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Original Paper

LncRNA KCNQ1OT1 Mediates Pyroptosis in Diabetic Cardiomyopathy

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Key Words
Long non-coding RNA • KCNQ1OT1 • Diabetic cardiomyopathy • miR-214-3p • Pyroptosis • Inflammation

Abstract
Background/Aims: Diabetic cardiomyopathy (DCM) is a common complication of diabetes and can cause heart failure, arrhythmia and sudden death. The pathogenesis of DCM includes altered metabolism, mitochondrial dysfunction, oxidative stress, inflammation, cell death and extracellular matrix remodeling. Recently, pyroptosis, a type of programmed cell death related to inflammation, was proven to be activated in DCM. However, the molecular mechanisms underlying pyroptosis in DCM remain elusive. The long non-coding RNA (lncRNA) Kcnq1ot1 participates in many cardiovascular diseases. This study aims to clarify whether Kcnq1ot1 affects cardiac pyroptosis in DCM. ***Methods:*** AC16 cells and primary cardiomyocytes were incubated with 5.5 and 50 mmol/L glucose. Diabetic mice were induced with streptozotocin (STZ). Kcnq1ot1 was silenced both *in vitro* and *in vivo*. qRT-PCR was used to detect the expression level of Kcnq1ot1. Immunofluorescence, qRT-PCR and western blot analyses were used to detect the degree of pyroptosis. Echocardiography, hematoxylin and eosin staining, and Masson's trichrome staining were used to detect the cardiac function and morphology in mice. Cell death and function were detected using TUNEL staining, immunofluorescence staining and Ca^{2+} measurements. ***Results:*** The expression of Kcnq1ot1 was increased in patients with diabetes, high glucose-induced cardiomyocytes and diabetic mouse cardiac tissue. Silencing Kcnq1ot1 alleviated pyroptosis by targeting miR-214-3p and caspase-1. Furthermore, silencing Kcnq1ot1 reduced cell death, cytoskeletal structure abnormalities and

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calcium overload *in vitro* and improved cardiac function and morphology *in vivo*. ***Conclusion:*** Kcnq1ot1 is overexpressed in DCM, and silencing Kcnq1ot1 inhibits pyroptosis by influencing miR-214-3p and caspase-1 expression. We clarified for the first time that Kcnq1ot1 could be a new therapeutic target for DCM.

Introduction

Diabetic cardiomyopathy (DCM), which is characterized by ventricular dysfunction, myocardial interstitial fibrosis and myocardial hypertrophy, is a myocardial microvascular complication of diabetes and occurs independently of coronary artery disease, hypertension and some other heart diseases [1, 2]. DCM results in a high-risk of heart failure, arrhythmia and sudden death, and is the major cause of mortality in patients with diabetes [3, 4]. The pathogenesis of DCM includes altered metabolism, mitochondrial dysfunction, oxidative stress, inflammation, cell death and remodeling of the extracellular matrix [5]. However, the diagnosis and therapy of DCM are limited because the molecular mechanisms of DCM remain elusive. Therefore, it is necessary to identify the pathological mechanisms and effective therapeutic targets of DCM.

Diabetes mellitus is a chronic inflammatory state. Pyroptosis, which is characterized by apoptosis and necrosis, is associated with cell death and the inflammatory reaction. In diabetes, hyperglycemia-activated expression of the Nod-like receptor protein 3 (NLRP3) inflammasome is followed by the activation of cleaved caspase-1. These processes lead to the conversion of interleukin-1 beta precursors (pro-IL-1β) and pro-IL-18 into mature IL-1β and IL-18 [6]. Emerging studies have shown that pyroptosis is associated with DCM [7-10]. Westermann et al. [9] indicated that cardiac dysfunction and inflammation in DCM were alleviated by a caspase-1 inhibitor. Luo et al. [10] found that the NLRP3 inflammasome played an important role in the process of DCM. Silencing NLRP3 might protect cardiac structures and functions from hyperglycemia-induced heart damage. Thus, it is vital to determine appropriate methods of alleviating pyroptosis in DCM. But little is known about the mechanisms regulating pyroptosis.

Long non-coding RNAs (lncRNAs) are RNAs that are longer than 200 nucleotides and have no protein encoding function [11]. LncRNAs can regulate gene expression and have vital effects on many physiological and pathological processes. The competitive endogenous RNA (ceRNA) hypothesis is a common mechanism by which lncRNAs function. LncRNAs can bind to the complementary binding site of microRNAs (miRNAs), which regulate the expression of genes by sponging the 3'-UTR of downstream target genes [12]. Understanding this RNA regulatory network provides a new perspective on the regulatory mechanisms of genes. The lncRNA Kcnq1ot1, the full name of which is *KCNQ1* overlapping transcript 1, is located in the *KCNQ1* locus at 11p15.5 [13]. Kcnq1ot1 has been reported to be associated with many disorders, particularly those related to heart disease [14-17]. Li et al. [14] indicated that suppression of Kcnq1ot1 might prevent myocardial I/R injury following acute myocardial infarction via regulating AdipoR1. Jiang et al. [15] elucidated that silencing Kcnq1ot1 could regulate QT interval prolongation via inhibiting *Kcnq1* gene expression. These researches demonstrated that Kcnq1ot1 plays a significant role in the heart, but the expression and mechanisms of Kcnq1ot1 in DCM are still unknown.

A recent study found that Kcnq1ot1 might act as a ceRNA to regulate the expression of caspase-1 via sponging miR-214-3p to influence cataract formation [18]. However, whether Kcnq1ot1 can regulate pyroptosis in DCM is poorly understood. In the present study, we attempted to clarify whether Kcnq1ot1 could affect cardiac pyroptosis in DCM and defined the mechanisms involved. We demonstrated that Kcnq1ot1 was overexpressed in DCM models and that silencing Kcnq1ot1 attenuated pyroptosis via miR-214-3p and caspase-1 in high glucose-induced cardiomyocytes and STZ-induced diabetic mice. Our research suggests that Kcnq1ot1 may be a novel therapeutic target for DCM.

Materials and Methods

Ethics statement

Serum samples from eight patients with diabetes and eight healthy subjects were obtained from the Second Affiliated Hospital of Harbin Medical University. None of the patients had coronary artery disease, hypertension or other heart diseases. All patients participating in this study signed informed consent forms. This study received approval from the ethical committee of Harbin Medical University (KY2017-189).

Animals and establishment of the diabetic model

Male C57BL/6 mice weighting 18-20 g were purchased from the animal experiment center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). All mice were fed adaptively for 1 week with food and water ad libitum and were divided randomly into the following 4 groups: the control group (Control), the diabetes group (DM), the diabetes with Kcnq1ot1 lentivirus-shRNA group (DM+KCNQ1OT1-shRNA) and the diabetes with caspase-1 inhibitor group (DM+caspase-1 I). Diabetic mice received 50 mg/kg streptozotocin (STZ, Sigma, St. Louis, MO) in citrate buffer (pH=4.6) by intraperitoneal injection for 5 days. After 1 week, glucose levels greater than 16.7 mmol/L in blood taken from the tail vein as measured by a Contour glucose meter (Roche, Germany) were considered evidence of successful establishment of diabetic models. Subsequently, some diabetic mice were treated with Kcnq1ot1 lentivirus-shRNA or caspase-1 inhibitor. The lentivirus-shRNA for Kcnq1ot1 was purchased from GenePharma (GenePharma, Shanghai, China); $1x10^9$ TU lentivirus-shRNA was dissolved in 50 μL of saline and injected into the tail vein of diabetic mice. Mice in the DM+caspase-1 I group were administered 0.1 mg/kg caspase-1 inhibitor Ac-YVAD-CMK (Cayman Chemical, MI, USA) by intraperitoneal injection every day. All mice were maintained for 8 weeks (n=5 for each group).

Echocardiography

After 8 weeks, mice were anesthetized with avertin for echocardiography. Two-dimensional M-mode echocardiography was performed with a Vevo1100 high-resolution imaging system (VisualSonics, Toronto, ON, Canada). The ejection fraction (EF) and fractional shortening (FS) of the left ventricle were derived using the machine.

Hematoxylin and eosin (HE) staining and Masson's trichrome staining

Tissue from the left ventricle fixed in 4% paraformaldehyde were embedded in paraffin and cut into 5-μm thick sections. The sections were stained separately with HE and Masson's trichrome. The morphology of the cardiomyocytes and the deposition of collagen were observed by fluorescence microscope.

Immunohistochemical analysis

Left ventricle 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), IL-1β (R&D Systems, Minneapolis, MN, 1:200) and IL-18 (R&D Systems, 1:200) at 4°C overnight before being washed three times with phosphate-buffered saline (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). Data were analyzed using Image-Pro Plus 6.0 (Media Cybemetics, USA).

Cell culture and transfection

AC16 human myocardial cells were obtained from the Shanghai Institute for Biological Sciences (SIBS, Shanghai, China). Primary cardiomyocytes were extracted from the hearts of one- to three-day-old neonatal C57BL/6 mice. The isolation and culture methods were described previously [15]. The cells were cultured with 5.5 mM glucose (Control) or 50 mM glucose (HG) for 24 hours in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Beit-Haemek, Israel) at 37°C and 5% CO_2. Primary cardiomyocytes in the HG group were treated with or without the caspase-1 inhibitor Ac-YVAD-CMK at 100 μM according

to the previous studies [19, 20]. The cells were transfected with small interfering RNA (siRNA) against Kcnq1ot1 (si-Kcnq1ot1), miR-214-3p mimics (miR-214-3p), the anti-miRNA oligonucleotide of miR-214-3p (AMO-214-3p) or the corresponding scrambled negative control (si-NC, NC, AMO-NC), which were designed and synthesized by RIOBIO (Guangzhou, China) using X-treme GENE siRNA transfection reagent (Roche, Germany). The procedure was performed according to the manufacturer's instructions. The sequences were as follows: si-Kcnq1ot1, GGTAGAATAGTTCTGTCTT; miR-214-3p mimics, forward, 5'-ACAGCAGGCACAGACAGGCAGU-3' and reverse, 3'-UGUCGUCCGUGUCUGUCCGUCA-5'; and miR-214-3p inhibitor, 5'-mAmCmUmGmCmCmUmGmUmCmUmGmUmGmCmCmUmGmCmUmGmU-3'.

RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

The total RNA was extracted from the human serum using TRIzol LS (Invitrogen, Carlsbad, CA, USA), and the total RNA of the cardiomyocytes and cardiac tissues was extracted using TRIzol (Invitrogen). The concentration of the RNA was detected using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA was reverse transcribed using a reverse transcription kit (Toyobo, Japan). The cDNA was amplificated and detected by the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA) using SYBR Green I (Yoyobo, Osaka, Japan). The PCR conditions were 95°C for 60 s, 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of genes. GAPDH served as the internal control for Kcnq1ot1 and the mRNAs. U6 was used as the internal control for miR-214-3p. The primers sequences were as follows:

Kcnq1ot1 Forward: 5'-GCACTCTGGGTCCTGTTCTC-3'
Kcnq1ot1 Reverse: 5'-CACTTCCCTGCCTCCTACAC-3'
miR-214-3p Forward: 5'-TATACATCAAACAGCAGGCACA-3'
miR-214-3p Reverse: 5'-CATTCGATCTTCTCCACAGTCTC-3'
caspase-1 Forward: 5'-ACACGTCTTGCCCTCATTATCT-3'
caspase-1 Reverse: 5'-ATAACCTTGGGCTTGTCTTTCA-3'
NLRP3 Forward: 5'-GTGGAGATCCTAGGTTTCTCTG-3'
NLRP3 Reverse: 5'-CAGGATCTCATTCTCTTGGATC-3'
IL-1β Forward: 5'-CCCTGCAGCTGGAGAGTGTGG-3'
IL-1β Reverse: 5'-TGTGCTCTGCTTGAGAGGTGCT-3'
IL-18 Forward: 5'-ACAACCGCAGTAATACGGAGCA-3'
IL-18 Reverse: 5'- TGTGCTCTGCTTGAGAGGTGCT-3'
GAPDH Forward: 5'-ATCACTGCCACCCAGAAGAC-3'
GAPDH Reverse: 5'-TTTCTAGACGGCAGGTCAGG-3'
U6 Forward: 5'-CTCGCTTCGGCAGCACATATACT-3'
U6 Reverse: 5'-ACGCTTCACGAATTTGCGTGTC-3'

Protein extraction and western blot analysis

Total protein samples were extracted and loaded on 10% SDS-PAGE. Then, the proteins were transferred to nitrocellulose membranes. After blocking with BSA for 2 h at room temperature, the membranes were incubated with primary antibodies against caspase-1, NLRP3, IL-1β, IL-18 and GAPDH (ZSGB-BIO, Beijing, China) (1:1000) on a shaking bed at 4°C overnight. Then, the membranes were washed with PBS containing 0.5% Tween 20 (PBS-T) three times and incubated with secondary antibodies for 1 h at room temperature. GAPDH served as the internal control. The bands were imaged using the GelDox XR System (Bio-Rad, CA, USA). Quantity One software (Bio-Rad) was used to determine the gray value of the bands.

Immunofluorescence staining

AC16 cells were treated with 4% buffered paraformaldehyde for 20 min at room temperature, after which 1% BSA and 0.1% Triton-X were used to block the cells for 2 h at room temperature, followed by incubation with primary antibodies against NLRP3, caspase-1, IL-1β, IL-18 and vimentin (R&D Systems, Minneapolis, MN) (1:200) at 4°C overnight. Then, the cells were washed with PBS and treated with the appropriate secondary antibody for 1 h at room temperature. The nuclei were stained with DAPI (Beyotime, Shanghai, China). The results were captured by fluorescence microscope or confocal microscope (ZEISS LSM-700) and analyzed using Image-Pro Plus 6.0.

TUNEL staining

Cell Death Detection Kits (Roche, IN, USA) were used to detect DNA damage. AC16 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.

Fluorescence measurement of $[Ca^{2+}]_i$ in cardiomyocytes

AC16 cells and primary cardiomyocytes were washed with standard Tyrode solution (in mmol/l: 12.6 NaCl, 0.54 KCl, 1 HEPES, 0.033 NaH_2PO_4, 0.1 $MgCl_2 \cdot 6H_2O$, 1.8 $CaCl_2$ and 10 glucose, pH=7.40) and loaded with 10 μM Fluo-4/AM (Invitrogen) and Pluronic F-127 (Invitrogen) at 37°C for 30 min. Then, the cells were washed with Tyrode solution twice and stored in 1 ml of calcium solution. Images were captured by confocal microscope (ZEISS LSM-700) for about 5 min. The excitation wavelength was 488 nm. During the process, the cells were stimulated with 50 μl of KCl. The fluorescence intensity (FI) of the cells was calculated using the machine. The relative changes in $[Ca^{2+}]_i$ were calculated as $\Delta F/F_0=(F_1-F_0) / F_0$.

Data analysis

The values were analyzed with SPSS 13.0 software and are presented as the mean±standard deviation (SD). Unpaired Student's t-tests and one-way ANOVA were performed to compare the differences between two groups or among different groups, respectively. A two-tailed $P<0.05$ was considered statistically significant. GraphPad Prism 6 was used to generate the graphs.

Results

Kcnq1ot1 and caspase-1 are overexpressed in high glucose-induced cardiomyocytes

We found that the expression of Kcnq1ot1 was higher in the serum of patients with diabetes than in the serum of the healthy controls (Fig. 1A). To measure the expression of Kcnq1ot1 *in vitro*, we cultured the human cardiomyocyte cell line AC16 with 5.5 mmol/L glucose (control) and 50 mmol/L glucose (high glucose, HG) to imitate the hyperglycemic state of DCM. It was found that Kcnq1ot1 expression was markedly increased in the HG group compared to the control group (Fig. 1B). The expression level of Kcnq1ot1 was also elevated in high glucose-treated primary cardiomyocytes compared to the control (Fig. 1C). Furthermore, qRT-PCR, immunofluorescence staining and western blot analyses were conducted to detect the expression of *NLRP3, caspase-1*, *IL-1β* and *IL-18*. The results showed that they were clearly overexpressed in the HG group compared to the control (Fig. 1D-J). Thus, we determined that Kcnq1ot1 is highly expressed in DCM models and that pyroptosis is activated in high glucose-treated cardiomyocytes.

Silencing Kcnq1ot1 by siRNA inhibits pyroptosis of cardiomyocytes

To further investigate the impact of silencing Kcnq1ot1 on pyroptosis in cardiomyocytes, high glucose-induced AC16 cells were transfected with control siRNA (si-NC) or siRNA against Kcnq1ot1 (si-Kcnq1ot1). Kcnq1ot1 expression was successfully repressed by si-Kcnq1ot1 (Fig. 2A). The mRNA and protein expression levels of *caspase-1* were significantly downregulated (Fig. 2B & C). These results were also verified in primary cardiomyocytes (Fig. 2D-F). Kcnq1ot1 is associated with pyroptosis in cardiomyocytes. The bioinformatics assay predicted that miR-214-3p has binding sites for both Kcnq1ot1 and *caspase-1* (Fig. 2G). The ceRNA networks between Kcnq1ot1 and miR-214-3p and between *caspase-1* and miR-214-3p were demonstrated in a previous study using the luciferase assay [18].

Furthermore, we detected the expression of miR-214-3p in the DCM model. These results suggested that miR-214-3p was reduced in the serum of patients with diabetes and AC16 cells incubated with high levels of glucose (Fig. 2H & I). Moreover, after transfection with si-Kcnq1ot1, the level of miR-214-3p was substantially increased (Fig. 2J). In addition, the expression levels of Kcnq1ot1 and *caspase-1* were markedly decreased after transfection with miR-214-3p mimics in high glucose-treated AC16 cells. In contrast, the expression

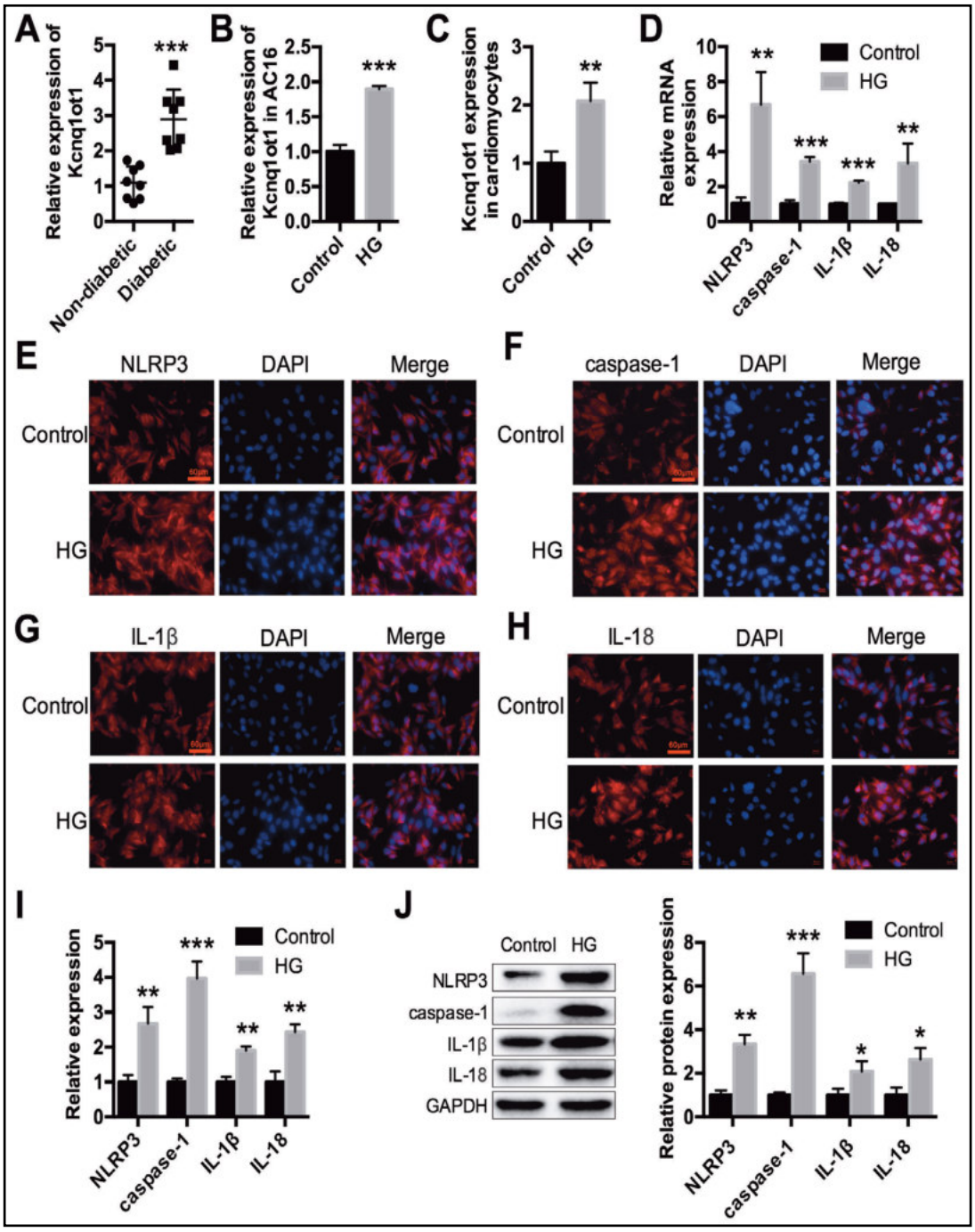

Fig. 1. The lncRNA Kcnq1ot1 and caspase-1 are overexpressed in high glucose-induced cardiomyocytes. (A) The relative expression levels of Kcnq1ot1 in the serum of non-diabetic and diabetic patients were detected by qRT-PCR. n=8. ***P<0.001 versus the non-diabetic group. (B) The relative expression levels of Kcnq1ot1 in AC16 cells incubated with 5.5 and 50 mmol/L glucose were detected by qRT-PCR. n=3. (C) The relative expression levels of Kcnq1ot1 in primary cardiomyocytes were detected by qRT-PCR. (D) The relative mRNA expression levels of NLRP3, caspase-1, IL-1β and IL-18 in AC16 cells were detected by qRT-PCR. n=3. Immunofluorescence analysis was performed to detect the expression of NLRP3 (E), caspase-1 (F), IL-1β (G) and IL-18 (H) in AC16 cells. Caspase-1, IL-1β, IL-18 and NLRP3, red; nuclei, blue. Scale bar, 60 μm. (I) The quantification and statistical analysis of immunofluorescence staining are shown. (J) The relative protein expression levels of caspase-1, IL-1β, IL-18 and NLRP3 in AC16 cells were detected by western blot. n=3. *P<0.05, **P<0.01, ***P<0.001 versus the control group.

levels of Kcnq1ot1 and *caspase-1* were increased after transfection with AMO-214-3p (Fig. 2K-N). We also obtained similar results in primary cardiomyocytes (Fig. 2O-S).

Kcnq1ot1 regulates pyroptosis by targeting caspase-1 via miR-214-3p in cardiomyocytes

To evaluate the regulation of caspase-1 by Kcnq1ot1 via miR-214, reverse experiments were performed. Immunofluorescence, qRT-PCR and western blotting showed that after transfection with si-Kcnq1ot1, the expression levels of *caspase-1*, *IL-1β*, *IL-18* and *NLRP3* were significantly downregulated, while that downregulation was prevented by cotransfection

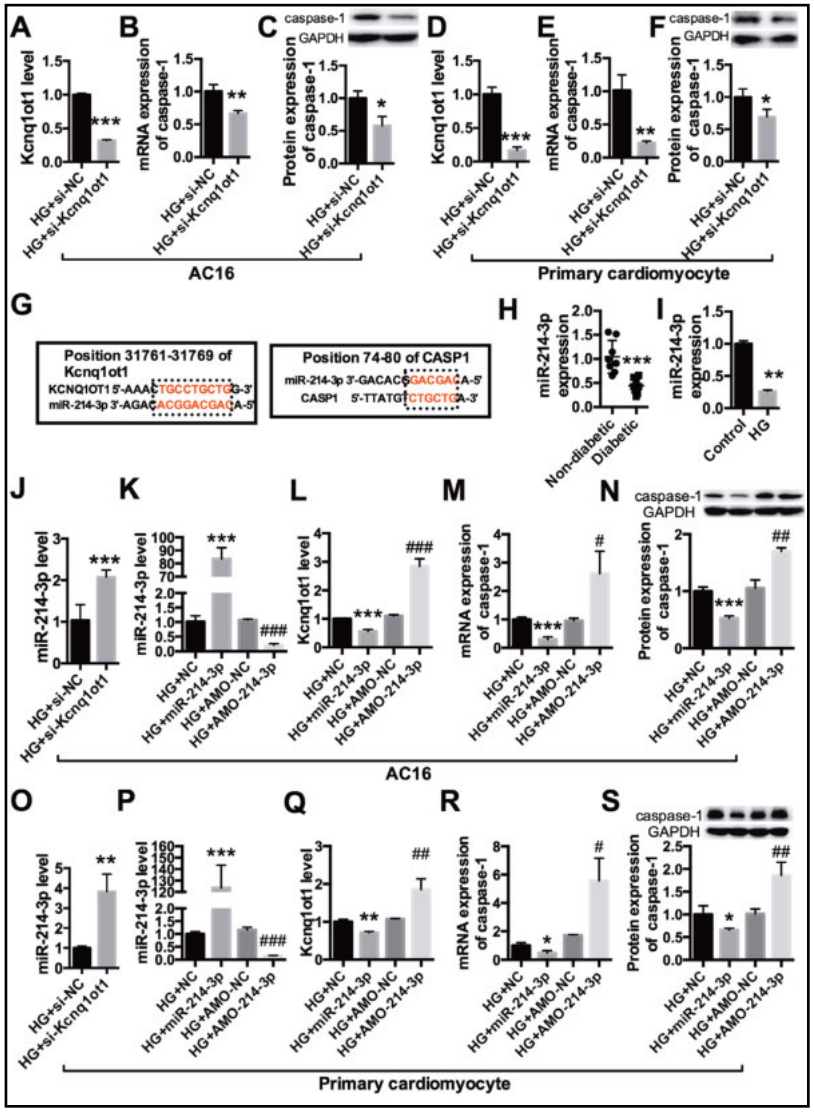

Fig. 2. Silencing Kcnq1ot1 by siRNA inhibits the pyroptosis of AC16 cells and primary cardiomyocytes. (A) The relative expression levels of Kcnq1ot1 in HG-treated AC16 cells transfected with si-NC and si-Kcnq1ot1. n=3. (B and C) The relative mRNA and protein expression levels of caspase-1 in AC16 cells were detected by qRT-PCR and western blot. n=3. (D) The relative expression levels of Kcnq1ot1 in HG-induced primary cardiomyocytes. n=3. (E and F) The relative mRNA and protein expression levels of caspase-1 in primary cardiomyocytes. n=3. (G) The binding sites between Kcnq1ot1 and miR-214-3p as predicted by the bioinformatics assay are shown in the left panel. The binding sites between miR-214-3p and caspase-1 are shown in the right panel. (H) The expression levels of miR-214-3p in non-diabetic and diabetic patients were detected by qRT-PCR. n=8. (I) The relative expression levels of miR-214-3p in HG-induced AC16 cells were detected by qRT-PCR. n=3. (J) The relative expression levels of miR-214-3p in HG-induced AC16 cells transfected with si-NC and si-Kcnq1ot1. n=3. (K-N) HG-induced AC16 cells were transfected with NC, miR-214-3p mimics, AMO-NC and AMO-214-3p, and the expression levels of miR-214-3p (K), Kcnq1ot1 (L), and caspase-1 (M and N) were detected by qRT-PCR and western blot. n=3. (O) The relative expression of miR-214-3p in HG-induced primary cardiomyocytes transfected with si-NC and si-Kcnq1ot1. n=3. HG treated cardiomyocytes were transfected with NC, miR-214-3p mimics, AMO-NC and AMO-214-3p, and the expression levels of miR-214-3p (P), Kcnq1ot1 (Q), and caspase-1 (R and S) were detected by qRT-PCR and western blot. n=3. ***P<0.001 compared with the HG+NC group. #P<0.05, ##P<0.01, ###P<0.001 compared with the HG+AMO-NC group.

with AMO-214-3p (Fig. 3A-H). Therefore, silencing Kcnq1ot1 suppresses pyroptosis by inhibiting caspase-1 via miR-214-3p in cardiomyocytes.

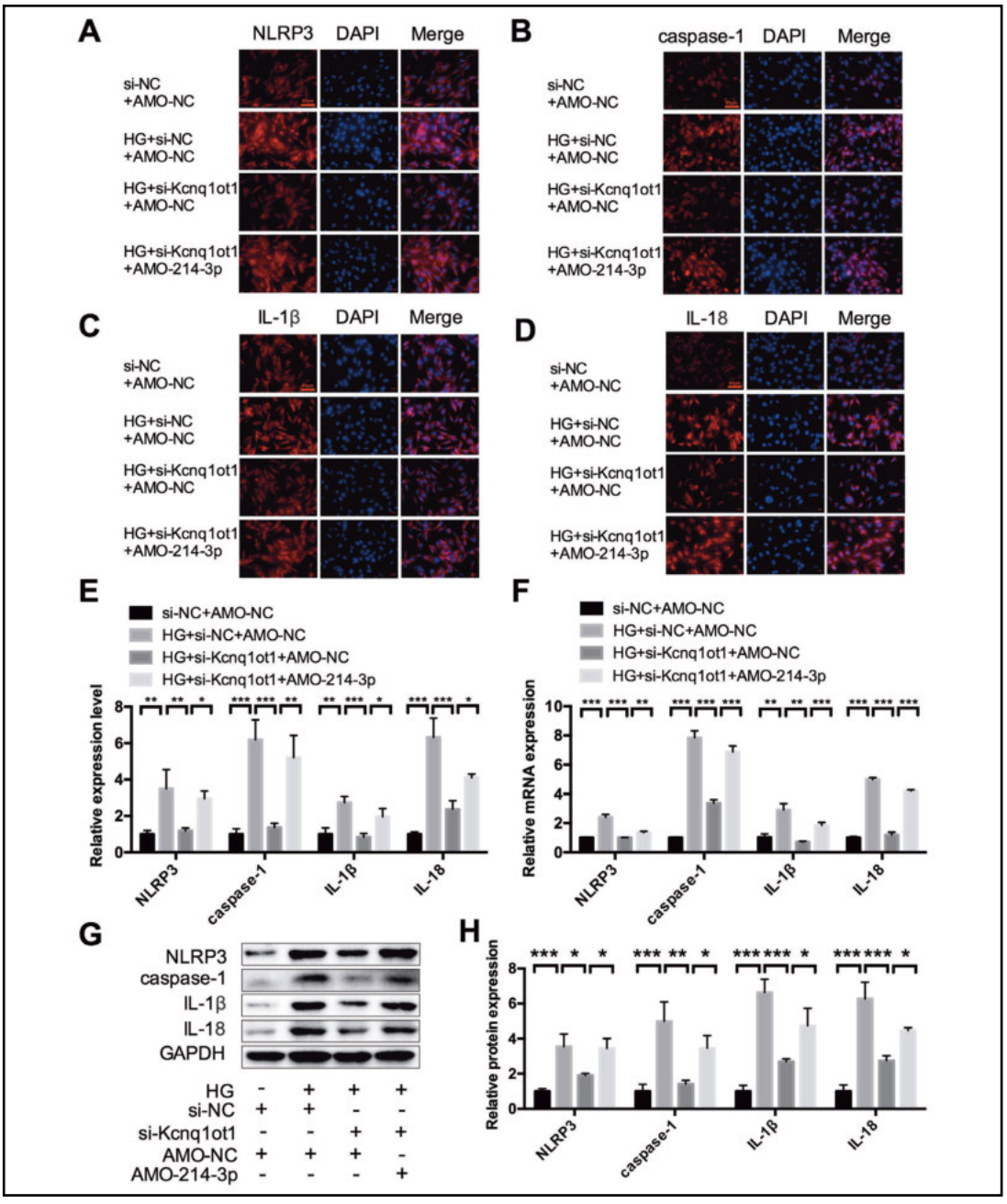

Fig. 3. Silencing Kcnq1ot1 suppresses caspase-1 via miR-214-3p in cardiomyocytes. AC16 cells were treated with si-NC + AMO-NC, HG + si-NC + AMO-NC, HG + si-Kcnq1ot1 + AMO-NC, or HG + si-Kcnq1ot1 + AMO-214-3p. Immunofluorescence analysis was conducted to detect the expression levels of NLRP3 (A), caspase-1 (B), IL-1β (C) and IL-18 (D). NLRP3, caspase-1, IL-1β and IL-18, red; nuclei, blue. Scale bar, 60 μm. (E) The quantification and statistical analysis of immunofluorescence staining are shown. (F) qRT-PCR was performed to detected the expression levels of NLRP3, caspase-1, IL-1β and IL-18. (G) Representative bands of NLRP3, caspase-1, IL-1β and IL-18 as detected by western blot are shown. (H) Relative protein expression levels of NLRP3, caspase-1, IL-1β and IL-18 in each group. n=3. "*" indicates significant differences between the marked groups.

Kcnq1ot1-miR-214-3p network is vital to cardiomyocyte function

We then verified the effects of Kcnq1ot1 on the structure and function of cardiomyocytes. To determine the role played by Kcnq1ot1/miR-214-3p/caspase-1 in cell death, a TUNEL assay was conducted. Silencing Kcnq1ot1 alleviated DNA fracture in AC16 cells treated with high levels of glucose. Cotransfection with AMO-214-3p partially eliminated the alleviation of cell death (Fig. 4A & B). In addition, the expression of vimentin, a cytoskeletal protein that could reflect the structure of cardiomyocytes, was detected by immunofluorescent assay. In the HG group, the expression of vimentin was pervasive and clustered compared with that of the control group. Kcnq1ot1 silencing weakened the damage done by hyperglycemia. However, miR-214-3p downregulation accelerated the damage, characterized by an irregular cytoskeletal structure (Fig. 4C). Moreover, repressing Kcnq1ot1 by siRNA ameliorated calcium overload, as shown in the peak fluorescence images and calculated FI, whereas it was reversed by cosilencing with miR-214-3p (Fig. 4D & E). The Kcnq1ot1-miR-214-3p network regulates cell death, calcium overload and cytoskeletal structure in high glucose-induced cardiomyocytes.

Caspase-1 inhibitor attenuates pyroptosis and reduces the expression of Kcnq1ot1 in primary cardiomyocytes

We showed that inhibiting Kcnq1ot1 could reduce the expression of caspase-1. Next, we identified whether the inhibition of caspase-1 could form a negative feedback network to regulate the expression of Kcnq1ot1 in primary cardiomyocytes. High glucose-induced cardiomyocytes were treated with 100 μmol/L caspase-1 inhibitor Ac-YVAD-CMK or

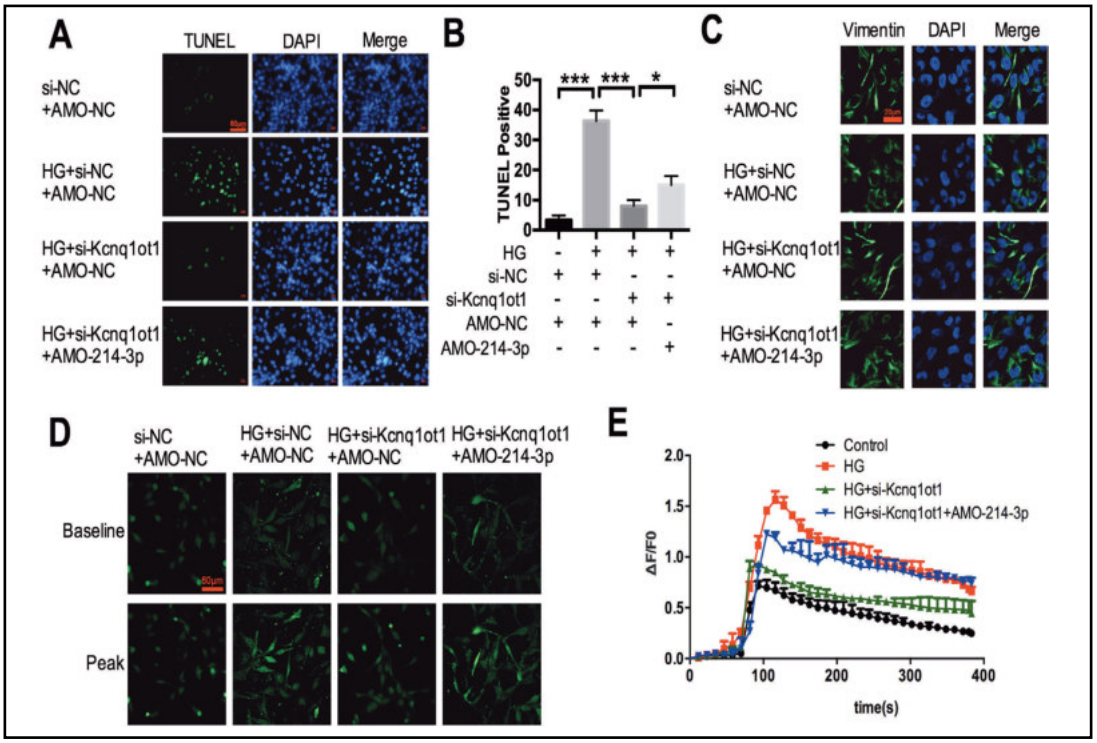

Fig. 4. Kcnq1ot1 regulates cell death, calcium overload and cytoskeletal structure via miR-214-3p and caspase-1 in high glucose-induced AC16 cells. (A and B) A TUNEL assay was performed in AC16 cells. TUNEL staining, green; nuclei, blue. Scale bar: 60 μm. (C) Immunofluorescence analysis was conducted to detect the expression of vimentin. Vimentin, green; nuclei, blue. Scale bar: 20 μm. (D) Fluorescence measurement of $[Ca^{2+}]_i$ was performed in AC16 cells. The baseline and peak fluorescence intensity images are shown. Scale bar: 60 μm. (E) The changes in fluorescence intensity of $[Ca^{2+}]_i$ in each group are shown. n=3. "*" indicates significant differences between the marked groups.

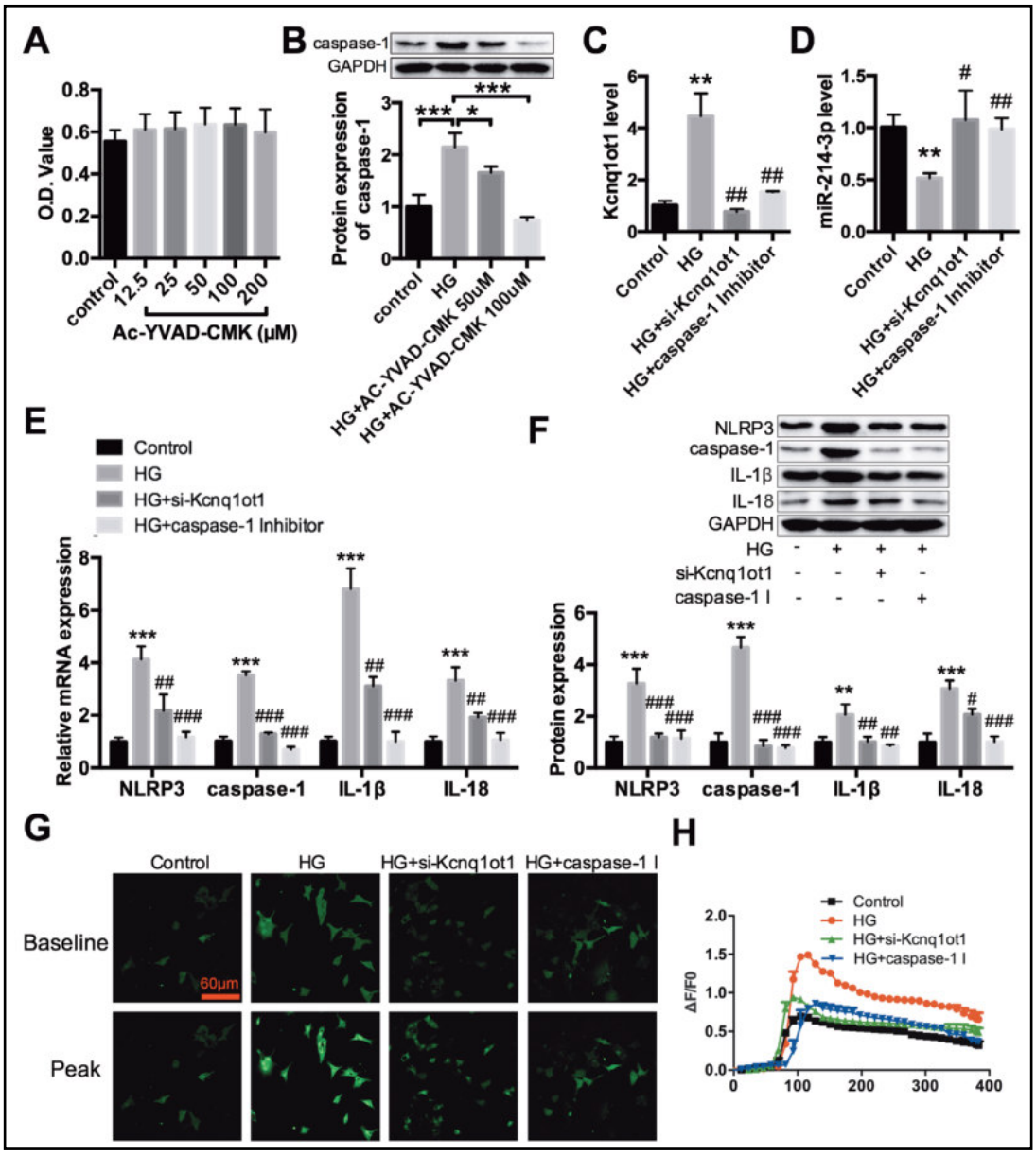

Fig. 5. Inhibiting caspase-1 decreases Kcnq1ot1 expression in primary cardiomyocytes. (A) The number of viable cells was determined by the MTT assay. (B) The relative protein expression levels of caspase-1 in cardiomyocytes were detected by western blot. (C) The relative expression levels of Kcnq1ot1 in the control, HG, HG + si-Kcnq1ot1, and HG + caspase-1 inhibitor Ac-YVAD-CMK groups. (D) The relative expression of miR-214-3p in each group. (E and F) qRT-PCR and western blotting were conducted to detect the mRNA and protein expression levels of NLRP3, caspase-1, IL-1β and IL-18. n=3. "*" indicates significant differences compared with the control group. "#" indicates significant differences compared with the HG group. (G) Fluorescence measurement of $[Ca^{2+}]_i$ was performed in cardiomyocytes. The baseline and peak fluorescence intensity images are shown. (H) The changes in fluorescence intensity of $[Ca^{2+}]_i$ in each group are shown.

transfected with siRNA against Kcnq1ot1. The concentration of caspase-1 inhibitor was proven to have no effect on cell activity and effectively inhibited caspase-1 expression in high glucose-treated cardiomyocytes using methylthiazoletetrazolium (MTT) and western blot analyses (Fig. 5A & B). After transfection with si-Kcnq1ot1, the expression levels of Kcnq1ot1, *NLRP3*, *caspase-1*, *IL-1β* and *IL-18* were markedly downregulated, and miR-214-3p expression was clearly increased after the administration of the caspase-1 inhibitor

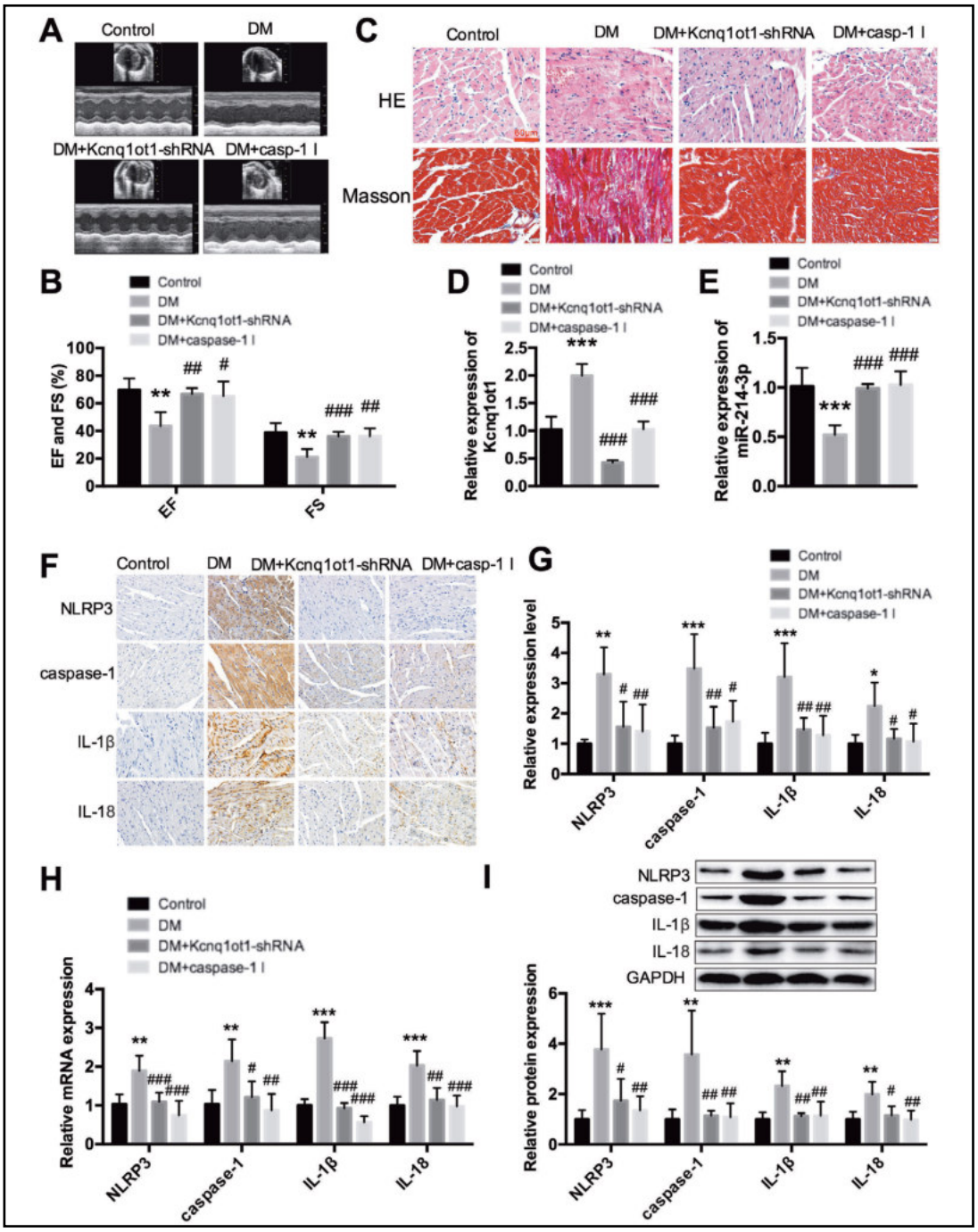

Fig. 6. Kcnq1ot1 regulates the pyroptosis of DCM *in vivo*. (A) M-mode echocardiograms of non-diabetic mice, diabetic mice, diabetic mice injected with Kcnq1ot1 lentivirus-shRNA, and diabetic mice injected with caspase-1 inhibitor Ac-YVAD-CMK. (B) The ejection fraction (EF) and shortening fraction (FS) in each group are shown. (C) HE and Masson's trichrome staining were performed for each group. Scale bar, 60 μm. (D) The relative Kcnq1ot1 expression levels were detected by qRT-PCR. (E) The relative expression levels of miR-214-3p were detected by qRT-PCR. (F) The expression levels of NLRP3, caspase-1, IL-1β and IL-18 in each group were detected by immunohistochemistry analysis. Scale bar, 60 μm. (G) The quantification and statistical analysis of immunohistochemistry staining are shown. (H & I) qRT-PCR and western blotting were conducted to detected the mRNA and protein expression levels of NLRP3, caspase-1, IL-1β and IL-18. n=5. "*" indicates significant differences compared with the control group. "#" indicates significant differences compared with the DM group.

(Fig. 5C-F). Functionally, calcium overload was significantly ameliorated after treatment with the caspase-1 inhibitor (Fig. 5G & H).

Silencing Kcnq1ot1 ameliorates pyroptosis in diabetic mice

After verifying the role of Kcnq1ot1 *in vitro*, we further established a type 1 diabetic model in C57BL/6 mice to detect the effect of silencing Kcnq1ot1 by lentivirus-shRNA in DCM (DM+Kcnq1ot1-shRNA). In addition, diabetic mice were also treated with caspase-1 inhibitor (DM+caspase-1 I) to confirm the effect of pyroptosis on DCM *in vivo*. M-mode ultrasound cardiogram indicated that the systolic and diastolic functions, ejection fraction (EF) and fractional shortening (FS) of the left ventricle in diabetic mice were deteriorated significantly in diabetic mice but ameliorated in the DM+Kcnq1ot1-shRNA and DM+caspase-1 I groups (Fig. 6A-B). Furthermore, HE and Masson's trichrome staining were performed to evaluate the histopathological changes. Kcnq1ot1-shRNA and the caspase-1 inhibitor attenuated the cardiomyocyte hypertrophy and collagen deposition induced by diabetes (Fig. 6C).

It was shown that Kcnq1ot1 was upregulated in diabetic mice and was clearly downregulated after injection with Kcnq1ot1-shRNA or the caspase-1 inhibitor (Fig. 6D). In contrast, miR-214-3p produced the opposite results (Fig. 6E). In addition, qRT-PCR, western blotting and immunohistochemistry indicated that the expression levels of *NLRP3*, *caspase-1, IL-1β* and *IL-18* were significantly upregulated in diabetic mice; that upregulation was partially reversed by Kcnq1ot1-shRNA and the caspase-1 inhibitor (Fig. 6F-I). These data demonstrate that Kcnq1ot1 can regulate cardiac function and structure via the Kcnq1ot1/miR-214-3p/caspase-1 pathway *in vivo*.

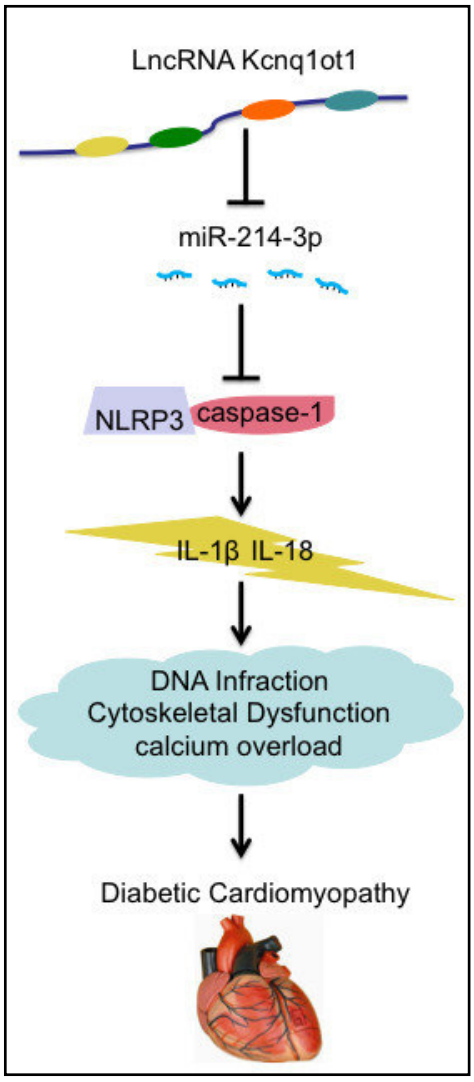

Fig. 7. The functional model underlying the mechanism by which the lncRNA Kcnq1ot1 acts in DCM. Kcnq1ot1 sponges miR-214-3p, which targets caspase-1 as a ceRNA and modulates the expression levels of NLRP3, IL-1β and IL-18. The elevated levels of inflammatory cytokines stimulate DNA infraction, cytoskeletal dysfunction and calcium overload in vitro and promote the pathological process of DCM *in vivo*.

Discussion

LncRNAs play important roles in many biological processes, including cardiovascular diseases, diabetes and related complications, but the relationship between lncRNAs and pyroptosis in DCM is largely unknown. In this study, we proved that the expression of the lncRNA Kcnq1ot1 was upregulated in the serum of patients with diabetes, high glucose-treated cardiomyocytes and the cardiac tissue of diabetic mice. Silencing Kcnq1ot1 alleviated pyroptosis by targeting miR-214-3p and caspase-1, thus reducing cell death, calcium overload and the abnormality of the cytoskeletal structure *in vitro* and improving cardiac function and morphology *in vivo*. The mechanism by which the lncRNA Kcnq1ot1 acts in DCM is shown in Fig. 7. We are the first to fully illuminate

the effect of Kcnq1ot1 on pyroptosis in DCM.

Previous studies indicated that many lncRNAs participate in the process of DCM. For example, the lncRNA H19 is downregulated in DCM and influences apoptosis via miR-675 and VDAC1 [21]. The lncRNA MIAT is upregulated in high glucose-treated cardiomyocytes and STZ-induced mice, and it sponges miR-22-3p to regulate DAPK2 expression [22]. The lncRNA MALAT1 is upregulated in DCM rats and is associated with the apoptosis of cardiomyocytes [23]. In addition, emerging studies have revealed that Kcnq1ot1 is vital in many diseases, especially in cardiovascular diseases, such as long QT syndrome and myocardial ischemia/reperfusion injury [14-17, 24, 25]. However, there have been no reports about Kcnq1ot1 and pyroptosis in DCM. In our study, Kcnq1ot1 was clearly upregulated in DCM. Silencing Kcnq1ot1 by siRNA and lentivirus-shRNA significantly reduced pyroptosis and improved cardiac function in DCM. Therefore, Kcnq1ot1 might be a novel mechanism and therapeutic target for DCM.

LncRNAs play biological roles through multiple mechanisms, including genetic imprinting, chromatin remodeling, cell cycle control, splicing regulation, mRNA decay, and translational regulation [26]. Kcnq1ot1, a widely studied lncRNA, could regulate multiple genes in the Kcnq1 domain by the recruitment of chromatin and DNA-modifying proteins [13]. Kcnq1ot1 was also proved to act as a ceRNA, targeting miRNAs to regulate the expression of mRNAs. Guo et al. [27] suggested that Kcnq1ot1 could regulate MET expression to promote melanoma growth via binding to miR-153. In our study, the bioinformatics assay predicted that miR-214-3p was a ceRNA of Kcnq1ot1 and had binding sites for both Kcnq1ot1 and caspase-1, which was consistent with the results of previous study on cataracts [18]. We elaborated for the first time the downregulation of miR-214-3p in high glucose-treated cardiomyocytes and the effect of Kcnq1ot1 on DCM via miR-214-3p and caspase-1.

Pyroptosis is programmed cell death associated with inflammation, and it participates in the development of DCM. High glucose-induced reactive oxygen species (ROS) can trigger the activation of the NLRP3 inflammasome and promote the production of cleaved caspase-1, thus accelerating the release of IL-1β and IL-18 [28]. This process can induce mitochondrial oxidative stress, promote apoptosis and influence the abnormal metabolism of glucose and adipose tissue [29]. Inflammation is a common mechanism that can accelerate other pathological changes [30, 31]. Increasing evidences have shown that repressing inflammation can reduce myocardial cell death, regulate calcium homeostasis and myocardial cytoskeletal structure [32-35]. Our research found that silencing Kcnq1ot1 ameliorated DNA fractionation, reduced calcium overload, and maintained the structure of cardiomyocytes via the miR-214 and caspase-1 axis. We speculate that this is closely related to the inhibition of inflammation. In this study, we elaborate the mechanisms by which Kcnq1ot1 acts in DCM from many aspects. In the future, more studies are needed to determine whether silencing Kcnq1ot1 can regulate other pathological processes in DCM, such as ROS, mitochondrial dysfunction and metabolic abnormalities.

Conclusion

In summary, our results indicate that the lncRNA Kcnq1ot1 is upregulated in DCM and that silencing Kcnq1ot1 inhibits pyroptosis by influencing miR-214-3p and caspase-1. We are the first to clarify that Kcnq1ot1 could be a new therapeutic target for DCM.

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experimental design. Y.F. wrote the manuscript and analyzed the data, while C.H., H.T. and L.A. revised the work. W.Y., S.X., L.A. and M.S. performed the mouse experiments. Q.Y., Y.F. and H.T. conducted the cell culture and transfections. Q.Y., S.X. and L.J. conducted the western blot and real-time PCR assays. Q.Y. conducted the immunofluorescence staining, TUNEL assay and vimentin detection. S.X. performed the cardiomyocyte fluorescence measurement of $[Ca^{2+}]_I$ and analyzed the data. All authors reviewed this manuscript.

Disclosure Statement

All authors declare that they have no competing interest.

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