

## Silencing circANKRD36 protects H9c2 cells against lipopolysaccharide-induced injury via up-regulating miR-138



Shengwei Shi<sup>a</sup>, Shaohui Zhang<sup>b,\*</sup>, Huiling Zhang<sup>c</sup>, Qifeng Jin<sup>c</sup>, Deguang Wu<sup>d</sup>

<sup>a</sup> Department of Cardiology I, People's Hospital of Juye County, Heze 274900, China

<sup>b</sup> Department of Cardiology III, Affiliated Hospital of Jining Medical University, Jining 272000, China

<sup>c</sup> Department of Cardiology I, Affiliated Hospital of Jining Medical University, Jining 272000, China

<sup>d</sup> Department of Cardiology V, Affiliated Hospital of Jining Medical University, Jining 272000, China

### ARTICLE INFO

#### Keywords:

circANKRD36  
miR-138  
Lipopolysaccharide  
p38MAPK/NF- $\kappa$ B pathway  
H9c2 cells

### ABSTRACT

**Background:** Myocarditis refers to the inflammatory process that affects the muscle tissue of the heart. Persistent myocardial inflammation promotes myocardial cell damage, which ultimately leads to heart failure or death. Therefore, we aimed to explore the functional impacts of circANKRD36 on myocarditis.

**Methods:** H9c2 cells were pre-treated with lipopolysaccharide (LPS). Viability and apoptosis were evaluated utilizing CCK-8 assay and flow cytometry. Inflammatory cytokines mRNA expression and production were assessed by qRT-PCR and ELISA. Western blot was utilized to distinguish apoptosis and inflammatory related factors expression. Sequentially, the above mentioned parameters were reassessed when overexpressed miR-138.

**Results:** LPS declined viability and as well as raised apoptosis and inflammatory injury in H9c2 cells. Silencing circular RNA ANKRD36 (si-circANKRD36) alleviated apoptosis and inflammatory injury induced by LPS. MiR-138 expression was suppressed by LPS and elevated by si-circANKRD36. Increase of viability and inflammatory injury induced by si-circANKRD36 was alleviated by down-regulation of miR-138. Eventually, si-circANKRD36 inhibited p38MAPK/NF- $\kappa$ B pathway which activated by LPS via up-regulating miR-138.

**Conclusion:** Si-circANKRD36 exerted its anti-apoptosis and anti-inflammatory function under the condition of LPS in H9c2 cells through p38MAPK/NF- $\kappa$ B pathway and up-regulation of miR-138.

### 1. Introduction

Myocarditis refers to the inflammatory process that affects the muscle tissue of the heart (Bracamonte-Baran and Cihakova, 2017). Moreover, myocarditis is the cause of non-diagnosed cause of acute heart failure, chronic dilated cardiomyopathy and sudden death (Sagar et al., 2012). In addition, a number of clinical experiments revealed that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and other inflammatory cytokines participated in the pathogenesis of cardiac injury in myocarditis (Matsumori et al., 1994). Thus, the regulation of inflammatory response is considered to be an underlying treatment strategy for myocarditis (Zhou et al., 2015).

Circular RNAs (circRNAs) are innovative races of RNAs belong to non-coding RNA (ncRNA) (Xu et al., 2018). CircRNAs have been widely informed to play critical roles in multifarious biological processes and regulate multiple cellular mechanisms. For instance, circBCRC4 raised apoptosis and reduced viability in bladder cancer (Li et al., 2017). Another study demonstrated circRNA\_010567 facilitated myocardial

fibrosis *in vitro* experiments (Zhou and Yu, 2017). For the past few years, circRNAs were investigated to be participated in heart development and diseases (Devaux et al., 2017; Werfel et al., 2016). Wang et al. disclosed that circHRCR could protect the heart against heart failure and pathological myocardial hypertrophy (Wang et al., 2016). Additionally, Pan's study revealed that several circRNAs such as hsa\_circ\_0089378, hsa\_circ\_0083357 and so forth exerted their function in coronary artery disease patients (Pan et al., 2017). Moreover, circANKRD36 was explored and testified to be associated with apoptosis and regulating inflammatory responses in type 2 diabetes mellitus (Fang et al., 2018). Thus, we speculated circANKRD36 had the similar functions in myocarditis.

MicroRNAs (miRNAs) are small ncRNAs molecules that control gene expression level after transcription (Lee et al., 1993). Accumulating evidence shows that miRNAs represent abnormal expression in many types of diseases, such as lung cancer (Sun et al., 2013), type 2 diabetes (Ma et al., 2017) and myocarditis (Chen et al., 2017). More than that, miRNAs are participated in numerous biological, pathological

\* Corresponding author at: Department of Cardiology III, Affiliated Hospital of Jining Medical University, No.89 Guhuai Road, Jining 272000, Shandong, China.  
E-mail address: [zhang19sh@163.com](mailto:zhang19sh@163.com) (S. Zhang).

<https://doi.org/10.1016/j.yexmp.2019.104300>

Received 30 May 2019; Received in revised form 26 July 2019; Accepted 20 August 2019

Available online 20 August 2019

0014-4800/ © 2019 Elsevier Inc. All rights reserved.

proceedings, physiological perturbations and disease conditions (LaPierre and Stoffel, 2017). For instance, miRNAs showed anti-apoptosis effect in T-ALL cells (Fan et al., 2016). Besides, miRNAs could mediate inflammatory response *in vivo* and *in vitro* experiments. Fu's research reported that miR-30a-5p alleviated spinal cord injury-induced inflammatory responses *via* mitogen-activated protein kinases (MAPK)/ERK pathway (Fu et al., 2018). In addition, abundant studies revealed that miRNAs played vital roles in myocarditis. For instance, miR-155 inhibition suppressed autoimmune myocarditis and immune response in CD4+ cells (Yan et al., 2016). More than that, miR-138 was investigated to play diverse roles in cardiomyocytes. As an illustration, miR-138 inhibited hypoxia-induced apoptosis in cardiomyocytes (He et al., 2013). Here, we aimed to investigate circANKRD36 and miR-138 impact and interaction mechanism in lipopolysaccharide (LPS)-induced H9c2 cells.

## 2. Materials and methods

### 2.1. Cell culture

H9c2 cells (ATCC, Manassas, VA, USA) were derived from rat embryonic ventricular cardiomyocytes and hatched in Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin (Solarbio, Beijing, China) and 100 µg/mL streptomycin (Wuhan Fortuna Chemical Co., Ltd., Wuhan, China) in an incubator that contains 95% air and 5% CO<sub>2</sub> at 37 °C.

### 2.2. Cell treatment

LPS (Solarbio) solution at the concentration of 10 µg/mL was prepared for cell culture. Experimental cells were pre-treated with LPS for 6 h in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C.

### 2.3. Cell counting kit-8 (CCK-8) assay

Cell viability was determined utilizing CCK-8 (Dojindo Laboratories, Tokyo, Japan). H9c2 cells were inoculated in a 96-well plate at the density of  $5 \times 10^3$  cells/well. When the treatments were completed, the used cell culture medium was substituted with fresh cell culture media containing 10 µL CCK-8, and then the cultures were incubated for 1 h. The absorbance at 450 nm was determined utilizing a Microplate Reader (Bio-Rad, Hercules, CA, USA).

### 2.4. Apoptosis assay

H9c2 cells were inoculated in a 6-well plate. After cells had been subjected to the treatments as described above, cells were rinsed twice gently with cold phosphate buffered saline (PBS, Thermo Scientific, Waltham, MA, USA) and re-suspended in binding buffer. The rates of H9c2 cells apoptosis were analyzed by flow cytometry (Beckman Coulter, Atlanta, GA, USA) following Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) protocols.

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

Cells were cultured in 24-well plates and the supernatant was gathered. The concentrations of inflammatory cytokines measured by ELISA according to protocols supplied on the basis of user's manual delivered by the producer (R&D Systems, Abingdon, UK) and normalized to cell protein concentrations.

### 2.6. Transfection

Small interfering RNA (siRNA) of circANKRD36, miR-138 inhibitor, miR-138 mimic, negative control (NC) mimic and NC inhibitor were

incorporated (Life Technologies, Carlsbad, MD, USA) and cells were cultured in 6-well plates. Experimental cells were transfected with circANKRD36 siRNAs, miR-138 inhibitor and NC inhibitor for 48 h. All transfections were completed using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol. Because the highest transfection efficiency was occurred at 48 h, thus 48 h was considered as the harvest time in the subsequent experiments.

### 2.7. Quantitative reverse transcription PCR (qRT-PCR)

RNA from transfected cells was separated utilizing Trizol reagent (Invitrogen, San Diego, CA, USA) as stated in the manufacturer's protocols. RNA concentration and purity was measured by UV spectrophotometer at 260 nm and 280 nm. MiRNA reverse transcription was worked utilizing the MultiscribeRTkit (Biosystems, Barcelona, Spain). The PCR was utilized of the SYBR® Green Master Mix (TaKaRa, Tokyo, Japan) following the protocols. The relative expression levels were calculated utilizing the  $2^{-\Delta\Delta Ct}$  method, all experiments replied thrice.

### 2.8. Luciferase reporter assay

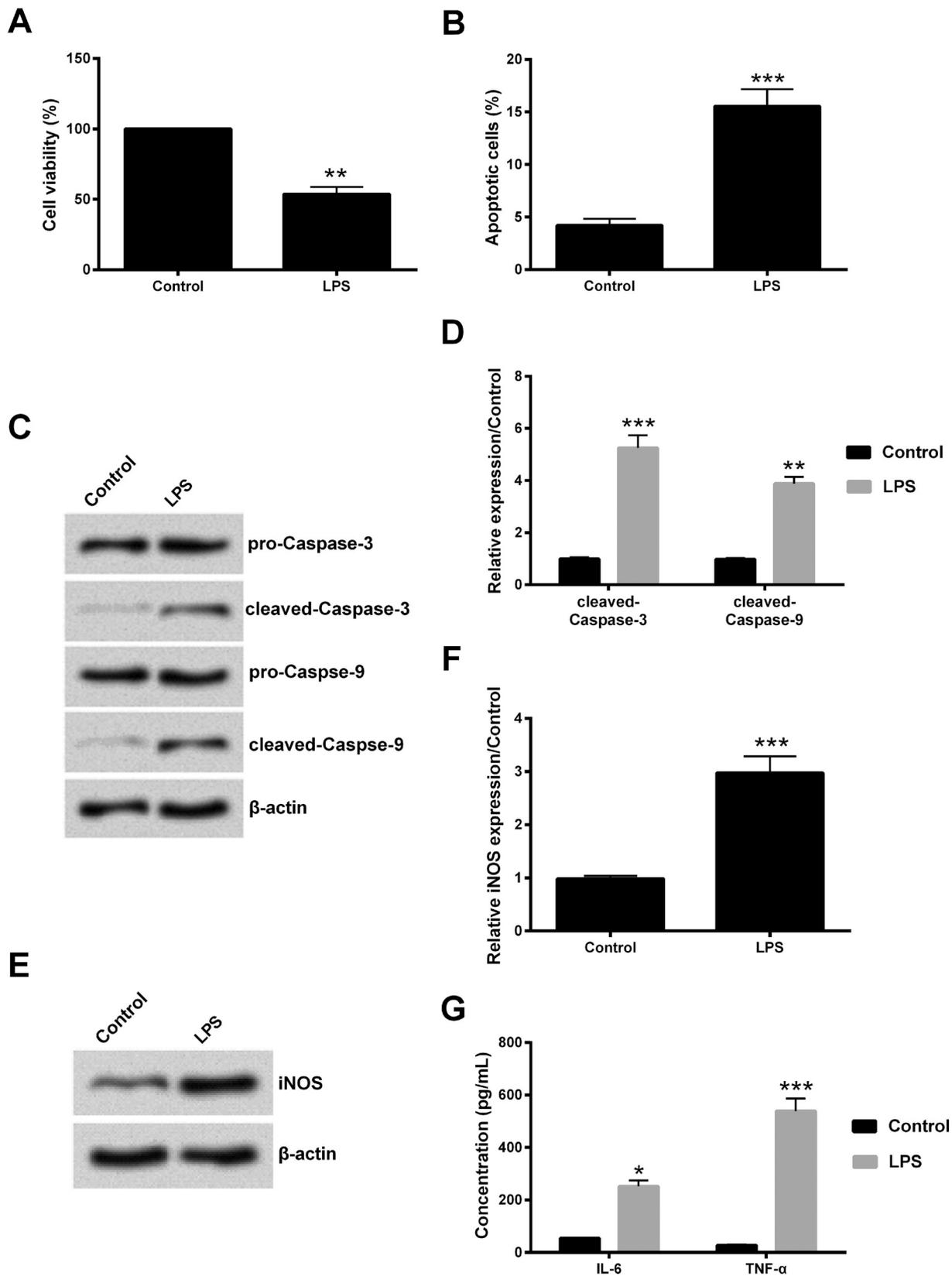
For luciferase reporter assay, the mutant circANKRD36 (circANKRD36-mut) and wild-type circANKRD36 (circANKRD36-wt) were constructed into pMIR-Report Luciferase vector (Promega, Madison, WI, USA). Cells were seeded in 12-well plates, which were divided NC mimic group and miR-124 mimic group. Each group was transfected with circANKRD36-wt and circANKRD36-mut vectors by Lipofectamine 3000 (Invitrogen, San Diego, CA, USA) in antibiotic-free medium for 48 h. Luciferase activity was measured with the Dual-Luciferase® Reporter Assay System (Promega). The relative luciferase activity was normalized.

### 2.9. Western blot assay

Proteins of experimental cells were separated utilizing RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) fortified with protease-inhibitor (Roche, Basel, Switzerland). An equal amount of extracted protein was evaluated utilizing the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Each protein bands were detached by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (Millipore, Boston, MA, USA). Afterwards, the appropriate primary antibodies were diluted and conducted with the membranes at 4 °C overnight. Primary antibodies were listed as shown below: cleaved-Caspase-3 (No.ab2302, Abcam, Cambridge, MA, USA), cleaved-Caspase-9 (No.ab2324, Abcam), pro-Caspase-3 (No. ab32150, Abcam), pro-Caspase-9 (No. ab135544, Abcam), iNOS (No. ab3523, Abcam), p-nuclear factor kappa-B (NF-κB) p65 (No. ab106129, Abcam), t-p65 (No. ab16502, Abcam), p-p38MAPK (No. AM063, Beyotime, Shanghai, China), t-p38MAPK (No. SAB4500492, Sigma-Aldrich, St. Louis, MO, USA) and β-actin (No. sc-47778, Santa Cruz Biotechnology) was utilized as an endogenous protein for normalization. After the treated membranes were rinsed, they were conjunct with suitable secondary antibodies marked with horseradish peroxidase for 1 h at room temperature. Ultimately, the membranes with protein bands were removed into the Bio-Rad ChemiDoc™ XRS system. Finally, each area of protein band was detected and assessed by Image Lab™ Software (Bio-Rad). Each test was performed in triplicate experiments.

### 2.10. Statistical analysis

Each analysis was replied at least triple times. The evidence of various experiments was performed as the mean + SD and measured utilizing SPSS 19.0 statistical software (SPSS, Chicago, IL, USA). The *p*-values were determined utilizing a one-way analysis of variance (ANOVA) or Student *t*-test. A *p*-value of < 0.05 was appraised statistically significant.



**Fig. 1.** Apoptosis and viability was raised by LPS exposure. (A) Viability was reduced by LPS. (B) Apoptosis was raised by LPS. (C–D) Cleaved-Caspase-3 and cleaved-Caspase-9 expression was remarkably elevated when H9c2 cells were induced by LPS. (E–F) iNOS expression was notably improved by LPS. (G) IL-6 and TNF- $\alpha$  secretion was increased when H9c2 cells were exposed with LPS. \* $p < .05$ , \*\* $p < .01$  or \*\*\* $p < .001$  compared with marked group in the graph.

### 3. Results

#### 3.1. LPS induces apoptosis and inflammatory response in H9c2 cells

To manifest the effectiveness of LPS on H9c2 cells, we detected viability, apoptosis, apoptosis-related protein expression, iNOS expression and inflammatory cytokines. As the data demonstrated in Fig. 1A, viability was remarkably declined when induced by LPS ( $p < .01$ ). Whereas apoptotic cells were conspicuously elevated by LPS in H9c2 cells (Fig. 1B,  $p < .001$ ). Meanwhile, Western blot results indicated that cleaved-Caspase-3 and cleaved-Caspase-9 expression was notably raised by LPS ( $p < .01$  or  $p < .001$ , Fig. 1C–D). Furthermore, iNOS expression was remarkably raised in H9c2 cells ( $p < .001$ , Fig. 1E–F). Sequentially, ELISA results implied that secretion of IL-6 and TNF- $\alpha$  was significantly escalated by LPS in H9c2 cells ( $p < .05$  or  $p < .001$ , Fig. 1G).

#### 3.2. LPS up-regulates circANKRD36 expression

In order to identify the association of circANKRD36 and LPS, qRT-PCR was utilized to examine circANKRD36 expression in H9c2 cells. After experimental cells were treated with LPS, circANKRD36 expression was prominently raised ( $p < .01$ , Fig. 2).

#### 3.3. Silencing circANKRD36 alleviates LPS-induced apoptosis and inflammatory injury

To investigate whether si-circANKRD36 was transfected into the experimental cells successfully, qRT-PCR was carried out to assess the expression level of experimental cells. CircANKRD36 expression was significantly reduced under the condition of knocking down circANKRD36 in H9c2 cells ( $p < .01$ , Fig. 3A). We silenced circANKRD36 expression to identify the effect of circANKRD36 on apoptosis and inflammatory in H9c2 cells. CCK-8 assay was utilized to detect viability in H9c2 cells. Viability was elevated when silencing circANKRD36 expression under LPS-pretreated in H9c2 cells ( $p < .05$ , Fig. 3B). On the other hand, apoptosis was declined when silencing circANKRD36 expression under LPS-pretreated in H9c2 cells ( $p < .05$ , Fig. 3C). Furthermore, cleaved-Caspase-3 and cleaved-Caspase-9 expression was notably reduced when circANKRD36 was silenced after

experimental cells were treated by LPS ( $p < .01$ , Fig. 3D–E). Moreover, iNOS expression was diminished when circANKRD36 was silenced after experimental cells were treated utilizing LPS ( $p < .05$ , Fig. 3F–G). What's more, we also assessed inflammatory cytokines secretion in H9c2 cells. The data demonstrated that secretion of IL-6 and TNF- $\alpha$  was lessened as well when silenced circANKRD36 expression ( $p < .05$  or  $p < .01$ , Fig. 3H).

#### 3.4. Regulating miR-138 expression mediated LPS-induced apoptosis and inflammatory injury

In order to identify the effect of miR-138 on H9c2 cells, miR-138 mimic and miR-138 inhibitor were transfected into H9c2 cells, respectively. To identify whether miR-138 was transfected into experimental cells successfully, qRT-PCR was utilized to evaluate miR-138 expression. MiR-138 expression was strongly up-regulated by miR-138 mimic in Fig. 4A ( $p < .001$ ). Conversely, miR-138 expression was notably reduced when miR-138 inhibitor was transfected into H9c2 cells successfully ( $p < .01$ , Fig. 4B). As the data represented in Fig. 4C, viability was raised in a certain degree when up-regulating miR-138 and further reduced ( $p < .05$ ) when down-regulating miR-138 expression in experimental cells ( $p < .05$ ). On the other hand, apoptotic cells were lessened when up-regulating miR-138 ( $p < .05$ ) and obviously escalated by down-regulating miR-138 in H9c2 cells ( $p < .05$ , Fig. 4D). Moreover, secretion of IL-6 and TNF- $\alpha$  was partially decreased by up-regulating miR-138 ( $p < .05$  or  $p < .01$ ) and remarkably elevated by down-regulating miR-138, respectively ( $p < .01$ , Fig. 4E). Moreover, the expression of apoptosis-associated proteins and iNOS were examined. Expression of cleaved-Caspase-3 and cleaved-Caspase-9 was dramatically declined by up-regulating miR-138 in H9c2 cells ( $p < .01$ ). On the contrary, the expression was markedly raised by down-regulating miR-138 ( $p < .01$ , Fig. 5A–B). Similarly, iNOS expression was suppressed by down-regulating miR-138 ( $p < .05$ ) and the suppression was reversed by up-regulating miR-138 ( $p < .05$ , Fig. 5C–D). Furthermore, we investigated the effect of miR-138 on p38MAPK/NF- $\kappa$ B pathway in H9c2 cells. p-NF- $\kappa$ B p65 expression was repressed by up-regulating miR-138 ( $p < .01$ ) and escalated by down-regulating miR-138 ( $p < .01$ , Fig. 5E–F). In addition, p-38MAPK expression showed the same trend as p-NF- $\kappa$ B p65 ( $p < .05$  or  $p < .01$ , Fig. 5G–H).

#### 3.5. Silencing circANKRD36 up-regulates miR-138 expression

To confirm the relationship between circANKRD36 and miR-138, miR-138 expression was tested by qRT-PCR. As the evidence displayed in Fig. 6, LPS declined miR-138 expression at first ( $p < .05$ ). Sequentially, miR-138 expression was remarkably elevated by silencing circANKRD36 expression ( $p < .01$ ).

#### 3.6. MiR-138 is a direct target of circANKRD36

To demonstrate whether miR-138 directly targets circANKRD36, we constructed luciferase reporter assay and predicted the binding sites of circANKRD36 and miR-138. The binding sites of circANKRD36 and miR-138 were shown in Fig. 7A. The results of luciferase reporter assay indicated normalized fluorescence intensity was significantly lower in H9c2 cells co-transfected with circANKRD36-wt and miR-138 mimic compared to control group ( $p < .05$ ). By contrast, no significant difference was detected between the control group and experimental cells co-transfected with miR-138 mimic and circANKRD36-mut (Fig. 7B).

#### 3.7. Silencing circANKRD36 alleviates LPS-induced apoptosis and inflammatory injury via up-regulating miR-138

To analyze the combined action of circANKRD36 and miR-138 on H9c2 cells, circANKRD36 and miR-138 inhibitor were co-transfected

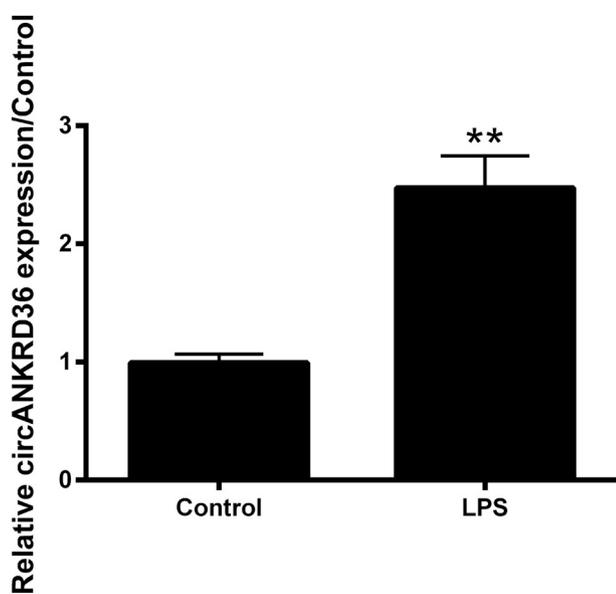


Fig. 2. CircANKRD36 expression was elevated by LPS. After H9c2 cells were induced by LPS, circANKRD36 expression was strongly enhanced.  $**p < .01$  compared with marked group in the graph.

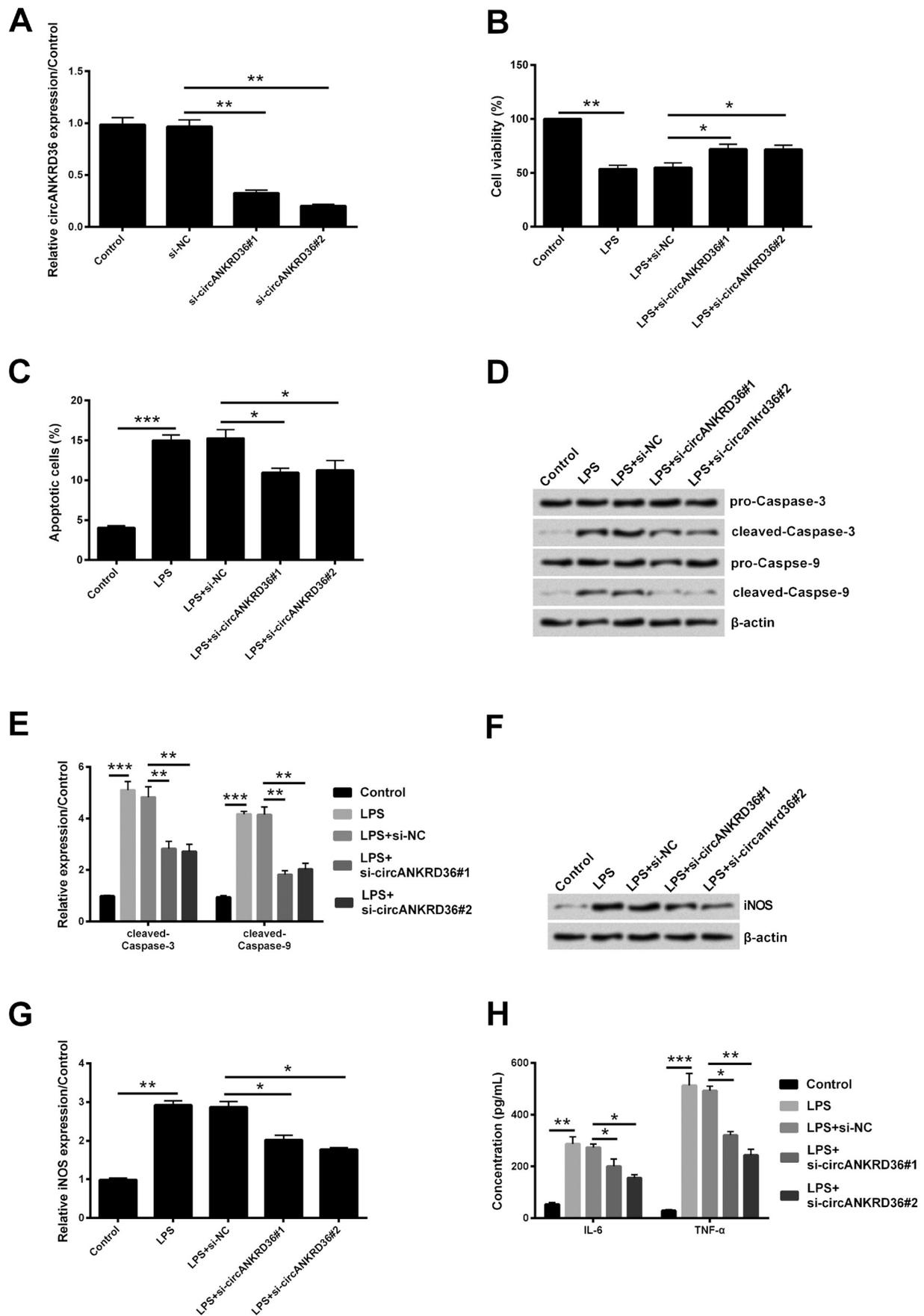
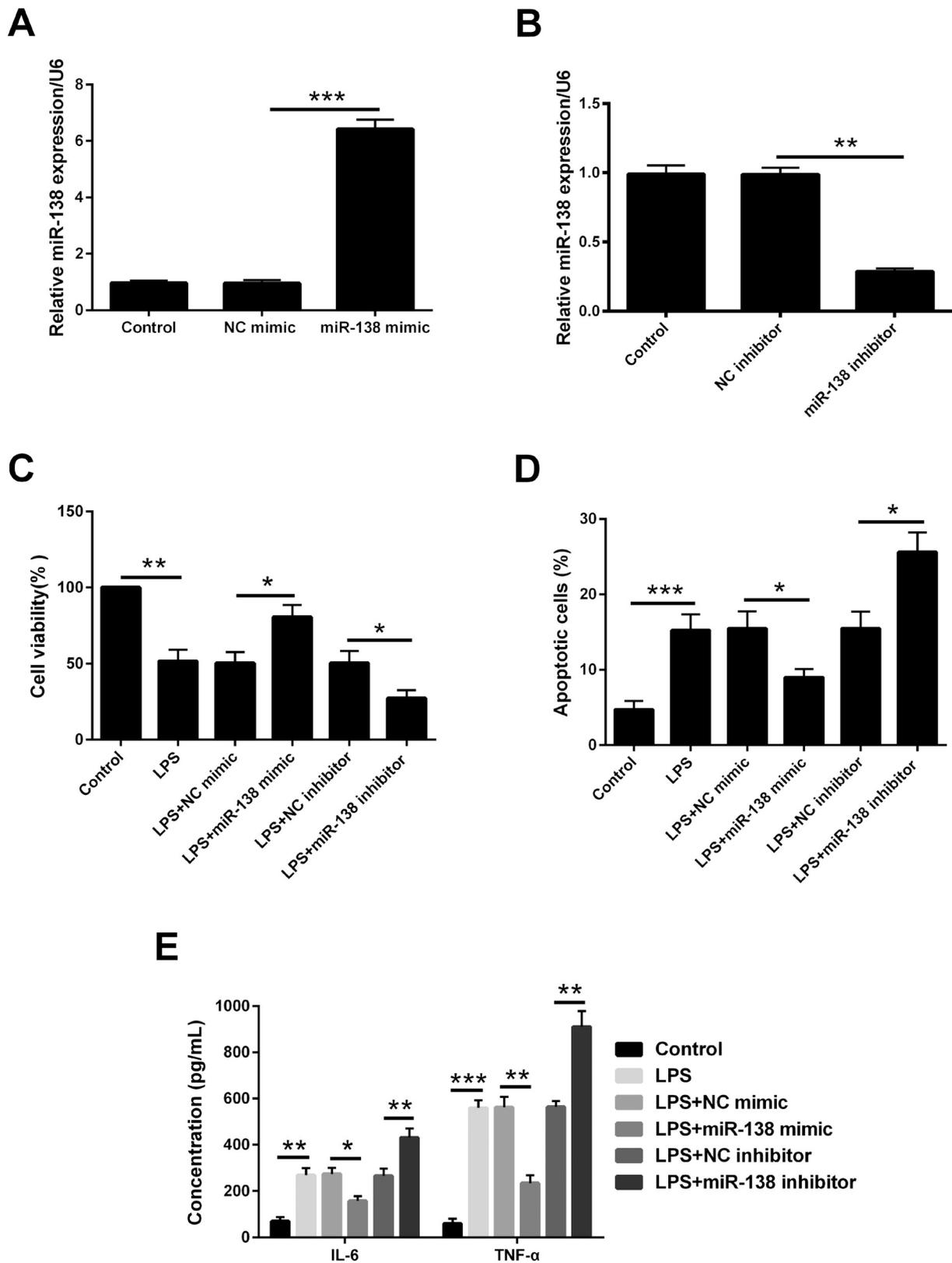
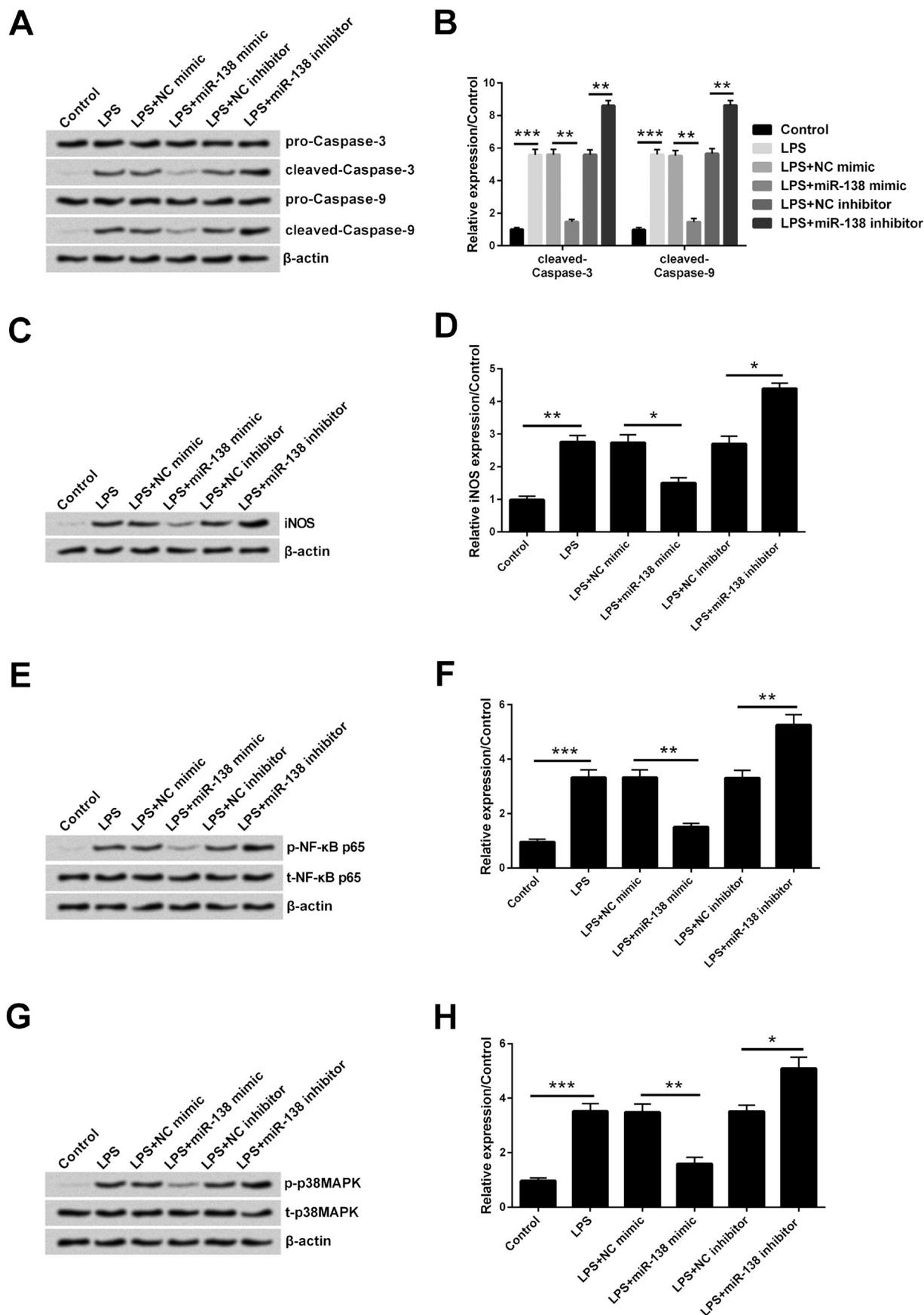


Fig. 3. LPS-induced apoptosis and inflammatory injury was alleviated when silenced circANKRD36. (A) When si-circANKRD36 was transfected into H9c2 cells successfully, circANKRD36 was silenced in H9c2 cells. (B) Viability was promoted when circANKRD36 was silenced. (C) Apoptosis was assuaged when circANKRD36 was silenced in H9c2 cells. (D–E) Cleaved-Caspase-3 and cleaved-Caspase-9 expression was reduced when circANKRD36 was silenced. (F–G) iNOS expression was declined when circANKRD36 was silenced. (H) IL-6 and TNF- $\alpha$  secretion was strongly decreased when circANKRD36 was silenced in H9c2 cells. \* $p < .05$ , \*\* $p < .01$  or \*\*\* $p < .001$  compared with marked group in the graph.



**Fig. 4.** LPS-induced apoptosis and inflammatory injury was regulated by miR-138. (A) MiR-138 expression was up-regulated by miR-138 mimic. (B) MiR-138 expression was down-regulated by miR-138 inhibitor. (C) Viability was elevated when up-regulating miR-138 and reduced when down-regulating miR-138. (D) Apoptotic cells were decreased by up-regulating miR-138 and escalated by down-regulating miR-138. (E) Secretion of IL-6 and TNF- $\alpha$  were alleviated by up-regulating miR-138 and raised by down-regulating miR-138. \* $p < .05$ , \*\* $p < .01$  or \*\*\* $p < .001$  compared with marked group in the graph.



**Fig. 5.** MiR-138 mediated the expression of apoptosis-related proteins and p38MAPK/NF-κB pathway. (A-B) Expression of cleaved-Caspase-3 and cleaved-Caspase-9 was decreased by up-regulating miR-138 and increased by down-regulating miR-138. (C-D) iNOS expression was reduced by up-regulating miR-138 and raised by down-regulating miR-138. (E-H) Expression of p-NF-κB p65 and p-p38MAPK were decreased by up-regulating miR-138 and elevated by down-regulating miR-138. \*p < .05, \*\*p < .01 or \*\*\*p < .001 compared with marked group in the graph.

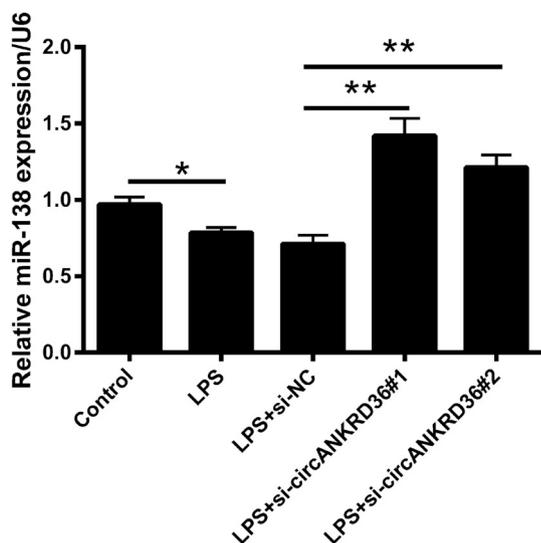


Fig. 6. MiR-138 expression was notably enhanced by silencing circANKRD36. MiR-138 was declined by LPS and increased when circANKRD36 was silenced. \* $p < .05$  or \*\* $p < .01$  compared with marked group in the graph.

into cells. Viability of H9c2 cells strongly reduced when cells were transfected with miR-138 inhibitor ( $p < .05$  or  $p < .01$ ). In addition, viability was reduced when down-regulating miR-138 expression and silencing circANKRD36 in the meantime ( $p < .05$ , Fig. 8A). What's more, apoptosis was remarkably increased by miR-138 inhibitor ( $p < .01$  or  $p < .001$ ) and escalated when down-regulating miR-138 expression and silencing circANKRD36 ( $p < .05$ , Fig. 8B). Additionally, IL-6 and TNF- $\alpha$  secretion was markedly promoted ( $p < .01$ ) and then enhanced when knocking down miR-138 expression and silencing circANKRD36 ( $p < .05$ , Fig. 8C). Furthermore, expression of cleaved-Caspase-3, cleaved-Caspase-9 and iNOS was firstly elevated by miR-138 inhibitor ( $p < .05$ ) and improved when knocking down miR-138 expression and silencing circANKRD36 ( $p < .05$ , Fig. 9A–D).

### 3.8. Silencing circANKRD36 suppresses p38MAPK/NF- $\kappa$ B pathway via up-regulating miR-138

In order to identify the underlying mechanism of which

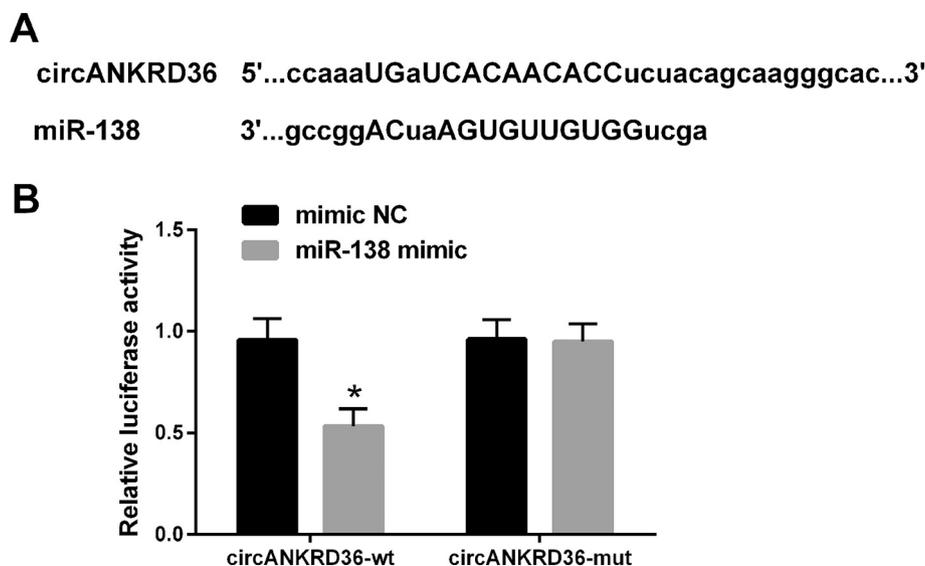


Fig. 7. MiR-138 was a direct target of circANKRD36. (A) The binding sites of miR-138 and circANKRD36 were predicted. (B) Relative luciferase activity was determined by Luciferase reporter assay. \* $p < .05$  compared with marked group in the graph.

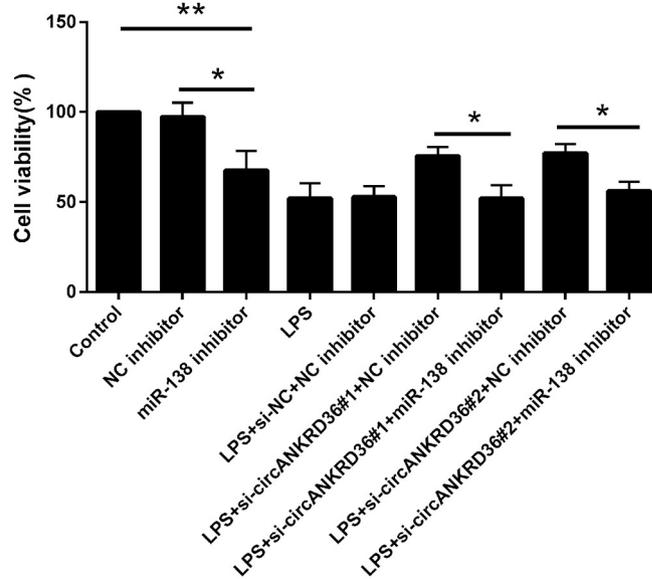
circANKRD36 and miR138 effected in H9c2 cells. The expression of p-NF- $\kappa$ B p65, t-NF- $\kappa$ B p65, p-p38MAPK and t-p38MAPK was assessed. The rates of p/t-NF- $\kappa$ B p65 and p/t-p38MAPK were remarkably raised by miR-138 inhibitor and LPS, respectively ( $p < .01$  or  $p < .001$ ). More than that, the rates of above-mentioned proteins were also elevated when silencing circANKRD36 expression after pretreated utilizing LPS ( $p < .05$  or  $p < .01$ ). The data implied that LPS activated p38MAPK/NF- $\kappa$ B pathway and the activation was suppressed by silencing circANKRD36. On the other hand, when silencing circANKRD36 and knocking down miR-138, the rates were raised in H9c2 cells ( $p < .05$ , Fig. 10A–D). The results suggested that the suppression of circANKRD36 attenuated mobilization of p38MAPK/NF- $\kappa$ B pathway by down-regulating miR-138 expression. All these consequences manifested that silencing circANKRD36 inhibited p38MAPK/NF- $\kappa$ B pathway when up-regulating miR-138 expression.

## 4. Discussion

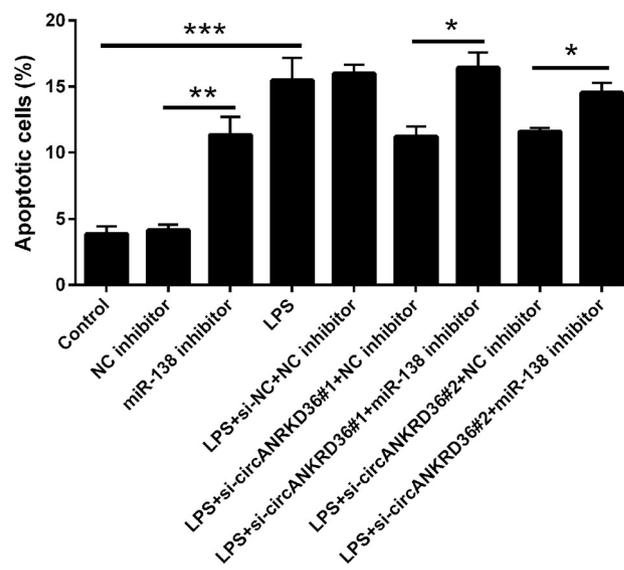
In the current study, we explored a circRNA, circANKRD36, which showed that silencing circANKRD36 alleviated LPS-induced apoptosis and inflammatory responses by regulating miR-138 in H9c2 cells. Furthermore, the relation of circANKRD36 and miR-138 was revealed.

Most myocarditis is caused by drug infections, bacterial infections, viral infections and autoimmune diseases (Hekimian and Combes, 2017). Persistent myocardial inflammation promotes myocardial cell damage, which ultimately leads to heart failure or death. Therefore, finding effective anti-inflammatory drugs is of great significance in the treatment of myocarditis. In our research, LPS was utilized to stimulate inflammatory injury and establish *in vitro* stimulation of myocarditis. In line with previous results (Ren et al., 2018), our study confirmed that LPS remarkably suppressed viability and raised apoptosis, expression level and production of inflammatory factors like IL-6 and TNF- $\alpha$  in H9c2 cells. CircRNAs have been found > 40 years. However, the functions of circRNAs are recognized in recent years. Accumulating researches illustrated that circRNAs played vital roles in various biological processes. For instance, hsa\_circ\_0020397 regulated viability, apoptosis and invasion by up-regulating miR138 expression (Zhang et al., 2017). Another report discovered that silencing hsa\_circ\_0007534 repressed proliferation and activated apoptosis in colorectal cancer cells (Zhang et al., 2018). Contrary to these findings, our study indicated that when circANKRD36 was muted, viability was raised and apoptosis was reduced. Furthermore, circRNAs are associated with inflammatory

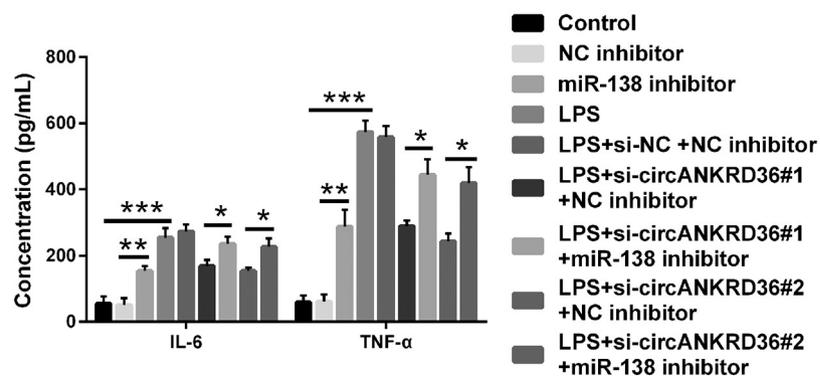
**A**



**B**

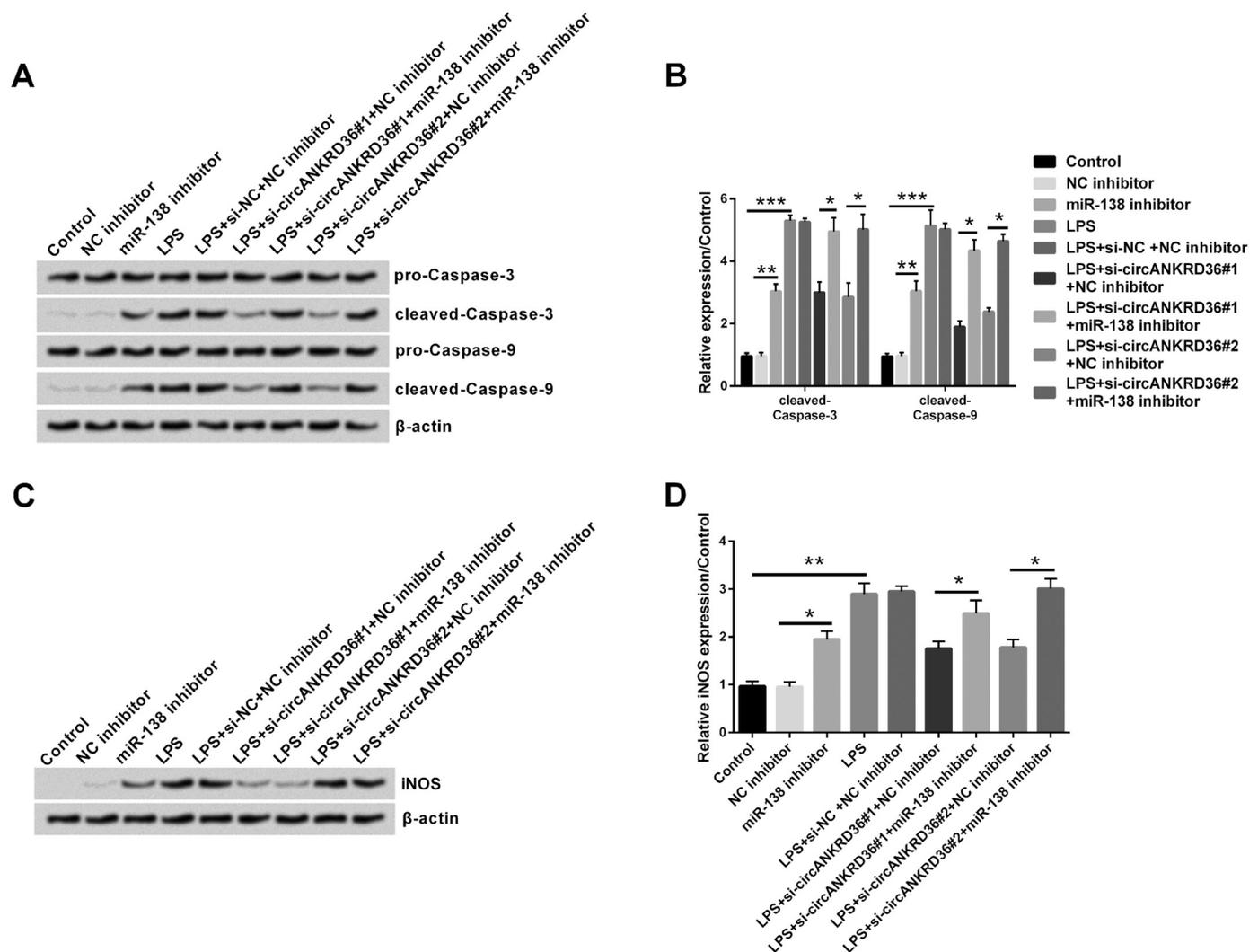


**C**



(caption on next page)

**Fig. 8.** CircANKRD36 exerted its function on LPS-induced cells via regulating miR-138 expression. (A) Viability was reduced by down-regulating miR-138. Moreover, viability was lessened by silencing circANKRD36 and down-regulating miR138 in the meantime. (B) Apoptosis was escalated when down-regulating miR-138. Furthermore, apoptosis was improved by silencing circANKRD36. (C) Secretion of IL-6 and TNF- $\alpha$  was raised by down-regulating miR-138 and promoted by silencing circANKRD36. \* $p < .05$ , \*\* $p < .01$  or \*\*\* $p < .001$  compared with marked group in the graph.

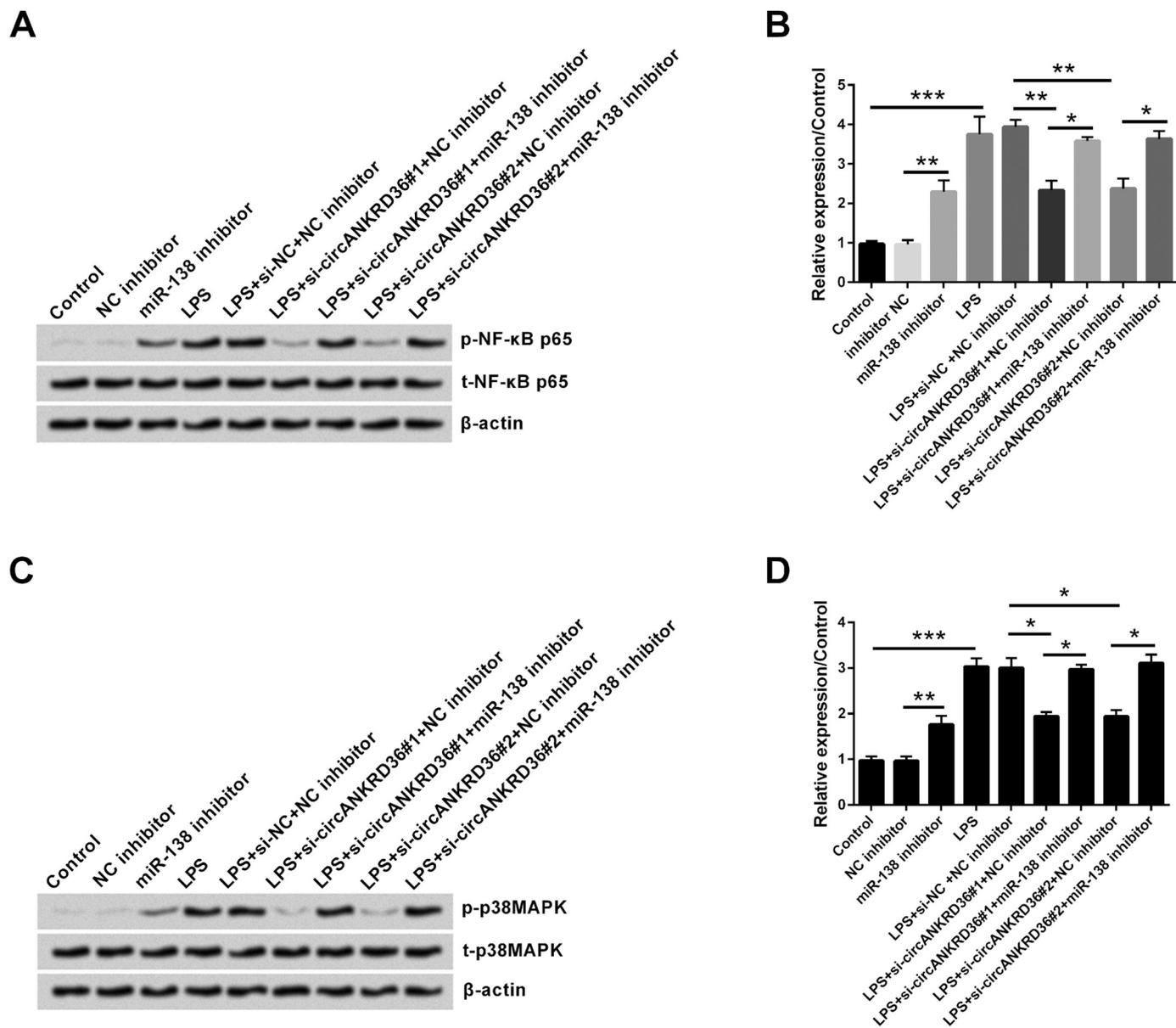


**Fig. 9.** CircANKRD36 mediated the expression of Caspase-3, Caspase-9 and iNOS by down-regulating miR-138. (A–B) Cleaved-Caspase-3 and cleaved-Caspase-9 expression was raised when down-regulated miR-138 and promoted by silencing circANKRD36. (C–D) iNOS expression was also raised when down-regulated miR-138 and improved by silencing circANKRD36. \* $p < .05$ , \*\* $p < .01$  or \*\*\* $p < .001$  compared with marked group in the graph.

response. Peng's investigations indicated that circ-Sirt1 regulated inflammatory responses in vascular smooth muscle cells via NF- $\kappa$ B pathway (Kong et al., 2019). Furthermore, a similar investigation of circANKRD36 explored that circANKRD36 was associated with inflammatory responses and inflammation-associated pathways in type 2 diabetes mellitus (Fang et al., 2018). However, the relationship between circANKRD36 and myocarditis remains widely unknown. We examined the effect of circANKRD36 on H9c2 cells for the first time in the current investigation. Consistent with Yuan's research (Fang et al., 2018), we investigated the IL-6 and TNF- $\alpha$  secretion changes. The conclusion can be implied according to the data that circANKRD36 raised IL-6 and TNF- $\alpha$  production in H9c2 cells. Moreover, apoptosis was raised by LPS and promoted by circANKRD36 in H9c2 cells. MiRNAs are participated in numerous biological and pathological proceedings, such as proliferation, apoptosis, inflammatory responses and so on (Wang et al., 2017). Besides, miR-138 has also been discriminated extensively and it played vital part in a variety of cells and diseases. And the function of miR-138 in apoptosis has been widely

reported. Manafi et al. proved that up-regulation of miR-138 prohibited cell growth and advocated apoptosis mediated by Caspase family (Manafi Shabestari et al., 2018). According to Requenez-Contreras's research, miR-138 inhibited IL-6 and TNF- $\alpha$  mRNA expression and secretion. Meanwhile, miR-138 constrained NF- $\kappa$ B/p65 expression (Requenez-Contreras et al., 2017). Nevertheless, there is no research on the function of miR-138 in myocarditis at present. In our study, we confirmed that silencing circANKRD36 suppressed apoptosis and LPS-treated inflammatory response via up-regulating miR-138. Combined with previous studies (Fang et al., 2018; Manafi Shabestari et al., 2018), we identified the role of circANKRD36 and miR-138 in H9c2 cells and confirmed the relationship between circANKRD36 and miR-138.

p38MAPK is a common pathway for intracellular signal transduction and is closely related to a variety of cellular processes such as cell growth, development, proliferation and apoptosis (Jian et al., 2017). Liu's investigation explored that inhibition of renin receptor-mediated p38MAPK signaling reduced hypoxia/reoxygenation-induced apoptosis in H9c2 cells (Liu et al., 2015). Similar with his results, after H9c2 cells



**Fig. 10.** Silencing circANKRD36 inhibited p38MAPK/NF-κB pathway via regulating miR-138. (A–D) The rates of p/t-NF-κB p65 and p/t-p38MAPK were noteworthy raised by LPS and were declined by silencing circ-ANKRD36. Finally, the decline was reversed when down-regulating miR-138. \* $p < .05$ , \*\* $p < .01$  or \*\*\* $p < .001$  compared with marked group in the graph.

were pretreated utilizing LPS, cleaved-Caspase-3, cleaved-Caspase-9 and p-p38MAPK expression was remarkably escalated. And this escalation was suppressed by si-circANKRD36 via up-regulating miR-138. In addition, p38MAPK is a principle intracellular signaling molecules associated with secretion of various inflammatory cytokines, such as TNF- $\alpha$  and IL-16 (Bode et al., 2012). Thus, we investigated IL-6 and TNF- $\alpha$  secretion changes in current experiments. The secretion of these two inflammatory factors was stimulated utilizing LPS and prohibited when silencing circANKRD36 via up-regulating miR-138. NF- $\kappa$ B is another signaling molecule that participates in LPS-induced injury. In addition, NF- $\kappa$ B is a centrally acting transcription factor that is participated in the expression of a number of genes related to immune and inflammatory responses (Komatsu et al., 2017). Activated NF- $\kappa$ B is transferred to the nucleus where it regulated transcription of its target genes, containing TNF- $\alpha$ , IL-6 and iNOS. Furthermore, NF- $\kappa$ B is activated by p38MAPK (a member of the MAPK family) in cardiomyocytes (Guo et al., 2013). Moreover, recent reports have confirmed that p38MAPK was taken part in the secretion of pro-inflammatory factors (Chen et al., 2016). Thus,

inhibition of NF- $\kappa$ B mobilization and signal cascade controlled by MAPKs may work on anti-inflammatory activity. To identify the function mechanism of circANKRD36 in H9c2 cells, we evaluated NF- $\kappa$ B p65 and p38MAPK expression. The data suggested that si-circANKRD36 suppressed p38MAPK/NF- $\kappa$ B pathway. Moreover, the rates of p/t-NF- $\kappa$ B p65 and p/t-p38MAPK were partially reversed by down-regulating miR-138, which indicated that circANKRD36 suppressed p38MAPK/NF- $\kappa$ B pathway via up-regulating miR-138. On the whole, the present study is the first to indicate that circANKRD36 and miR-138 could affect LPS-treated apoptosis and inflammatory response on myocarditis. Firstly, si-circANKRD36 suppressed apoptosis and inflammatory in LPS-treated H9c2 cells. Moreover, si-circANKRD36 exerted its anti-apoptosis and anti-inflammatory function by regulating miR-138. These findings uncovered a novel molecular mechanism of circANKRD36 and miR-138 effect on myocarditis, and provided an innovative target for clinical treatment. Additionally, further *in vivo* experiments still need to be conducted to prove the effect of circANKRD36. Furthermore, molecular mechanism of myocarditis involved in circANKRD36 and miR-138 will

be verified in a 3D *in vitro* model in the future.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Fundings

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Authors' contributions

Conceived and designed the experiments: Shengwei Shi, Shaohui Zhang.

Performed the experiments and analyzed the data: Shengwei Shi, Shaohui Zhang, Huiling Zhang, Qifeng Jin, Deguang Wu.

Wrote the manuscript: Shengwei Shi, Shaohui Zhang.

### Declaration of Competing Interest

The authors declare that they have no competing interests.

### Acknowledgments

No.

### References

- Bode, J.G., et al., 2012. The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. *Cell. Signal.* 24, 1185–1194.
- Bracamonte-Baran, W., Cihakova, D., 2017. Cardiac autoimmunity: myocarditis. *Adv. Exp. Med. Biol.* 1003, 187–221.
- Chen, J., et al., 2016. Inflammatory MAPK and NF-kappaB signaling pathways differentiated hepatitis potential of two agglomerated titanium dioxide particles. *J. Hazard. Mater.* 304, 370–378.
- Chen, X., et al., 2017. MicroRNA-98 plays a critical role in experimental myocarditis. *Int. J. Cardiol.* 229, 75–81.
- Devaux, Y., et al., 2017. Circular RNAs in heart failure. *Eur. J. Heart Fail.* 19, 701–709.
- Fan, S.J., et al., 2016. miRNA-149\* promotes cell proliferation and suppresses apoptosis by mediating JunB in T-cell acute lymphoblastic leukemia. *Leuk. Res.* 41, 62–70.
- Fang, Y., et al., 2018. Screening of circular RNAs and validation of circANKRD36 associated with inflammation in patients with type 2 diabetes mellitus. *Int. J. Mol. Med.* 42, 1865–1874.
- Fu, X., et al., 2018. MiR-30a-5p ameliorates spinal cord injury-induced inflammatory responses and oxidative stress by targeting Neurod 1 through MAPK/ERK signalling. *Clin. Exp. Pharmacol. Physiol.* 45, 68–74.
- Guo, R.M., et al., 2013. Activation of the p38 MAPK/NF-kappaB pathway contributes to doxorubicin-induced inflammation and cytotoxicity in H9c2 cardiac cells. *Mol. Med. Rep.* 8, 603–608.
- He, S., et al., 2013. miR-138 protects cardiomyocytes from hypoxia-induced apoptosis via MLK3/JNK/c-jun pathway. *Biochem. Biophys. Res. Commun.* 441, 763–769.
- Hekimian, G., Combes, A., 2017. Myocarditis. *Rev. Med. Interne* 38, 531–538.
- Jian, C.Y., et al., 2017. Naringin protects myocardial cells from doxorubicin-induced apoptosis partially by inhibiting the p38MAPK pathway. *Mol. Med. Rep.* 16, 9457–9463.
- Komatsu, W., et al., 2017. Nasunin inhibits the lipopolysaccharide-induced pro-inflammatory mediator production in RAW264 mouse macrophages by suppressing ROS-mediated activation of PI3 K/Akt/NF-kappaB and p38 signaling pathways. *Biosci. Biotechnol. Biochem.* 81, 1956–1966.
- Kong, P., et al., 2019. circ-Sirt1 controls NF-kappaB activation via sequence-specific interaction and enhancement of SIRT1 expression by binding to miR-132/212 in vascular smooth muscle cells. *Nucleic Acids Res.* 47, 3580–3593.
- LaPierre, M.P., Stoffel, M., 2017. MicroRNAs as stress regulators in pancreatic beta cells and diabetes. *Mol. Metab.* 6, 1010–1023.
- Lee, R.C., et al., 1993. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75, 843–854.
- Li, B., et al., 2017. Overexpression of CircRNA BCRC4 regulates cell apoptosis and MicroRNA-101/EZH2 signaling in bladder cancer. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 37, 886–890.
- Liu, Y., et al., 2015. Inhibiting (pro)renin receptor-mediated p38 MAPK signaling decreases hypoxia/reoxygenation-induced apoptosis in H9c2 cells. *Mol. Cell. Biochem.* 403, 267–276.
- Ma, J., et al., 2017. Comparisons of serum miRNA expression profiles in patients with diabetic retinopathy and type 2 diabetes mellitus. *Clinics (Sao Paulo)* 72, 111–115.
- Manafi Shabestari, R., et al., 2018. Overexpression of MiR-138 inhibits cell growth and induces caspase-mediated apoptosis in acute promyelocytic leukemia cell line. *Int. J. Mol. Cell. Med.* 7, 24–31.
- Matsumori, A., et al., 1994. Increased circulating cytokines in patients with myocarditis and cardiomyopathy. *Br. Heart J.* 72, 561–566.
- Pan, R.Y., et al., 2017. Circular RNAs promote TRPM3 expression by inhibiting hsa-miR-130a-3p in coronary artery disease patients. *Oncotarget* 8, 60280–60290.
- Ren, Q., et al., 2018. Astragalus polysaccharide alleviates LPS-induced inflammation injury by regulating miR-127 in H9c2 cardiomyoblasts. *Int. J. Immunopathol. Pharmacol.* 32 (2058738418759180).
- Requenez-Contreras, J.L., et al., 2017. MiR-138 indirectly regulates the MDR1 promoter by NF-kappaB/p65 silencing. *Biochem. Biophys. Res. Commun.* 484, 648–655.
- Sagar, S., et al., 2012. Myocarditis. *Lancet* 379, 738–747.
- Sun, Q., et al., 2013. Expression and significance of miRNA-21 and BTG2 in lung cancer. *Tumour Biol.* 34, 4017–4026.
- Wang, K., et al., 2016. A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. *Eur. Heart J.* 37, 2602–2611.
- Wang, M., et al., 2017. miRNA-34c inhibits myoblasts proliferation by targeting YY1. *Cell Cycle* 16, 1661–1672.
- Werfel, S., et al., 2016. Characterization of circular RNAs in human, mouse and rat hearts. *J. Mol. Cell. Cardiol.* 98, 103–107.
- Xu, Z., et al., 2018. The potential role of circRNA in tumor immunity regulation and immunotherapy. *Front. Immunol.* 9, 9.
- Yan, L., et al., 2016. Inhibition of microRNA-155 ameliorates experimental autoimmune myocarditis by modulating Th17/Treg immune response. *J. Mol. Med. (Berl.)* 94, 1063–1079.
- Zhang, X.L., et al., 2017. Overexpressing has\_circ\_0020397 increased viability and invasion as well as suppressed apoptosis by up-regulating miR-138 expression in colorectal cancer cells. *Cell Biol. Int.* 41, 1056–1064.
- Zhang, R., et al., 2018. Silencing of hsa\_circ\_0007534 suppresses proliferation and induces apoptosis in colorectal cancer cells. *Eur. Rev. Med. Pharmacol. Sci.* 22, 118–126.
- Zhou, B., Yu, J.W., 2017. A novel identified circular RNA, circRNA\_010567, promotes myocardial fibrosis via suppressing miR-141 by targeting TGF-beta1. *Biochem. Biophys. Res. Commun.* 487, 769–775.
- Zhou, L., et al., 2015. Inhibition of histone deacetylase activity aggravates coxsackievirus B3-induced myocarditis by promoting viral replication and myocardial apoptosis. *J. Virol.* 89, 10512–10523.