

## Original Article

# MiR-663a/MiR-423-5p are involved in the pathogenesis of lupus nephritis via modulating the activation of NF- $\kappa$ B by targeting TNIP2

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**Abstract:** Lupus nephritis (LN) is a kidney disorder resulting from systemic lupus erythematosus (SLE), an autoimmune inflammatory disease. MicroRNAs (miRNAs) have emerged as a new class of therapeutic targets in LN treatment, but how they specifically contribute to the disease development remains unknown. In this study, the expression of miR-663a/miR-423-5p and TNIP2 were compared between human renal biopsy tissues from LN patients and renal cell carcinoma patients. Additionally, the LN mouse model was used to measure the levels of miR-663a/miR-423-5p and TNIP2 in the control group and the experiment group. Dual luciferase reporter assay was used to validate TNIP2 as the target of miR-663a/miR-423-5p. MiR-663a/miR-423-5p were highly expressed in kidney tissues from LN patients as compared to kidney tissues from SLE patients and normal tissues. TNIP2 showed comparatively low expression in tissues from LN patients. In the LN mouse model, the levels of miR-663a/miR-423-5p were improved whereas TNIP2 was reduced in response to renal injury stimulated by pristine. MiR-663a/miR-423-5p mimics and inhibitors triggered decrease and increase of TNIP2 levels, respectively. Dual luciferase assay showed that TNIP2 was a direct target of miR-663a/miR-423-5p. In addition, detection of inflammatory factors confirmed that miR-663a/miR-423-5p and TNIP2 fundamentally contributed to LPS-induced NF- $\kappa$ B activation. Our findings suggested the involvement of miR-663a/miR-423-5p-TNIP2-NF- $\kappa$ B axis in the development of LN, thereby providing new therapeutic targets for LN treatment.

**Keywords:** miR-663a/miR-423-5p, lupus nephritis, NF- $\kappa$ B, TNIP2, inflammatory

## Introduction

Systemic lupus erythematosus (SLE), also known as lupus, is an autoimmune inflammatory disease, which predominantly affects females. Most patients with SLE are likely to develop lupus nephritis (LN) within 10 years, which might cause premature death. Immunosuppressive drugs have significantly advanced the survival of patients with LN. However, due to drug resistance through activation of signaling cascade, these broad-spectrum drugs are usually inefficient in controlling disease symptoms. Thus, identifying more therapeutic targets is critical for the treatment of LN.

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling significantly contributes to the inflammation process by regulating transcription of pro-inflammatory genes [1]. In their inactive form, NF- $\kappa$ B family mem-

bers are sequenced in the cytoplasm by I $\kappa$ B $\alpha$  and p100. When activated by pro-inflammatory stimulation by TNF $\alpha$  and LPS, I $\kappa$ B $\alpha$  is degraded to release NF- $\kappa$ B into the nucleus [2]. Overactive NF- $\kappa$ B signaling has been associated with different inflammatory and autoimmune diseases such as LN.

TNIP2 (also known as ABIN2) was initially discovered in a yeast two-hybrid screen as the binding partner of A20, a negative regulator of NF- $\kappa$ B signaling [3]. By increasing IKK $\alpha$  autophosphorylation and kinase activity, TNIP2 increases IKK $\alpha$ -mediated NF- $\kappa$ B activation to induce transcription of NF- $\kappa$ B target genes [4]. Over-expression of TNIP2 was reported to inhibit TNF $\alpha$ -induced NF- $\kappa$ B activation and contribute to cell proliferation in human hepatocellular carcinoma [5]. Even though the TNIP2 homolog TNIP1 was linked to SLE pathogenesis [6], the

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**Table 1.** Clinical and histopathological data of LN patients

Classification	Number	Average age (years)	Proteinuria (g/day)	Serum creatinine (umol/L)
III	3	30.20±3.56	1.35±0.06	92.18±15.21
IV	21	36.55±6.75	3.45±0.10	189.68±101.31
V	5	31.76±8.45	2.84±1.38	174.12±73.36

Total LN: 29. Data are expressed as mean ± SD.

relation between TNIP2 and LN is not completely understood.

MicroRNAs (miRNAs) are small non-coding RNAs, which function in post-transcriptional regulation of gene expression. Deregulation of miRNAs is associated with many inflammatory diseases such as SLE [7-9]. MiR-663 was shown to be involved in many biological processes including carcinogenesis development and inflammation [10-12]. Nevertheless, little is known about its role in LN. MiR-423-5p was associated with the proliferation and chemoresistance of glioblastomas [13]. Whether and how miR-423-5p functions in the inflammatory process remain largely unknown.

In the current study, we assessed the levels of miR-663/miR-423-5p and TNIP2 in tissue samples from LN patients. Furthermore, we demonstrated that TNIP2 was the direct target of miR-663/miR-423-5p in 293T cells. NF-κB signaling activated by miR-663/miR-423-5p through down-regulation of TNIP2 was also evaluated. Our data suggested a miR-663/miR-423-5p-TNIP2-NF-κB axis in LN, offering new therapeutic targets for its treatment.

### Material and methods

#### *Clinical samples*

The utilization of renal biopsy tissues was approved by the ethics committee of Cangzhou Central Hospital. A total of 56 female SLE patients were selected for the study between 2010 and 2013. A patient was confirmed to have SLE if she had biopsy-proven LN by ANA or anti-dsDNA and met at least four criteria of the American College of Rheumatology (ACR), including at least one clinical and one immunological criterion. Of the 56 SLE patients, 29 met the criteria for LN [14]. All participants voluntarily signed an informed consent form to participate in this study. The consent procedure was approved by the ethics committee of

Cangzhou Central Hospital. No patient was treated with immunosuppressive drugs before diagnostic renal biopsy. The diagnosis of LN biopsies was performed as per the criteria of the International Society of Nephrology/Renal Pathology Society (ISN/RPS)

[15]. Pathological grading of LN patients is summarized in **Table 1**. A total of 25 normal kidney tissues, acquired from nephrectomies of renal cell carcinoma patients, served as normal controls.

#### *Generation of LN mouse model*

Female BALB/c mice, aged 6-8 weeks, were purchased from the Vital River Company (Beijing, China). The Principles of Laboratory Animal Care (NIH publication number 85Y23, revised 1996) were followed. The experimental protocol was approved by the Animal Care Committee, Peking University Health Science Center (LA2010-059). The mice were randomly divided into the experiment group and the control group. In the experiment group, mice were intraperitoneally (i.p.) injected with 0.5 ml pristine (Sigma-Aldrich), while the control group mice were i.p. injected with 0.5 ml PBS. Five months after treatment, the mice were sacrificed, their kidney tissues were fixed with 10% formalin overnight and then paraffin-embedded. Paraffin-embedded renal tissue sections were stained using hematoxylin and eosin (H&E). The pathological changes in the kidney tissues were established by analyzing the glomerular and tubulointerstitial activities as previously reported [16].

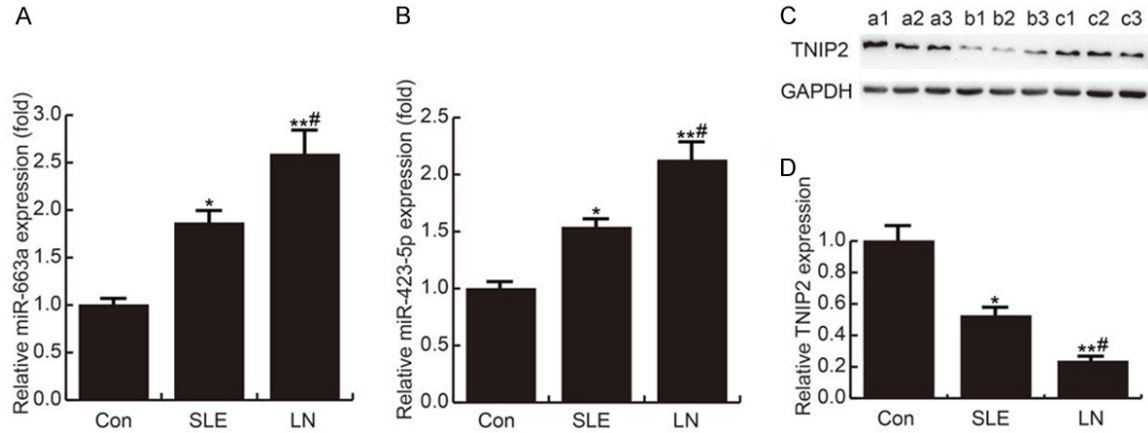
#### *Cell culture and agent*

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) enhanced with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin at 37°C with 5% CO<sub>2</sub>. Recombinant human LPS was purchased from Invitrogen (Carlsbad, CA, USA).

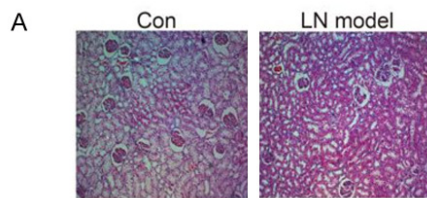
#### *LPS treatment*

HEK293T cells were seeded into 6-well plates at 1×10<sup>6</sup> cells/well. NF-κB signaling was activated by 1 μg/ml LPS treatment for an estimated 24 hours.

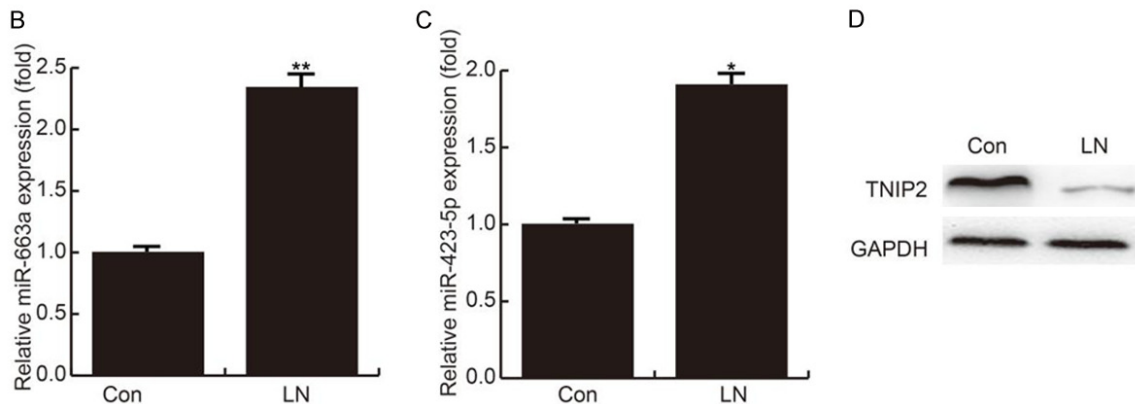
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**Figure 1.** Highest level of miR-663a/miR-423-5p were accompanied by lowest protein level of TNIP2 in kidney tissues of LN patients compared with tissues from SLE patients and normal control. A, B: Highest level of miR-663a/miR-423-5p was observed in kidney tissues of LN patients in comparison with tissues of SLE patients and normal control. C: Lowest protein level of TNIP2 was observed in kidney tissues of LN patients in comparison with tissues of SLE patients and normal control; D: Lowest relative mRNA level of TNIP2 was observed in kidney tissues of LN patients in comparison with tissues of SLE patients and normal control; Lowest protein/mRNA level of TNIP2 was observed in kidney tissues of LN patients in comparison with tissues of SLE patients and normal control. \* $P < 0.05$ , \*\* $P < 0.01$  vs Con; # $P < 0.05$  vs SLE.



**Figure 2.** Elevated miR-663a/miR-423-5p were accompanied by decrease of TNIP2 in kidney tissues of LN model mice. A: H&E staining of the glomeruli of mice. B: Elevated miR-663a was detected in kidney tissues of LN model mice; C: Elevated miR-423-5p was detected in kidney tissues of LN model mice; D: Decrease expression of TNIP2 was detected in kidney tissues of LN model mice. \* $P < 0.05$ , \*\* $P < 0.01$  vs Con.



### RNA extraction and quantitative real-time PCR

Total RNA was extracted from the tissue samples by Trizol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. For detection of miRNA levels, reverse transcription was performed using the All-in-One miRNAqRT-PCR detection kit (Genecopoeia, Rockville, MD, USA). For TNIP2, RNA was subjected to cDNA synthesis using PrimeScript RT reagent kit (TaKaRa, Otsu, Shiga, Japan). Real-

time PCR was then performed with SYBR Premix Ex Taq (TaKaRa) in CFX96 RT-PCR system (Bio-Rad, Hercules, CA, USA). GAPDH was used as an internal control.

### Western blotting

The protein lysates were prepared using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Antibodies against TNIP2 (AINB2) and NF- $\kappa$ B (p65) were obtained from Santa Cruz

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Biotechnology (Santa Cruz, CA, USA). The Phospho-NF- $\kappa$ B (p-p65) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA) and GAPDH mouse monoclonal antibody was acquired from Kangchen (Shanghai, China). Briefly, the protein lysates were separated by SDS-PAGE, transferred to a PVDF membrane and then incubated with the designated antibodies. The blots were treated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce/Thermo Scientific, Rockford, IL) and imaged using ImageQuant LAS 4000 (GE healthcare, Little Chalfont, England).

### *Enzyme-linked immunosorbent assay (ELISA)*

The levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in HEK293T cells were detected using specific ELISA kits (RayBiotech, GA, USA) as per the manufacturer's protocols. The samples were added into 96-well plates coated with primary antibodies for about 2.5 hours. After washing, biotinylated antibodies were added into every well, and detected using HRP-conjugated streptavidin and chromogen reagent. Then the absorbance was immediately read at 450 nm in an ELISA reader (BioTekPowerWave XS, Winooski, VT, USA). Every experiment was repeated three times.

### *Dual luciferase assay*

The full length TNIP2 3'-UTR was cloned into pGL3-Control Vector (Promega, Madison, WI, USA) to create the luciferase reporter. MiRNA mimic and inhibitor were synthesized by GenePharma Co., Ltd (Shanghai, China). pRL-TK served as an internal control and was co-transfected into the cells. For luciferase reporter assays, HEK293T cells were seeded in 24-well plates. Luciferase reporter vectors were transfected into the cells along with miRNA mimic or inhibitor using lipofectamine 2000 (Invitrogen). After two days, the cells were harvested and analyzed using the dual-luciferase assay kit (Promega). Each experiment was repeated three times. The results were presented as relative luciferase activity (Firefly luciferase/Renilla luciferase).

### *Statistical analysis*

All data were analyzed with Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) and expressed as means  $\pm$  SE. The variations between the two groups were statistically

examined using the Student's t-test.  $P < 0.05$  indicated significant variations.

## Results

### *Up-regulated miR-663a/miR-423-5p were accompanied by decreased TNIP2 in kidney tissues of LN patients*

To analyze the expression of miR-663a/miR-423-5p and TNIP2 in LN patients, we detected the transcription level of miR-663a/miR-423-5p and protein level of TNIP2 in kidney samples from normal control, SLE patients and LN patients. As illustrated in **Figure 1**, highest expression of miR-663a/miR-423-5p was observed in the samples from LN patients as compared to the other two groups. Meanwhile, the protein level of TNIP2 was reduced in the LN patients as compared to the other two groups. Low levels of miR-663a/miR-423-5p and high levels of TNIP2 were detected in normal controls. These data indicated a negative correlation between miR-663a/miR-423-5p and TNIP2, which may be linked to the advancement of LN.

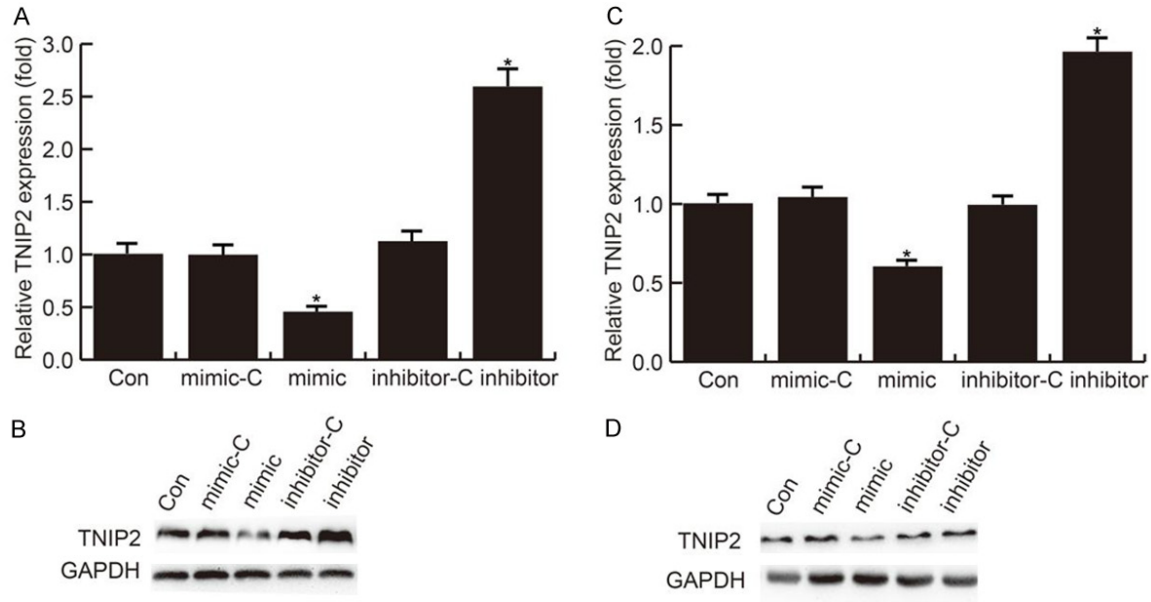
### *High miR-663a/miR-423-5p levels were accompanied by low TNIP2 level in kidney tissue of LN mouse model*

To further verify the negative correlation between miR-663a/miR-423-5p and TNIP2, we generated a lupus mouse model by i.p. injection of pristane to BALB/c mice. After five months, mesangial and endocapillary proliferation were observed along with an increase in the thickness of mesangial membranes in glomeruli of the model mice (**Figure 2A**). This indicated successful establishment of the lupus model. qPCR showed that the relative level of miR-663a/miR-423-5p was considerably higher in kidney tissues of the model group as compared to the control group (**Figure 2B, 2C**). Conversely, low protein expression of TNIP2 was detected in kidney tissues from lupus mice (**Figure 2D**). Therefore, in line with the data from the patient samples, the mouse model also showed a negative correlation between miR-663a/miR-423-5p and TNIP2.

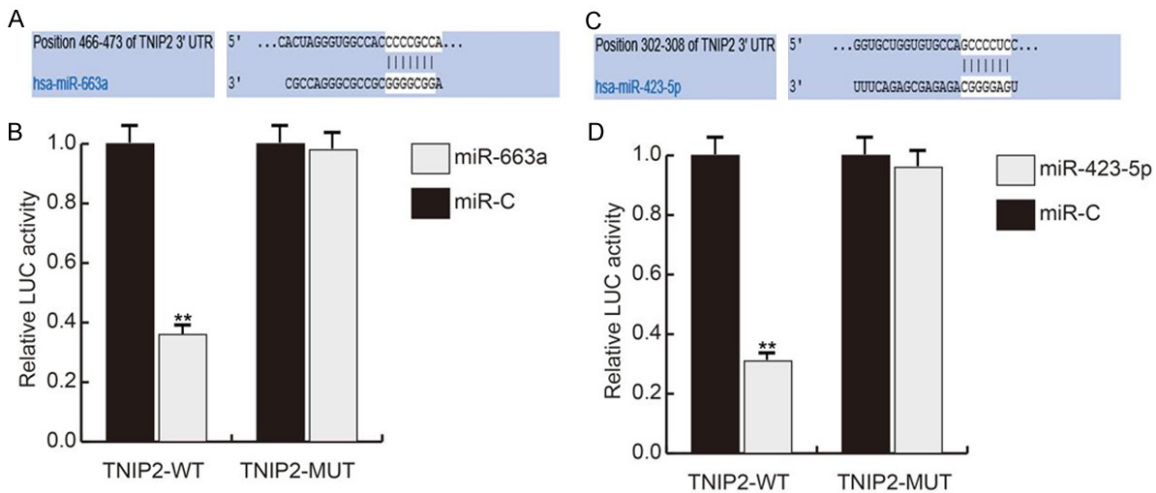
### *Validation of TNIP2 as the target gene of miR-663a/miR-423-5p in HEK293T cells*

To establish whether TNIP2 expression was modulated by miR-663a/miR-423-5p, HEK293T cells were transiently transfected with miR-

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**Figure 3.** TNIP2 expression was affected by miR-663a/miR-423-5p in HEK293T. A, B: In HEK293T, over-expression of miR-663a by miR-663a mimics represses the mRNA/protein expression of TNIP2. Inhibitor of miR-663a promoted the expression of TNIP2. Con: cells without any treatment; mimic-C: cells transfected with the control of miR-663a mimics; mimic: cells transfected with miR-663a mimics; inhibitor-C: cells transfected with the control of miR-663a inhibitor; inhibitor: cells transfected with miR-663a inhibitor. C, D: In HEK293T, over-expression of miR-423-5p by miR-423-5p mimics represses the mRNA/protein expression of TNIP2. Inhibitor of miR-423-5p promoted expression of TNIP2. Con: cells without any treatment; mimic-C: cells transfected with the control of miR-423-5p mimics; mimic: cells transfected with miR-423-5p mimics; inhibitor-C: cells transfected with the control of miR-423-5p inhibitor; inhibitor: cells transfected with miR-423-5p inhibitor. \* $P < 0.05$  vs Con.

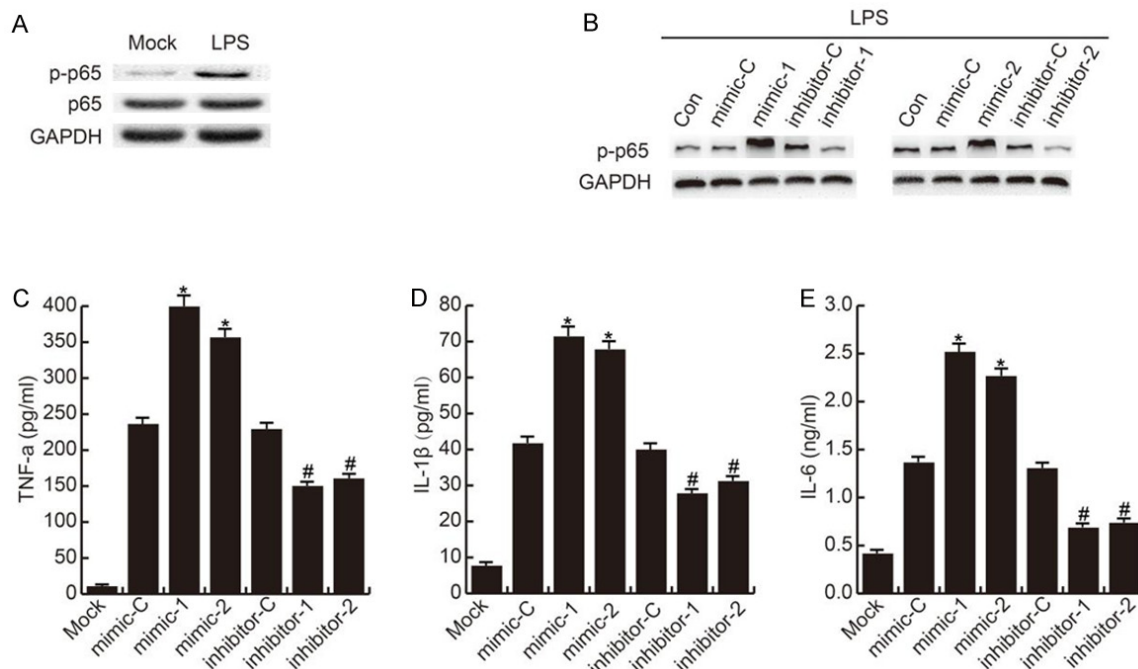


**Figure 4.** miR-663a/miR-423-5p directly targets TNIP2. A, C: Interaction between miR-663a/miR-423-5p and 3'UTR of TNIP2 was predicted using TargetScan; B, D: Luciferase activity was assessed by Dual luciferase assay. Here, "MUT" indicates the TNIP2 3'UTR with a mutation in the miR-663a/miR-423-5p binding site. UTR, untranslated region. All data are presented as the mean  $\pm$  SD. \*\* $P < 0.01$  vs control.

663a/miR-423-5p mimics and inhibitors. The mRNA and protein levels of TNIP2 decreased in cells transfected with miR-663a or miR-423-5p mimic. In contrast, inhibition of miR-663a or

miR-423-5p considerably amplified TNIP2 level (**Figure 3A-D**). To prove that TNIP2 was directly inhibited by miR-663a/miR-423-5p, a dual-luciferase reporter system was utilized. MiR-

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**Figure 5.** Over-expression of miR-663a/miR-423-5p enhanced LPS-induced NF-κB activation and promoted release of inflammatory factors. A: Protein levels of p65 and p-p65 were elevated in cells treated with LPS; B: Forced over-expression of miR-663a or miR-423-5p augmented increase of p-p65; lower-expression of miR-663a or miR-423-5p decrease p-p65 expression; C-E: Levels of inflammatory factors (TNFα, IL-1β and IL-6) were detected by ELISA. Secretion of inflammatory factors were observed in cells treated with LPS, forced over-expression of miR-663a or miR-423-5p further promoted secretion of inflammatory factors. Mock: cells without any treatment; mimic-1/inhibitor-1: cells transfected with miR-663a mimics or inhibitors; mimic-2/inhibitor-2: cells transfected with miR-423-5p mimics or inhibitors; mimic-C/inhibitor-C: cells transfected with the control of mimics/inhibitor. \*P<0.05 vs mimic-C, #P<0.05 vs inhibitor-C.

663a and miR-423-5p inhibited the firefly luciferase reporter activity of wild-type TNIP2 3'UTR. However, this inhibition was less obvious for 3'UTR with mutated binding sites (Figure 4A-D). Thus, TNIP2 was a direct target gene of miR-663a and miR-423-5p.

### *MiR-663a/miR-423-5p enhanced LPS-induced NF-κB activation by regulation of TNIP2*

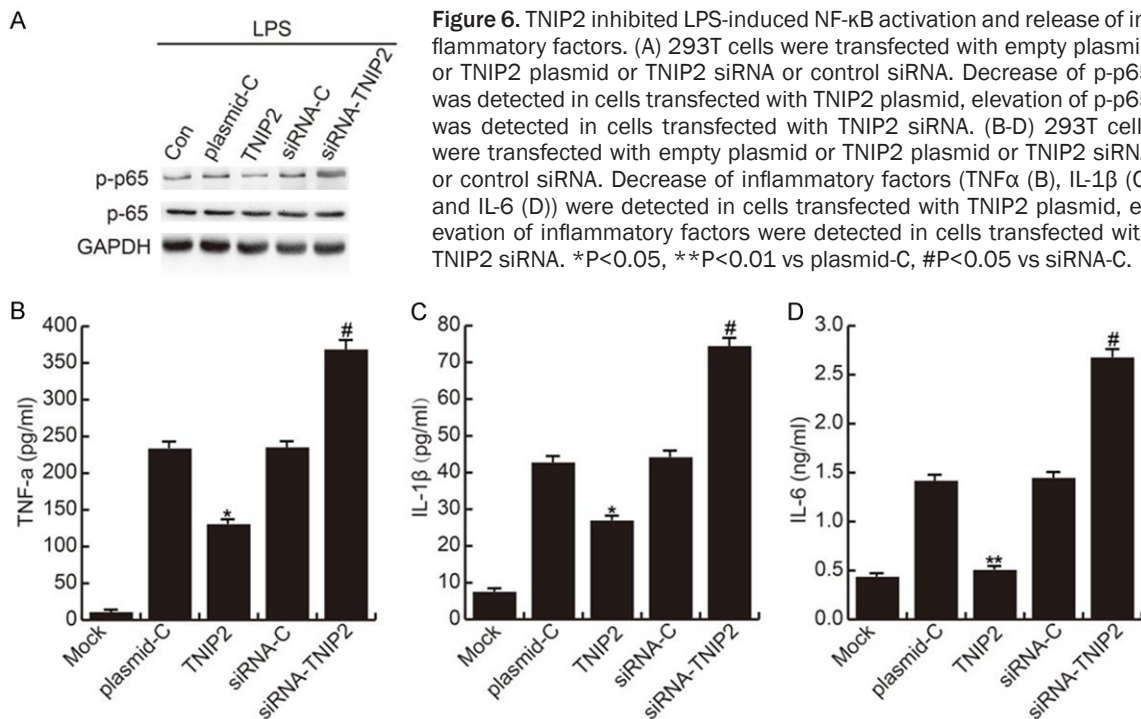
Next, we tested the effect of miR-663a/miR-423-5p over-expression on LPS-induced NF-κB activation. In HEK293T cells, LPS treatment increased p65 as well as p-p65 protein levels, depicting activation of NF-κB pathway (Figure 5A). Additionally, LPS triggered secretion of TNFα, IL-1β and IL-6 in the cells. Forced over-expression of miR-663a or miR-423-5p considerably enhanced p-p65 protein level, and secretion of TNFα, IL-1β and IL-6 (Figure 5B-E). Down-regulation of miR-663a or miR-423-5p also significantly enhanced p-p65 protein level, and secretion of TNFα, IL-1β and IL-6 (Figure 5B-E). TNIP2 knockdown by siRNA also incre-

ased p-p65, TNFα, IL-1β and IL-6 levels, whereas TNIP2 over-expression reduced p-p65, TNFα, IL-1β and IL-6 levels (Figure 6). These data indicated that miR-663a/miR-423-5p were involved in the inflammatory process by regulation of TNIP2.

### Discussion

Lupus nephritis (LN) is intimately linked with morbidity and mortality of the patients with systemic lupus erythematosus (SLE). The therapeutic impact of the current drugs is limited. MiRNAs are known to be involved in the pathogenesis of SLE. Identifying the specific miRNAs is significant for deep understanding of SLE and facilitating advancement of new targets. Our study indicated that tissues of LN patients had highest miR-663a and miR-423-5p levels, whereas SLE patients had moderate miR-663a and miR-423-5p levels. The lowest miR-663a and miR-423-5p levels were found in the control group. Additionally, in the LN model mice, miR-663a and miR-423-5p were also found to

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be considerably higher as compared to the control mice. These results showed that miR-663a and miR-423-5p were associated with LN.

Inflammatory factors play a fundamental role in the pathogenesis of kidney diseases, including LN [17]. The NF- $\kappa$ B signaling pathway is intimately related to initiation and advancement of LN through the transcriptional regulation of inflammatory factors [18]. TNIP2 was found to regulate NF- $\kappa$ B by binding to A20 (TNFAIP3), a well-known anti-inflammatory signaling molecule [19]. Earlier studies majorly focused on TNIP1, the homolog of TNIP2. The present study showed that protein level of TNIP2 was elevated in LN patients and LN model mice, suggesting its potential role in the pathogenesis of LN. Knockdown of TNIP2 in cells treated with LPS significantly blocked the NF- $\kappa$ B signaling activation and inhibited the release of inflammatory factors. Conversely, in cells treated with LPS, over-expression of TNIP2 increased NF- $\kappa$ B activation and enhanced the release of inflammatory factors. These data confirmed that TNIP2 was a potential driver of LN by regulating the NF- $\kappa$ B signaling pathway.

In hepatocellular carcinoma, miR-1180 was reported to activate the NF- $\kappa$ B signaling pathway by directly targeting TNIP2 [5]. The present

study showed a negative correlation between miR-663a/miR-423-5p and TNIP2 in the samples from LN patients as well as the mouse model. MiRNA mimics and inhibitors showed that TNIP2 was negatively controlled by miR-663a/miR-423-5p. In addition, TNIP2 was confirmed as a direct target of miR-663a/miR-423-5p through the dual luciferase assay. During the inflammatory process induced by LPS, the activation of NF- $\kappa$ B and the release of inflammatory factors was improved through the over-expression of miR-663a/miR-423-5p. Therefore, the present study confirmed a miR-663a/miR-423-5p-TNIP2-NF- $\kappa$ B axis in the pathogenesis of LN.

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### Disclosure of conflict of interest

None.

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