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ORIGINAL PAPER

Circ_0091702 serves as a sponge of *miR-545-3p* to attenuate sepsis-related acute kidney injury by upregulating *THBS2*

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Abstract

Circular RNA (circRNA) has been shown to play an important function in the progression of human diseases, including sepsis with acute kidney injury (AKI). However, the role and mechanism of *circ_0091702* in sepsis-induced AKI have yet to be confirmed. Lipopolysaccharide (LPS) was used to induce HK2 cells to construct AKI cell models in vitro. Quantitative real-time PCR was used to measure the expression of *circ_0091702*, inflammatory cytokines, microRNA (*miR*)-*545-3p* and thrombospondin 2 (*THBS2*). Cell counting kit 8 assay and flow cytometry were used to assess cell viability and apoptosis. Besides, the protein levels of apoptosis markers and *THBS2* were evaluated by western blot analysis. In addition, the concentrations of inflammatory cytokines were detected by enzyme-linked immunosorbent assay (ELISA). Cell oxidative stress was determined by detecting the contents of oxidative stress markers. Dual-luciferase reporter assay and RIP assay were used to confirm the relationship between *miR-545-3p* and *circ_0091702* or *miR-545-3p* and *THBS2*. *Circ_0091702* was downregulated in septic AKI patients and LPS-induced HK2 cells. *Circ_0091702* could attenuate LPS-induced HK2 cell injury, while its silencing had an opposite effect. In the terms of mechanism, *circ_0091702* could act as a sponge of *miR-545-3p*, and *miR-545-3p* could directly target *THBS2*. Functional experiments revealed that *miR-545-3p* could reverse the alleviating effect of *circ_0091702* on LPS-induced HK2 cell injury, and *THBS2* knockdown also could overturn the suppressing effect of *miR-545-3p* inhibitor on LPS-induced HK2 cell injury. Additionally, we also suggested that *circ_0091702* could sponge *miR-545-3p* to regulate *THBS2* expression. In conclusion, our results showed that *circ_0091702* could suppress LPS-induced HK2 cell injury via the *miR-545-3p*/*THBS2* axis, indicating that *circ_0091702* might be an important biomarker for relieving sepsis-related AKI.

Keywords Sepsis · Acute kidney injury · LPS · *circ_0091702* · *miR-545-3p* · *THBS2*

Introduction

Sepsis is a common complication of critically ill patients and an important risk factor for acute kidney injury (AKI; Bellomo et al. 2017; Poston and Koyner 2019). The inflammatory injury and oxidative stress of cells is considered to be an important cause of sepsis-related AKI (Al-Harbi et al. 2019; Jia et al. 2018). Compared with non-septic AKI, patients with septic AKI have a worse clinical prognosis due to the more rapid onset and more severe kidney injury (Hamzic-Mehmedbasic et al. 2015; Pinheiro et al. 2019). Therefore, identifying promising biomarkers of sepsis-related AKI is of great significance for the early detection and treatment of AKI.

Non-coding RNA (ncRNA) is a kind of RNA that has a regulatory effect and does not encode protein, which is widely present in organisms (Mattick and Makunin 2006). Circular RNA (circRNA) is a new type of special ncRNA molecule that forms a circular structure by covalent bonds, which is a key molecule that maintains normal cell functions and participates in the development of diseases (Chen and Yang 2015; Patop et al. 2019). In addition, existing studies have also shown that circRNA has an important gene expression regulation effect at the transcription and post-transcription level. It can act as a sponge of microRNA (miRNA) and indirectly relieve the inhibitory effect of miRNA on

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its target, which has been confirmed in many researches (Wang et al. 2019; Yu and Liu 2019). At present, many circRNAs have been found to be abnormally expressed in sepsis and associated with the progression of sepsis, including *circ-PRKCI* (Wei and Yu 2020) and *circ_0001105* (Liu et al. 2020). Studies have suggested that the knockdown of *circ_0001679* and *circ_0001212* could alleviate the sepsis-induced acute lung injury (Zou et al. 2020). However, the role of many circRNA in sepsis-related AKI remains unclear.

Lipopolysaccharide (LPS) is an endotoxin that is often used to induce a variety of cell injury models including AKI (Chen et al. 2017; Riemondy et al. 2019). *Circ_0091702* is located at chrX and derived from the vacuolar ATPase assembly factor (*VMA21*) genic, so it is also called *circVMA21*. In past studies, *circ_0091702* was found to alleviate septic AKI rat models and inhibit LPS-induced tubular epithelial cell apoptosis and inflammation, so it might be a target for sepsis-related AKI (Shi et al. 2020c). Therefore, more studies are needed to confirm the function of *circ_0091702* and provide more theoretical basis for it to become a potential therapeutic target of sepsis-induced AKI. This study is to explore the role of *circ_0091702* in LPS-induced AKI cell model, and reveal its potential molecular mechanism through bioinformatics analysis.

Materials and methods

Blood samples collection

The blood of 33 patients diagnosed with septic AKI was collected from People's Hospital of Tiantai County. In addition, we collected the blood from 33 non-affected individuals who were undergoing a healthy medical examination at this hospital, matched to the age and sex of the patients with septic AKI. The serum was collected after centrifugation and stored at − 20 °C. The inclusion criteria for patients with sepsis are as follows: hypothermia or fever (< 36 °C or > 38.5 °C), tachycardia (> 90 beats/min), leukopenia (< 4000/mm^3), leukocytosis (> 12,000/mm^3), tachypnea (> 20 breaths/min or $PaCO_2$ < 32 mmHg) and increase of immature cells (> 10%). The exclusion criteria for patients are as follows: cancer, end-stage renal disease, acquired immunodeficiency syndrome, history of kidney transplantation, and high-dose steroid treatment. All patients and non-affected individuals signed informed consent. The ethical approval was obtained from People's Hospital of Tiantai County.

Cell culture and LPS treatment

Human tubular epithelial cells (HK2) were purchased from ATCC (Manassas, MA, USA) and cultured in K-SFM medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin–Streptomycin (Gibco) at 37 ℃ with 5% CO_2. For LPS treatment, HK2 cells were treated with different concentrations (0, 1, 5 and 10 μg/mL) of LPS (Solarbio, Beijing, China) for 24 h.

Cell transfection

HK2 cells were transfected with the oligonucleotides (oligos) or vectors using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). All oligos and vector were synthesized by Genepharma (Shanghai, China), including *circ_0091702* pcDNA overexpression vector and small interfering RNA (siRNA) (pcDNA-*circ_0091702* and si-*circ_0091702*) or their controls (pcDNA-control and si-NC), *miR-545-3p* mimic and inhibitor (*miR-545-3p* and anti-*miR-545-3p*) or their controls (miR-NC and anti-miR-NC), the siRNA of thrombospondin 2 (*THBS2*) (si-*THBS2*) and its control (si-NC). The amount of pcDNA was 4.0 μg, and that of the miRNA mimic, inhibitor and siRNA were 50 nM.

Quantitative real-time PCR (qRT-PCR)

TRIzol Reagent (Invitrogen) was performed to extract total RNA from the serum and cells. Using the TIANscript RT Kit (Tiangen, Beijing, China), the RNA was reverse-transcribed into cDNA. Afterwards, qRT-PCR was carried out with SYBR Green (Invitrogen). *GAPDH* or *U6* was used as internal control, and data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The specific sequences of primers were shown as below: *circ_0091702*, F 5′-CCGTTTTCTGGATGGATTTT-3′, R 5′-ATACACAAAGAGGGCCAGCA-3′; *VMA21*, F 5′-CGCTCCTGTTCTTCACAGCTT-3′, R 5′-CCTATTGGACATCCCAAGGGC-3′; *TNF-α*, F 5′-GACAAGCCTGTAGCCCATGT-3′, R 5′-GGAGGTTGACCTTGGTCTGG-3′; *IL-6*, F 5′-CCACCGGGAACGAAAGAGAA-3′, R 5′-GAGAAGGCAACTGGACCGAA-3’; *IL-1β*, F 5′-AGCCATGGCAGAAGTACCTG-3’, R 5′-TGAAGCCCTTGCTGTAGTGG-3′; *miR-545-3p*, F 5′-GCCGAGTCAGCAAACATTTATT-3′, R 5′-CAGTGCGTGTCGTGGAGT-3′; *THBS2*, F 5′-GACACGCTGGATCTCACCTAC-3′, R 5′-GAAGCTGTCTATGAGGTCGCA-3′; *GAPDH*, F 5′-CAATGACCCCTTCATTGACC-3′, R 5′-TGGAAGATGGTGATGGGATT-3′; *U6*, F 5′-ATTGGAACGATACAGAGAAGATT-3′, R 5′-GGAACGCTTCACGAATTTG-3′.

Cell counting kit 8 (CCK8) assay

HK2 cells were seeded in 96-well plates (2000 cells/well). After culturing for 48 h at 37 ℃, the cells were added with 10 μL CCK8 reagent (Beyotime, Shanghai, China) and

further incubated for 2 h. The absorbance at 450 nm was measured to evaluate cell viability using a microplate reader.

Flow cytometry

After culturing for 48 h, HK2 cells (5×10^5 cells) were collected and washed with PBS (Beyotime). According to the instructions of Annexin-V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Vazyme, Nanjing, China), the cell suspensions were re-suspended with binding buffer and stained with Annexin-V-FITC and PI. Cell apoptosis rate was analyzed on a flow cytometer.

Western blot (WB) analysis

HK2 cells were lysed by RIPA lysis buffer (Beyotime) to obtain total protein. After quantified protein concentration, the protein sample was separated using 10% SDS-PAGE gel and transferred into PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked by skim milk, and then incubated with anti-Bax (1:1,000, ab53154, Abcam, Cambridge, CA, USA), anti-Bcl-2 (1:1000, ab194583, Abcam), anti-Cleaved caspase-3 (1:500, ab2302, Abcam), anti-THBS2 (1:1000, ab84469, Abcam) or anti-GAPDH (1:2500, ab9485, Abcam). After the membrane was hatched with Goat Anti-Rabbit IgG (1:10,000, ab205718, Abcam), the protein bands were visualized by the BeyoECL Plus (Beyotime). Relative protein expression was analyzed using the Image J Software (NIH, Bethesda, MD, USA). Relative protein expression was normalized by *GAPDH*.

Enzyme-linked immunosorbent assay (ELISA)

Human serum creatinine (SCr), blood urea nitrogen (BUN), TNF-α, IL-6 and IL-1β ELISA Kits were purchased from Beyotime. According to the manufacturer's instructions, the levels of SCr and BUN in the serum of patients and the concentrations of TNF-α, IL-6 and IL-1β in the culture medium of HK2 cells were determined by corresponding ELISA Kits, respectively.

Detection of cell oxidative stress

The contents of reactive oxygen (ROS), malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) were determined to assess cell oxidative stress. According to the instructions of Human ROS, MDA, GSH and SOD Assay Kits (Jincheng Bioengineering Institute, Nanjing, China), the contents of ROS, MDA, GSH and SOD in the culture medium of HK2 cells were measured, respectively.

Dual-luciferase reporter assay

The binding sites and designed mutate sites of *miR-545-3p* in *circ_0091702* or *THBS2* 3′-UTR were cloned to pmirGLO vector (Promega, Madison, WI, USA), generating the corresponding wild-type (WT) and mutated-type (MUT) vectors. The vectors (50 ng) were transfected into HEK293 cells (ATCC) with *miR-545-3p* mimic (20 nM) or miR-NC (20 nM). The luciferase activity was examined through the Dual-Luciferase Reporter Assay System (Promega).

RIP assay

HK2 cells were transfected with *miR-545-3p* mimic or miR-NC for 48 h. According to the instructions of Magna RIP Kit (Millipore), the cells were lysed by RIP buffer, and then the cell lysates were incubated with magnetic beads combined with anti-Ago2 (RIP-Ago2, 2.5 μg, SAB4200085, Sigma-Aldrich, St. Louis, MO, USA) or anti-IgG (RIP-IgG, 2.5 μg, 14131, Sigma-Aldrich). After incubated at 4℃ overnight, the magnetic bead mixture was hatched with Proteinase K to obtain immunoprecipitated RNA. The expression of *circ_0091702* and *THBS2* was examined by qRT-PCR.

Statistical analysis

All data were expressed as mean ± standard deviation based on at least 3 independent experiments. GraphPad Prism 7 (GraphPad, La Jolla, CA, USA) was used for statistical analysis. Comparisons were performed using Student's *t*-test or one-way ANOVA. Pearson correlation analysis was used for analyzing the correlation among *circ_0091702*, *miR-545-3p* and *THBS2*. $P < 0.05$ was considered statistically significant.

Results

Circ_0091702 was downregulated in septic AKI patients and LPS-induced AKI cell models

Compared to the normal group, the serum levels of SCr and BUN were upregulated in septic AKI patients, confirming that AKI patients had indeed suffered kidney injury (Supplementary Fig. 1a, b). In the serum of septic AKI patients, *circ_0091702* was found to be lowly expressed compared with that in normal humans (Fig. 1a). In LPS-induced HK2 cells, the expression of *circ_0091702* also was decreased significantly with the increase of LPS concentration (Fig. 1b). To evaluate the success of the LPS-induced AKI cell model, we examined the injury degree of HK2 cells treated with different concentrations of LPS. The results showed that with the continuous increase of LPS concentration, HK2 cell viability was gradually reduced (Fig. 1c), and the apoptosis

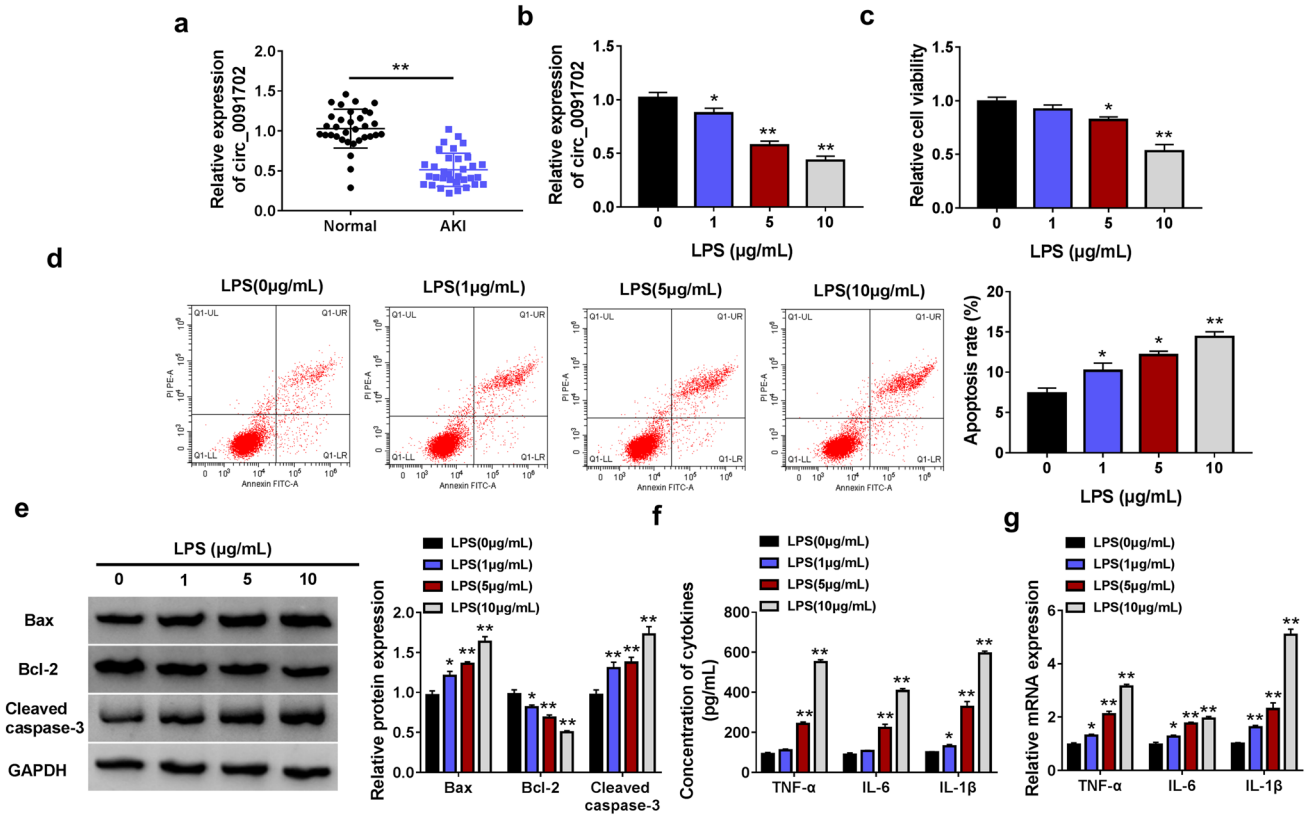

Fig. 1 *Circ_0091702* was downregulated in septic AKI patients and LPS-induced sepsis cell models. **a** The expression of *circ_0091702* in the serum of septic AKI patients (n = 33) and normal humans (n = 33) was measured by qRT-PCR. **b**–**g** HK2 cells were treated with different concentrations of LPS (0, 1, 5 and 10 μg/mL). **b** qRT-PCR was used to detect *circ_0091702* expression in HK2 cells under treatment with different concentrations of LPS (n = 3). CCK8 assay (**c**) and flow cytometry (**d**) were used to assess cell viability and apoptosis. **e** The protein levels of Bax, Bcl-2 and Cleaved caspase-3 were determined by WB analysis. **f** and **g** ELISA assay and qRT-PCR were performed to examine the concentrations and mRNA expression levels of TNF-α, IL-6 and IL-1β. $^{*}P < 0.05$, $^{**}P < 0.01$

rate was gradually increased (Fig. 1d). In addition, under the treatment of LPS, the expression of apoptotic proteins Bax and Cleaved caspase-3 was significantly promoted, while the expression of anti-apoptotic protein Bcl-2 was obviously decreased in a concentration-dependent manner (Fig. 1e). Moreover, we also used ELISA assay to detect the expression of inflammatory cytokines. The results showed that the concentrations of TNF-α, IL-6 and IL-1β were remarkably enhanced in HK2 cells with the increase of LPS concentration (Fig. 1f), and we got consistent results at the mRNA level (Fig. 1g). Our data showed that LPS did induce HK2 cell injury. Because the cell injury induced by 10 μg/mL LPS was better, the treatment concentration of LPS in subsequent experiments was 10 μg/mL.

Circ_0091702 alleviated LPS-induced HK2 cell injury

To explore the role of *circ_0091702* in AKI, we constructed the pcDNA overexpression vector and siRNA for *circ_0091702* to evaluate the effect of *circ_0091702* on LPS-induced HK2 cell injury. By detecting *circ_0091702* expression, we confirmed that pcDNA-*circ_0091702* could indeed promote *circ_0091702* expression and si-*circ_0091702* also could inhibit *circ_0091702* expression (Fig. 2a). Also, we confirmed that the si-*circ_0091702* and pcDNA-*circ_0091702* could only affect the expression of *circ_0091702*, but not the corresponding linear VMA21 mRNA expression (Supplementary Fig. 2a, b). The detection of cell viability and apoptosis rate showed that *circ_0091702* overexpression could enhance the viability and reduce the apoptosis rate of LPS-induced HK2 cells, and its knockdown had an opposite effects (Fig. 2b, c). Besides, *circ_0091702* overexpression also hindered the protein levels of Bax and Cleaved caspase-3, and improved the protein level of Bcl-2 in LPS-induced HK2 cells (Fig. 2d). However, silenced *circ_0091702* increased Bax and Cleaved caspase-3 protein levels, and decreased Bcl-2 protein level in HK2 cells treated with LPS (Fig. 2d). In addition, we also found that the concentrations and the mRNA expression levels of TNF-α, IL-6 and IL-1β in LPS-induced HK2 cells could be inhibited by *circ_0091702* overexpression and augmented by *circ_0091702* knockdown (Fig. 2e, f). Moreover, we

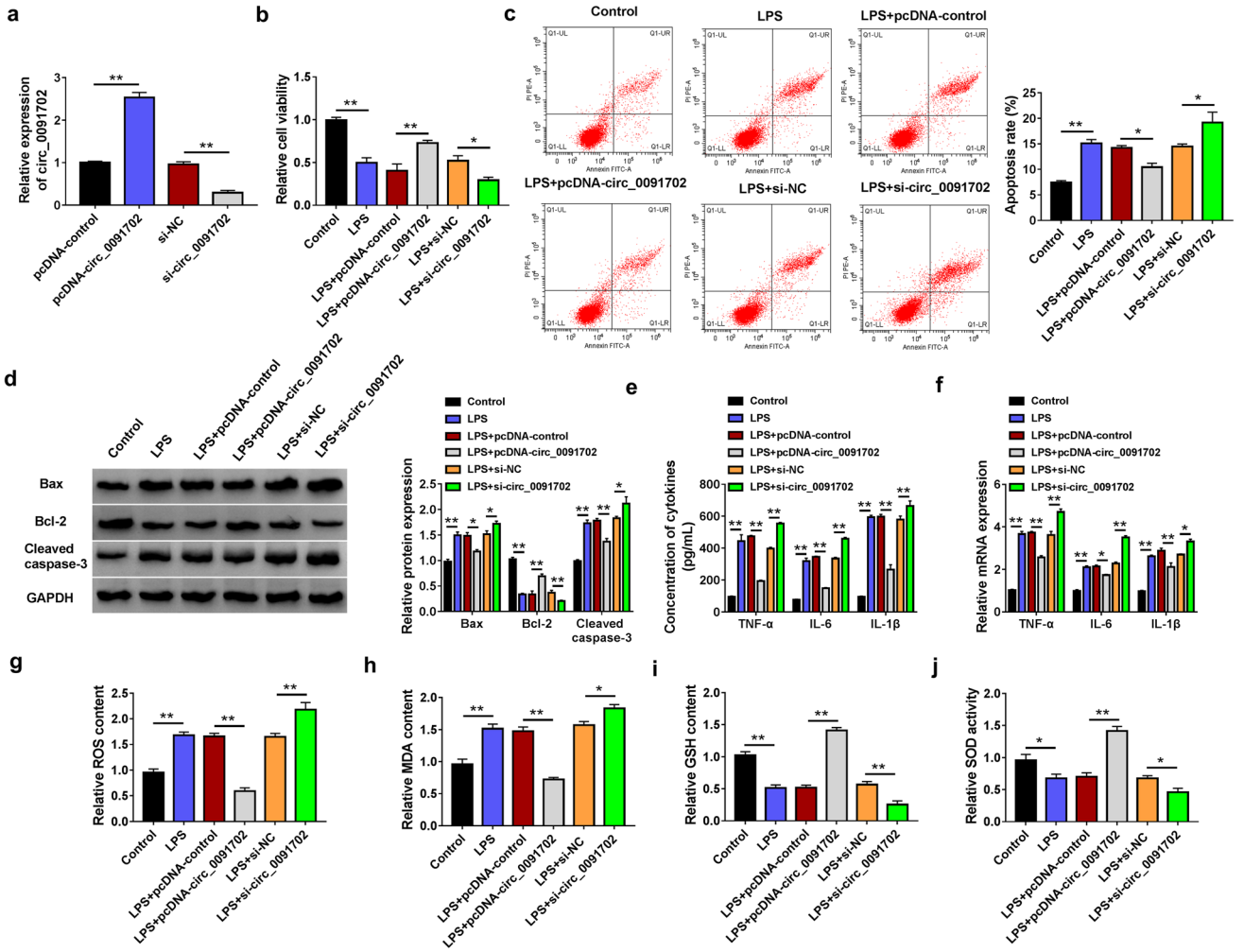

Fig. 2 *Circ_0091702* alleviated LPS-induced HK2 cell injury. **a** HK2 cells were transfected with pcDNA-control, pcDNA-*circ_0091702*, si-NC or si-*circ_0091702* (n = 3). The expression of *circ_0091702* was detected by qRT-PCR. **b–j** HK2 cells were transfected with pcDNA-control, pcDNA-*circ_0091702*, si-NC or si-*circ_0091702*, followed by treatment with 10 μg/mL LPS. Non-transfected and non-treated HK2 cells were used as Control. The viability and apoptosis of cells (n = 3) were determined by CCK8 assay (**b**) and flow cytometry (**c**). **d** WB analysis was used to test the protein levels of Bax, Bcl-2 and Cleaved caspase-3 (n = 3). **e** and **f** The concentrations and mRNA expression levels of TNF-α, IL-6 and IL-1β (n = 3) were determined using ELISA assay and qRT-PCR. **g–j** The contents of ROS, MDA, GSH and SOD (n = 3) were measured using ELISA assay to assess the oxidative stress of cells. *$P < 0.05$, **$P < 0.01$

detected the contents of oxidative stress markers and found that LPS could promote the contents of ROS and MDA and repress the contents of GSH and SOD in HK2 cells, while these effects could be abolished by *circ_0091702* overexpression and could be aggravated by *circ_0091702* silencing (Fig. 2g–j). Therefore, we confirmed that *circ_0091702* could relieve LPS-induced HK2 cell injury.

Circ_0091702 could serve as a sponge of *miR-545-3p*

The circular RNA interactome (https://circinteractome.nia.nih.gov/mirna_target_sites.html) tool was used to predict the targeted miRNA of circRNA (Dudekula et al. 2016). And *miR-545-3p* was found to have binding sites with *circ_0091702* (Fig. 3a). To further explore the regulatory relationship between *circ_0091702* and *miR-545-3p*, we constructed *miR-545-3p* mimic and confirmed that they could indeed promote the expression of *miR-545-3p* (Fig. 3b). In dual-luciferase reporter assay, we discovered that *miR-545-3p* mimic could inhibit the luciferase activity of *circ_0091702*-WT vector without affecting that of the *circ_0091702*-MUT vector (Fig. 3c). Moreover, overexpressed *miR-545-3p* also could increase the enrichment of *circ_0091702* in RIP-Ago2 (Fig. 3d). Additionally, we found that *miR-545-3p* expression was highly expressed in the serum of septic AKI patients (Fig. 3e), and its expression was negatively correlated with *circ_0091702* expression (Fig. 3f). In HK2 cells

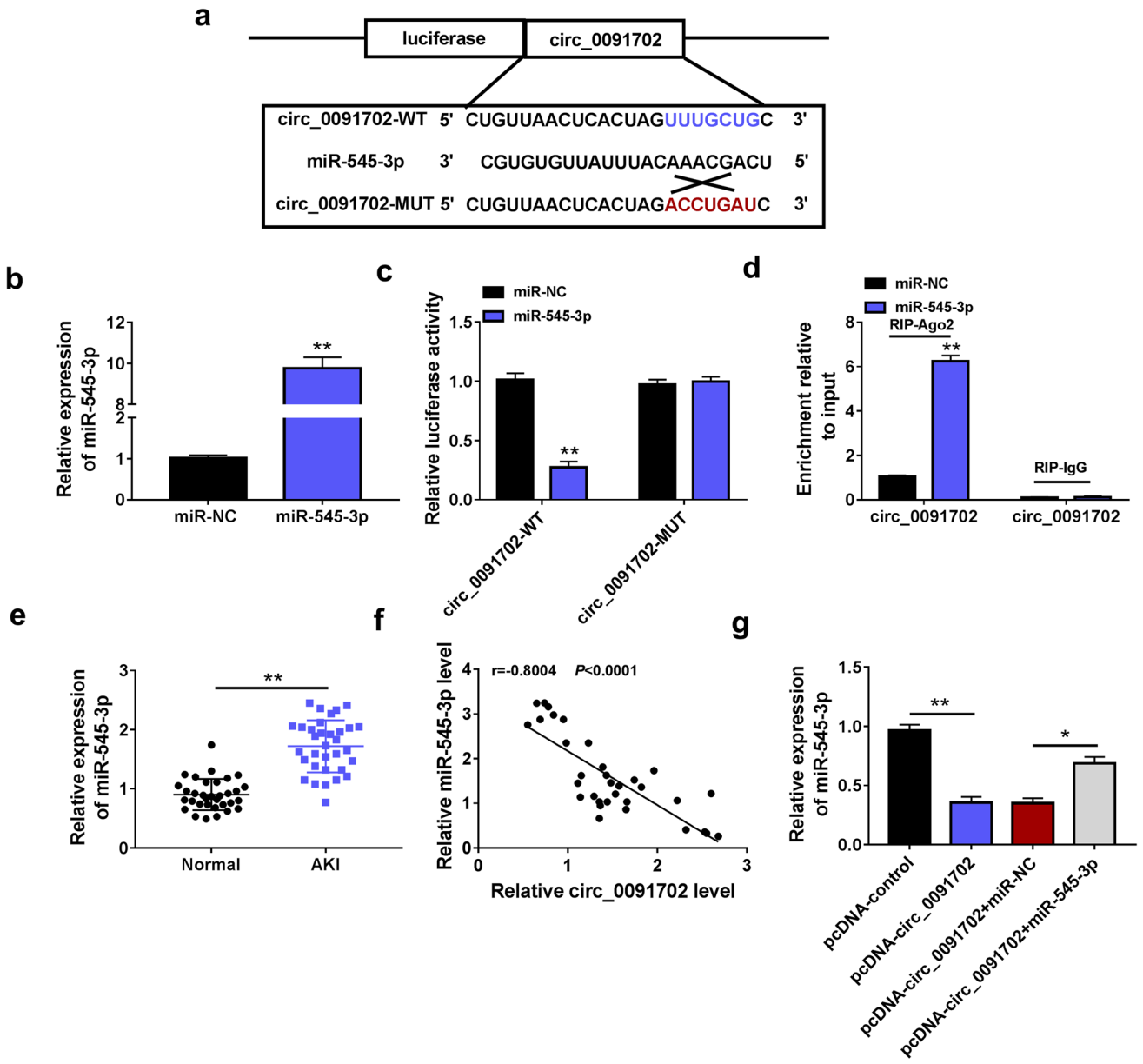

Fig. 3 *Circ_0091702* could serve as a sponge of *miR-545-3p*. **a** The binding sites and mutated sites between *circ_0091702* and *miR-545-3p* were shown. **b** The transfection efficiency of *miR-545-3p* mimic was confirmed by detecting *miR-545-3p* expression (n = 3) using qRT-PCR. The interaction between *circ_0091702* and *miR-545-3p* was verified by dual-luciferase reporter assay (n = 3) (**c**) and RIP assay (n = 3) (**d**). **e** The expression of *miR-545-3p* in the serum of septic AKI patients (n = 33) and normal humans (n = 33) was determined using qRT-PCR. **f** Pearson correlation analysis was used to assess the correlation between *circ_0091702* and *miR-545-3p* in the serum of septic AKI patients (n = 33). **g** HK2 cells were transfected with pcDNA-control, pcDNA-*circ_0091702*, pcDNA-*circ_0091702* + miR-NC or pcDNA-*circ_0091702* + *miR-545-3p*. qRT-PCR was used to detect *miR-545-3p* expression (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$

transfected with pcDNA-*circ_0091702* and *miR-545-3p* mimic, we uncovered that *circ_0091702* overexpression could markedly reduce *miR-545-3p* expression, while these effect could be reversed by *miR-545-3p* mimic (Fig. 3g). These data showed that *miR-545-3p* could be sponged by *circ_0091702*.

The inhibition effect of *circ_0091702* on LPS-induced HK2 cell injurycould be reversed by *miR-545-3p*

Subsequently, in LPS-induced HK2 cells co-transfected with pcDNA-*circ_0091702* and *miR-545-3p* mimic, we assessed cell viability, apoptosis, inflammatory response

and oxidative stress. CCK8 assay and flow cytometry results showed that the promotion effect of *circ_0091702* overexpression on the viability and the suppressive effect of it on the apoptosis of LPS-induced HK2 cells could be reversed by *miR-545-3p* (Fig. 4a, b). Besides, the decreasing Bax and Cleaved caspase-3 protein expression and the increasing Bcl-2 protein expression in LPS-induced HK2 cells regulated by *circ_0091702* overexpression also could be overturned by *miR-545-3p* mimic (Fig. 4c). In addition, *miR-545-3p* overexpression also reversed the inhibitory effects of *circ_0091702* on the concentrations and mRNA expression levels of TNF-α, IL-6 and IL-1β in LPS-induced HK2 cells (Fig. 4d, e). Meanwhile, the repressing effect of *circ_0091702* on the contents of ROS and MDA, as well as the enhancing effect on the contents of GSH and SOD in LPS-induced HK2 cells also could be recovered by *miR-545-3p* (Fig. 4f–i). These results

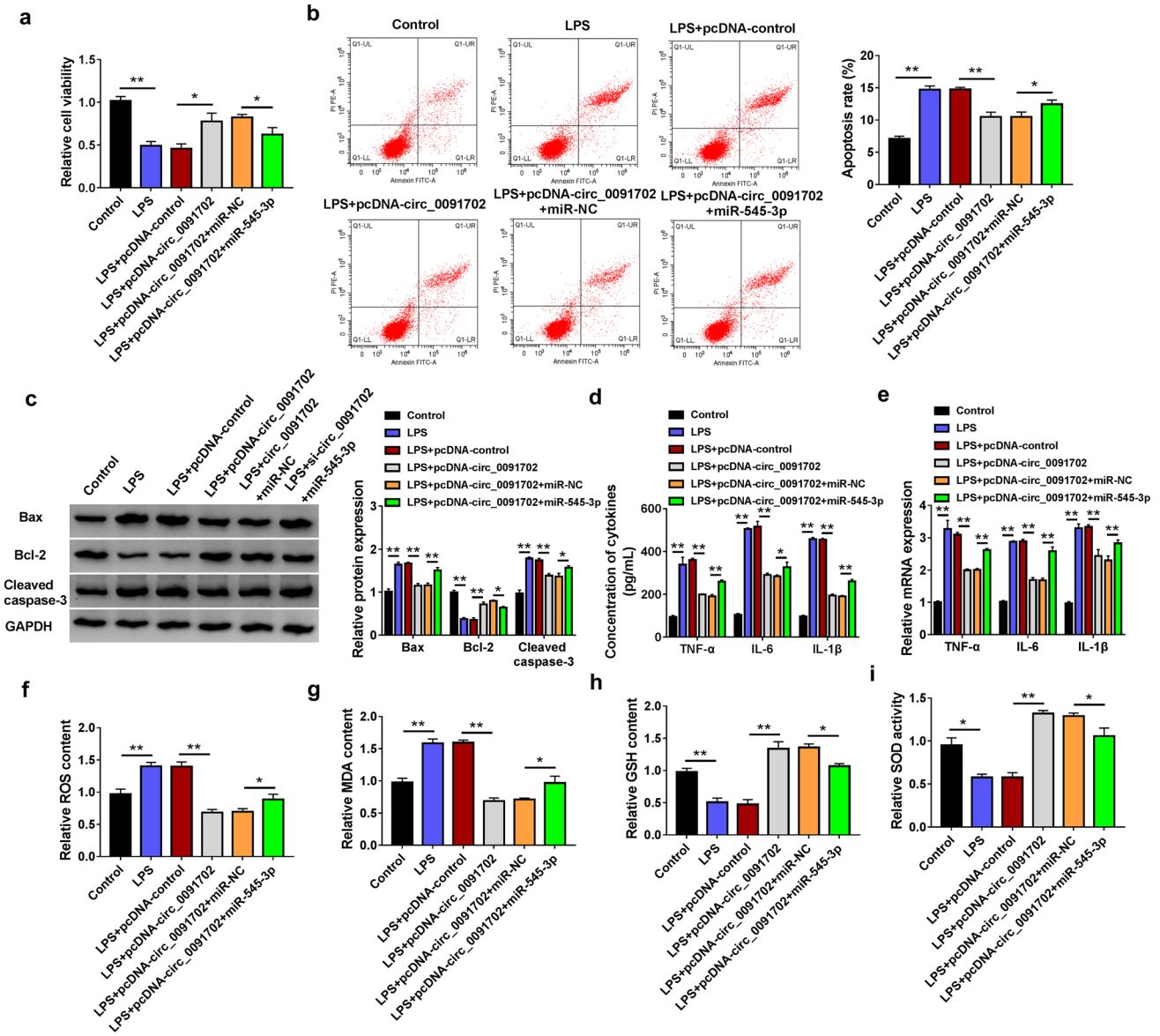

Fig. 4 The inhibition effect of *circ_0091702* on LPS-induced HK2 cell injury could be reversed by *miR-545-3p*. HK2 cells were transfected with pcDNA-control, pcDNA-*circ_0091702*, pcDNA-*circ_0091702* + miR-NC or pcDNA-*circ_0091702* + *miR-545-3p*, followed by treatment with 10 μg/mL LPS. Non-transfected and non-treated HK2 cells were used as Control. CCK8 assay (**a**) and flow cytometry (**b**) were employed to measure the viability and apoptosis of cells (n = 3). **c** The protein levels of Bax, Bcl-2 and Cleaved caspase-3 (n = 3) were assessed using WB analysis. **d** and **e** The concentrations and mRNA expression levels of TNF-α, IL-6 and IL-1β (n = 3) were evaluated by ELISA assay and qRT-PCR. **f–i** Cell oxidative stress was determined by detecting the contents of ROS, MDA, GSH and SOD (n = 3) using ELISA assay. *$P < 0.05$, **$P < 0.01$

suggested that *circ_0091702* regulated LPS-induced HK2 cell injury by sponging *miR-545-3p*.

miR-545-3p could target *THBS2*

The Targetscan software (http://www.targetscan.org/vert_71/) was used to investigate the targets of miRNA (Agarwal et al. 2015). As presented in Fig. 5a, there are complementary binding sites between *miR-545-3p* and the 3′-UTR of *THBS2*. The results of dual-luciferase reporter assay showed that the luciferase activity of *THBS2* 3′-UTR-WT vector could be reduced by *miR-545-3p* mimic, while the luciferase activity of *THBS2* 3′-UTR-MUT vector was not affected (Fig. 5b). Furthermore, the expression of *THBS2* also was enriched in RIP-Ago2 in HK2 cells transfected with *miR-545-3p* mimic (Fig. 5c). In the serum of septic AKI patients, we discovered that the mRNA and protein expression of *THBS2* was notably lower than that in normal humans (Fig. 5d, e), and its mRNA expression was negatively correlated with *miR-545-3p* expression (Fig. 5f). To further explore the regulation of *miR-545-3p* on *THBS2*, anti-*miR-545-3p* and si-*THBS2* were constructed. Through measuring the *miR-545-3p* expression and *THBS2* protein expression after transfection, we confirmed the transfection efficiencies of anti-*miR-545-3p* and si-*THBS2* (Fig. 5g, h). In HK2 cells transfected with anti-*miR-545-3p* and si-*THBS2*, we found that *miR-545-3p* inhibitor significantly promoted *THBS2* protein expression, and these effect could be abolished by *THBS2* knockdown (Fig. 5i). All data indicated that *THBS2* was a target of *miR-545-3p*.

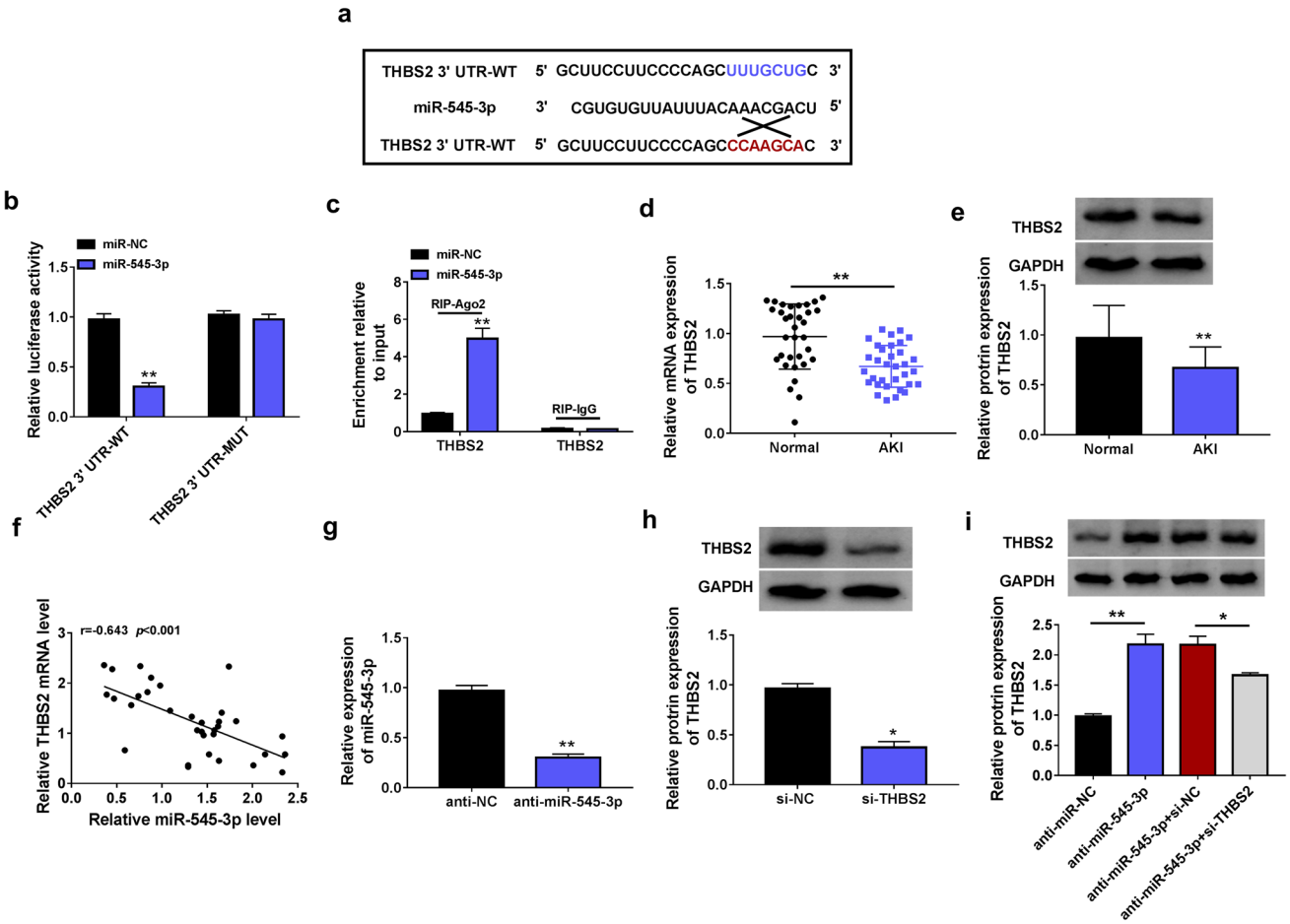

Fig. 5 *miR-545-3p* could target *THBS2*. **a** The binding sites and mutated sites between *miR-545-3p* and *THBS2* 3′-UTR were exhibited. Dual-luciferase reporter assay (n = 3) (**b**) and RIP assay (n = 3) (**c**) were performed to assess the interaction between *miR-545-3p* and *THBS2*. **c** and **e** The mRNA and protein expression levels of *THBS2* in the serum of septic AKI patients (n = 33) and normal humans (n = 33) were measured by qRT-PCR and WB analysis, respectively. **f** The correlation between *miR-545-3p* and *THBS2* in the serum of septic AKI patients (n = 33) was evaluated by Pearson correlation analysis. **g** and **h** The transfection efficiencies of *miR-545-3p* and si-*THBS2* were confirmed by measuring *miR-545-3p* expression and *THBS2* protein expression (n = 3) using qRT-PCR and WB analysis (n = 3), respectively. **i** HK2 cells were transfected with anti-miR-NC, anti-*miR-545-3p*, anti-*miR-545-3p* + si-NC or anti-*miR-545-3p* + si-*THBS2*. WB analysis was performed to test the protein expression of *THBS2* (n = 3). *$P < 0.05$, **$P < 0.01$

THBS2 knockdown reversed the suppressive effect of *miR-545-3p* inhibitor on LPS-induced HK2 cell injury

Then, anti-*miR-545-3p* and si-*THBS2* were co-transfected into HK2 cells followed by treatment with LPS. The detection results of cell viability and apoptosis rate indicated that *miR-545-3p* inhibitor could enhance the viability and repress the apoptosis of LPS-induced HK2 cells, while this effect could be overturned by *THBS2* silencing (Fig. 6a, b). Meanwhile, the inhibition effect of *miR-545-3p* inhibitor on the Bax and Cleaved caspase-3 protein expression and the promotion effect on Bcl-2 protein expression in LPS-induced HK2 cells also could be reversed by *THBS2* knockdown (Fig. 6c). Additionally, *miR-545-3p* inhibitor also inhibited the concentrations and mRNA expression levels of inflammatory cytokines, decreased the contents of ROS and MDA, and augmented the contents of GSH

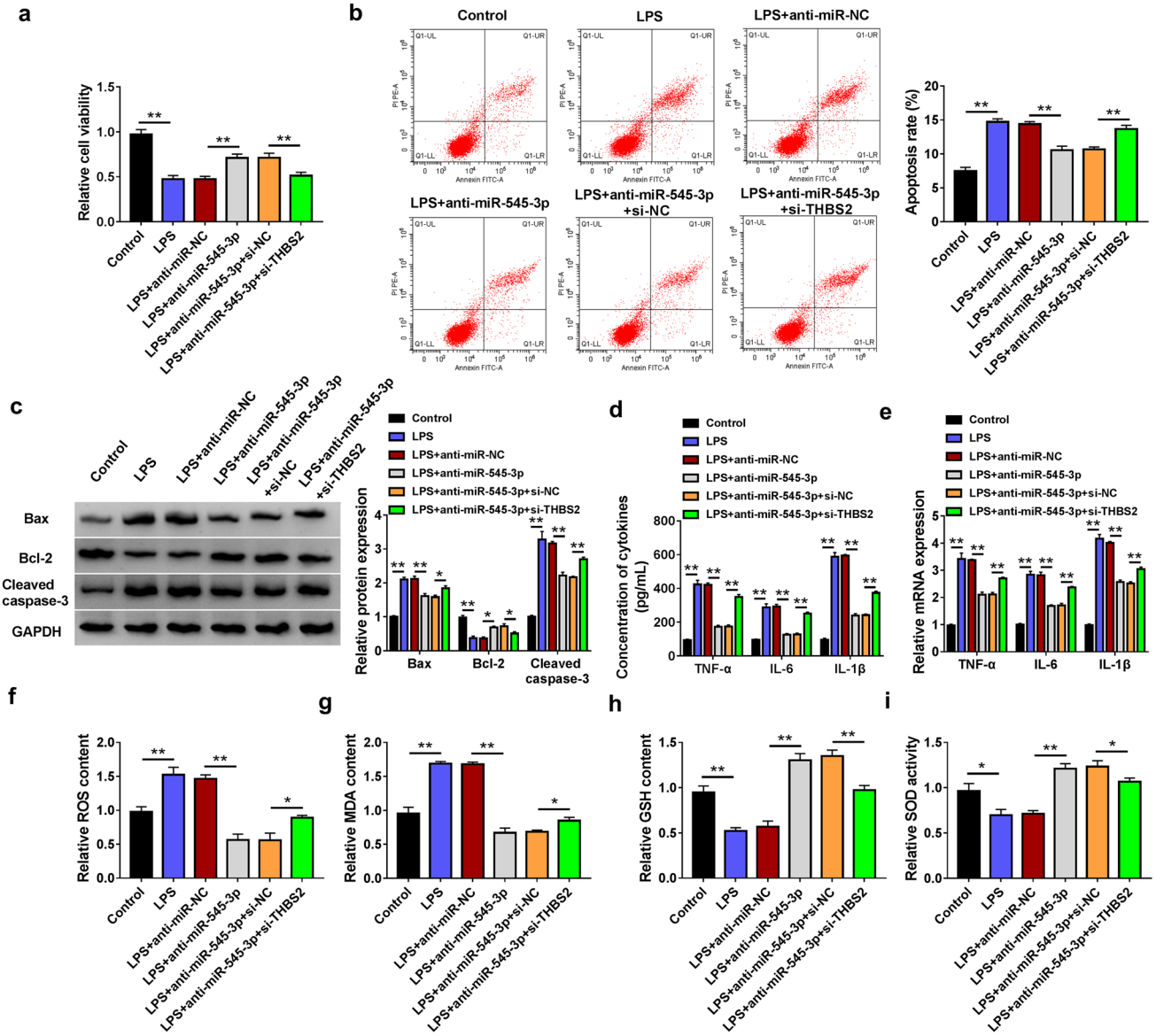

Fig. 6 *THBS2* knockdown reversed the suppressive effect of *miR-545-3p* inhibitor on LPS-induced HK2 cell injury. HK2 cells were transfected with anti-miR-NC, anti-*miR-545-3p*, anti-*miR-545-3p* + si-NC or anti-*miR-545-3p* + si-*THBS2*, followed by treatment with 10 μg/mL LPS. Non-transfected and non-treated HK2 cells were used as Control. The viability and apoptosis rate of cells (n = 3) were determined using CCK8 assay (**a**) and flow cytometry (**b**). **c** WB analysis was used to detect the protein levels of Bax, Bcl-2 and Cleaved caspase-3 (n = 3). **d** and **e** ELISA assay and qRT-PCR were used to measure the concentrations and mRNA expression levels of TNF-α, IL-6 and IL-1β (n = 3). **f**–**i** The contents of ROS, MDA, GSH and SOD (n = 3) were detected using ELISA assay to evaluate cell oxidative stress. $^{*}P < 0.05$, $^{**}P < 0.01$

and SOD in LPS-induced HK2 cells (Fig. 6d–i). However, knockdown of *THBS2* also reversed the regulation of *miR-545-3p* inhibitor on the levels of inflammatory cytokines and oxidative stress markers in LPS-induced HK2 cells (Fig. 6d–i). These results revealed that *miR-545-3p* targeted *THBS2* to regulate LPS-induced HK2 cell injury.

THBS2 expression was regulated by *circ_0091702* and *miR-545-3p*

Our above data showed that *circ_0091702* could sponge *miR-545-3p* and *miR-545-3p* could target *THBS2*. To uncover whether *circ_0091702* sponged *miR-545-3p* to regulate *THBS2*, we detected the mRNA and protein expression of *THBS2* in LPS-induced HK2 cells transfected with pcNDA-*circ_0091702* and *miR-545-3p*. Our results suggested that *circ_0091702* overexpression could markedly enhance *THBS2* expression in LPS-induced HK2 cells, but *miR-545-3p* mimic also could reverse this effect (Fig. 7a, b). These data indicated that *circ_0091702* regulated *THBS2* by sponging *miR-545-3p*.

Discussion

AKI is a common critical condition in which kidney function declines rapidly over a short period of time, with an incidence of up to 50% in sepsis (Forni et al. 2015; Liu et al. 2019). At present, the commonly used indicators for clinical diagnosis of AKI include SCr and BUN, but the diagnostic sensitivity and specificity of these indicators are often low (Lu et al. 2018; Uchino et al. 2012). Therefore, the development of new targets for judging AKI can provide a new theoretical strategy for reducing the mortality of septic AKI. Although the important role of circRNA in human diseases has been confirmed in many studies, the roles of many circRNAs in sepsis-associated AKI are unknown.

Previous studies have suggested that *circ_0091702* may be a potential target for sepsis-related AKI (Shi, Sun 2020c), but the evidence is limited. In our study, we investigated the effect of *circ_0091702* on cell injury using LPS-induced AKI cell model. Our data once again confirmed that *circ_0091702* was indeed significantly underexpressed in septic AKI patients and decreased in LPS-induced HK2 cells, which was consistent with the reported study (Shi, Sun 2020c). Further functional experiments revealed that

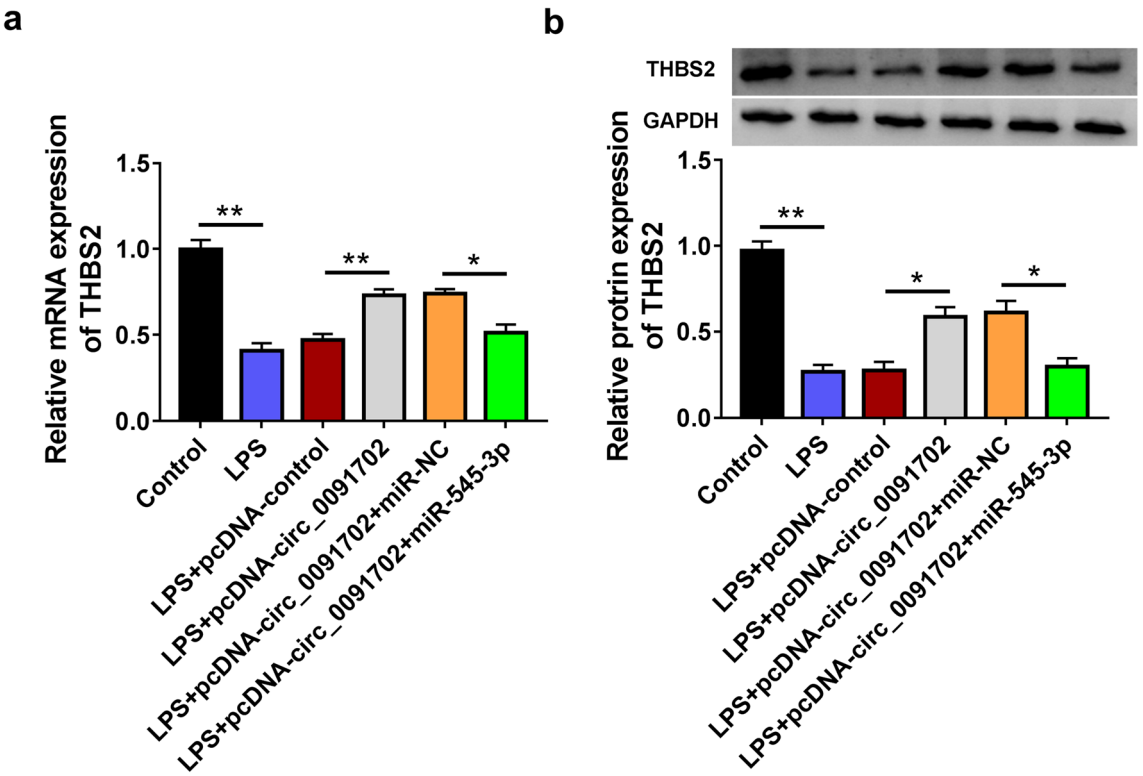

Fig. 7 *THBS2* expression was regulated by *circ_0091702* and *miR-545-3p*. HK2 cells were transfected with pcDNA-control, pcDNA-*circ_0091702*, pcDNA-*circ_0091702* + miR-NC or pcDNA-*circ_0091702* + *miR-545-3p*, followed by treatment with 10 μg/mL LPS. Non-transfected and non-treated HK2 cells were used as Control. qRT-PCR (**a**) and WB analysis (**b**) were used to detect the mRNA and protein expression levels of *THBS2* (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$

circ_0091702 overexpression suppressed LPS-induced HK2 cell apoptosis, inflammatory response, oxidative stress, and promoted cell viability, while its silencing had a pro-apoptosis, pro-inflammatory response and pro-oxidative stress in LPS-induced HK2 cells. These data confirmed that *circ_0091702* could alleviate LPS-induced cell injury, and once again proposed that *circ_0091702* might be an effective target for sepsis induced AKI.

Many studies have shown that circRNA can participate in the regulation of cell progression as a competitive endogenous RNA (ceRNA) of miRNA (Wang, Guo 2019; Yu and Liu 2019). Shi et al. suggested that *circ_0091702* regulated sepsis-associated AKI by acting as a ceRNA of *miR-9-3p* (Shi, Sun 2020c). Here, we discovered that *circ_0091702* could function as a ceRNA for *miR-545-3p*. In reported studies, *miR-545-3p* had been found to inhibit the proliferation and metastasis of epithelial ovarian cancer, thus inhibiting cancer malignant progression (Shi et al. 2020a). Also, *miR-545-3p* could suppress osteoblasts proliferation and differentiation to hinder bone formation (Hao et al. 2020). Cheng et al. suggested that *miR-545* inhibitor could restrain HO-induced primary neuron death and apoptosis to alleviate neuronal cell injury (Cheng et al. 2019). In past studies, *miR-545* had been shown to be associated with sepsis risk, clinical disease severity, and 28-day death risk, and it also could exacerbate LPS-induced HK2 cell injury (Shi et al. 2020b; Wei and Yu 2020). Our study showed that *miR-545-3p* overexpression could reverse the suppressive effect of *circ_0091702* on LPS-induced HK2 cell injury, while its inhibitor could relieve LPS-induced HK2 cell injury. Our findings illuminated that *miR-545-3p* played a role in promoting cell injury, and further suggested that *circ_0091702* might target *miR-545-3p* to alleviate sepsis-induced AKI.

THBS2 is a member of thrombospondin family that mediate intercellular interactions and has also been shown to be an important regulator for tumor growth and angiogenesis (Wu et al. 2019; Zhou et al. 2019). Previous studies proposed that *THBS2* could be used as a biomarker for the diagnosis, treatment, and prognosis of many cancers, such as gastric cancer (Zhuo et al. 2016), lung cancer (Weng et al. 2016), and colorectal cancer (Fei et al. 2017). Shen et al. showed that *THBS2* was lowly expressed in sepsis patients and could alleviate LPS-induced kidney epithelial cell inflammation and apoptosis (Shen et al. 2019). Consistent with the previous study, our data indicated that *THBS2* was downregulated in septic AKI patients and LPS-induced cell injury. Furthermore, the reversal effect of *THBS2* silencing on *miR-545-3p* inhibitor verified that *THBS2* knockdown could promote LPS-induced cell injury and *miR-545-3p* indeed targeted *THBS2* to regulate cell injury. The positively regulation of *circ_0091702* on *THBS2* expression further confirmed that *circ_0091702* sponged *miR-545-3p* to mediate *THBS2* expression.

In conclusion, our evidence revealed that *circ_0091702* alleviated LPS-induced cell injury by *miR-545-3p*/*THBS2* axis, showing that *circ_0091702* might be a potential target for sepsis-induced AKI.

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Declarations

Conflict of interest The authors declare that they have no financial conflicts of interest.

Ethical approval Our study was approved by the Ethics Committee of People's Hospital of Tiantai County and was conducted in accordance with the Declaration of Helsinki.

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