and cardiac fibroblasts. Our study shows that downregulation of DNMT3A can lead to increased expression of Lnc RNAGAS5, thus inactivating the NLRP3 signaling pathway. LPS can trigger the activation of NLRP3 inflammasome and promote the production of cleaved caspase 1 [39]. Our study found that DNMT3A methylation silencing of Lnc RNA GAS5 may lead to myocardial fibroblast focusing and cardiac fibrosis through activation of the NLRP3 axis. In addition, this study provides evidence that Lnc RNA GAS5 and DNMT3A can be used as novel biomarkers for cardiac fibrosis and provide new ideas for future treatment of cardiac fibrosis.

CONCLUSION

After myocardial fibrosis in SD rats, the expression of Lnc RNA GAS5 was low and NLRP3 was high, and the expression of Lnc RNA GAS5 was decreased in cardiac fibrosis and affected the pyrodeath of myocardial fibroblasts. The down-regulation of DNMT3A could increase the expression of Lnc RNA GAS5. The NLRP3 level of myocardial fibroblasts was significantly decreased. We speculated that the occurrence and development of myocardial fibroblast pyrodeath and cardiac fibrosis were related to the activation of NLRP3 pathway by Lnc RNA GAS5 after DNMT3A silencing.

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