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ORIGINAL RESEARCH

Long Noncoding RNA SOX2OT Ameliorates Sepsis-Induced Myocardial Injury by Inhibiting Cellular Pyroptosis Through Mediating the EZH2/Nrf-2/NLRP3 Signaling Pathway

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Objective: Cellular pyroptosis is a pro-inflammatory mode of programmed cell death that has been identified in recent years, and studies have shown that the LncRNA SOX2OT regulates myocardial injury during sepsis, but the exact regulatory mechanism is unclear. The aim of this study was to assess the role of SOX2OT in regulating cardiomyocyte injury during sepsis cardiomyopathy.
Methods: Rat cardiomyocytes, C57BL/6 mice, and transgenic mice were divided into four groups: control, LPS, LPS+ knockout LncRNA SOX2OT, and LPS+ overexpression LncRNA SOX2OT. Inflammatory factor levels were detected by qPCR. Associated proteins and gene expression were detected by Western blotting and qPCR. Dual luciferase was used to detect the target genes of SOX2OT. Nrf2 and EZH2 knockdown and overexpression cell lines were established, and the expression of related genes was detected by qPCR.
Results: Results In this study, we found that SOX2OT knockdown exacerbated LPS-induced levels of inflammatory factors and procalcitoninogen (PCT), and increased the expression of pyroptosis-related proteins and LDH. The results of dual luciferase reporter gene assay showed that EZH2 is the target gene of SOX2OT, and overexpression of SOX2OT decreased the expression of EZH2; we also found that knockdown of EZH2 in H9c2 cells decreased the expression of Nrf2, which was positively correlated with the expression level of NLRP3. Further in vivo results showed that overexpression of SOX2OT attenuated SIMD (sepsis-induced myocardial dysfunction), as evidenced by improved myocardial structural integrity and reduced inflammatory cell infiltration. The expression of pyroptosis-related proteins and LDH was significantly increased in the mice in the LPS group; this effect was reversed by overexpression of SOX2OT, and potentiated by knockdown of SOX2OT.
Conclusion: Our data reveal a novel mechanism by which SOX2OT inhibits cardiomyocyte sepsis through the EZH2/Nrf-2/NLRP3 pathway, thereby attenuating septic myocardial injury, which may contribute to the development of new therapeutic strategies.
Keywords: SOX2OT, EZH2, Nrf2, sepsis, pyroptosis

Introduction

Sepsis is a complex multifactorial disease and one of the leading causes of death and critical illness worldwide; sepsis poses serious health and economic burdens for patients and health care systems around the world.[1] Epidemiological data show that cardiac dysfunction occurs in approximately 40% to 60% of patients with septic shock; the 28-day mortality rate among hospitalized sepsis patients with cardiac insufficiency is as high as 47%, and this rate among hospitalized sepsis patients with normal cardiac function is 16%.[2] The most common forms of sepsis-induced cardiac dysfunction are myocardial injury and myocardial depression, suggesting that myocardial function plays an important role in sepsis-induced cardiac

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insufficiency.[3] The "Surviving Sepsis Campaign Guidelines" (SSC) state that diagnosing sepsis early in its course and intervening correctly and in a timely manner improves prognosis.[4] Therefore, the search for promising early detection markers and more effective and safer therapies has great potential to improve clinical strategies and outcomes in the treatment of septic myocardial injury.

Long noncoding RNAs (LncRNAs) are a group of important RNA molecules with lengths ranging from 200–100,000 nucleotides. LncRNAs are involved in the regulation of a variety of important molecular and physiological processes ranging from X-chromosome silencing to genome imprinting and chromatin modification.[5] In addition, many noncoding RNAs, including LncRNAs, are found not only in tissues but also in the peripheral circulatory system, such as the blood, where they can be easily sampled and detected; and the levels of these molecules in the blood are often correlated with the course of disease.[6] More importantly, efficient and specific regulation of LncRNA expression can be achieved by constructing antisense RNA expression vectors. SOX2 overlapping transcript, chromosome 3q26.33 (SOX2OT) is a LncRNA located in the intronic region of the SOX2 gene.[7] Recently, increasing evidence has shown that SOX2OT is a powerful biomarker that is strongly associated with myocardial injury, such as myocardial ischemia/reperfusion injury[8] and coronary microembolism.[9] However, the clinical significance and biological function of SOX2OT in septic myocardial injury have not been clearly elucidated.

Cellular pyroptosis is a type of programmed cell death, also known as cellular inflammatory necrosis; pyroptosis is dependent on inflammatory cysteoaspartic enzymes (mainly caspase-1, caspase-4, caspase-5, and caspase-11) and is accompanied by the release of large quantities of proinflammatory factors that induce strong inflammatory responses.[10] The morphological features, occurrence and regulatory mechanisms of cellular pyroptosis are different from those of other modes of cell death, such as apoptosis and necrosis, and the fact that pyroptosis does not require the specific segmentation of DNA fragments allows cell lysis to occur faster than in this process than in apoptosis.[11] When infection is not controlled, recognition of pathogen-associated molecular patterns (PAMPs) activates the cellular pyroptosis pathway on a large scale, and pyroptotic cells release large amounts of danger signaling molecular patterns (DAMPs), which also activate the cellular pyroptosis pathway, thus creating a positive feedback loop that can lead to infectious shock in severe cases.[12] Sepsis-induced abnormalities in cardiac function often occur due to excessive inflammatory responses, and NLRP3-mediated release of inflammatory vesicles/caspase-1/IL-1β likely participate in this process by regulating cardiomyocyte pyroptosis. Therefore, in our study, we used rat cardiomyocyte (H9c2) and C57/BL mouse models of sepsis to explore the relevant molecular mechanisms and potential therapeutic targets. We identified SOX2OT as a key player and revealed the specific mechanism by which it participates in septic cardiomyocyte pyroptosis; this molecule could serve as a potential therapeutic target.

Materials and Methods

Cell Culture and Stimulation

Rat cardiomyocytes (H9c2 cells) were purchased from the Cell Bank of the Typical Cultures Preservation Committee of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (Corning). The cells were incubated in a cell culture incubator at 37 °C in 5% CO_2. The medium was changed every 2 days, and the cells were passaged until they reached 70–80% confluence. H9c2 cells were treated with different concentrations (0.1 or 0.5 μg/mL) of LPS (Sigma) for 6 hours at 37 °C. In some experiments, shSOX2OT, SOX2OT-OE shEZH2, shNrf2, and Nrf2-OE were transfected into H9c2 cells using Lipofectamine 2000 according to the manufacturer's instructions. The sequences are shown in the accompanying table.

Reverse Transcription-Quantitative PCR (RT–qPCR)

Total RNA was extracted from H9c2 cells using FastPure Cell/Tissue Total RNA Isolation Kit V2 reagent. HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper) was used for reverse transcription. ChamQ Universal SYBR qPCR Master Mix was used for RT–qPCR. (Novozymes Biotechnology Stock Co., Ltd., Nanjing, China) The relative expression levels of all the target genes were calculated using the 2-ΔΔCq calculation method. The following primer sequences were used. The sequences of primers used for PCR are shown in Table 1.

Table 1 PCR Primer Sequences

SOX2OT	forward: TTTCTATTCCAGGGATTGCAGAGG
	reverse: GGTGTTGTCTTGTAGCAGTTTGATT
EZH2	forward: TGAAGCAAATTCTCGGTGTC
	reverse: CAATGGCACAAAAGTTATCGT
Nrf2	forward: CTACATTTCAGTCGCTTGCCCT
	reverse: AAATAGCTCCTGCCAAACTTGC
GAPDH	forward: CCCTTCATTGACCTCAACTACA
	reverse: ATGACAAGCTTCCCGTTCTC
NLRP3	forward: CCTTCTGAACCGAGACGTGA
	reverse: CCAAAGAGGAAGCGTACAACA
IL-6	forward: TCTGCTCTGGTCTTCTGGAGT
	reverse: GCCACTCCTTCTGTGACTCT
IL-1β	forward: CCTATGTCTTGCCCGTGGAG
	reverse: CACACACTAGCAGGTCGTCA
TNF-α	forward: GGCGTGTTCATCCGTTCTCT
	reverse: CCCAGAGCCACAATTCCCTT

Elisa

The level of IL-1β was measured by ELISA. The cell culture medium was centrifuged at 1000 × G for 10 min at 4 °C, and the supernatant was collected. Experiments were performed using a commercially available IL-1β ELISA kit (Rat IL-1β ELISA Kit, E-EL-R0012c, Elabscience) according to the manufacturer's instructions.

Lactate Dehydrogenase (LDH) Measurements

Serum levels of LDH were determined by commercial kits (Biyuntian Biotechnology Co., Ltd., C0016, Shanghai, China) using standard techniques, and each procedure was performed according to the manufacturer's protocol.

Western Blotting

Total protein was extracted with RIPA lysis buffer (Doctoral Bioengineering Co., Ltd., AR0102-100, Wuhan, China). The protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit. Equal quantities of proteins (20 μg/lane) were separated on 10% SDS–PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk in 1X Tris-buffered saline and 0.1% Tween-20 at room temperature for 1 h. The membranes were then incubated overnight at 4 °C with primary antibodies against NLRP3 (Solepol Technology Co., Ltd., K008087P, Beijing, China), caspase-1 and H2A.X (Zhengneng Biotech Co., Ltd. 342,947, Chengdu, China), ASC, pro-IL-1β and IL-1β (Biyuntian Biotech Co., Ltd., Shanghai, China), and GAPDH (1:1:1). Chengdu, China). The membranes were then probed with appropriate secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG H&L) at room temperature for 2 h. The target protein levels were detected using enhanced chemiluminescence reagents (Doctoral Bioengineering Co., Ltd., AR1174, Wuhan, China) and analyzed with ImageJ.

Dual-Luciferase Reporter Assay

To confirm the relationship between EZH2 and SOX2OT, an online bioinformatics software called miRDB (http://mirdb.org/) was used to predict the binding site of SOX2OT in the 3'UTR of EZH2. To confirm the interaction between EZH2 and

SOX2OT, a dual-luciferase reporter assay was performed. The wild-type EZH2 3'UTR and the mutated EZH2 3'UTR sequences were inserted into the pGL4 vector containing the firefly luciferase reporter gene. H9c2 cells were plated in a 24-well plate at a density of 5×10^5 cells per well. One hundred nanograms of pGL4-WT-CNN1 or pGL4-MUT-CNN1, 100 nM SOX2OT mimic or negative control, and Renilla vector were cotransfected into the H9c2 cells. The relative luciferase activities were determined using the dual-luciferase assay system after the cells were incubated for 48 h.

Establishment and Grouping of Animal Models

Specific pathogen-free male C57BL/6 mice aged 6–8 weeks were purchased from Ningxia Medical University (Yinchuan, China). SOX2OT transgenic mice were generated according to the standard protocol of Cyagen Transgenic Animal Center (China). SOX2OT+/− male mice were mated with SIRT6+/− female mice to obtain knockout (SOX2OT −/−) and wild-type (SOX2OT+/+) mice. Total protein was isolated from the tails to confirm the levels of SOX2OT overexpression or knockout by qPCR. Prior to the experiment, the mice were housed under suitable conditions (23 ± 1 °C, light/dark cycle) and were allowed free access to food and water. The mice were divided into 4 groups (N = 6): the control group, LPS model group, SOX2OT overexpression group, and SOX2OT knockout group. First, the mice in the LPS model group, SOX2OT overexpression group, and SOX2OT knockout group were intraperitoneally injected with LPS (5 mg/kg), and the mice in the control group were intraperitoneally injected with physiological saline. Twenty-four hours after LPS administration, the mice were sacrificed, and their blood and hearts were collected for subsequent analysis. The protocol of this study was approved by the Ethics Committee of Ningxia Medical University. All the methods were performed in accordance with the US Public Health Service Guide for the Care and Use of Laboratory Animals, and every effort was made to minimize the suffering and number of animals used in this study.

Hematoxylin–Eosin (H&E) Staining

Immediately after blood collection, the heart of each rat was removed and then placed in 4% formaldehyde solution. The tissues were dehydrated and paraffin-embedded, and pathological sections were made. HE staining was performed, and myocardial pathological changes were observed under a light microscope. We used a myocardial histological scoring system of grade 0, no lesions; grade I, focal subendocardial lesions; grade II, extensive focal myocardial lesions with myofibrillar swelling and rupture; grade III, extensive fused myocardial lesions; and grade IV, myocardial degeneration and necrosis.

Immunofluorescence

Mouse heart tissues were fixed in 4% paraformaldehyde solution for 48 hours, then put into a disposable tissue embedding box for dehydration, dipped in wax and embedded, then the tissue wax blocks were sectioned and baked. The sections were sequentially placed in xylene I for 15 min-xylene II for 15 min-anhydrous ethanol I for 5 min-anhydrous ethanol II for 5 min-85% alcohol for 5 min-75% alcohol for 5 min-distilled water wash. The sections were placed in a repair cassette with 0.1 M sodium citrate (pH 6.0) (PP-1056, Shanghai Amazing Bioscience Co., Ltd.) antigen repair solution in a microwave oven for antigen repair. After natural cooling, the slides were washed in PBS for 3 times/5 min, and the sections were incubated in 3% hydrogen peroxide solution for 25 min at room temperature, protected from light, and washed in PBS for 3 times/5 min. The sections were shaken off and dried, and then a circle was drawn around the tissue with a histochemical pen, and a drop of BSA (A8020, Solarbio) was added inside the circle to cover the tissue uniformly, and the sections were closed at room temperature for 30 min, and then the closure solution was shaken off and a drop of anti-H2S was added on the sections. The sections were incubated with anti-H2A.X antibody (381,558, Chengdu Zhengneng Biotechnology Co., Ltd.) dropwise at 4°C overnight. On the next day, the sections were washed 3 times/5min with PBS, dried slightly, and then immunohistochemical secondary antibody (HRP-labeled) (K5007, DAKO) was added dropwise to cover the tissues in the circle, and the sections were incubated for 50min at room temperature. Ltd.) for re-staining. All the samples were examined by microscope and analyzed by image acquisition.

Paraffin-embedded heart sections were sequentially incubated with 0.1 M sodium citrate (pH 6.0) at 100 °C for dewaxing, hydration, and antigen repair. Cardiac sections or fixed cells were then permeabilized with 0.1% Triton X-100 and incubated in containment buffer. The samples were sequentially incubated with anti-H2A.X antibodies (381,558,

Chengdu Zhengneng Biotechnology Co., Ltd.) at 4 °C overnight, FITC- or rhodamine-conjugated secondary antibodies for 1 hour at room temperature, and DAPI. All the samples were examined by laser scanning confocal microscopy.

Statistical Analysis

The data are presented as the mean±SD. GraphPad Prism version 9.0 statistical software was used for statistical analysis. One-way analysis of variance was followed by Tukey's post hoc test for multiple group comparisons. The results were considered significant at $P<0.05$.

Results

Identification of the Optimal Concentrations and Time Points for LPS Challenge

To determine the optimal concentrations and time points for LPS challenge that should be used in the following validation experiments, the expression level of γ-H2A was measured by Western blotting, and the levels of tumor necrosis factor (TNF)-α, interleukin 6 (IL-6), interferon γ (IFN-γ), and procalcitonin (PCT) were measured by qPCR. As shown in Figure 1A and B, the LPS group was treated with different concentrations of LPS (100 ng/mL or 500 ng/mL) for 6 hours. We chose the concentration of 500 ng/mL LPS and the timepoint of 6 hours for the following experiments.

Knockdown of LncRNA SOX2OT Promotes LPS-Induced Pyroptosis in Rat H9c2 Cardiomyocytes

To verify the effect of SOX2OT on LPS-induced pyroptosis in H9c2 cells, we transfected SOX2OT shRNA into H9c2 cells. We used Western blotting to analyze pyroptosis in H9c2 cells transfected with shSOX2OT. Histone γH2AX is an important player in the DNA damage response, and the expression of γH2AX was significantly increased in shRNA2-transfected H9c2 cells compared with the control group and the shRNA3-transfected group (Figure 2A). shRNA2-transfected H9c2 cells were used for subsequent experiments. Compared with those in the control group, the levels of LDH, IL-1β, IL-6, and TNF-α were significantly increased in the supernatants of shSOX2OT-transfected cells (Figure 2B–D), and the expression of the pyroptosis-related proteins NLRP3, ASC, caspase-1, and IL-1β was significantly increased in shSOX2OT-transfected H9c2 cells, as shown by Western blotting (Figure 3). These results suggest that knockdown of SOX2OT in H9c2 cells can increase LPS-induced cell damage.

LncRNA SOX2OT Targets EZH2

To determine whether EZH2 is indeed a target of SOX2OT, we transfected SOX2OT-overexpressing lentivirus into H9c2 cells. Our results showed that the relative RNA levels of SOX2OT were increased in H9c2 cells transfected with SOX2OT-OE compared with H9c2 cells transfected with NC RNA (Figure 4A); Further experiments verified that

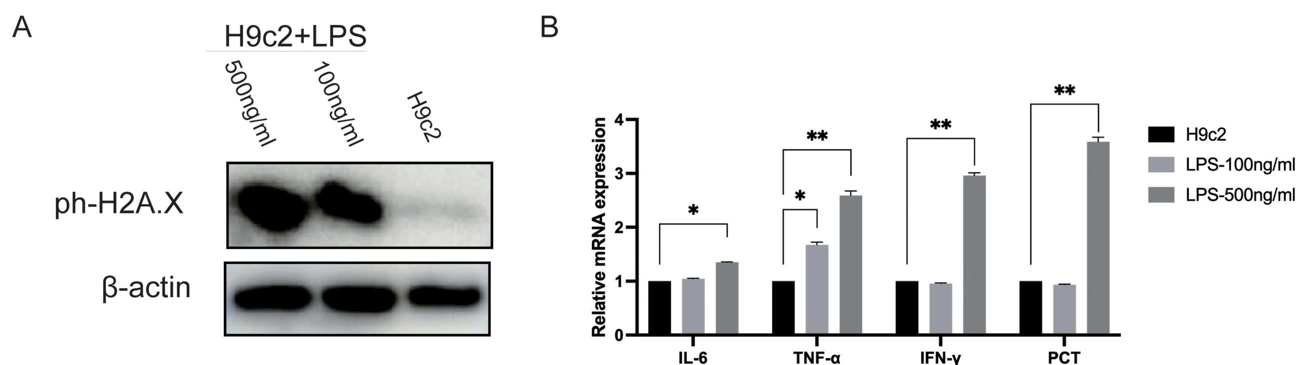

Figure 1 Identification of Optimal Concentrations and Time Points for LPS Challenge.
Notes: (**A**) Western blot assessment of LPS-induced H9c2 cell damage. (**B**) Assessment of the levels of the inflammatory factors TNF-α, IL-6, IFN-γ and PCT by qPCR. (*P < 0.05 compared with the corresponding control group, **P < 0.01 compared with the corresponding control group).

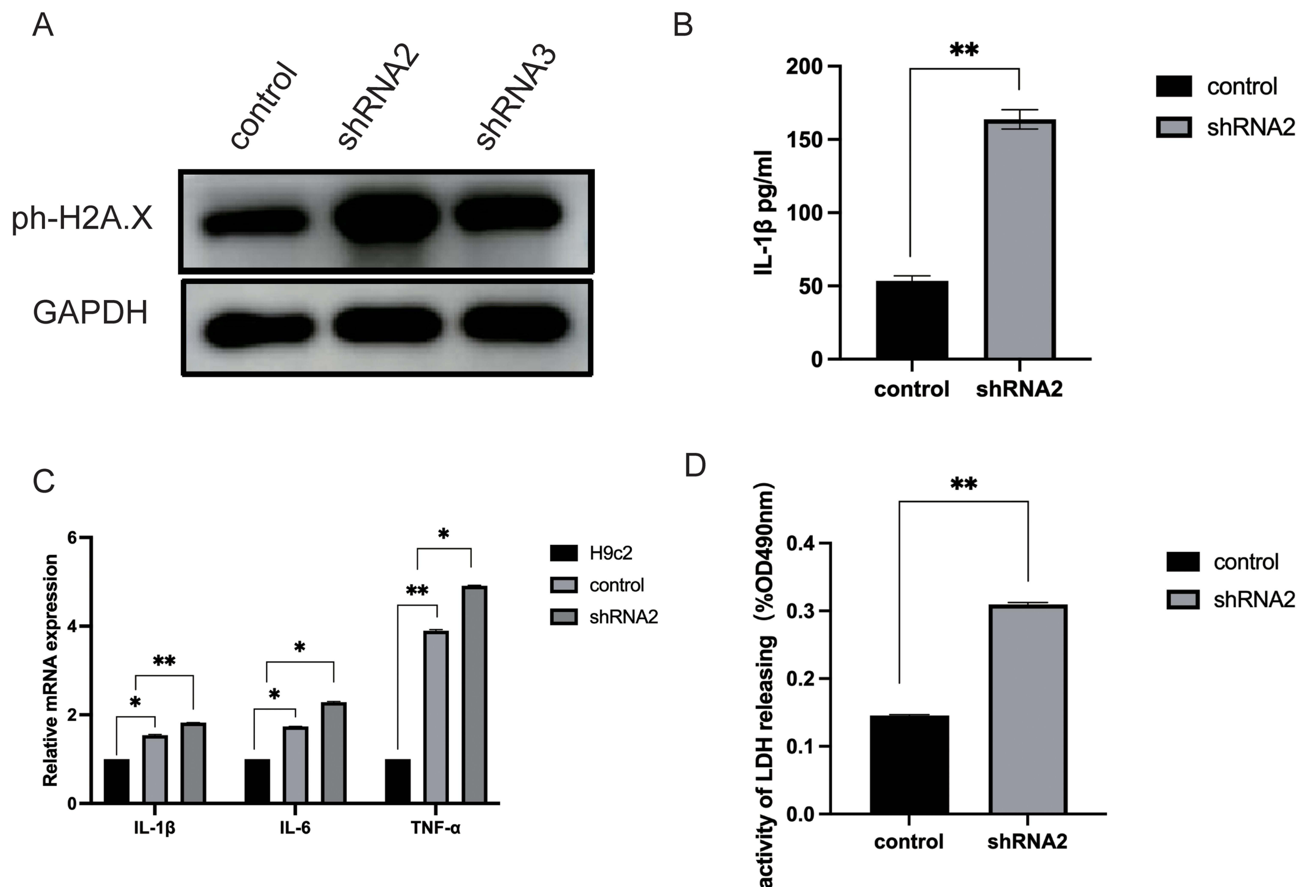

Figure 2 Knockdown of lncRNA SOX2OT promotes LPS-induced pyroptosis in H9c2 cells.
Notes: (**A**) Western blotting analysis of cellular damage in H9c2 cells transfected with shSOX2OT. (**B**–**C**) Levels of IL-1β, IL-6 and TNF-α in supernatants from shRNA-transfected H9c2 cells. (**D**) LDH in supernatants from shRNA-transfected H9c2 cells. (*P < 0.05 compared with the corresponding control group, **P < 0.01 compared with the corresponding control group).

compared to the control group, EZH2 expression levels were reduced in H9c2 cells transfected with SOX2OT-OE (Figure 4B); then, we performed luciferase microRNA target reporter gene assays. Cotransfection of the SOX2OT inhibited the luciferase expression of the reporter construct carrying the EZH2 3'-UTR (Figure 4C). Overall, these results suggest that the SOX2OT may directly target EZH2 to inhibit its expression during sepsis.

LncRNA SOX2OT Mediates Septic Cardiomyocyte Pyroptosis Through the EZH2/Nrf-2 Axis

To further determine that **SOX2OT** mediates septic cardiomyocyte pyroptosis through the EZH2/Nrf-2 axis, the shEZH2 cell line was constructed and shEZH2 expression was verified by qPCR; our results showed that the relative mRNA level of EZH2 was reduced in H9c2 cells transfected with shEZH2 compared with control, and shEZH2 1 was chosen for subsequent experiments (Figure 5A). Next, we established Nrf2 knockdown and overexpression cell lines, and verified the expression of Nrf2 by the qPCR method; the expression of Nrf2 was increased in the Nrf2-OE group compared to the control group (Figure 5B). The relative mRNA level of Nrf2 was decreased in H9c2 cells transfected with Nrf2-sh and Nrf2-sh2 was selected for the subsequent experiments (Figure 5C); Further qPCR experiments verified the correlation between EZH2 and Nrf2, and the expression of Nrf2 was decreased in the shEZH2 group compared to the control group (Figure 5D). The effect of Nrf2-sh/ Nrf2-OE on NLRP3 gene expression was further verified, and the results showed that the Nrf2 and NLRP3 expression levels were positively correlated (Figure 5E). Finally, the effect of SOX2OT on Nrf2 gene expression was verified, and Nrf2 expression was decreased after SOX2OT overexpression (Figure 5F). These results suggest that SOX2OT mediates septic cardiomyocyte pyroptosis through the EZH2/Nrf-2 axis.

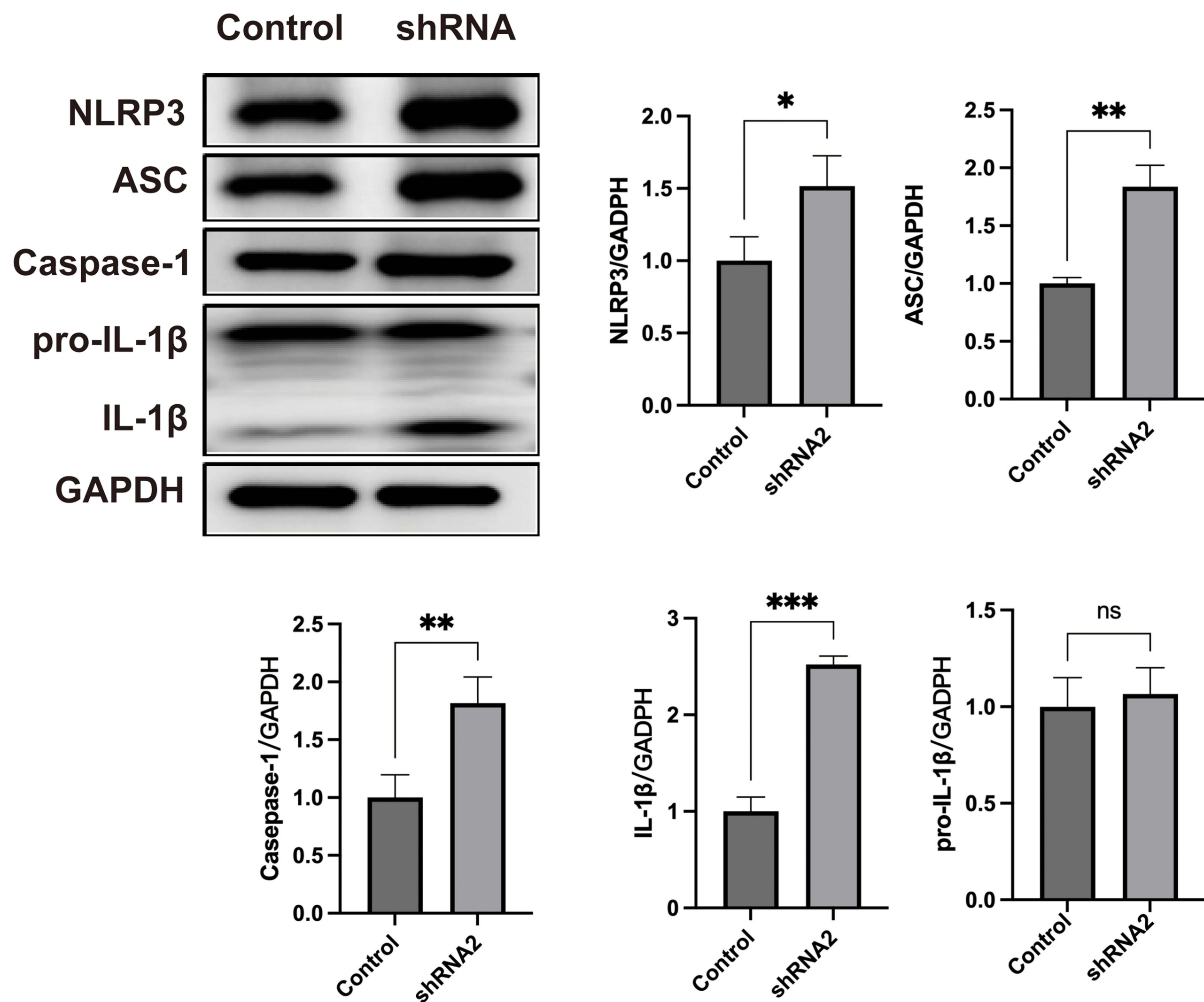

Figure 3 The expression of the pyroptosis-related proteins was increased in shSOX2OT-transfected H9c2 cells.
Notes: Protein expression of NLRP3, ASC, caspase-1, proIL-1β, and IL-1β in shRNA-transfected H9c2 cells. (**ns** indicates no statistically significant difference compared to the corresponding control group, *P < 0.05 compared with the corresponding control group, **P < 0.01 compared with the corresponding control group, ***P < 0.001 compared with the corresponding control group).

Effects of LncRNA SOX2OT on Cardiomyocyte Pyroptosis in Animal Models

An in vivo animal model of sepsis was established by the intraperitoneal injection of LPS, and the effect of SOX2OT on the cardiomyocytes of septic mice was investigated.

A kit was used to measure LDH expression levels, and SOX2OT overexpression ameliorated LDH expression after myocardial injury, as shown by the significant reduction in LDH levels (Figure 6A). The ELISA method was used to measure IL-1β expression levels, and SOX2OT overexpression decreased IL-1β expression compared with that observed in the LPS group, while SOX2OT silencing exerted the opposite effect (Figure 6B). The expression of pyroptosis-related proteins, namely, NLRP3, ASC, caspase-1, pro-IL-1β, and IL-1β was measured by Western blotting. The expression of pyroptosis-related proteins was reduced in the SOX2OT overexpression group compared with the LPS group, whereas the opposite trend was observed in the SOX2OT-silenced group (Figure 7).

As we mainly focused on the pathological changes in the heart, H&E staining was considered sufficient to conclude that overexpression of lncRNA SOX2OT reduced myocardial necrosis and resulted in well-arranged myocardial fibers. HE staining was performed to observe morphological changes induced by LPS and SOX2OT during myocardial injury. Pathological sections from rats from the 4 groups were stained with H&E, and the results are shown in Figure 8. The

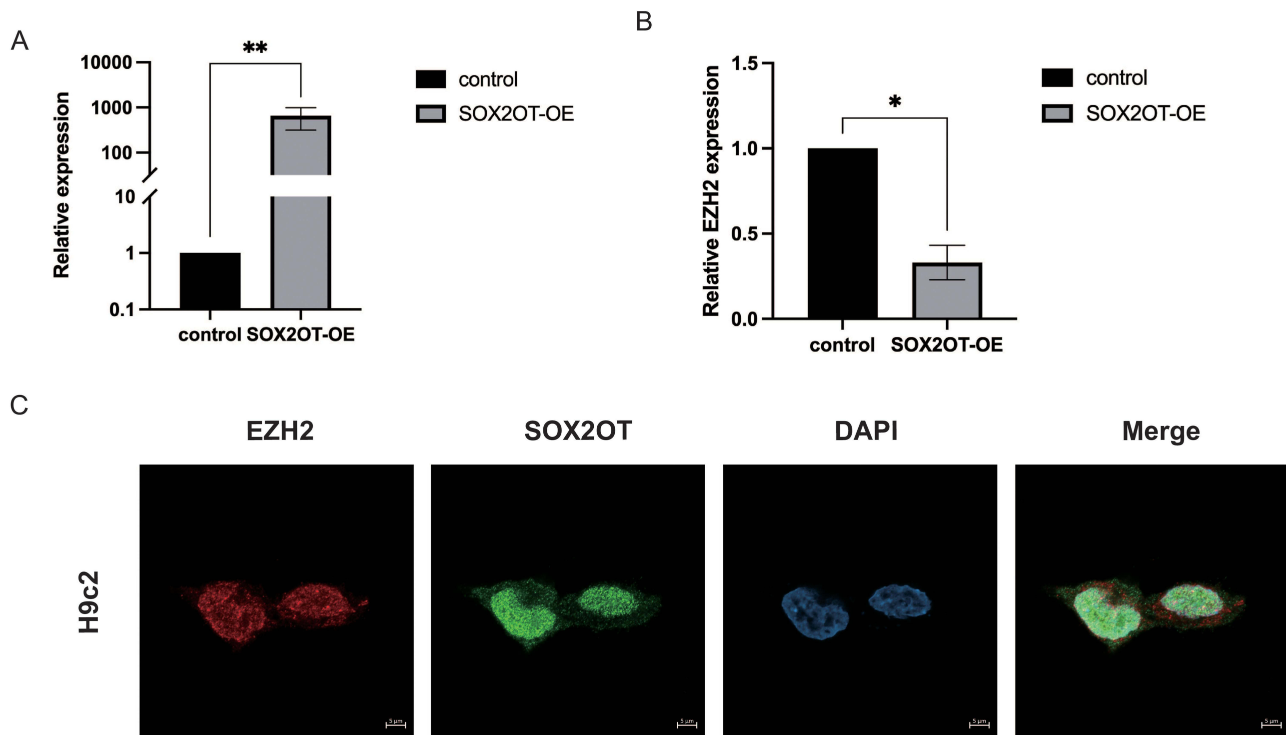

Figure 4 LncRNA SOX2OT targets EZH2.

Notes: (**A**) qPCR analysis showed that the expression level of SOX2OT in the SOX2OT-OE-transfected group was higher than that of the NC group. (**B**) The relative expression of EZH2 in the SOX2OT overexpression group was lower than that in the NC group. (**C**) SOX2OT specifically reduces luciferase expression through pMIR-REPORT EZH2 3'-UTR mimicry, Scale line value of 5μm. (*P < 0.05 compared with the corresponding control group, **P < 0.01 compared with the corresponding control group).

results revealed that compared with the control group, the LPS group exhibited disorganized myocardial cell arrangement and inflammatory cell infiltration. The SOX2OT-KO group showed more abnormalities than the LPS group. In contrast, the SOX2OT-KI group had uniform cardiomyocyte interstitial spaces with clear boundaries and clearly arranged myocardial fibers, and only a small amount of inflammatory cell infiltration was observed by H&E staining. These histological results showed that silencing SOX2OT promoted myocardial injury in septic mice, whereas overexpression of SOX2OT attenuated myocardial injury in septic mice. In addition, IHC experiments also verified that silencing SOX2OT resulted in more cellular DNA damage, whereas overexpression of SOX2OT attenuated cellular.

Discussion

Myocardial depression is one of the main indicators of poor prognosis in patients with sepsis; however, the cardiac dysfunction that occurs in sepsis is unique because it can be reversed within 7–10 days.[13] Sepsis combined with myocardial damage (SIMD) is difficult to treat in the clinic, and affected patients have a poor prognosis; however, there is no specific treatment probably because the molecular mechanism underlying myocardial inhibition in sepsis has not yet been studied in depth. Therefore, exploring the mechanism underlying sepsis-induced myocardial injury and searching for genes related to this condition have great clinical importance and value.

Previous studies have found that SOX2OT is an oncogene that plays an important role in promoting tumor development and cancer cell proliferation. In recent years, SOX2OT has been shown not only to be closely related to tumor development but also to play a role in myocardial tissues; additionally, the Co-LncRNA database (http://www.bio-bigdata.com/Co-LncRNA/) shows that SOX2OT is associated with biological processes such as muscle contraction and cardiomyocyte proliferation. It has been shown that SOX2OT regulates the expression of miR-122, which is present at high levels in the serum of patients with sepsis and remains at high levels for up to 14 days after intensive care.[14] These findings suggest that l SOX2OT plays a regulatory role in septic myocardial injury. Consistent with this, the present

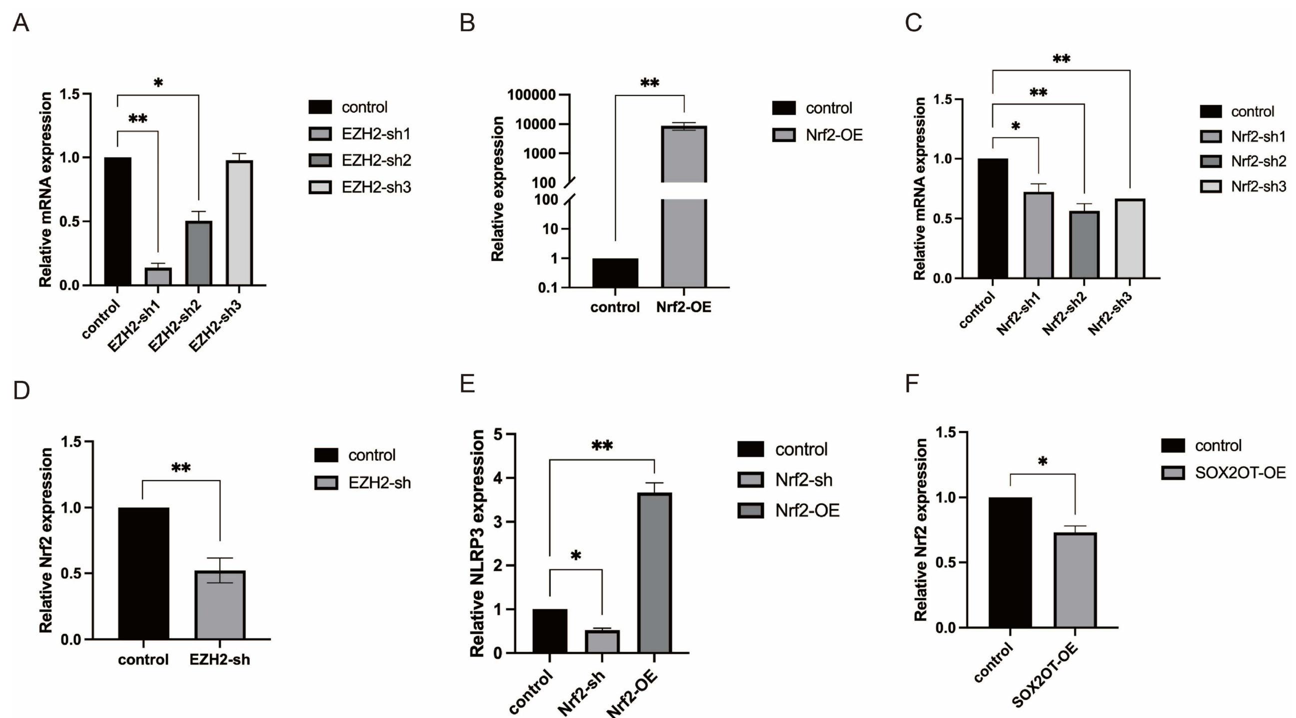

Figure 5 LncRNA SOX2OT mediates septic cardiomyocyte pyroptosis through the EZH2/Nrf-2 axis.
Notes: (**A**) qPCR analysis showed that the expression level of in the EZH2-sh transfection group was lower than that in the control group. (**B**) qPCR analysis showed that the expression level of NRF2 in the NRF2-OE transfection group was higher than that in the control group. (**C**) qPCR analysis showed that the expression level of Nrf2 in the Nrf2-sh transfection group was lower than that in the control group. (**D**) Compared with that in the control group, Nrf2 expression was decreased in the shEZH2 group. (**E**) Nrf2 and NLRP3 expression levels were positively correlated. (**F**) Nrf2 expression was decreased in the SOX2OT-OE group compared to the control group. (*P < 0.05 compared with the corresponding control group, **P < 0.01 compared with the corresponding control group).

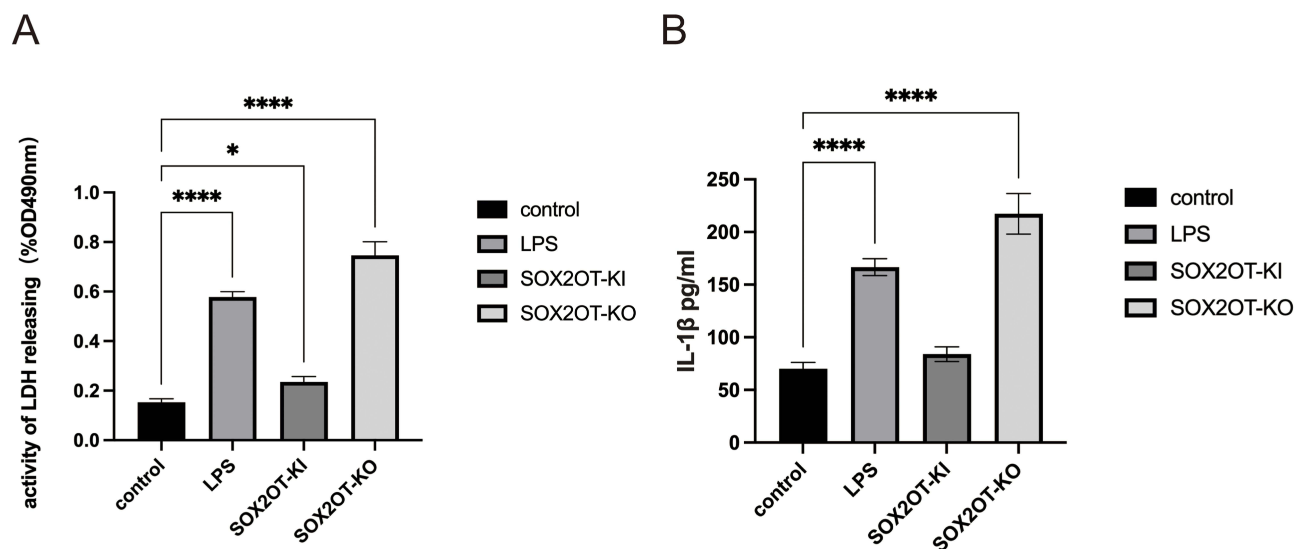

Figure 6 Effects of SOX2OT on cardiomyocyte pyroptosis in animal models.
Notes: (**A**) LDH levels. (**B**) The expression levels of IL-1β were analyzed by ELISA. (**ns** indicates no statistically significant difference compared to the corresponding control group, *P < 0.05 compared with the corresponding control group,****P < 0.0001 compared with the corresponding control group).

results suggest that knockdown of SOX2OT increases the expression of the DNA damage-associated proteins γH2AX and lactate dehydrogenase and promotes the secretion of inflammatory cytokines; these results suggest that SOX2OT may be a potential therapeutic target for SIMD treatment.

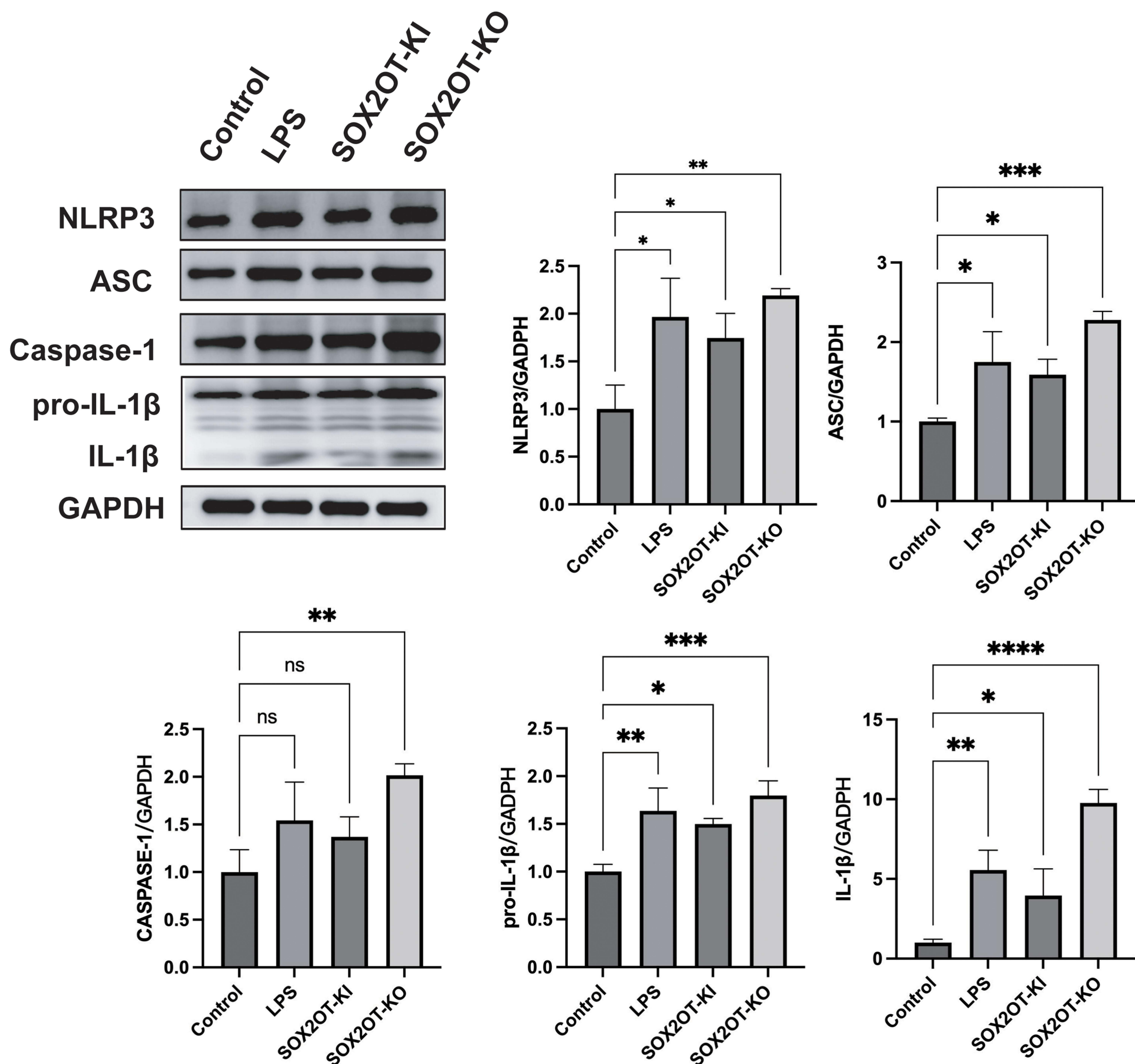

Figure 7 Effect of SOX2OT on the expression of pyroptosis-related proteins in an animal model.

Notes: Western blotting revealed the expression of pyroptosis-related proteins in mouse heart tissues. GAPDH was used as an internal control. (**ns** indicates no statistically significant difference compared to the corresponding control group, *P < 0.05 compared with the corresponding control group, **P < 0.01 compared with the corresponding control group, ***P < 0.001 compared with the corresponding control group, ****P < 0.0001 compared with the corresponding control group).

EZH2, a key histone methyltransferase (HMTase), is the enzymatic subunit of polycomb repressive complex 2 (PRC2), which catalyzes the trimethylation of lysine 27 of histone H3 (H3K27me3), leading to transcriptional silencing.[15] Recently, several studies have revealed the involvement of EZH2 in the development and progression of sepsis.[16,17] EZH2 has been shown to accelerate inflammation and apoptosis, and EZH2 inhibition has been shown to protect against sepsis.[18] Bioinformatics analysis indicated that SOX2OT may interact with EZH2.[19] In addition, under oxidative stress conditions, SOX2OT is involved in the regulation of the G2/M phase by modulating the expression of the EZH2 gene.[20] In addition, EZH2 is an important methylase that regulates nuclear factor-erythroid 2-related factor 2 (Nrf2); specifically, it binds to the Nrf2 promoter region, enhances Nrf2 H3K27me3 methylation, and inhibits its expression.[21] It has been shown that Nrf2 expression in MPVECs could be inhibited by circEXOC5 via the recruitment of EZH2.[22] In this study, we verified that SOX2OT targets EZH2 and negatively regulates EZH2 expression by dual

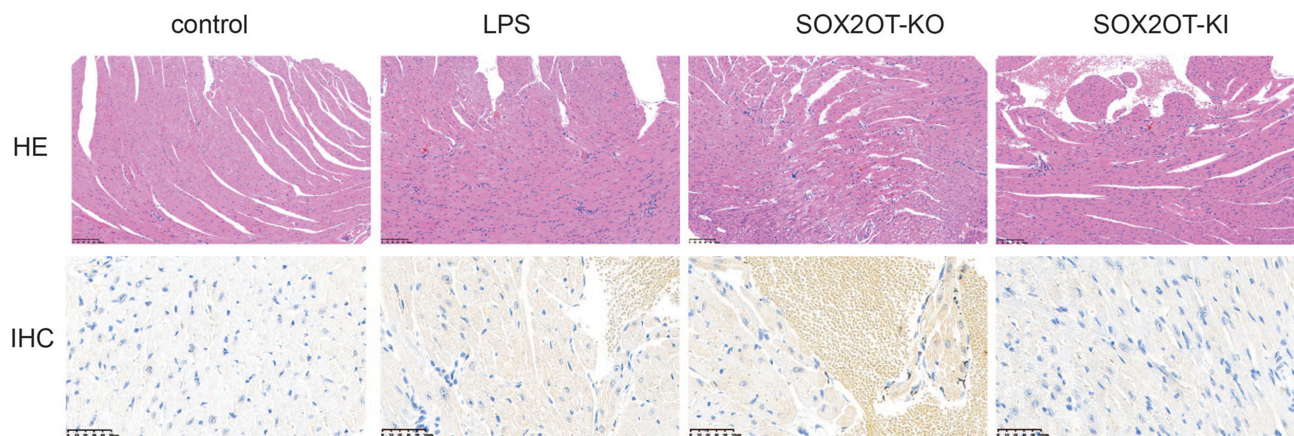

Figure 8 Histological validation of the effect of SOX2OT on cardiomyocytes in animal models.
Notes: Images of H&E staining and images of γ-H2A IHC experiments. According to the cardiac pathology scores the results were 0 for the control group, 1 for the LPS model group, 2 for the SOX2OT-KO group and 1 for the SOX2OT-KI group. HE's Scale bar = 100 μm. n = 6; IHC's Scale bar = 400 μm. n = 6.

luciferase reporter gene assay and qPCR. It was also demonstrated that Nrf2 expression was significantly decreased after EZH2 knockdown.

Nrf2 is a key transcription factor in the cellular oxidative stress response. It has a highly conserved basic leucine zipper structure, and through by to the cis-acting enhancer sequence of antioxidant response elements (AREs), it regulates the expression of genes related to redox regulation, inflammation, autophagy, metabolism, protein homeostasis, and the unfolded protein response (UPR).[23] A previous study by our group showed that Nrf2-mediated antioxidant effects are important for protecting cardiomyocytes from sepsis-induced injury.[24] However, the exact mechanism by which Nrf2 promotes cardiomyocyte survival is not fully understood. Studies have shown that Nrf2 regulates cellular pyroptosis, and in vascular endothelial cells, it has been shown that dihydromyricetin activates the Nrf2 signaling pathway, which in turn inhibits NLRP3 inflammatory vesicle-dependent cellular pyroptosis.[25] Another study showed that PCB also protects the heart from DOX-induced cardiotoxicity by reducing cardiomyocyte pyroptosis through Nrf2/Sirt3 signaling pathway activation.[26] Thus, the above experiments suggest that Nrf2 also plays an important role in regulating cellular focal death.

Of all the types of cell death, pyroptosis is most closely associated with infection and inflammation. N-terminal gasdermin (GSDMD-NT) expression is increased in sepsis models, whereas GSDMD-knockout (GSDMD-/-) mice with sepsis exhibit improved survival and reduced cardiac dysfunction; additionally, transcriptomics analysis reveals that deletion of GSDMD significantly reduces the expression of NLPRP3 and caspase-1, although the exact regulatory mechanisms remain unclear at present.[27,28] Further studies showed that pyroptosis plays an important role in SIMD and that activation of the innate immune system, induction of mitochondrial damage, and increases in oxidative stress during sepsis can mediate cardiomyocyte pyroptosis.[29] In the present study, it was found that the expression of pyroptosis-associated proteins was increased after Nrf2 knockdown, and the opposite effect was observed after Nrf2 overexpression. Further in vivo and in vitro experiments verified that the expression of focal death-associated proteins was increased after SOX2OT knockdown, and the opposite effect was observed after SOX2OT overexpression. Taken together, the present results suggest that SOX2OT responds to SIMD-associated inflammation through the EZH2/Nrf2/NLRP3 signaling pathway. Of course, our research also has certain limitations. Due to limitations in experimental conditions, we did not detect the cardiac function of septic cardiomyopathy mice through ultrasound. We did not detect the protein expression level of other cytokines in the blood except of IL-1β, though we detected the relative mRNA level. In addition, regarding other proteins involved in pyroptosis, such as Gasdermin D, we did not conduct any tests. Also, we did not test caspase-1 activity to verify the degree of pyroptosis. We look forward to more relevant research in the future to clarify the pathogenesis of septic cardiomyopathy at the molecular level to provide more possible therapeutic targets for the clinical diagnosis and treatment of septic cardiomyopathy.

Conclusion

We found that SOX2OT overexpression inhibitory NLRP3 inflammatory vesicle-mediated cardiomyocyte pyroptosis and cytokine overproduction by inhibiting the expression of EZH2, and thus inhibiting the expression of Nrf-2 through in vivo and in vitro experiments. Knockdown of the SOX2OT opposite these effects. Thus, our data reveal a novel mechanism by which SOX2OT alleviate septic myocardial injury by inhibiting cardiomyocyte pyroptosis through the EZH2/Nrf-2/NLRP3 pathway, which may contribute to the development of new therapeutic strategies.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of The People's Hospital of the Ningxia Hui Autonomous Region (Yinchuan, China; accession No. [2019] Luncheon Review [Scientific] No. (053)). Animal experiments were conducted in accordance with the guidelines for the use of laboratory animals (38) and were approved by the Institutional Animal Care and Use Committee of Ningxia Medical University (Yinchuan, China; accession no. IACUC-NYLAC-2020-188. The study endeavored to reduce the number of animals used and animal suffering.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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