Upregulation of miR-126 inhibits podocyte injury in sepsis via EGFL6/DKC1 signaling pathway

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Abstract. Sepsis-induced cardiorenal syndrome is one of the multiple organ dysfunctions observed in sepsis. It is determined by a primary dysfunction in one organ that leads to secondary injury to another organ. Studies have shown the involvement of microRNAs (miRs) in the diagnosis and prognosis of several pathologies. However, the implication of miR-126 in the podocyte damage associated with sepsis has not been evaluated until now. In the current study, the miR-126 expression was downregulated in a podocyte injury model together with downregulation of nephrin expression. The transfection of podocytes from podocyte injury group with miR-126 mimics demonstrated an increase in cell proliferation and a decrease in cell apoptosis. Bioinformatics analysis predicted that the target of miR-126 was epidermal growth factor-like domain multiple 6 (EGFL6) and dyskeratosis congenita 1 (DKC1) and these were confirmed by dual-luciferase reporter assay. miR-126 upregulation determined EGFL6 and DKC1 upregulation and prevented podocyte injury. The current study demonstrated that overexpression of miR-126 could protect podocytes from sepsis-induced injury through an EGFL6/DKC1 signaling pathway.

Introduction

Sepsis is a pathological condition caused by a number of factors, including severe infections, burn, trauma and surgery (1-5). An imbalance between the secretion of pro-inflammatory and anti-inflammatory factors that lead to multiple organ failure appears in the pathophysiological process of sepsis (6). Sepsis is one of the leading causes of mortality in critical patients (7). Higher mortality in sepsis appears when cardiac injury or acute kidney injury develops, especially in intensive care unit patients (8,9). Sepsis-induced cardiorenal syndrome is one of the multiple organ dysfunctions observed in sepsis. It is determined by a primary dysfunction in one organ that leads to secondary injury to another organ (10). Its main clinical manifestation is myocardial inhibition leading to decreased left ventricular systolic function and cardiac output (10). Podocytes are differentiated cells anchored on the basement membrane of the glomerulus. They maintain the structure of glomerulus, which is an important part of the glomerular filtration membrane (11). Toxic effects on podocytes that lead to podocyte losing integrity or functional damage are the main causes of proteinuria and glomerulosclerosis involved in the occurrence and development of a number of kidney diseases (12).

MicroRNAs (miRs) are endogenous small molecules of non-coding RNAs. Their biological effects are associated with gene expression regulation by modifying mRNA protein synthesis. miRNAs influence the protein synthesis at one or more mRNAs genes by targeting the 3' untranslated region (UTR), 5'UTR region and the coding sequence of the gene (13). Bioinformatics and research studies demonstrate that there are thousands of target genes for human miRs and more >5,000 target genes are regulated by miRs, indicating their involvement in various physiological and pathological processes (14-20). Environmental factors including diet, lifestyle, pollutants and carcinogens can influence the expression of miRs (21-25) and modulate important molecular pathways implicated in the pathogenesis of chronic diseases. Studies have shown that the profile of miRs has a great implication in the diagnosis and prognosis of several pathologies, including types of cancer (26-31). Disease-associated miRs can also serve as targets for personalized therapy (32-34). In the model of Dicer knockout mice, where miRs were knocked out, proteinuria, serious renal damage and changes in podocyte cytoskeleton proteins of podocytes are observed, showing the association between miR and podocyte integrity (35).

Studies have associated miR-126 with multiple functions in the organism, including the development of cardiovascular pathologies, Parkinson's disease, diabetes, diabetic nephropathy and types of cancer (36-40).

The present study hypothesized that miR-126 might be involved in the podocyte damage associated with sepsis. Lipopolysaccharide (LPS) is a part of the gram-negative bacterial cell wall that mediates systemic inflammation leading

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to sepsis. LPS induces podocyte injury by mediating the release of pro-inflammatory factors (41). An *in vitro* model of LPS-induced injury in conditioned immortalized mouse podocytes was used to provide a theoretical basis for further studies clarifying the pathogenesis of sepsis-induced nephrotoxicity.

Materials and methods

Cell culture and transfection. Mouse podocyte cells (SV40 MES 13 cell line; cat. no. CRL-1927; American Type Culture Collection) were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone; Cytiva) containing 10% fetal bovine serum (FBS; HyClone; Cytiva) and $8x10^4$ U/l recombinant mouse γ-interferon (Beijing Solarbio Science & Technology Co., Ltd.) in 5% CO_2 at 37˚C in an incubator (Binder GmbH). The cells were cultured for 3-5 days and were then maintained in RPMI-1640 medium containing 5% FBS.

Mouse podocyte cells with a density of $1.0x10^5$ cells/well were inoculated in a 6-well plate overnight. The podocyte injury model was established by stimulation with 10 mg/l LPS for 12, 24 and 36 h. The normal control group was treated with the same volume of phosphate-buffered saline (PBS). The plates were incubated at 37˚C for 12, 24 and 36 h. The expression of nephrin protein was detected by western blotting. According to the results of western blotting, the optimal time of LPS stimulation was selected.

The cells in the miR-126 mimic group and miR-mimic negative control (NC) group were treated separated with miR-126-mimic (5'-ACCTCCAGCTG GGTCGTAC CGTGAGTAATAATG-3') and miR-mimic NC (5'-CTCAAC TGGTGTCCTGGA-3'), then mixed Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) until the final concentration in the RPMI-1640 medium was 20 nmol/l. Cells were incubated for 1 h then cultured in complete medium for 24-48 h. All reagents were purchased from Shanghai GenePharma Co., Ltd. and all experiments were performed in triplicate.

Reverse transcription-quantitative (RT-q) PCR. RT-PCR Eastrop™ Super total RNA Extraction kit (Promega Corporation) was used to extract total RNA from samples. RNA was reverse transcribed into cDNA using an RT kit (Thermo Fisher Scientific, Inc.). RNA extraction, cDNA synthesis and qPCR were performed according to the manufacturer's protocol. When cell density reached 80%, culture medium was discarded, and 1 ml lysate was added to each well of a 6-well plate for 10 min at room temperature. The reaction system (20 µl) included: 1.0 cDNA, 10.0 SYBR-Green Master Mix (Thermo Fisher Scientific, Inc.), 0.5 upstream and downstream primers (SANGON Biotech Co., Ltd.; Table I) and 8 µl ddH_2O (Milli-q Academic A10; EMD Millipore). Each experiment was performed three times and U6 was used as the internal reference. The reaction conditions were as follows: 95˚C pre denaturation for 10 min, 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec (40 cycles). Quantification was performed via the $2^{-\Delta\Delta Cq}$ method (42).

Western blotting. Mouse podocyte cells ($5x10^5$) were collected, rinsed with PBS, mixed with 250 µl RIPA Lysis Buffer (Thermo Fisher Scientific, Inc.) and left on ice for 5 min. Then the mixture was transferred into a new EP tube and centrifuged at 3,000 x g, 4˚C for 10 min. The supernatant obtained was used to determine the total protein concentration of the cells by the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). The protein samples were diluted with 5X loading buffer (Thermo Fisher Scientific, Inc.) and PBS, boiled in water bath for 5 min and denatured. The protein liquid after denaturation was added to 12% SDS-PAGE gel (Beijing Solarbio Science & Technology Co., Ltd.) and underwent 80 V constant pressure electrophoresis for 60-120 min, each well was loaded with 20 µg protein. The SDS-PAGE gel was placed onto a nitrocellulose membrane to perform the constant current transfer. Bovine serum albumin (BSA; 3%; HyClone; Cytiva) was used for blocking at 4˚C for 1 h. Following blocking, the nitrocellulose membrane was washed with PBS and incubated with monoclonal Anti-nephrin (1:1,000; rabbit antibody (Y17-R); cat no. ab136894, Abcam), anti-epidermal growth factor-like domain multiple 6 (EGFL6; 1:1,000; rabbit polyclonal mouse antibody 51182-T16, Abcam), anti-dyskeratosis congenita 1 (DKC1; 1:1,000; rabbit monoclonal antibody, EPR10398; Abcam) and anti-GAPDH (1:1,000; mouse monoclonal antibody; cat. no. K106390M, Beijing Solarbio Science & Technology Co., Ltd.), respectively. The membrane was incubated overnight at 4˚C. Then the nitrocellulose membrane was washed with PBS buffer and incubated with Anti-mouse IgG for IP (HRP; 1:5,000; cat no. ab131368, Abcam) labeled with horseradish peroxidase at room temperature for 30 min. Then, the nitrocellulose membrane was washed with PBS buffer and Chemistar ECL Western Blotting Substrate (Ultra-sensitive ECL luminescent solution; Hanbio Biotechnology Co., Ltd.) was used for chemiluminescence. Kodak X (Kodak) was used to expose the nitrocellulose membrane for ~3 min. The X-ray film was scanned (Oxford Instruments plc) and the grey analysis of the strip was processed by Gel-Pro Analyzer software 4.0 (Media Cybernetics, Inc.). The grey value of each band was measured by software in three independent repeated experiments and the ratio of the grey value of the target gene product to that of the β-actin product was used as the relative expression of the protein.

Cell viability assay using cell counting kit-8 (CCK8). The treated cells of each group were inoculated into 96-well plates at a density of $1x10^4$ cells/well. The final volume of each well was 100 µl. Each experiment was performed in triplicate. After 24 h, when the cells adhere to the wall, the original culture medium was replaced and the cells were incubated with the treatment according to their time points. Following incubation, the cell viability was determined using a CCK-8 kit (Thermo Fisher Scientific, Inc.). A volume of 10 µl CCK8 solution was added to each well and cultured in 5% CO_2 at 37˚C for 2 h. The absorbance (OD) was determined at 450 nm using a microplate reader. Cell survival rate was calculated using the following formula: Cell survival rate: OD value of experimental group-OD value of blank group/OD value of the control group-OD value of blank group x100%.

Terminal deoxynucleotidyl transferase dUTP nick labeling (TUNEL) assay. Cell apoptosis was determined using TransDetect® *In Situ* Fluorescein TUNEL Cell Apoptosis Detection kit (TransGen Biotech Co., Ltd.), according to the manufacturer's instructions. Briefly, the cells were washed

Table I. Primer sequence information (Sangon Biotech Co., Ltd.).

Name	Primer sequence (5'-3')	
Nephrin	Upstream primers	CCCTCCGGGACCCTACTG
	Downstream primers	TCTGGGAGGATGGGATTGG
miR-126	Upstream primers	CGGCAGGAACCTCCTTACTC
	Downstream primers	TGTGCCCTAGGGACGAAGGA
EGFL6	Upstream primers	TCTGTTTGCTCTTTGATTACCG
	Downstream primers	TTCCCTGTCTTCCACTTTTCAT
DKC1	Upstream primers	GCTAAGTTGGACACGTCTCAG
	Downstream primers	TGCAAGAGGTGTATAGTGTGTTG
GAPDH	Upstream primers	TGTGTCCGTCGTGGATCTGA
	Downstream primers	TTGCTGTTGAAGTCGCAGGAG

miR, microRNA; EGFL6, epidermal growth factor-like domain multiple 6; DKC1, dyskeratosis congenita 1.

with PBS and fixed with 4% paraformaldehyde or biyuntian (p0098) for 30-60 min. Then the cells were washed with PBS or Hank's balanced salt solution, followed by the addition of PBS containing 0.1% Triton X-100 and incubation on an ice bath for 2 min. To each sample, 50 μl TUNEL detection solution was added and incubated for 60 min at 37˚C, washed again with PBS and incubated with DAPI in PBS for 30 min at 30˚C. After incubation, the samples were washed with PBS for three successive times. The samples were observed under the IX73 fluorescence microscope (Olympus Corporation; x400 magnification). The excitation wavelength range was 450-500 nm and the emission wavelength range was 515-565 nm (green fluorescence).

Dual-luciferase reporter assay. For the dual-luciferase reporter assay, the TransDetect® Double-Luciferase Reporter Assay kit (Firefly Luciferase and *Renilla* Luciferase and plasmids; TransGen Biotech Co., Ltd.) was used. The cells were cultured and transfected for 48 h with EGFL6 3'UTR wild-type (Wt)/mutant (Mut) reporter plasmids and DKC1 3'UTR Wt/Mut reporter plasmids with or without miR-126 mimic/control precursor plasmids using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Each experiment was performed in triplicate. Following transfection, the medium was discarded and the cells were lysed with cell lysis buffer. The luciferase activity was measured using a GloMax 96 microplate luminescent detector (Promega Corporation). Luciferase activity was normalized to that of *Renilla*.

Statistical analysis. SPSS 22.0 statistical software (IBM Corp.) was used for data analysis. The data were analyzed by t-test for the differences between 2 groups and one-way analysis of variance (ANOVA) for the comparison of >2 groups. ANOVA with Dunnett's post hoc test was used to compare the data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Podocyte injury model. LPS (10 μg/ml) was used to treat mouse immortalized podocytes for sepsis model establishment. RT-qPCR demonstrated that the mRNA expression of nephrin was significantly downregulated with the prolongation of LPS treatment, reaching the lowest level after 36 h of culture (Fig. 1A), showing a time-dependent effect. The results of western blotting demonstrated that the protein expression of nephrin also significantly decreased 36 h after LPS stimulation (Fig. 1B). In addition, the expression level of miR-126 was downregulated with the prolongation of LPS treatment (Fig. 1C). These results confirmed the successful establishment of podocyte injury model and suggested that miR-126 served a role in podocyte function.

Increased miR-126 inhibited the podocyte injury. To investigate the specific role of differentially expressed miR-126 in septic podocyte injury, miR-126 mimics and miR-mimic NC were transfected into podocytes. Following transfection with miR-126 mimics and miR-mimic NC, RT-qPCR demonstrated that the expressions of miR-126 in the miR-mimic NC group and the control group were in the same level (Fig. 2A). Following transfection with miR-126 mimics, RT-qPCR demonstrated that the expression of miR-126 (Fig. 2A) and nephrin (Fig. 2B) in podocytes was promoted, indicating that upregulated miR-126 might mediate inhibition of podocyte injury. CCK-8 assay was used to detect the effect of miR-126 on the proliferation of podocytes. Results demonstrated that the growth of podocyte cells transfected with miR-126 was significantly promoted compared with podocyte injury group (Fig. 2C). Following transfection with miR-126 mimics, the growth of podocytes was promoted and the proliferation ability was increased.

Increased miR-126 inhibited the podocyte apoptosis. TUNEL assay results demonstrated that in the podocyte injury group the number of apoptotic cells was significantly increased compared with the control group, while the overexpression of miR-126 can alleviate injury and reduce the number of apoptotic cells (Fig. 3).

miR-126 target 3'UTR binding sites of EGFL6/DKC1 mRNA to regulate their expression and inhibit podocyte injury. Following co-transfection of miR-126 and Wt 3'UTR of EGFL6, luciferase

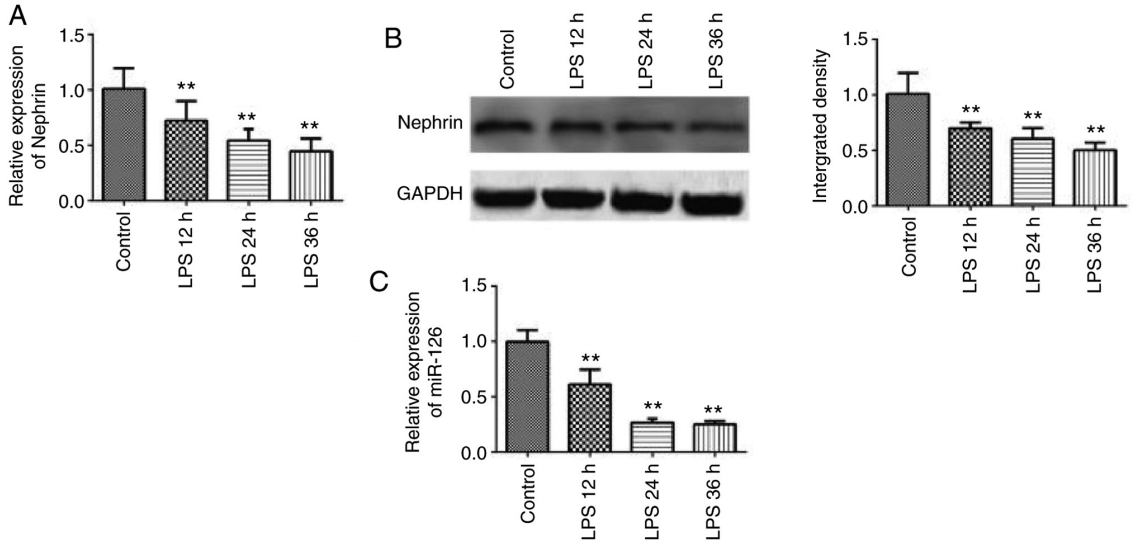

Figure 1. Podocyte injury model. (A) RT-qPCR results of the expression of nephrin in LPS-induced sepsis model of podocytes . (B) The expression of nephrin in LPS-induced sepsis model of podocytes by western blotting. (C) RT-qPCR results of the expression of miR-126 in LPS-induced sepsis model of podocytes. **P<0.01 vs. control. RT-qPCR, reverse transcription-quantitative PCR; LPS, lipopolysaccharide.

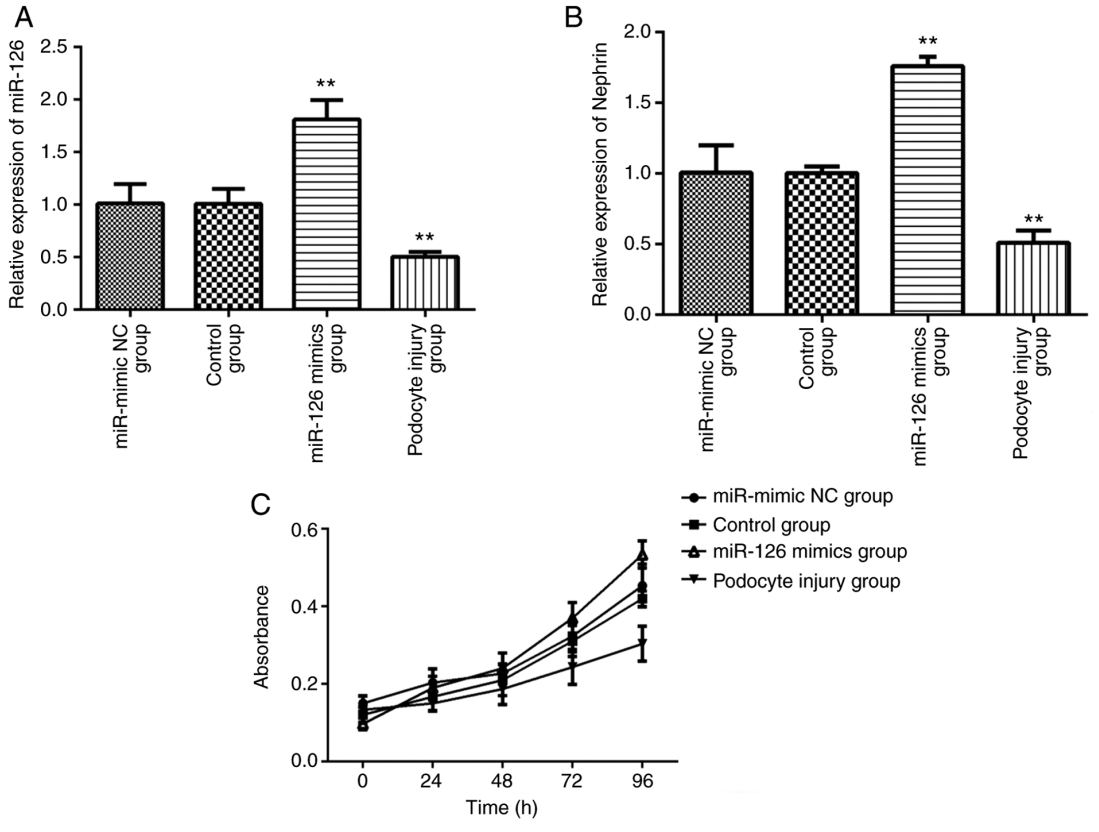

Figure 2. Increased miR-126 inhibits podocyte injury. (A) The expression of miR-126 following transfection with miR-126 mimics by RT-qPCR. (B) The expression of nephrin following transfection with miR-126 mimics by RT-qPCR. (C) Cell proliferation following transfection with miR-126 mimics by CCK8 assays. **P<0.01 vs. control. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

activity was significantly reduced (P<0.001; Fig. 4A). Similarly, as shown in Fig. 4B, luciferase activity was significantly reduced compared with the control group (P<0.001) after co-transfection of miR-126 and Wt 3'UTR of DKC1.When miR-126 was upregulated, the protein levels of both EGFL6 and DKC1 as determined by western blotting were increased (Fig. 5).

Discussion

Glomerular podocytes are differentiated terminal cells with a weak capability of division and regeneration. Podocytes, endothelial cells and basement membrane act together as glomerular filtration barrier. Negative charge of podocytes can

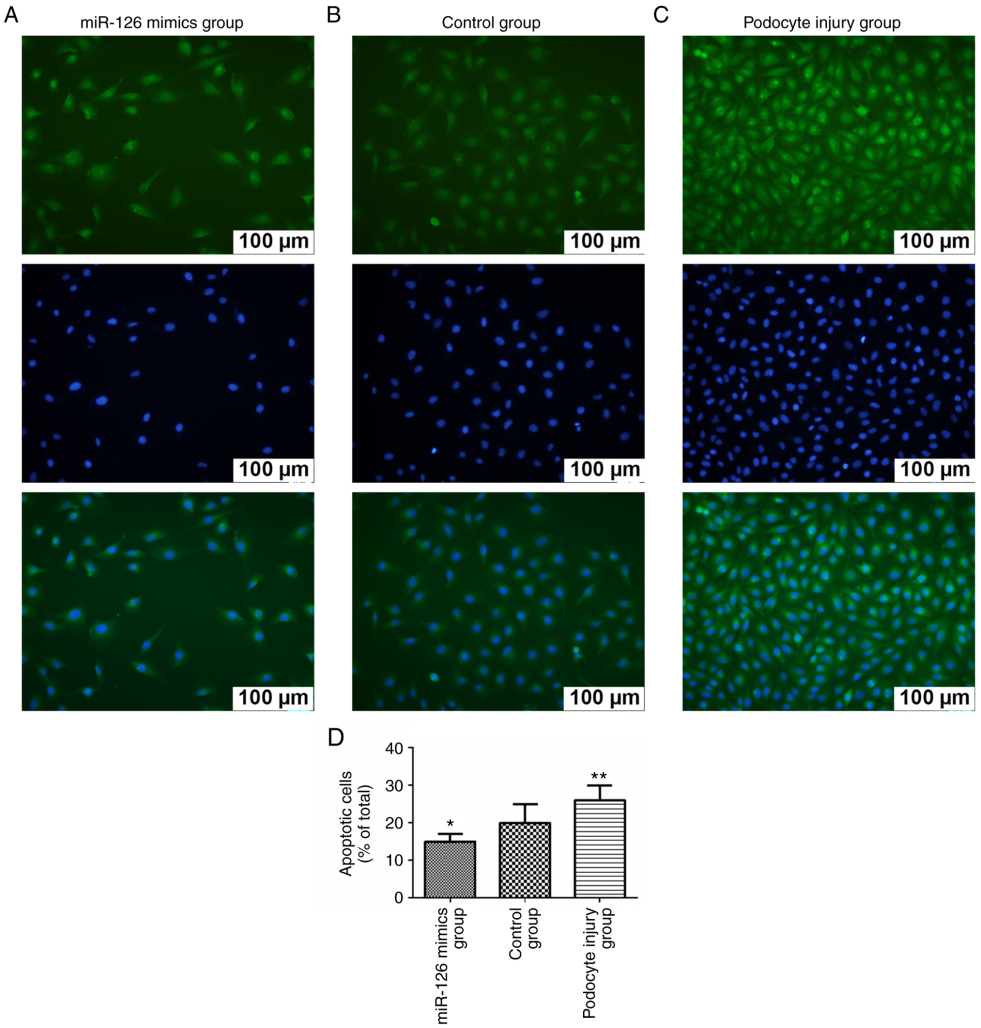

Figure 3. Apoptosis evaluation with TUNEL assay. (A) The TUNEL positive cells in miR-126 mimics group. (B) The TUNEL positive cells in control group. (C) The TUNEL positive cells in podocyte injury group. (D) The percentage of apoptotic cells. *P<0.05, **P<0.01.

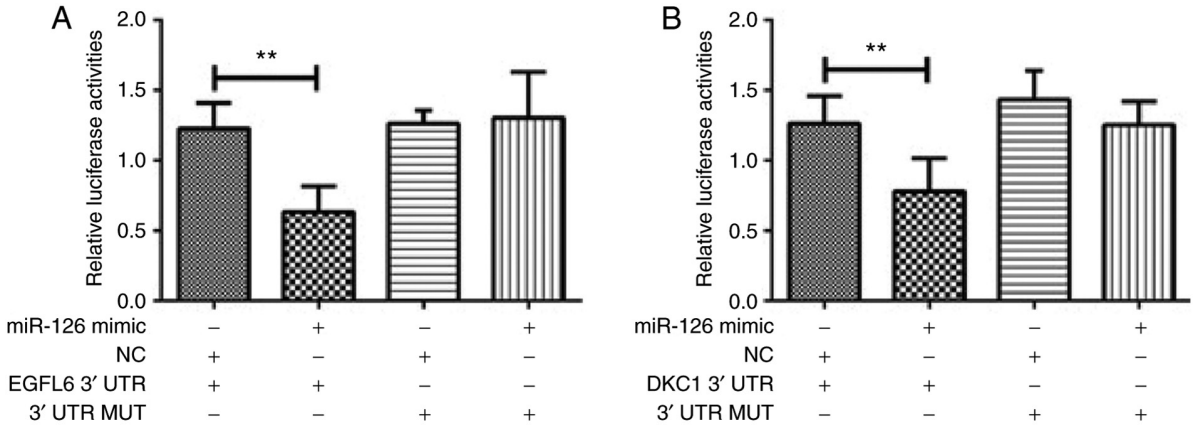

Figure 4. Target gene of miR-126 in the podocytes. (A) Dual-luciferase reporter assay: The luciferase activities were detected after co-transfected with EGFL6 3'UTR Wt/Mut reporter plasmids and miR-126/NC mimic for 48 h in the podocyte; (B) Dual-luciferase reporter assay: The luciferase activities were detected after co-transfected with DKC1 3'UTR Wt/Mut reporter plasmids and miR-126/NC mimic for 48 h in the podocyte. miR, microRNA; EGFL6, epidermal growth factor-like domain multiple 6; UTR, untranslated region; Wt, wild-type; Mut, mutant; DKC1, dyskeratosis congenita 1; NC, negative control group. **P<0.01.

prevent loss (or alternatively deprivation) of albumin and other macromolecules (11,12). Inflammation is an important factor in promoting damage of podocytes and proteinuria formation (43). The loss of podocyte structure and function is closely associated with inflammatory factors (11) and its mechanism has remains to be elucidated. The implication of inflammatory factors in podocyte damage has attracted the attention of a number of researchers aiming to find new therapeutic targets

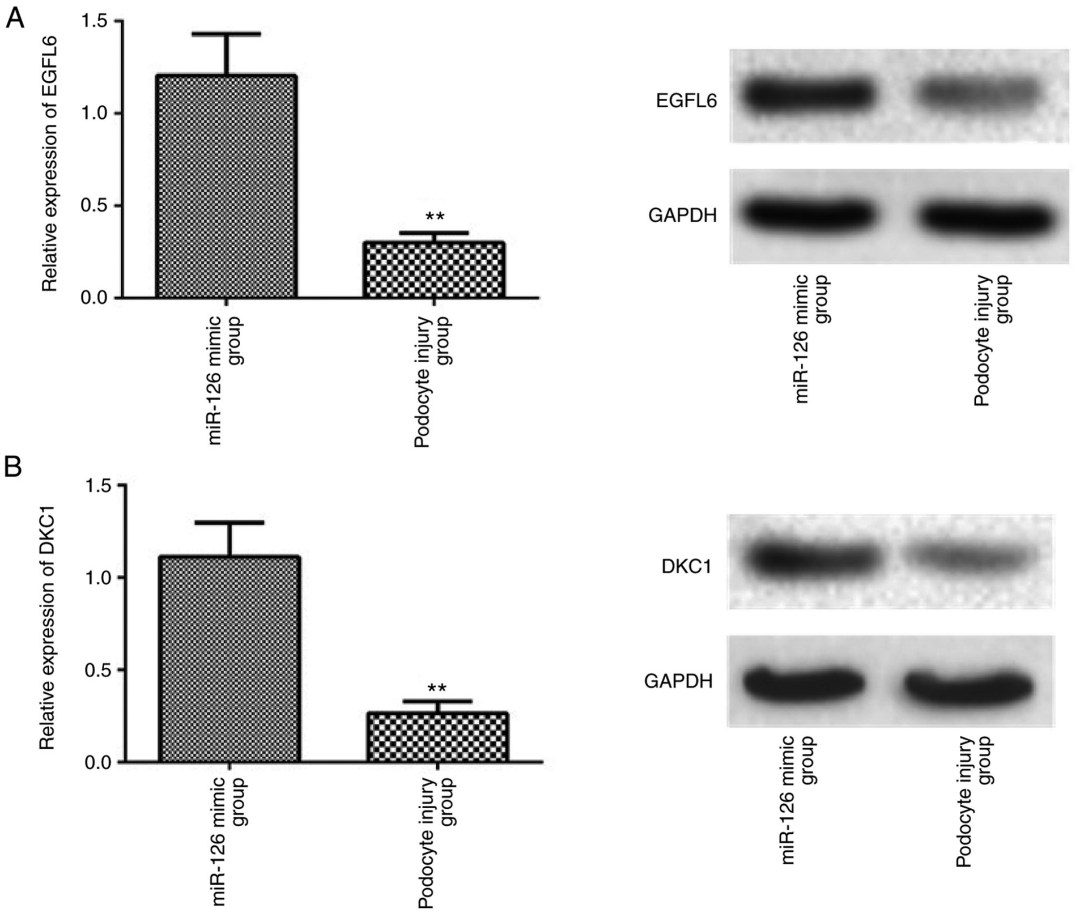

Figure 5. Protein levels of EGFL6 and DKC1 are increased when miR-126 is upregulated. Expression of (A) EGFL6 and (B) DKC1 proteins in miR-126 mimic group and podocyte injury group. EGFL6, epidermal growth factor-like domain multiple 6; DKC1, dyskeratosis congenita 1; miR, microRNA. **P<0.01.

which can provide a fundamental theoretical basis for the diagnosis and treatment of kidney diseases.

The present study found that miR-126 was downregulated in sepsis-induced podocyte injury. Overexpression of miR-126 could protect LPS-induced podocytes damage through an EGFL6/DKC1 signaling pathway. Bioinformatics analysis and dual-luciferase reporter assay demonstrated that EGFL6 and DKC1 are the key targets of miR-126.

miRs serve a key role in the post-transcriptional regulation of gene expression and are involved in normal and pathological renal function (44). Upregulation of miR-27a, miR-21 and miR-370 and downregulation of miR-15b-5p and miR-34c is associated with podocytes damage in diabetic nephropathy (45-49). Downregulation of miR-120a-5p is associated with increased expression of M-type phospholipase A2 receptor, which determines podocyte apoptosis and the progression of membranous nephropathy (50). Henique *et al* (51) demonstrate that upregulation of miRNA-92a is associated with the development and progression of glomerulonephritis. Sepsis-induced acute kidney disease is associated with the miR-15a-5p-XIST-CUL3 regulatory axis (52). Upregulation of miR-27b is associated with puromycin aminonucleoside-induced podocytes damage by targeting adenosine receptor 2B (53). The present study focused attention on miR-126 as one of the main regulators of sepsis-induced podocytes injury, potentially by targeting EGFL6 and DKC1 proteins.

The EGFL6 gene is a member of the EGF superfamily. The members of EGF superfamily are implicated in a wild spectrum of functions in the organism including cell proliferation, cell cycle and developmental processes (54). EGFL6 has been associated with tumor angiogenic functions in several types of cancers, including hepatocellular carcinoma, ovarian and breast cancer (54-56). Its role in preventing LPS-induced podocytes damage has yet to be investigated. The present study is the first, to the best of the authors' knowledge, that associates increased EGFL6 protein expression with a protective kidney effect. Further studies are required to elucidate these findings and potential utilization as a therapeutic strategy.

DKC1 is a gene that provides instruction for dyskerin protein involved in telomere integrity (57). A previous study demonstrated that downregulation of miR-126 is associated with high glucose-induced ageing to human glomerular mesangial cells and transfection with miR-126 mimics can act as an inhibitor of telomere-p-53-p21-RB signaling pathway and delay the effects (58). The current study supported these findings, showing that the modulation of DKC1 could be involved in the protective mechanism of miR-126 against podocytes damage.

The signal pathway regulated by various miRs could be interpreted as the key pathophysiological mechanism of nephropathy. These miR-related signal pathway inhibitors or inducers are expected to become clinical therapeutic drugs in the future (59). Understanding the key mechanism of miRNA in the development and progression of renal injury will help

to identify new potential therapeutic targets and design new therapeutic strategies. At present, it has been found that these miRNAs serve a regulatory role in some specific molecular pathways (59). It is necessary to expand the interaction between different pathways of miRNA and construct the miRNA interaction network in nephrotic patients. This will contribute to the understanding of the specific mechanism of miRNA in functional renal injury and structural renal injury.

From the perspective of treatment, miR as a new diagnostic and therapeutic marker of nephropathy is a new (or recent) trend (51). However, it is still necessary to fully understand the exact regulatory mechanism and specific functions of each miR at the transcription and translation level.

In conclusion, the present study found that miR-126 was downregulated in sepsis-induced podocyte injury and that overexpression of miR-126 could protect podocytes through an EGFL6/DKC1 signaling pathway. However, more research is needed to clarify the exact function and mechanism of miRNAs as a specific treatment for nephropathy.

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Availability of data and materials

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

Authors' contributions

JS and LD performed the experiments, analyzed and interpreted data and wrote the manuscript. Both authors were responsible for confirming the authenticity of the data. Both authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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