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LncRNA TUG1 affects the inflammatory factors and apoptosis of glomerular mesangial cells through targeting the miR-16-5p regulated cGAS-STING pathway in mice with lupus nephritis

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Abstract

Our study intends to investigate the effect and mechanisms of lncRNA TUG1 on the inflammatory factors secretion and apoptosis of glomerular mesangial cells of mice with lupus nephritis. lncRNA TUG1 in glomerular mesangial cells from normal or lupus nephritis mice were knocked down with siRNA followed by measuring TUG1 and miR-16-5p expression by RT-qPCR and TNF-α, IL-1β, IL-6, cGAS, STING, and TBK1 protein expression by Western blot and cell apoptosis by flow cytometry. The online bioinformatics database and dual-luciferase reporting system assessed the relationship between TUG1 and miR-16-5p. Compared with normal mesangial cells, TUG1 was up-regulated and miR-16-5p expression was decreased in LN glomerular mesangial cells. Knockdown of TUG1 or miR-16-5p overexpression inhibited TNF-α, IL-1β, and IL-6 secretion and promoted apoptosis. miR-16-5p inhibition reversed the TUG1's effect on apoptosis, cGAS-STING pathway, and TNF-α, IL-1β, and IL-6 secretion in glomerular mesangial cells. cGAS-STING pathway activator CDN reversed miR-16-5p and TUG1's effect on inflammatory factors and apoptosis of glomerular mesangial cells. In conclusion, TUG1 can promote the production of inflammatory factors and inhibit glomerular mesangial cells apoptosis via miR-16-5p-regulated cGAS-STING signaling.

Keywords: lncRNA TUG1; miR-16-5p; cGAS - STING pathways; lupus nephritis

Introduction

The systemic lupus erythematosus (SLE) is a complicated disease and is caused by both genetic and environmental factors, resulting in autoimmune disorder and further complications of organs. As one of the SLE complications, the morbidity and mortality of lupus nephritis (LN) are related to the poor prognosis [1,2]. If LN is not treated in time, it will cause kidney tissue damage [3]. The drugs or immunosuppressants to treat LN generally have side effects such as infection and liver damage. Therefore, it is critical and urgent to seek new treatments for LN.

With the rapid development of next-generation sequencing technology, the pathogenetic involvement of LncRNA in LN pathogenesis becomes a hot spot. It had been reported that the abnormal expression of lncRNAs such as RP11-2B6.2, linc0949, and linc0597 were involved in anti-cancer or carcinogenetic activities [4,5]. lncRNA Taurine upregulated gene 1 (TUG1) is abnormally expressed in a variety of tumors. In LN cells, TUG1 regulates the expression of miR-223/Sirt1, thereby activating the downstream PI3K/AKT and NF-κB signaling pathway to protect LN cells from LPS-induced inflammatory damage [6]. Studies have shown that in prostate cancer, TUG1 activates Wnt/β-catenin signaling pathway by regulating miR-496 [7]. miR-16-5p has been reported to be dysregulated in multiple diseases such as breast cancer, colorectal cancer, and spinal cord injury, through affecting cell proliferation, apoptosis, and inflammatory response [8-10]. A similar finding was observed in the cGAS-STING signaling pathway, which also participates in the proliferation, apoptosis, and inflammation of cancer cells [11,12]. The excessive proliferation of glomerular mesangial cells is recognized as the primary pathological indicator of LN and an important cause of kidney

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injury [13]. As a result, this study established the glomerular mesangial cell line from LN mice model to explore TUG1's role, aiming to provide a novel therapeutic target and a diagnostic basis for the treatment of LN.

Methods

Cell line establishing in LN mice model

18 MRL/faslpr and 6 healthy MRL/MPJ 12-week-old male mice (Institute of Animal Modeling, Nanjing University) weighted 22.0-26.0 g were used to establish the LN model for Mesangial cells and normal control cells, respectively. Mice were fed in SPF animal room under standard conditions with the relative temperature of 20±2℃, relative humidity of 50±2%, light and dark conditions for 12 hrs. 8-items urine dipstick was used to measure the urine protein in the urine for one week until the urine protein concentration level reached 0.1~1 mg/L, which is an indicator of the successfully established LN model.

The two sides of mice kidney tissues were obtained and cut into approximate 1 mm×1 mm pieces followed by digestion using 1 mg/ml collagenase. the digested tissue was subjected to grind, centrifuge, and discard the supernatant. The DMEM medium with 10% FBS (Invitrogen, USA) re-suspend glomerular mesangial cells which were then cultured. When the cell density reached 70% in a 6-well plate, the cells were transfected according to the Lipofectamine TM2000 (TaKaRa, Dalian) manufacturers' protocol with siRNAs. All the follow-up experiments were carried out 48hrs post-transfection.

RT-qPCR

RNA of glomerular mesangial cells was extracted with Trizol kit (TaKaRa, Dalian). According to the manufacturers' protocol, cDNA was synthesized using the reverse transcription kit (TaKaRa, Dalian) with 40 cycles of amplification: 95 ℃ for 30s, 94 ℃ for 15s, 56 ℃ for 45s, 72 ℃ for 45s. Real time PCR was detected and analyzed with the CFX96 Touch qPCR System (Bio-Rad, U.S.)

Western blotting (WB)

Proteins were extracted from the conditioned glomerular mesangial cells using the lysis buffer and transfer to polyvinylidene fluoride (PVDF) membrane by gel electrophoresis (SDS-PAGE). After 1hr of blocking with skim milk at room temperature, the following antibodies (Abcam, USA) were added: anti-TNF-α (1:1000), anti-IL-1β (1:1000), anti-IL-6 (1:1000), anti-cGAS (1:1000), anti-STING (1:1000) and anti-TBK1 (1:1000). The membrane was developed with chemiluminescent solution after 1hr of secondary antibody incubation and images were captured on Bio Spectrum platform (UVP, U.S.)

Flow Cytometry (FC)

Each group of conditioned cells was digested with trypsin and centrifuged at 1200 r/min for 2 mins to remove the supernatant. 1 ml of cell suspension was washed with PBS and cell apoptosis was assessed using Annexin-V-FITC/PI kit (Beijing Soleibao).

Dual-luciferase reporter gene assay

The online open-source platform starBase (Sun Yat-sen University) was serviced to predict the binding sites of TUG1 and miR-16-5p. Plasmid constructs of the wild-type vector (TUG1-WT) and binding site mutant vector (TUG1-MUT) were generated for dual-luciferase assay. TUG1 vectors were co-transfected with miR-NC or miR-16-5p in glomerular mesangial cells. After, the luciferase activity was detected 48 hrs post-transfection using dual-luciferase activity detection kit (Beijing Soleibao).

Statistical Analysis

SPSS 23.0 software analyzed data which were expressed as mean ± standard deviation (SD) and assessed by one-way ANOVA or t-test. $P<0.05$ indicates a significance.

Results

TUG1 impacts the inflammatory factors and apoptosis of glomerular mesangial cells

To firstly verify TUG1's expression in LN models, RT-qPCR was used to measure lncRNA TUG1 level in glomerular mesangial cells and found it was significantly elevated in glomerular mesangial cells from LN mice (Fig.1A). To detect whether the downstream inflammatory factors were affected by TUG1, a transient knockdown of TUG1 by siRNA in control glomerular mesangial cells was verified in RNA (Fig.1B) and protein level (Fig.1C), which showed a significant downregulation of TNF-α, IL-1β, and IL-6 (Fig.1C). Flow cytometry demonstrated that the knock-down of TUG1 significantly increased the apoptotic rate of glomerular mesangial cells (Fig.1D).

miR-16-5p effects inflammatory factors and glomerular mesangial cell apoptosis

Similar to the finding in TUG1 using LN mice model, RT-qPCR showed significantly down-regulated miR-16-5p in glomerular mesangial cells

from LN mice (Fig.2A). Ti assess miR-16-5p's role, we further overexpressed miR-16-5p in glomerular mesangial cells (Fig.2B) and found miR-16-5p overexpression was able to suppress the secretion of TNF-α, IL-1β, and IL-6 (Fig.2C) as well as significantly decrease the apoptotic rate in glomerular mesangial cells (Fig.2D).

TUG1 regulates miR-16-5p expression

To verify the potential regulatory role of TUG1 in miR-16-5p, we first used the online database starBase to predict the potential binding of TUG1 in miR-16-5p (Fig.3A). Then based on the predicted binding sequences, a dual-luciferase assay was carried out to further prove the interaction between TUG1 and miR-16-5p. miR-16-5p overexpression in TUG1-WT significantly decreased luciferase activity, while the luciferase activity in the mutated TUG1 was not changed (Fig.3B). In turn, the knocking down of TUG1 removed miR-16-5p's suppression effect, resulting in an increase of miR-16-5p RNA level (Fig.3C).

miR-16-5p inhibition reverses the effects of TUG1 knockdown on inflammatory factors and apoptosis of glomerular mesangial cells

To prove the regulation of TUG1 and miR-16-5p in roles of inflammatory factors and apoptosis, we tested the TUG1's effect when the miR-16-5p signaling pathway was inhibited. Single Knocking down of TUG1 resulted in the increase of miR-16-5p expression, which was reversed by a combination of miR-16-5p inhibitor (Fig.4A). In parallel with the changes in the miR-16-5p RNA level, the inflammatory factors and apoptosis rate of glomerular mesangial cells showed a similar trend (Fig. 3B, 3C). Inhibition of TUG1 induced cell apoptosis and reduced the secretion of the inflammatory factors. However, all the effects were reversed after inhibition of the downstream target of TUG1, the miR-16-5p, (Fig. 3B, 3C). These provided direct evidence that the regulation effects of TUG1 on inflammatory factors and apoptosis in glomerular mesangial cells was through miR-16-5p.

TUG1 regulates the cGAS-STING signaling pathway by targeting miR-16-5p

The knocking down of TUG1 significantly increased the expression of cGAS, STING, and TBK1 in glomerular mesangial cells (Fig.5). To prove whether TUG1's effects on the cGAS-STING pathway were also through miR-16-5p, the same strategy was applied by blocking miR-16-5p expression. As expected, the regulatory effect of TUG1 on the cGAS-STING pathway was rescued after miR-16-5p knockdown (Fig.5).

Effect of Astin C pathway inhibitor on cell inflammatory factors and apoptosis

The STING pathway is predominantly activated by cyclic dinucleotide (CDN), which is the second messenger generated by cGAS. To further address whether TUG1's effect on glomerular mesangial cells is through targeting miR-16-5p regulated cGAS-STING pathway, proteins level of cGAS, STING and TBK1 were measured under the conditions of single knocking down of TUG1, a combination of TUG1 and miR-16-5p, or additionally pathway activation by CDN (Fig.6A). The knocking down of miR-16-5p was able to rescue the up-regulation effect in the cGAS-STING pathway induced by TUG1 knockdown (Fig.6A, left panel). Additional cGAS-STING pathway activation partially reversed the effect of TUG1-miR-16-5p dual-knockdown on cGAS-STING pathway (Fig.6A, left panel). As a result, the consistent restore trends induced by CDN re-activation were observed in the cGAS-STING downstream regulatory targets, the inflammatory factors and apoptosis in glomerular mesangial cells (Fig. 6B, 6C).

Discussion

The pathogenesis of LN is very complicated and still remains unclear. Early diagnosis and treatment can control the inflammation in advance to prevent postoperative recurrence and metastasis, which can then improve the 5-year prognostic survival rate. LN is an autoimmune disease which is ranged as the most common glomerular disease in China [13,14]. Therefore, it is urgent to reveal the pathogenesis and development mechanism of LN to provide novel directions for early diagnosis and treatment. lncRNA can regulate the expression of several downstream miRNAs, thereby affecting the growth and development of cancer cells [6]. Several studies have shown that lncRNAs were abnormally expressed in SLE, such as GAS5, MALAT1, and NEAT1 [15]. However, there are still existing several contraries or confusing study findings compared to our study. Cao et al. [16] found that compared with normal tissues, TUG1 was down-regulated in the peripheral blood of patients with SLE, while our study demonstrated an up-regulation in LN glomerular mesangial cells from mice model. Consistent with our study, Zhang et al. [17] suggested that NEAT1 can target miR-146b to up-regulate TRAF6 and activate NF-κB signaling in mesangial cells. Studies have shown that miR-130b, miR-200b-5p, miR-141-5p and miR-200c-5p are down-regulated in LN [18,19]. miR-203 was also

reported to be down-regulated in LN tissues and cells and its overexpression could inhibit the inflammatory response of TRAF6 in LN [20]. As the main pathological manifestations of glomerular mesangial cells, apoptosis and inflammatory factors were focused on in our study. In our mice LN model, TUG1 in glomerular mesangial cells was up-regulated while miR-16-5p was decreased significantly.

cGAS is a recently discovered cytoplasmic DNA sensor that can activate stimulator of interferon genes (STING) to regulate and promote the expression of type I interferon (IFN) and further affects innate immune response [21,22]. Studies have shown that the cGAS-STING pathway participates in the physiological processes of tumors and inflammation [23]. It was reported that in patients with LN, lncRNA RP11-2B6.2 was up-regulated to promote IFN-1 signaling pathway [4]. This study further demonstrated that miR-16-5p inhibition reversed the inhibitory effect of TUG1 on the cGAS, STING, and TBK1 protein expression in glomerular mesangial cells. Further experiments indicated that addition of CDN can rescue the effect of TUG1-miR-16-5p dual-inhibition induced inflammatory factors up-regulation and apoptosis inhibition in glomerular mesangial cells. However, whether TUG1 is dysregulated in patients with LN was not assessed in our study, which is the main study limitation and requires further investigations to further demonstrate the clinical relevance of abnormal TUG1 expression to LN pathogenesis.

In summary, TUG1 negatively regulates miR-16-5 expression, leading to cGAS-STING signaling activation to promote inflammatory factors secretion as well as inhibit glomerular mesangial cell apoptosis, indicating that lncTUG1 might be a novel potential target for treating LN.

Acknowledgments

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Disclosure of conflict of interest

None.

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Figure legends

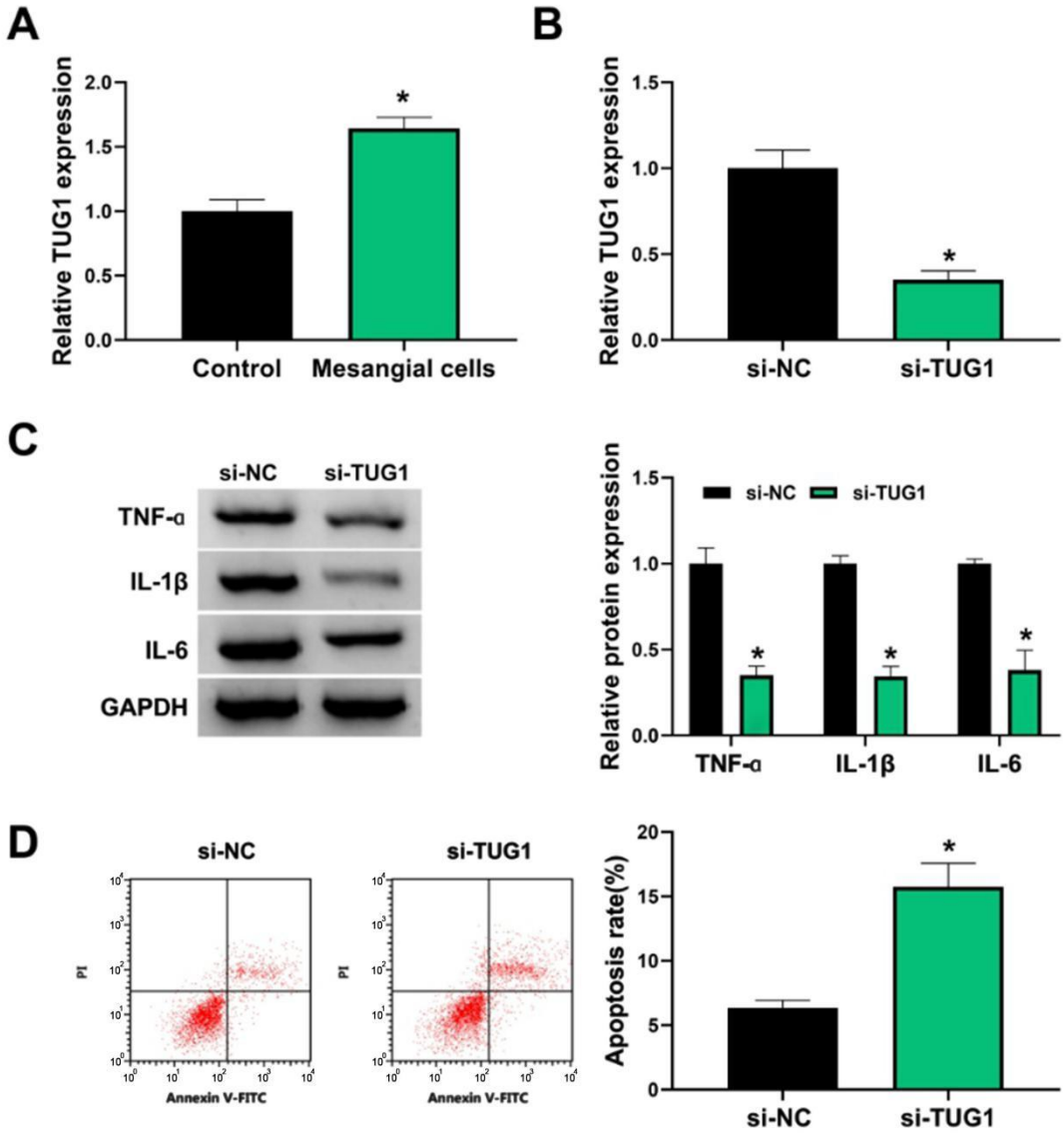

Figure 1**. The effect of TUG1 on the inflammatory factors and apoptosis of glomerular mesangial cells**

(A) RT-qPCR measurement of TUG1 lncRNA expression in glomerular mesangial cells comparing the health with LN mice models (n=6 and 18, respectively). (B) knockdown of lncRNA TUG1 was verified by RT-qPCR in glomerular mesangial cells. (C) WB measurement the expression of inflammatory factors TNF-α, IL-1β, and IL-6 affected by TUG1. (D) The effect of knocking down TUG1 on the apoptosis of glomerular mesangial cells.

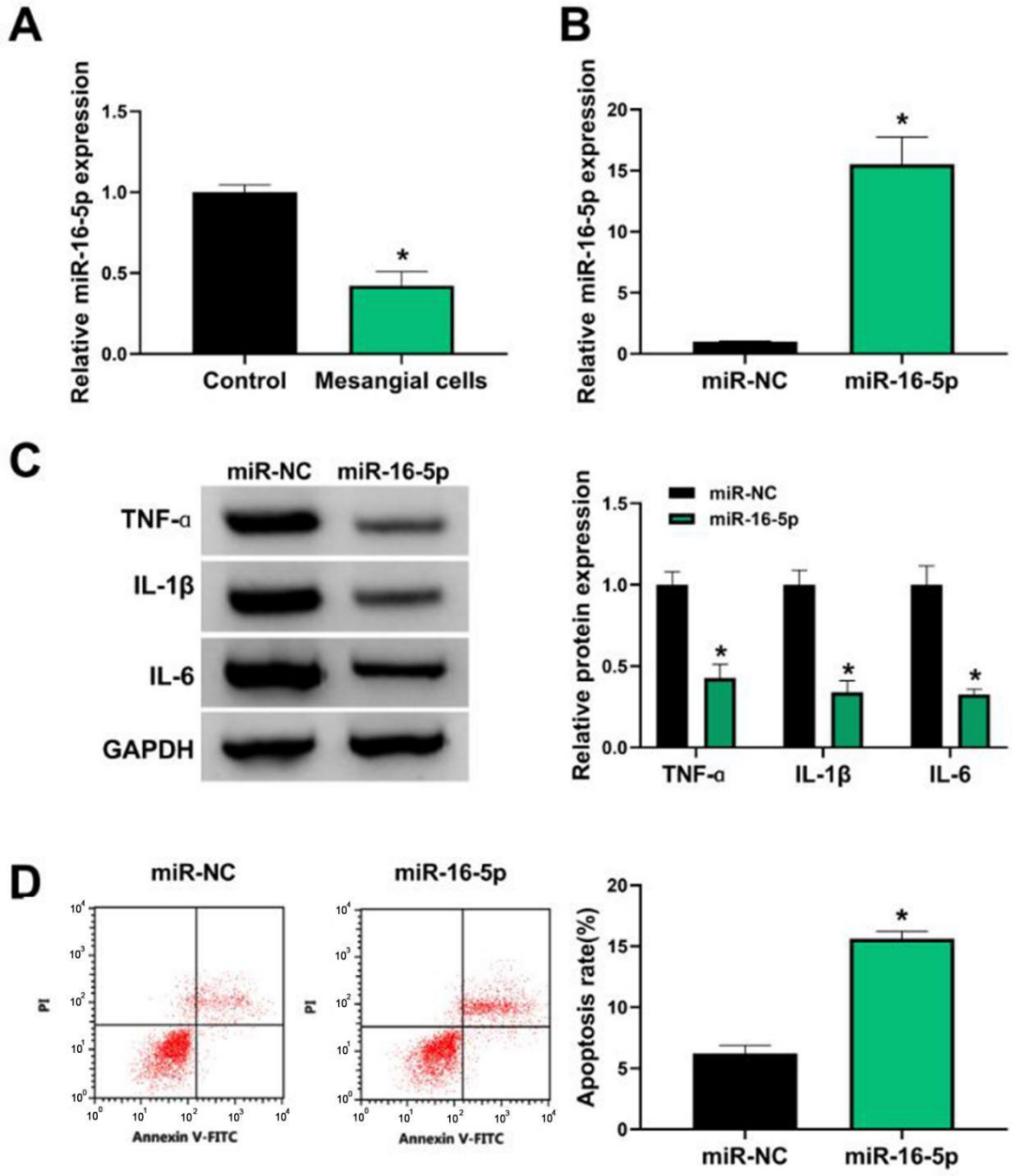

Figure 2. **Effects of miR-16-5p on inflammatory factors and apoptosis of glomerular mesangial cells**

(A)The expression of miR-16-5p in glomerular mesangial cells from health or LN mice measured by RT-qPCR (n=6 and 18, respectively). (B) The expression of overexpressed miR-16-5p in glomerular mesangial cells measured by RT-qPCR. (C) Inflammatory factors TNF-α, IL-1βAnd IL-6 expression level under the miR-16-5p overexpression measured by WB. (D) The effect of overexpression of miR-16-5p on the apoptosis of glomerular mesangial cells.

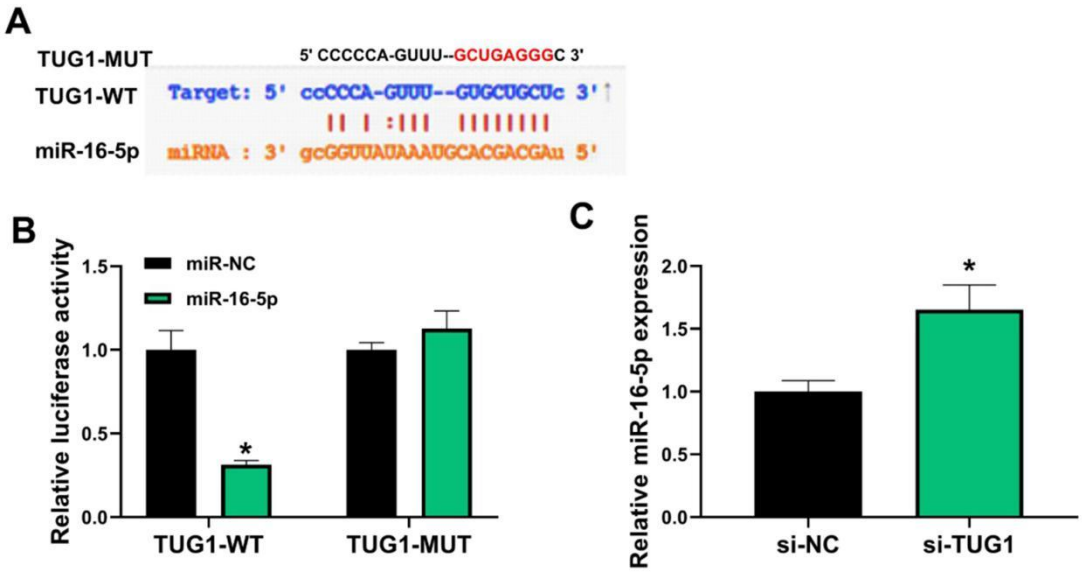

Figure 3. **TUG1 regulates the expression of miR-16-5p**

(A) starBase predicts the binding site of TUG1 and miR-16-5p; (B) Targeting relationship between dual-luciferase TUG1 and miR-16-5p; (C) miR-16-5p expression detected by RT-qPCR after Knocking down of TUG1.

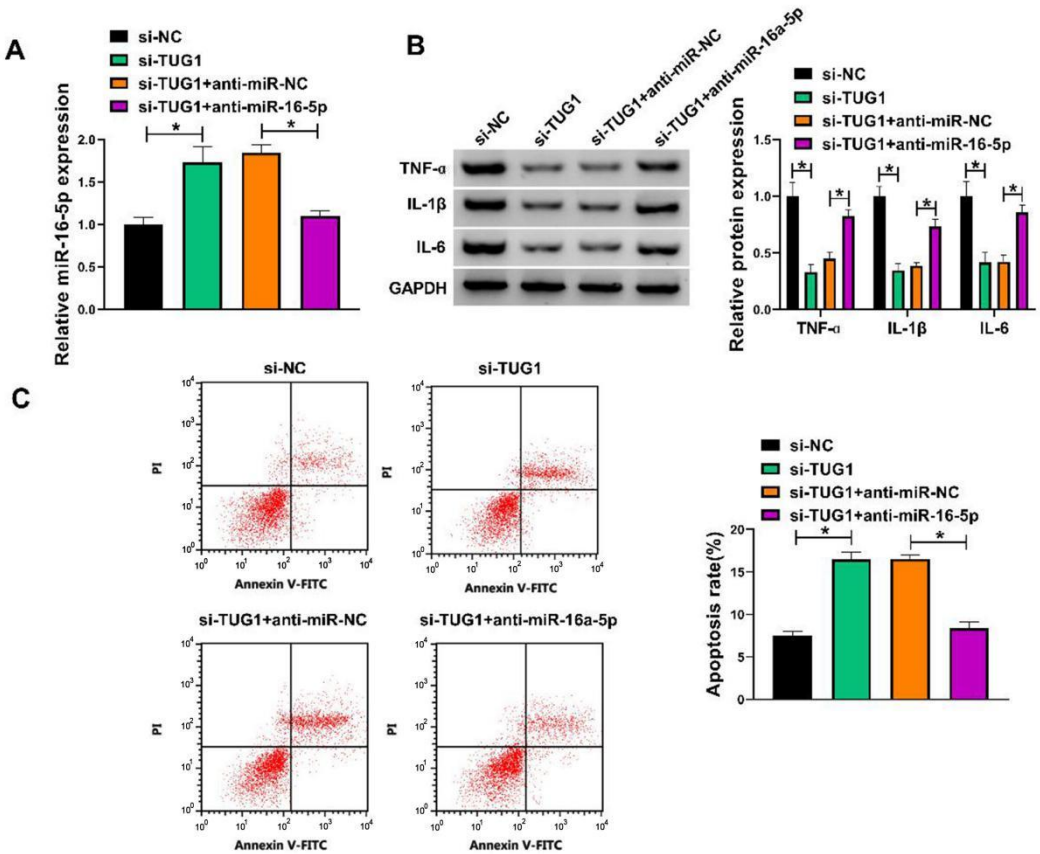

Figure 4. **Inhibition of miR-16-5p reverses the effect of knockdown of TUG1 on inflammatory factors and apoptosis of glomerular mesangial cells**

(A) Inhibition of miR-16-5p or TUG1 verified by RT-qPCR. (B) Protein's level of inflammatory factors TNF-α, IL-1β, and IL-6 were detected by WB after knocking down of TUG1 with or without the combination of miR-16-5p inhibition in glomerular mesangial cells. (C) The apoptosis rate was detected after the knocking down of TUG1 with or without the combination of miR-16-5p inhibition in glomerular mesangial cells.

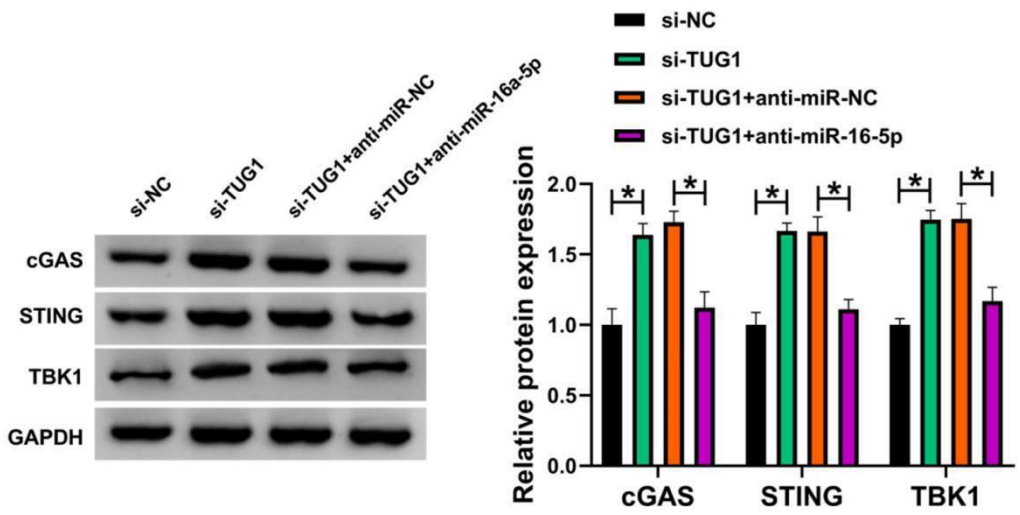

Figure 5. **TUG1 regulates the cGAS-STING pathway by targeting miR-16-5p.** Protein's level of cGAS, STING, and TBK1 were detected by WB after knocking down of TUG1 with or without the combination of miR-16-5p inhibition in glomerular mesangial cells

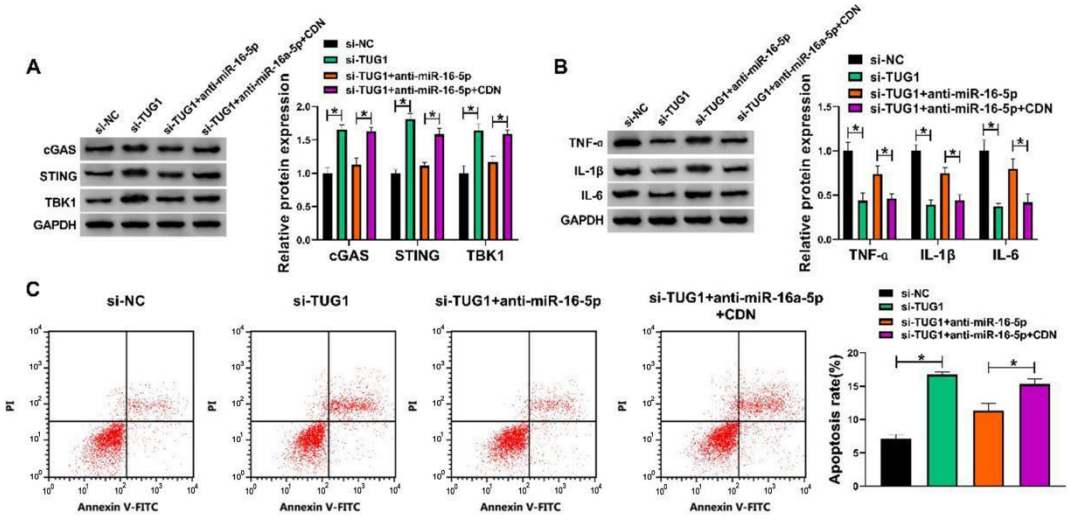

Figure 6. **Effect of CDN pathway activator on cell inflammatory factors and apoptosis** (A) The effect of CDN on cGAS/STING/TBK1 protein expression measured by WB. (B) The effect of CDN on inflammatory factors TNF-α, IL-1β, and IL-6 protein expression measured by WB. (C) The effect of CDN on cell apoptosis measured by FC.