



CircELK4 Contributes to Lupus Nephritis by Acting as a miR-27b-3p Sponge to Regulate STING/IRF3/IFN-I Signaling

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Abstract— Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease and a common complication of SLE is lupus nephritis (LN) during which lupus autoantibodies and proinflammatory cytokines attack the kidney and cause renal dysfunction. The current treatments to LN are limited due to a poor understanding of the pathogenesis. Here, we studied the molecular mechanisms of LN by investigating the function of circELK4/miR-27b-3p axis. MRL/lpr mice and LPS-treated HK-2 cells were used as the mouse model and cell model of LN, respectively. Blood samples were collected from LN patients. qRT-PCR and western blot were used to measure expression levels of circELK4, miR-27b-3p, apoptosis-related proteins, cytokines, and STING/IRF-3/IFN-I signaling. ELISA was performed to examine levels of cytokines including IL-6 and TNF- α . H&E staining was used to examine kidney morphology. TUNEL staining and flow cytometry were used to determine cell apoptosis. Dual luciferase activity assay and RNA pull down were employed to validate the interactions of circELK4/miR-27b-3p and miR-27b-3p/STING. CircELK4 was elevated in LN mice, patients, and LPS-treated HK-2 cells. Knockdown of circELK4 attenuated renal injury in LN mice and LPS-induced HK-2 cell injury. CircELK4 directly bound to miR-27b-3p while miR-27b-3p targeted STING. Moreover, overexpression of circELK4 could partially reverse the effects of miR-27b-3p mimics on cell apoptosis and inflammation. Furthermore, circELK4/miR-27b-3p regulated renal cell damage *via* modulating STING/IRF3/IFN-I signaling. CircELK4 contributes to renal injury by promoting inflammation and cell apoptosis *via* acting as a miR-27b-3p sponge to modulate STING/IRF3/IFN-I signaling in LN.

KEY WORDS: circELK4; miR-27b-3p; lupus nephritis; STING/IRF3/IFN-I signaling.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease featured by systemic inflammatory responses that result in damages to multiple organ systems [19, 24]. Lupus nephritis (LN) is the most common and serious complication of SLE and also the leading cause to morbidity and mortality of SLE patients [1, 32]. It occurs when lupus autoantibodies and proinflammatory cytokines attack the kidney and cause renal dysfunction [31]. The current overall treatment to SLE and LN involves inflammation suppression and steroids [23]. However, due to the unclear etiology, the prognosis of LN remains very poor. Searching for novel treatments is very necessary for better outcomes and it requires a good understanding of the mechanisms.

Circular RNAs are a relatively new class of non-coding RNAs that have important roles in many cellular processes, such as development, cell growth, and survival [15, 30]. They can regulate gene expression by acting as microRNA (miRNA) or protein inhibitors [30]. Accumulating evidence suggests that dysregulated circRNAs contribute to the development of many diseases, including LN [3, 11]. For example, a previous study indicates that circELK4 level correlates with LN disease activity [18]. Nevertheless, what is the function of circELK4 in LN and how circELK4 regulates LN remain largely unknown.

The majority of circRNAs exert their functions by binding miRNAs to disinherit the regulation of downstream targets [3, 15, 30]. Our preliminary bioinformatic analysis through starBase suggests that circELK4 might bind to miR-27b-3p. Regarding miR-27b-3p, many previous studies have shown that miR-27b-3p regulates kidney injury [6, 27]. For example, miR-27b-3p is decreased in the kidney following acute kidney injury and rescue of miR-27b-3p level promotes the recovery of acute kidney injury [27], suggesting that miR-27b-3p plays a protective role in kidney injury. Moreover, through TargetScan, we found that miR-27b-3p might target STING (stimulator of interferon genes), an endoplasmic reticulum (ER)-resident transmembrane protein crucial for innate immune response [2, 20]. Upon stress, cytoplasmic DNA sensor activates STING and secreted STING from ER functions to promote ER stress and inflammation by activating downstream elements such as interferon regulatory factors (IRF3) [2]. IRF3 regulates the transcription of type I interferon (IFN-I: IFN- α and IFN- β), as well as inflammatory cytokines [12]. Based on the aforementioned clues, we hypothesized that circELK4 might participate in LN by binding with miR-27b-3p to allow for the activation of STING/IRF3/IFN-I signaling.

In the present study, we aimed to investigate the function of circELK4 in LN, with the focus on the miR-27b-3p/STING/IRF3/IFN-1 pathway. We found that circELK4 was elevated in LN mice and patients. Knockdown of circELK4 ameliorated the renal injury in LN mice, as well as LPS-induced inflammation and cell apoptosis. We validated that circELK4 directly bound miR-27b-3p while miR-27b-3p targeted STING. CircELK4/miR-27b-3p regulated LPS-induced cell injury *via* STING/IRF3/IFN-I signaling. Our study reveals that circELK4/miR-27b-3p/STING/IRF3/IFN-I axis plays a key role in LN, shedding light on the mechanisms of LN and provides avenues for the development of new therapeutic strategies for LN.

MATERIALS AND METHODS

Plasmids and Lentivirus

The full length of circELK4 was subcloned into the overexpression plasmid pcDNA3.1. Sh-circELK4, control sh-NC, miR-27b-3p mimics, miR-27b-3p inhibitor, mimics NC, and inhibitor NC were synthesized from Genepharma (Shanghai, China).

To generate sh-circELK4 lentivirus, sh-circELK4 was subcloned into the lentivirus vector pLV-CMV. The construct was then transfected into the HEK293T cells with helper vectors pSPAX2 and pMD2G to generate lentivirus.

Mouse Model

The mouse MRL lymphoproliferation strain (MRL/lpr) is a widely used congenital model of autoimmune disease resembling systemic lupus [10]. The MRL/lpr mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (China). MRL/lpr mice and control wild-type mice were kept in the standard animal facility and animal assays were performed with the approval of the Institutional Animal Ethics Committee following the Animal Care Guidelines for the Care and Use in The Second Xiangya Hospital, Central South University. Urine of each mouse was collected using metabolic cages. Mice with the urinary protein concentration higher than 10 mg/L were considered mice with LN. The mice were sacrificed for serum collection and serum creatinine level was measured with the automatic biochemical analyzer (Uritest, China). To assess the functional role of circELK4 in development of LN, MRL/lpr mice were intravenously injected with 5 mg/kg sh-circELK4 lentivirus once a week for 4 weeks.

Human Blood Samples

Human blood samples (plasma) were acquired from 12 diagnosed LN patients from our hospital. The blood samples from age-matched and gender-matched healthy people were used as control. The procedure and protocol have been reviewed and approved by the ethics committee of The Second Xiangya Hospital, Central South University. All patients were informed of the study and signed the written consent. The clinical characteristics of patients with LN were listed in Table 1.

H&E and TUNEL Staining

Kidney tissues from mice were dissected out and then placed in 4% PFA overnight for fixation at 4°C. Optimal cutting temperature compound (OCT, Fisher HealthCare, USA) was added for tissue embedding. Embedded tissues were cut into 10- μ m-thick slices and then subjected for hematoxylin and eosin (H&E) staining or used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining with an *In Situ* Cell Death Detection Kit (Roche Applied Science, USA) as the manufacturer's protocol described. The stained sections were mounted on glass slides with DAPI containing mounting medium.

HK-2 Cell Culture, Transfection, and Treatment

Human-derived proximal tubule epithelial cell line HK-2 was used and purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). The medium used for cell culture was composed of Dulbecco's modified Eagle medium (DMEM) (Gibco, CA, USA) plus 10% fetal bovine serum (Thermo-Fisher Scientific, MA, USA) and

1% penicillin-streptomycin (Gibco, USA). The cells were maintained in the cell culture CO₂ incubator at 37°C.

Lipofectamine 3000 (Invitrogen, MO, USA) was utilized as the reagent for cell transfection. In brief, cells were cultured to ~70% confluence, and then construct was added together with Lipofectamine 3000 at a ratio of 1:1. Lipopolysaccharide (LPS; 1 μ g/mL, 24 h) was added to treat cells and cells were harvested for further analysis after 48 h's transfection.

Cell Counting Kit-8

Cell proliferation was measured using the standard cell counting kit-8 (CCK-8 kit) according to the manufacturer's instruction. Cells were plated in the 96-well plates and cultured in the incubator. A 10 μ L CCK-8 solution was added to each well and incubated at 37°C for 2 h. The absorbance at 450 nm was analyzed with the standard microplate reader.

RNA Extraction and qRT-PCR

Trizol (Invitrogen, China) was employed to extract total RNAs from tissues or cells as the manufacturer's instructions described. DNaseI was included into the lysis buffer to avoid the contamination of DNA. Commercial kit of cDNA synthesis kits (Thermo-Fisher, China) was utilized to generate cDNAs through reverse transcription. SYBR Green Master Mix (Invitrogen, China) was used for the quantitative PCR. Relative expression levels of circELK4/miR-27b-3p or STING mRNA were normalized to U6 or GAPDH mRNA, respectively, as internal controls. The relative expression level was calculated by

Table 1. The Clinical Characteristics of Patients with LN

Patient	Age (years)	Gender (male/female)	SLEDAI (score)	eGFR (mL/min/1.73 m ²)	URTP (g/day)
1	28	Female	26	135.6	0.8
2	51	Female	22	46.7	9.8
3	32	Female	22	52.1	2.9
4	41	Female	18	48.3	15.6
5	37	Male	25	128.1	11.4
6	43	Male	20	117.5	0.3
7	48	Female	21	113.4	4.2
8	34	Female	25	124.7	10.8
9	46	Female	23	118.2	8.6
10	53	Female	21	110.6	5.7
11	46	Male	19	48.3	11.4
12	47	Female	23	120.8	12.3

SLEDAI systemic lupus erythematosus disease activity index; eGFR estimated glomerular filtration rate based on CKD-EPI formula; URTP urinary total proteinuria

$2^{-\Delta\Delta Ct}$ method. The primers listed as follows were from Genepharma (Shanghai, China):

circELK4 forward (human): 5'-TGTGCAACTAGATATGGACAGC-3',
 circELK4 reverse: 5'-ACTTGAGCAGTTTACACCAGA-3';
 miR-27b-3p forward: 5'-CGGCTTCACAGTGGCTAAG-3',
 miR-27b-3p reverse: 5'-CGGCCAGTGTTCAGACTAC-3';
 STING forward: 5'-CAGGCACTGAACATCCTCCT-3',
 STING reverse: 5'-ATATACAGCCGCTGGCTCAC-3';
 U6 forward: 5'-CTCGCTTCGGCAGCACA-3',
 U6 reverse: 5'-AACGCTTCACGAATTTGCGT-3';
 GAPDH forward: 5'-CCAGGTGGTCTCCTCTGA-3',
 GAPDH reverse: 5'-GCTGTAGCCAAATCGTTGT-3';
 circELK4 forward (mouse): 5'-TTGGGGATTACAGCGTCTCT-3',
 circELK4 reverse: 5'-TGCTCCAAAAGTGGATGCTGG-3';
 GAPDH forward: 5'-AGCCCAAGATGCCCTTCAGT-3',
 GAPDH reverse: 5'-CCGTGTTCTTACCCTCCAATG-3'.

Western Blot Analysis

Proteins from kidney tissues or HK-2 cells were extracted by utilizing the RIPA lysis buffer (Abcam, China) according to standard protocol. DC Protein Assay Kit (Bio-Rad, China) was utilized to quantify the protein concentrations. Equal protein from each sample was loaded into SDS-polyacrylamide gels and separated through electrophoresis. Later proteins in the gels were transferred to PVDF membranes (Sigma-Aldrich, China). Three percent BSA was used to block the membranes for 30-60 min at room temperature and then specific primary antibodies were added to incubate at 4°C overnight. The antibodies were discarded and TBST was utilized to wash the membranes 3 times before incubation with specific secondary antibodies for 1-2 h at room temperature. Protein band intensities were detected by using the ECL kit (Bio-Rad). Primary antibodies used in the study were purchased from Abcam: anti-cleaved Caspase3 antibody (c-Caspase3; 1:500, ab49822); anti-Bax antibody (1:5000, ab32503);

anti-Bcl-2 antibody (1:1000, ab196495); anti-IL-6 antibody (1:1000, ab6672/ab208113); anti-TNF- α antibody (1:1000, ab9739); anti-STING antibody (1:500, ab179775); anti-p-IRF3 (1:1000, AP0623; ABclonal, Wuhan, China); anti-IRF3 (1:1000, A0816, ABclonal); anti-IFN- β (1:1000, A1575, ABclonal); anti- β -actin (1:2000, ab8227).

Flow Cytometry Apoptosis Assay

Transfected cells were plated and cultured in the 6-well culture plate until 70-80% confluence. Cells were harvested with lysis buffer and then incubated with Annexin-V-FITC/PI (BD Pharmingen, NJ) for 20 min at 4°C. Flow cytometry was used to analyze the relative number of positive and negative cells.

RNA Pull-Down Assay

The biotinylated RNA pull-down assay was utilized to verify the direct binding between circELK4 and miR-27b-3p. In brief, the biotinylated wild-type (WT)/mutant (MUT) miR-27b-3p was transfected into HK-2 cells together with circELK4 by using Lipofectamine 3000. Two days after overexpression, cells were harvested and lysed using lysis buffer. Magnetic beads were added to incubate with cell lysates overnight at 4°C to pull down biotinylated RNAs. The next day, the cell lysates were discarded and the beads were washed with lysis buffer followed by elution. The elution was subjected to qRT-PCR to determine the expression levels of circELK4.

Enzyme-Linked Immunosorbent Assay

Commercial enzyme-linked immunosorbent assay (ELISA) kits (IL-6 and TNF- α ; R&D Systems) were utilized to measure secreted cytokine concentration in culture medium as the manufacturer's instructions described. Briefly, 96-well plates were coated with coating buffer overnight at 4°C and then washed with washing buffer followed by block with blocking buffer at room temperature for 1 h. The culture medium from each condition was collected and equal amounts of standards and culture medium samples were added to individual wells of the 96-well plate and then incubated at room temperature for 2 h. The medium was discarded and then washed with washing buffer. Detection antibody was added afterwards for incubation for another 2 h and then washed again followed by incubation with substrate solution at room temperature for 30 min. In the end, stop solution was added and the signal was analyzed with a microplate reader set to 450 nm.

Dual Luciferase Activity Assay

The wild-type sequences or mutated binding sites of miR-27b-3p in 3' untranslated region (UTR) of STING mRNA and the full length of circELK4 were cloned into the luciferase report vector (psiCHECK2). Commercial kit (The Phusion Mutagenesis kit, Thermo-Fisher Scientific, China) was utilized to mutate the predicted binding sites as the protocol described. HK-2 cells were seeded in individual wells of the 24-well plates first overnight and then recombinant constructs were transfected into HK-2 cells together with miR-27b-3p mimic or miR-NC by using the Lipofectamine 3000. Forty-eight hours after transfection, the cells were harvested in the Reporter Lysis Buffer from the commercial kit (Promega, China) and relative luciferase activities were measured.

Statistical Analysis

All experiments were carried out with at least three biological replicates and the data were analyzed in GraphPad Prism 7. Normal distribution of the data was confirmed by Shapiro-Wilk normality test. Statistical details were calculated by Student *t* test (two groups) or one-way ANOVA (multiple groups). The difference was considered significant if $P < 0.05$. All experimental data are presented as mean \pm standard deviation (SD).

RESULTS

CircELK4 Was Elevated in LN Mice and Patients

To study the function of circELK4 in LN, we first employed a mouse model of LN (MRL/lpr mouse strain). Compared to normal wild-type mice, we saw significantly higher levels of urine protein concentration and serum creatinine in MRL/lpr mice (Fig. 1A, B). H&E and TUNEL staining results showed that the kidney from MRL/lpr mice was severely injured with atrophic glomerulus, vacuolar degeneration, and increased percentage of apoptotic cells (Fig. 1C, D). Furthermore, cytokines including IL-6 and TNF- α in the kidneys of MRL/lpr mice were greatly elevated (Fig. 1E). These results show that MRL/lpr mouse is a successful LN model with severely damaged renal function. With this mouse model, we found that circELK4 level was significantly increased in LN mice compared to normal mice. Moreover, circELK4 level in LN patients was higher than that in healthy people (Fig. 1G). Therefore, circELK4 may play an important role in LN.

Knockdown of circELK4 Ameliorated Renal Injury of LN Mice

We then investigated whether circELK4 regulates LN. Injection of sh-circELK4 lentivirus reversed the increase of circELK4 level induced by LN in the kidney (Fig. 2A). Using TUNEL staining, we found that knockdown of circELK4 suppressed the increase of cell apoptosis in LN mice (Fig. 2B). At the molecular level, we showed that apoptosis-related proteins including c-Caspase 3 and Bax and cytokines like IL-6 and TNF- α were upregulated in LN mice while anti-apoptotic protein Bcl-2 was downregulated (Fig. 2C). Nevertheless, knockdown of circELK4 reversed those changes caused by LN (Fig. 2C). Interestingly, STING, p-IRF3, and IFN- β also went up in the kidneys of LN mice while knockdown of circELK4 inhibited those increases (Fig. 2D). Together, these results indicate that knockdown of circELK4 ameliorates LN-induced renal injury and STING/IRF3/IFN-I pathway may be involved in this process.

Knockdown of circELK4 Reduced LPS-Induced Cell Injury

To investigate the underlying mechanisms, we used the human-derived proximal tubule epithelial cell line (HK-2) and mimicked the renal injury by treating cells with LPS. We found that LPS treatment greatly increased circELK4 in HK-2 cells (Fig. 3A). Transfection of HK-2 cells with sh-circELK4 remarkably diminished circELK4 level (Fig. 3A). With CCK-8 assay to measure cell viability, we showed that LPS treatment greatly reduced cell viability while transfection with sh-circELK4 inhibited the reduction of cell viability caused by LPS (Fig. 3B). Consistently, with flow cytometry, we observed that LPS treatment significantly enhanced the percentage of apoptotic cells while knockdown of circELK4 suppressed that upregulation (Fig. 3C). The western blot results showed that LPS increased apoptosis-related proteins (c-Caspase3, Bax) and cytokines (IL-6 and TNF- α) but decreased anti-apoptotic protein Bcl-2 (Fig. 3D). However, knockdown of circELK4 reversed those changes (Fig. 3D). We also measured secreted IL-6 and TNF α levels and found that LPS treatment induced IL-6 and TNF- α secretion while knockdown of circELK4 suppressed their secretions (Fig. 3E). Taken together, these results demonstrate that circELK4 knockdown inhibits LPS-induced inflammatory responses and apoptosis of HK-2 cells.

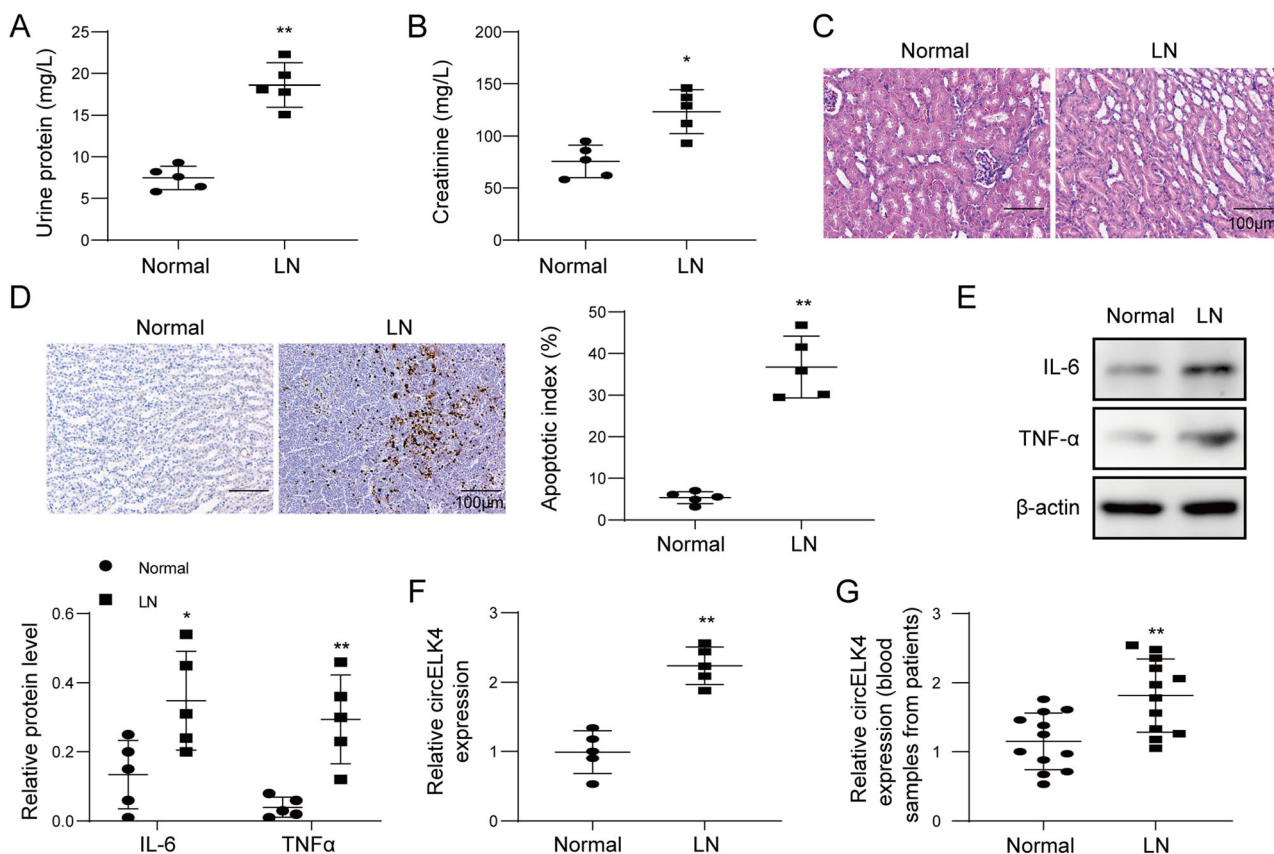


Fig. 1. CircELK4 was elevated in LN mice and patients. **A** Urine protein concentration in normal and LN mice, n = 5. **B** Serum creatinine level in normal and LN mice. **C** Representative images of H&E staining of kidney tissues from normal and LN mice. **D** TUNEL staining analysis of cell apoptosis in kidney tissues from normal and LN mice. **E** IL-6 and TNF- α levels in normal and LN mice. **F** Relative circELK4 level in normal and LN mice. **G** Relative circELK4 level in normal healthy people and LN patients, n = 12. Data were presented as means \pm SD. * p < 0.05, ** p < 0.01.

CircELK4 Acted as a miR-27b-3p Sponge

Through bioinformatic analysis, we observed some complementary binding sites between circELK4 and miR-27b-3p (Fig. 4A). Transfection of HK-2 cells with miR-27b-3p mimics largely increased miR-27b-3p level but its inhibitor reduced expression level (Fig. 4B). To directly examine their interaction, we employed RNA pull-down experiment and dual luciferase assay. Immunoprecipitation of biotin-miR-27b-3p-WT significantly pulled down a lot more circELK4 compared to immunoprecipitation of biotin-miR-27b-3p-MUT wherein the predicting binding sites with circELK4 were mutated (Fig. 4C). Similarly, miR-27b-3p mimics greatly diminished the relative luciferase activity of circELK4-WT but not the circELK4-MUT (Fig. 4D). Furthermore, knockdown of circELK4 with sh-circELK4 upregulated miR-27b-3p level in HK-2 cells (Fig. 4E). Therefore, we conclude that circELK4 binds miR-27b-3p to inhibit its expression.

CircELK4 Regulated LPS-Induced Cell Injury via miR-27b-3p

Next, we examined the function of circELK4/miR-27b-3p interaction in LPS-induced cell injury. LPS treatment downregulated miR-27b-3p level in HK-2 cells while overexpression of miR-27b-3p recovered the level (Fig. 5A). Co-transfection of circELK4 in miR-27b-3p-transfected cells following LPS treatment reduced miR-27b-3p level again (Fig. 5A). Using CCK-8 assay, we found that miR-27b-3p mimics suppressed the reduced cell viability induced by LPS treatment (Fig. 5B). However, co-overexpression of circELK4 reversed the effect of miR-27b-3p mimics (Fig. 5B). With flow cytometry, we showed that miR-27b-3p mimics inhibited the LPS-induced increase of cell apoptosis while circELK4 blocked the effects of miR-27b-3p (Fig. 5C). Western blot results indicated that miR-27b-3p mimics suppressed the LPS-induced expression changes in apoptosis-related proteins

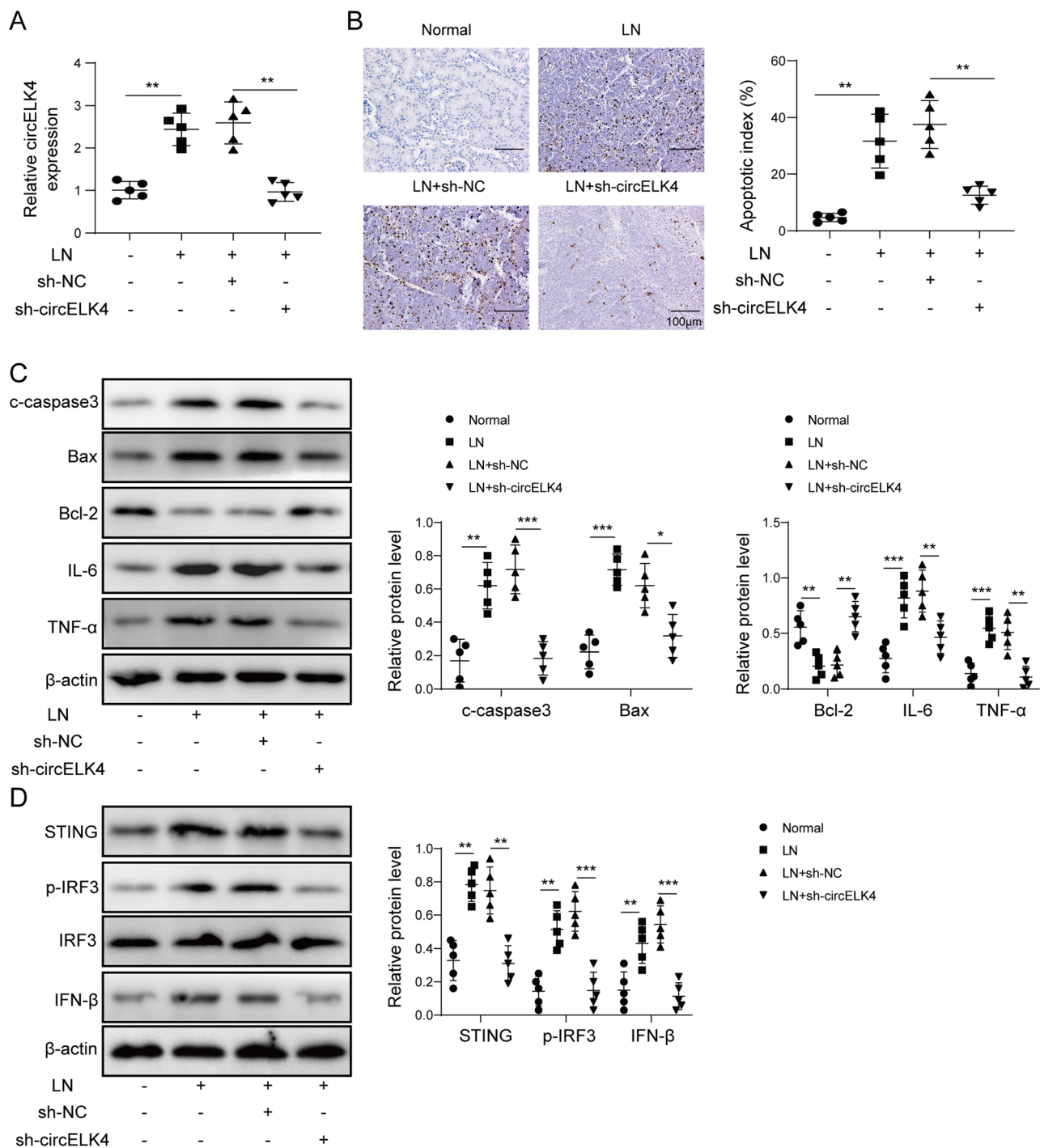


Fig. 2. Knockdown of circELK4 ameliorated renal injury of LN mice. **A** Relative circELK4 levels in kidneys from each group of mice, $n = 5$. **B** TUNEL staining analysis of cell apoptosis in kidney tissues from each group of mice. **C** Western blotting to determine levels of apoptosis-related proteins and cytokines. **D** Western blotting to measure levels of STING/IRF3/IFN-I signaling. Data were presented as means \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

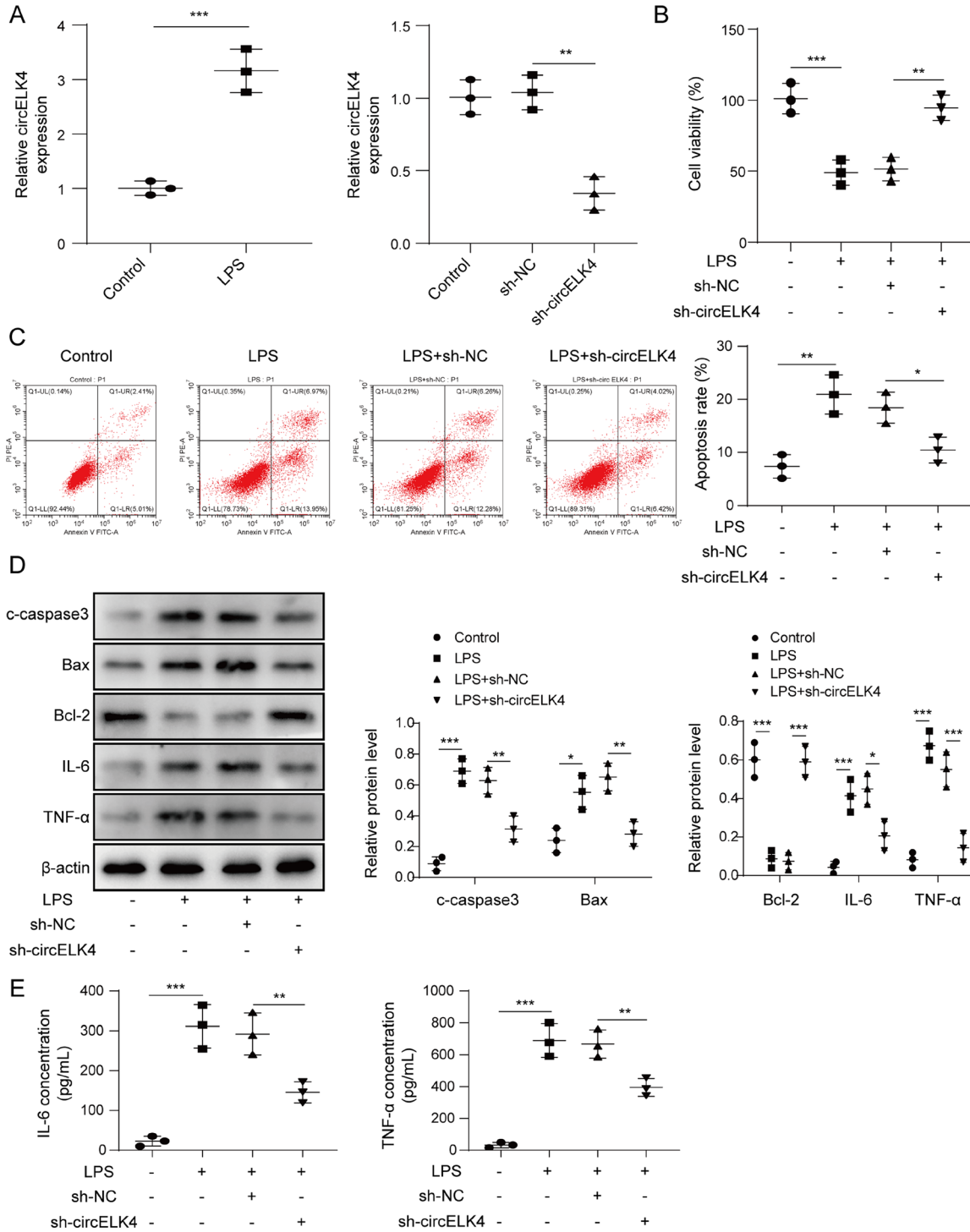


Fig. 3. Knockdown of circELK4 reduced LPS-induced cell injury. **A** Relative circELK4 level in HK-2 cells following LPS treatment or transfection with sh-circELK4. **B** CCK-8 analysis to evaluate cell viability of transfected HK-2 cells following LPS treatment. **C** Flow cytometry analysis to assess cell apoptosis in transfected HK-2 cells following LPS treatment. **D** Western blotting to determine levels of apoptosis-related proteins and cytokines in transfected HK-2 cells following LPS treatment. **E** ELISA analysis of secreted IL-6 and TNF-α levels from transfected HK-2 cells following LPS treatment. Data were presented as means ± SD, n = 3. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

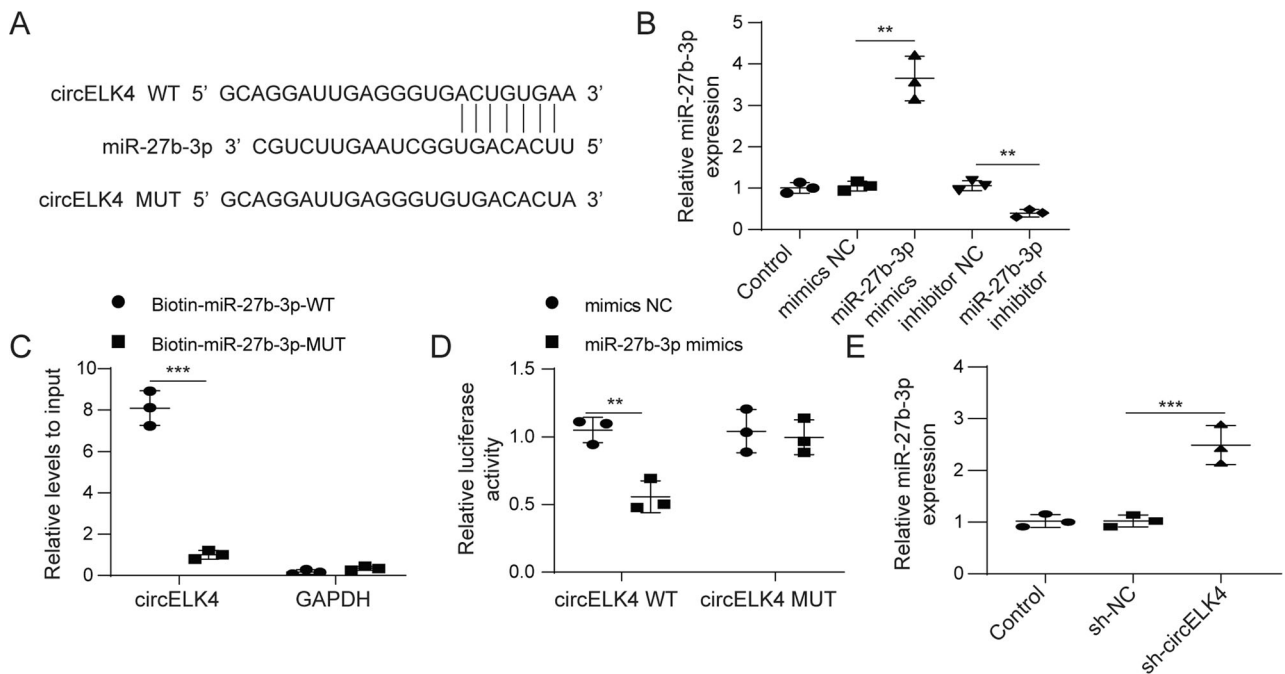


Fig. 4. CircELK4 acted as a miR-27b-3p sponge. **A** Complementary binding sites between circELK4 and miR-27b-3p. **B** Relative miR-27b-3p levels in transfected cells. **C** Relative circELK4 levels pulled down by immunoprecipitation of miR-27b-3p-WT or miR-27b-3p-MUT. **D** Relative luciferase activity of WT-circELK4 and MUT-circELK4. **E** Relative miR-27b-3p levels in transfected cells. Data were presented as means \pm SD, $n = 3$. $**p < 0.01$, $***p < 0.001$.

and cytokines while circELK4 reversed the effects of miR-27b-3p (Fig. 5D). Regarding secreted IL-6 and TNF- α levels, similarly we saw that miR-27b-3p inhibited LPS-induced secretions of IL-6 and TNF- α while co-transfection of circELK4 enhanced their secretions again (Fig. 5E). Altogether, these results indicate that circELK4 regulates LPS-mediated inflammatory responses and apoptosis *via* the repression of miR-27b-3p level.

CircELK4/miR-27b-3p Regulated STING/IRF3/IFN-I Signaling

STING/IRF3/IFN-I signaling has been shown to have a critical role in immune responses [2]. It is widely acknowledged that miRNAs function through negatively regulating gene expression [8]. Through bioinformatic analysis, we identified some complementary binding sites between miR-27b-3p and STING mRNA (Fig. 6A). To directly validate this interaction, we performed dual luciferase assay. As shown in Fig. 6B, miR-27b-3p mimics greatly decreased the relative luciferase activity of

STING-WT but not STING-MUT in which the predicted binding sites with miR-27b-3p were mutated, suggesting that miR-27b-3p directly binds STING mRNA. As expected, transfection of miR-27b-3p inhibitor significantly increased the mRNA level and protein level of STING (Fig. 6C). Consistent with our aforementioned results, LPS treatment increased expression levels of STING, p-IRF3, and IFN- β (Fig. 6D). However, overexpression of miR-27b-3p mimics suppressed those increased while co-transfection of circELK4 partially reversed the effects of miR-27b-3p mimics (Fig. 6D). As we presented before, knockdown of circELK4 restrained LPS-induced increase of cell apoptosis, but overexpression of STING blocked the effect of circELK4 (Fig. 6E). For secreted IL-6 and TNF- α , we observed similar regulations and overexpression of STING reversed the effects of circELK4 knockdown on LPS-induced IL-6 and TNF- α secretion (Fig. 6F). Collectively, these data demonstrate that circELK4/miR-27b-3p regulates LPS-induced inflammatory responses and cell apoptosis by modulating STING/IRF3/IFN-I signaling (Fig. 6G).

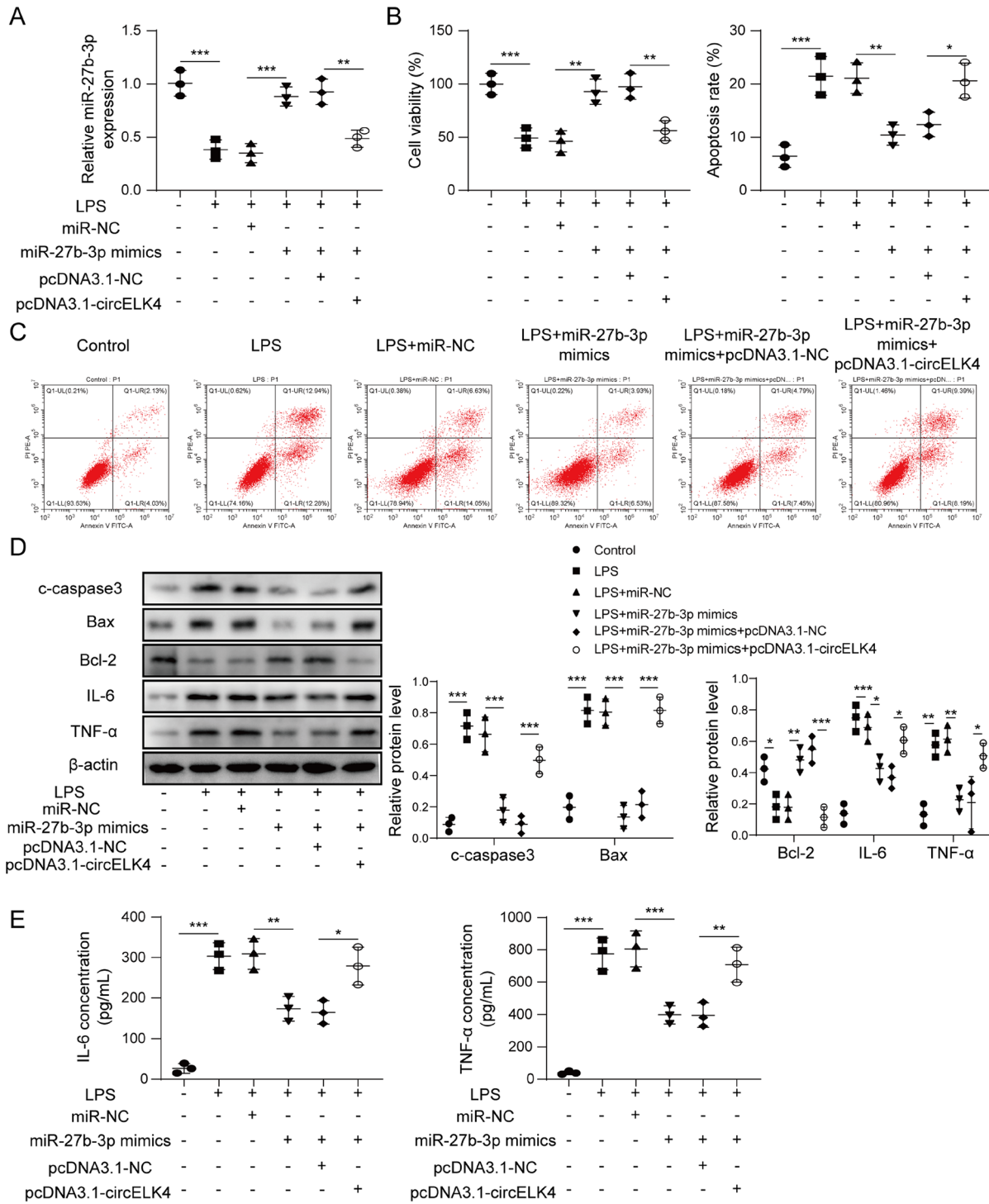


Fig. 5. CircELK4 regulated LPS-induced cell injury via miR-27b-3p. **A** Relative miR-27b-3p levels in transfected cells. **B** CCK-8 analysis to evaluate cell viability of transfected HK-2 cells following LPS treatment. **C** Flow cytometry analysis to assess cell apoptosis in transfected HK-2 cells following LPS treatment. **D** Western blotting to measure levels of apoptosis-related proteins and cytokines in transfected HK-2 cells following LPS treatment. **E** ELISA analysis of secreted IL-6 and TNF-α levels from transfected HK-2 cells following LPS treatment. Data were presented as means ± SD, n = 3. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

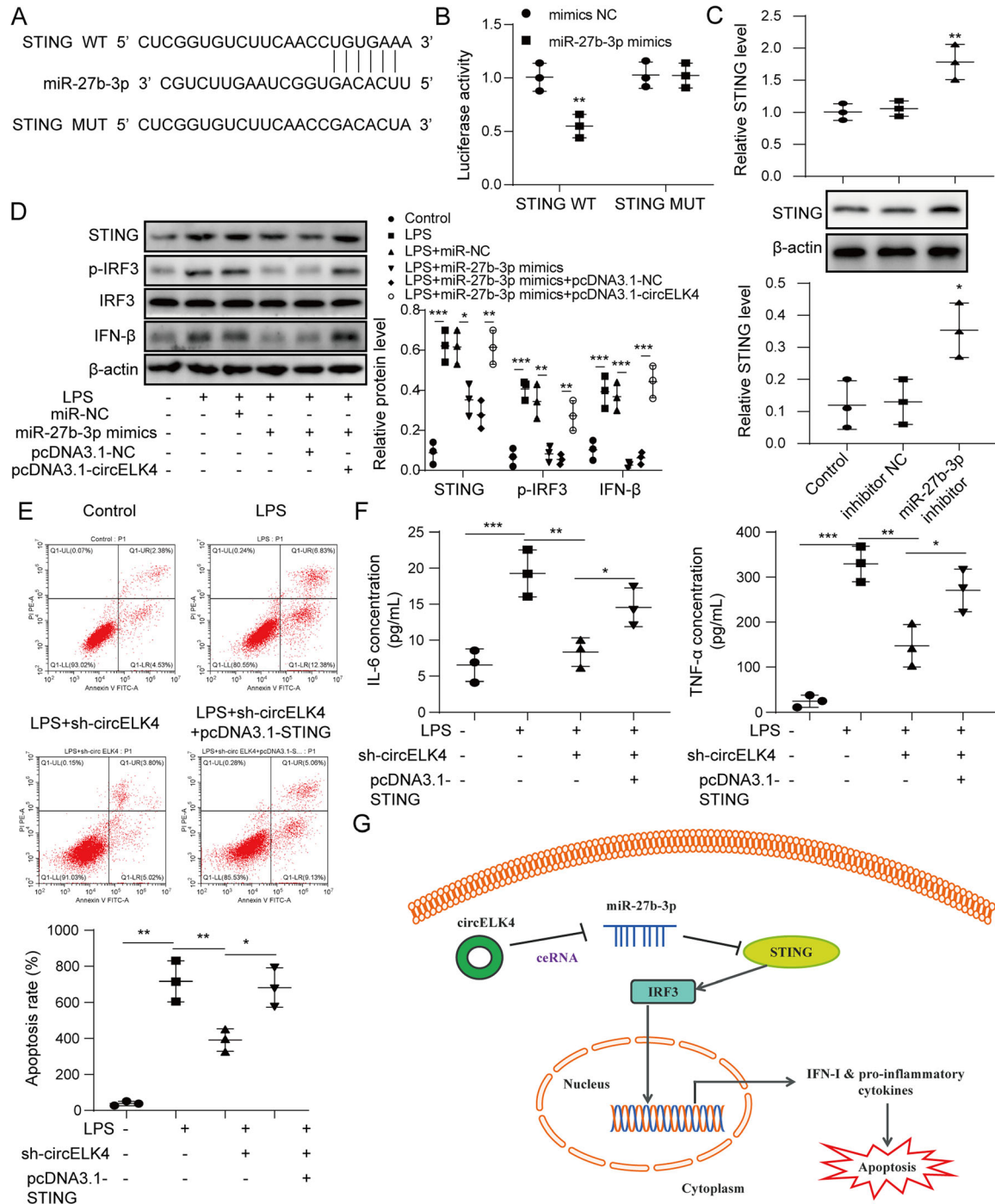


Fig. 6. CircELK4/miR-27b-3p regulated STING/IRF3/IFN-I signaling. **A** Complementary binding sites between STING mRNA and miR-27b-3p. **B** Relative luciferase activity of WT-STING and MUT-STING. **C** Relative STING mRNA and protein levels in transfected cells. **D** Western blotting to determine levels of apoptosis-related proteins and cytokines in transfected HK-2 cells following LPS treatment. **E** Flow cytometry analysis to assess cell apoptosis in transfected HK-2 cells following LPS treatment. **F** ELISA analysis of secreted IL-6 and TNF-α levels from transfected HK-2 cells following LPS treatment. **G** The mechanism of the regulatory network and function of circELK4. circELK4 contributes to renal injury by promoting inflammation and cell apoptosis *via* acting as a miR-27b-3p sponge to modulate STING/IRF3/IFN-I signaling. Data were presented as means \pm SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

Despite numerous advances made in research, the prognosis of LN is still poor with 10% of patients ending up in end-stage renal disease (ESRD) [1, 23]. The pathogenesis of LN is not well understood and thus effective treatments are lacking. In the current study, we investigate molecular mechanisms underlying renal injury during LN, with focus on circELK4/miR-27b-3p axis. We found that circELK4 was elevated in the LN mice and patients and that inhibition of circELK4 ameliorated renal injury and LPS-induced inflammation and cell apoptosis. Mechanistically, we identified that circELK4 directly bound miR-27b-3p while miR-27b-3p targeted STING. CircELK4/miR-27b-3p regulated the LPS-induced inflammation and cell apoptosis *via* STING/IRF3/IFN-I signaling. These results provide strong evidence that circELK4 contributes to renal injury during LN by acting as miR-27b-3p sponge to regulate STING/IRF3/IFN-I pathway.

It is acknowledged that circRNAs play important roles in many processes including physiological processes and pathological diseases [3, 15, 30]. LN is a condition caused by autoimmune responses and thus involves inflammation and cell apoptosis [9, 25]. Many studies have implicated circRNAs in immune regulation and cell death, such as circ-FNDC3B and circ-MYBL1 [4, 5, 22, 33]. Therefore, it is not surprising that varieties of circRNAs have been reported to play roles in LN [18, 29]. CircELK4 is a circRNA that is highly expressed in natural killer cells and T cells [22]. Previous studies have shown that circELK4 has critical roles in immune system [22, 33]. Moreover, a recent study reports that renal circELK4 correlated with LN disease activity [18]. However, the exact role of circELK4 in LN is not clear. Here, we fully elucidated the function of circELK4 in LN, showing that circELK4 promotes renal dysfunction in LN by enhancing inflammatory responses and cell apoptosis. Significantly, knockdown of circELK4 could suppress the levels of pro-inflammatory cytokines and cell apoptosis, protecting the kidney from the damage in LN. These results suggest that treatments targeting circLEK4 could be potentially used to treat LN.

Most circRNAs function by acting as ceRNAs (competing endogenous RNAs) [21, 30]. Through bioinformatic analysis, we identified miR-27b-3p as a target of circELK4. According to the existing evidence, miR-27b-3p may play a protective role in renal injury. In the present study, we showed that circELK4 regulated inflammation and cell apoptosis by sponging miR-27b-3p to modulate STING/IRF3/IFN-I signaling. STING is a well-known regulator of

innate immune responses and has key roles in regulating inflammation and apoptosis by targeting downstream element IRF3 [2, 16, 20]. Our results support the model and confirm that overexpression of STING could block the effects of sh-circELK4. Interestingly, we noticed that STING overexpression did not fully block the effects of circELK4 knockdown, suggesting that there may be other downstream targets of circELK4 that mediate its function in LN. Indeed, besides STING, miR-27b-3p targets multiple downstream genes [13, 14, 28]. For example, it can regulate cytokine production *via* targeting SPY (spleen tyrosine kinase) [7]. Also, besides, miR-27b-3p, circELK4 can bind to other miRNAs. Therefore, other targets of miR-27b-3p or circELK4 may mediate the roles of circELK4 in LN and future studies are necessary to test that hypothesis [17].

Our study was primarily carried out *in vitro* using cultured cells despite that we used MRL/lpr mouse as the *in vivo* model. There are multiple mouse models of LN and they have contributed a lot to our understanding of the disease [26]. Each model has its advantages and disadvantages. Therefore, it is necessary to confirm our results in other mouse models of LN. In addition, whether this regulation holds the same in human beings remains further validations.

In summary, with a combination of mouse model and cell model of LN, we demonstrate an essential role of circELK4/miR-27b-3p/STING axis in renal injury during LN. This study provides mechanistic insights into LN and thus helps us design better strategies to treat LN.

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AUTHOR CONTRIBUTION

Conception and study design: ZQX;

Data acquisition: YD;

Data analysis: XYH;

Manuscript drafting: WX;

Manuscript revising: XJH.

All authors have read and approved the final version of this manuscript to be published.

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DATA AVAILABILITY

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. **CODE**

AVAILABILITY

Not applicable. **DECLARATIONS**

Ethical Approval. Animal assays were performed with the approval of the Institutional Animal Ethics Committee following the Animal Care Guidelines for the Care and Use in The Second Xiangya Hospital, Central South University. The procedure and protocol have been reviewed and approved by the ethics committee of The Second Xiangya Hospital, Central South University. All patients were informed of the study and signed the written consent.

Consent to Participate. The informed consent was obtained from study participants.

Consent for Publication. The informed consent was obtained from study participants.

Conflict of Interest. The authors declare no competing interests.

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