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Original Article

MicroRNA-30a inhibits cell proliferation in a sepsis-induced acute kidney injury model by targeting the YAP-TEAD complex

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ABSTRACT

Background: Acute kidney injury (AKI) is a primary feature of renal complications in patients with sepsis. MicroRNA (miRNA/miR)-30a is an essential regulator of cardiovascular diseases, tumors, phagocytosis, and other physical processes, but whether it participates in sepsis-induced AKI (sepsis-AKI) is unknown. We aimed to elucidate the functions and molecular mechanism underlying miR-30a activity in sepsis-AKI.

Methods: The classical cecal ligation and puncture (CLP) method and lipopolysaccharide (LPS)-induced Human Kidney 2 (HK-2) cells were used to establish *in vivo* and *in vitro* sepsis-AKI models. Specific pathogen-free and mature male Sprague-Dawley (SD) rats, aged 6–8 weeks (weight 200–250 g), were randomly divided into five-time phase subgroups. Fluid resuscitation with 30 mL/kg 37 ˚C saline was administered after the operation, without antibiotics. Formalin-fixed, paraffin-embedded kidney sections were stained with hematoxylin and eosin. SD rat kidney tissue samples were collected for analysis by real-time quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. HK-2 cells were transfected with hsa-miR-30a-3p mimics or inhibitors, and compared with untreated normal controls. RNA, protein, and cell viability were evaluated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), western blot, and cell counting kit-8 methods. A Dual-Luciferase Assay Kit (Promega) was used to measure luciferase activity 48 h after transfection with miR-30a-3p mimics.

Results: Expression levels of miR-30a-3p and miR-30a-5p in renal tissues of the sepsis group were significantly reduced at 12 h and 24 h (P <0.05). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were significantly increased in renal tissue 3 h after the operation in rats (P <0.05), and gradually decreased 6 h, 12 h, and 24 h after CLP. Levels of miR-30a-5p and miR-30a-3p were significantly down-regulated at 3 h after LPS treatment (P <0.05), and gradually decreased in HK-2 cells. One hour after LPS (10 μg/mL) treatment, TNF-α and IL-1β levels in HK-2 cells were significantly up-regulated (P < 0.05), and they were markedly down-regulated after 3 h (P <0.05). IL-6 expression levels began to rise after LPS treatment of cells, peaked at 6 h (P <0.05), and then decreased to the initial level within a few hours. Stimulation with 10 μg/mL LPS promoted HK-2 cells proliferation, which was inhibited after miR-30a-3p-mimic transfection. Bioinformatics prediction identified 37 potential miR-30a-3p target genes, including transcriptional enhanced associate domain 1 (*TEAD1*). After transfection of HK-2 cells with miR-30a-3p mimics and miR-30a-3p inhibitor, *TEAD1* transcript was significantly up- and down-regulated, respectively (both P <0.05). After LPS treatment (24 h), expression of *TEAD1* in the inhibitors group was significantly increased (P <0.01), while that in the mimics group was significantly suppressed (P <0.01). In the dual luciferase reporter experiment, miR-30a-3p overexpression decreased fluorescence intensity (P <0.01) from *TEAD1-wt*-containing plasmids, but did not influence fluorescence intensity from *TEAD1-muta*-containing plasmids. LPS may promote HK-2 cells proliferation through the miR-30a-3p/*TEAD1* pathway.

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Conclusion: In a background of expression of inflammatory factors, including TNF-α, IL-1β, and IL-6, which were transiently increased in the sepsis-AKI model, miR-30a was down-regulated. Down-regulated miR-30a-3p may promote cell proliferation by targeting *TEAD1* in LPS-induced HK-2 cells, demonstrating its potential as a biomarker for early sepsis-AKI diagnosis.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection and a significant cause of death in critically ill patients.[1] Acute kidney injury (AKI) is a clinical syndrome characterized by a rapid decline of renal function and an accumulation of metabolic waste, with critical diagnostic criteria of azotemia and oliguria.[2] The kidney is among the most common organs affected by sepsis. Sepsis-induced AKI (sepsis-AKI), also referred to as sepsis-associated AKI or septic AKI, is a syndrome involving kidney injury followed by acute kidney dysfunction. Sepsis-AKI is the most common AKI syndrome in the intensive care unit, accounting for approximately half of all AKI in hospitals, and generally increases sepsis mortality.[3] The prognosis of patients with sepsis-AKI depends mainly on the degree of acute basal-level injury of the organ. Many research institutes have elucidated clinical risk factors, pathophysiological mechanisms, response to treatment, and factors affecting kidney recovery, which have improved the ability to prevent, detect, and treat sepsis-AKI. Over recent years, evidence has accumulated that non-coding RNAs, such as microRNAs (miRNAs/miR) and long non-coding RNAs, have significant potential for application in the development of diagnostic and therapeutic strategies for AKI.[4] miRNAs are endogenous, non-coding regulatory RNAs of approximately 23 nt that are associated with numerous human diseases and increasingly serve as clinical diagnostic and therapeutic targets. miRNA function involves the regulation of target protein-coding genes in animals and plants by pairing with their messenger RNAs (mRNAs), resulting in post-transcriptional inhibition.[5,6] Almost all miRNA family molecules are generated by a series of biogenetic steps to convert a primary miRNA transcript to a mature miRNA, which is loaded into the RNA-induced silencing complex to direct the complex to the target mRNA, resulting in translational inhibition and degradation. Gene expression profiling studies have demonstrated universal changes in miRNA expression in human diseases. Further, functional studies have frequently identified miRNA dysregulation as a cause of disease progression.

Many miRNAs are associated with AKI, some of which regulate apoptosis and inflammation, and contribute to AKI pathogenesis by amplifying or reducing acute injury responses. In contrast, other miRNAs regulate fibrosis and angiogenesis, participate in kidney recovery, or influence progression to fibrosis.[7] Wei et al.[8] reported the first evidence that miRNAs have a pathological role in AKI. They established a Dicer knockout mouse model in which Dicer was specifically removed from proximal tubular cells. These mice exhibited an overall down-regulation of miRNAs in the renal cortex and had normal renal function and histology under control conditions, but were resistant to AKI after bilateral renal ischemia–reperfusion injury. Further, Dicer knockout mice had significantly better renal function than wild-type mice, as well as reduced tissue damage, reduced tubular apoptosis, and higher survival rates.

Many biological and pathological processes involving miRNAs in AKI are yet to be fully understood. Further, some studies have yielded inconsistent data on miRNA expression patterns in various samples, species, disease models, and time points. Contrast-induced nephropathy (CIN) is a complex acute kidney disease syndrome that occurs after infusion of an intravascular contrast agent and is associated with an increased risk of adverse cardiovascular events. Gutierrez-Escolano et al.[9] demonstrated for the first time that plasma miRNAs, particularly three members of the miR-30 family (miR-30a, miR-30c, and miR-30e), can serve as early biomarkers for contrast agent-induced AKI (CI-AKI) and are candidate therapeutic targets in patients with CIN. Another study of CI-AKI identified three human-derived miRNAs, miR-188, miR-30a, and miR-30e, as possible early biomarkers for CI-AKI by screening miRNA profiles in mouse plasma and kidney tissues.[10] Hence, determining the target genes of miR-30 family molecules and their biological mechanisms in the context of Sepsis-AKI warrants further investigation.

Methods

Polymicrobial cecal ligation and puncture (CLP) sepsis-AKI

Animal experiments were complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

The CLP model, which consists of the perforation of the cecum allowing the release of fecal material into the peritoneal cavity to generate an exacerbated immune response induced by polymicrobial infection, was used to establish an animal model of sepsis-AKI.[11–14] Fluid resuscitation with 30 mL/kg 37 °C saline was administered after the operation without antibiotics. Specific pathogen-free and mature male Sprague-Dawley (SD) rats (aged, 6–8 weeks; weight 200–250 g) were maintained in an animal room with fresh air, sufficient light, standard feed, and clean water. The indoor temperature was controlled at 20–28 °C, and the humidity was 40%–60%. After 1 week of adaptive feeding, 38 active, healthy rats were selected for inclusion in the experiment and randomly divided into normal control group ($n = 6$) and CLP groups (3 h, 6 h, 12 h, and 24 h). After successful CLP, rats presented with fever, significantly increased heart and respiratory rates, increased oral and nasal discharge, malaise, lethargy, curling, hair erection, reduced movement, refusal or reduction of diet, and increased ocular discharge, accompanied by AKI, manifested as decreased urine output, increased creatinine (Cr), and overall 21.1% (8/38) natural mortality. Finally, 30 effective samples were included in the experiment, with 6 rats in each group. Eight rats that died before the target time were excluded. Mortality rates were 0 in the 0 h, 3 h, and 6 h groups, 25.0% (2/8) in the 12 h group, and 50.0% (6/12) in

the 24 h group. The symptoms described above are alleviated in surviving rats.

Evaluation of renal function and histology

Kidney sections fixed in 10% formalin and paraffin-embedded were stained with hematoxylin and eosin (Guangzhou Chemical Reagent Factory, China). Tubule damage was defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation.[15] Cr and blood urea nitrogen (BUN) serum levels were measured using an autoanalyzer (Creatinine and BUN determination kit, Beijing Beihua Kangtai Clinical Reagent Co., Ltd., OLYMPUS-AU400, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Kidney tissue samples (50 μg) from SD rats (n=6 per group) were shredded, ground, and centrifuged, and the supernatants were collected for quantification. TNF-α and interleukin-1β (IL-1β) levels were quantified by ELISA, using Rat TNF-α and Rat IL-1β ELISA kits (Invitrogen, USA), according to the manufacturer's protocols.

Cell culture and treatments

HK-2 cells were purchased from American Type Culture Collection (ATCC) (product number: CRL-2190) and adherent cultures grown *in vitro* in Dulbecco's modified Eagle's medium/F12 (HyClone, USA) containing 10% fetal bovine serum (Gibco, USA) and penicillin–streptomycin (HyClone) at 37 ˚C, 5% CO_2. When the cell density reached 80%, 0.25% trypsin (Trypsin-EDTA, Gibco) was used to dissociate cells for sub-culture. After approximately 2–3 passages, experiments were conducted using cells in a stable growth state. HK-2 cells were treated with varying concentrations of lipopolysaccharide (LPS) (L2880–25MG, O55:B5, Sigma, USA) at different time points.

Cell transfection

Based on mirbase.org data, the nucleotide sequence of miR-30a-3p is highly conserved between human and rat species, with 100% identity (both rno-miR-30a-3p and hsa-miR-30a-3p have the same sequence: "CUUUCAGUCGGAUGUUUGCAGC"); hsa-miR-30a-3p mimics, hsa-miR-30a-3p inhibitors, and negative controls (NC) were synthesized by GenePharma Co. (Shanghai, China) (Table S1–3). Reagents were transfected into HK-2 cells using Lipofectamine™ 3000 (Invitrogen, USA), according to the manufacturer's protocol. Cells were used for subsequent experiments 72 h after transfection.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from non-transfected and transfected HK-2 cells using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. A First Strand cDNA Synthesis Kit (Invitrogen, USA) was used to generate cDNAs, which were quantified using a DyNamo SYBR1 Green qPCR Kit (Takara, Dalian, China) and gene-specific primers (Table S1–2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The TaqMan MicroRNA Reverse Transcription Kit and TaqMan miRNA assay (Qiagen, China) were used to quantify miRNA expression; *U6* was used as an internal control. Expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Transfected or non-transfected HK-2 cells were harvested and lysed in radioimmunoprecipitation assay buffer (Meilunbio, Dalian, China), supplemented with a phenylmethanesulfonyl fluoride and protease inhibitor cocktail (Meilunbio). Extracted proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Beyotime, Shanghai, China). After blocking in 5% non-fat milk for 1 h at room temperature, the membrane was incubated overnight at 4 ˚C, with primary antibodies, as follows: anti-TEAD-1 (ab133533, 1:5000, Abcam, USA) and anti-GAPDH (AC002, 1:5000, ABclonal, Wuhan, China). After washing, membranes were treated with corresponding secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature, and finally incubated with enhanced chemiluminescence solution (Meilunbio). Densitometer technology and Quantity One software (Bio-Rad, Hercules, CA, USA) were used for signal visualization and quantification.

Cell viability

HK-2 cells were plated in 96-well plates at 5×10^4 cells/well. After treatment, the viability of cells in each group was examined by CCK-8 assay (AbMole, USA). Briefly, each well was supplemented with 10 μL CCK-8 solution and incubated at 37 ˚C for 4 h, after which absorbance was measured at 450 nm using a microplate reader.

Dual-luciferase reporter gene assay

A Dual-Luciferase Reporter Gene Assay Kit (Promega, Madison, WI, USA) was used to perform luciferase activity assays, according to the manufacturer's instructions. Briefly, wild-type and mutant 3′ untranslated region (3′UTR) fragments of the transcriptional enhanced associate domain 1 (*TEAD1*) gene were cloned into the pmirGLO luciferase reporter vector (Promega). HEK293T cells (ATCC; 2×10^4 cells/mL) were seeded into 24-well plates and co-transfected with the reporter plasmid and miR-30a-3p mimics after 5 h culture, using Lipofectamine™ 2000 (Invitrogen). A Dual-Luciferase Assay Kit (Promega) was used to measure the luciferase activity 48 h after transfection.

Statistical analysis

GraphPad Prism software version 7.0 for Mac (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze data. A non-paired t-test was applied to compare two independent samples, a one-way analysis of variance (ANOVA) to compare multiple means at different time points, a non-parametric test to determine the homogeneity of variance, and an F-test (P >0.05) to judge the homogeneity of variance.

Results

In vivo expression of miR-30a-3p, miR-30a-5p, TNF-α, and IL-1β was altered following CLP

Rats in the control group appeared in good condition, with stable breathing, quick movements, free consumption of food and water, and little or no secretion from their eyes. In contrast, rats in the CLP operation group appeared in poor condition postoperatively, with shortness of breath, chills, tremors, erect hair, slow movements, reluctant to consume water, and secretion from their eyes. There was no significant difference in body weight between groups before modeling (P >0.05) (Figure 1A). Compared with the control group, Cr levels were significantly higher in the 6 h, 12 h, and 24 h CLP groups (P <0.05) (Figure 1A). BUN gradually increased from 3 h after modeling and was significantly increased compared with the control group after 24 h (P <0.05) (Figure 1A). In the CLP groups, renal tubular epithelial cells were swollen, and tubular dilatation, tubular structure destruction, epithelial cell renal interstitial edema, glomerular capillary dilatation, and congestion were observed, with the pathological changes to renal tissue became more obvious over time (Figure 1B). ELISA showed that TNF-α and IL-1β were significantly increased in rat renal tissue 3 h after the operation (P <0.05), and then gradually decreased at 6 h, 12 h, and 24 h after CLP (Figure 1C). Expression levels of miR-30a-3p and miR-30a-5p in renal tissues from the CLP group were significantly lower than those in controls at 12 h and 24 h, (P <0.05 Figure 1D).

In vitro LPS stimulation transiently increased TNF-α, IL-1β, and IL-6, and decreased miR-30a expression in HK-2 cells

One hour after treatment with 10 μg/mL LPS, TNF-α and IL-1β levels were significantly up-regulated in HK-2 cells (P <0.05), while they were markedly down-regulated after 3 h (P <0.05). IL-6 expression began to rise after LPS treatment of cells, peaked at 6 h (P <0.05), and then decreased back to the initial level within a few hours (Figure 2A). Further, miR-30a-5p and miR-30a-3p expression levels were significantly down-regulated 3 h after LPS treatment (P <0.05), and gradually decreased with extended treatment duration (Figure 2B).

LPS stimulation promotes HK-2 cells proliferation

Stimulation with 10 μg/mL LPS promoted HK-2 cells proliferation; however, the proliferation was inhibited following transfection of miR-30a-3p-mimic (Figure 3).

Prediction of miR-30a-3p target genes

Using four miRNA target analysis databases, including miRWalk (http://mirwalk.umm.uni-heidelberg.de), TargetScan (http://www.targetscan.org/), miRDB (http://mirdb.org/miRDB/), and mirDIP (http://ophid.utoronto.ca/mirDIP/index.jsp), we retrieved potential hsa-miR-30a-3p target genes. Taking the intersection of the results from the four databases, we finally identified 37 candidate target genes, including *TEAD1* (Figure 4A and B); *TEAD1* was selected for subsequent experimental verification after literature screening.

MiR-30a-3p inhibits TEAD1 *expression by targeting its 3′UTR*

Using Lipofectamine™ 3000, miR-30a-3p mimics and miR-30a-3p inhibitors were transfected into HK-2 cells, incubated for 36 h, and then treated with LPS (10 μg/mL) for 12 h (Figure 5A). miR-30a-3p was significantly overexpressed in cells transfected with miR-30a-3p mimics (P <0.05) (Figure 5B), indicating that miR-30a-3p was successfully overexpressed after transfection.

The only *TEAD1* mRNA transcript was significantly up-regulated in the miR-30a-3p inhibitors group (P <0.05) and significantly down-regulated in the miR-30a-3p mimics group (P <0.05) (Figure 4B). Further, 24 h after LPS treatment, *TEAD1* expression was significantly increased in the inhibitors group (P <0.01), while that in the mimics group was significantly suppressed (P <0.01) (Figure 5C). These results indicate that miR-30a-3p inhibits *TEAD1* expression.

In dual luciferase reporter assays, when a plasmid carrying wild-type *TEAD* 3′UTR (*TEAD1-wt* 3′UTR) was introduced into 293T cells, fluorescence intensity decreased in response to miR-30a-3p overexpression (P <0.01); however, in cells transfected with a plasmid carrying mutated *TEAD* 3′UTR (*TEAD1-muta* 3′UTR) fluorescence intensity was unchanged after overexpression of miR-30a-3p (P >0.05) (Figure 5D and E). These findings indicate that miR-30a-3p inhibits *TEAD1* expression by targeting the *TEAD1* 3′UTR.

Discussion

Previous studies in the sepsis have shown that miR-30a is involved in vital phenotypes, including immunity, inflammation, apoptosis, and autophagy, as well as diseases, such as septic liver injury, renal ischemia–reperfusion injury, graft-*vs.*-host disease, and acute carbon monoxide poisoning, among others. Levels of circulating microRNAs, such as miR-30a-5p and miR-30d-5p, are positively correlated with those of the redox biomarker, peroxiredoxin-1 (Prdx-1), which is released by immune cells during inflammation, and reflect the severity of sterile systemic inflammatory response syndrome, but not sepsis.[16] miR-30a inhibits MD-2 expression by targeting signal transducer and activator of transcription 1 (STAT1) in human monocytes.[17] Adenosine deaminase acting on double-stranded RNA 1 (ADAR1) exerts a protective effect against sepsis by reducing inflammation and organ damage via the ADAR1-miR-30a-suppressor of cytokine signaling protein (SOCS)−3 axis.[18] miR-30a-3p overexpression may improve sepsis-induced cell apoptosis *in vitro* and *in vivo* via the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway.[19] miR-30a-5p mitigates autophagy by regulating the Beclin-1/autophagy-related gene 16 (*ATG16*) pathway in renal ischemia/reperfusion injury.[20] Further, miR-30a inhibits liver cell proliferation and promotes apoptosis by targeting and negatively regulating SOCS-1 via the Janus kinase (JAK)/STAT signaling pathway in rats with sepsis.[21] A specific plasma miRNA signature, including miR-30a, may serve as an independent biomarker for the prediction, diagnosis, and prognosis of acute graft-*vs.*-host disease.[22] Moreover, miR-30a may provide a reference, which is released for early diagnosis and prediction of disease progression and prognosis in cardiac injury following acute carbon monoxide poisoning.[23] While the role miR-30a plays in sepsis-AKI requires clarification.

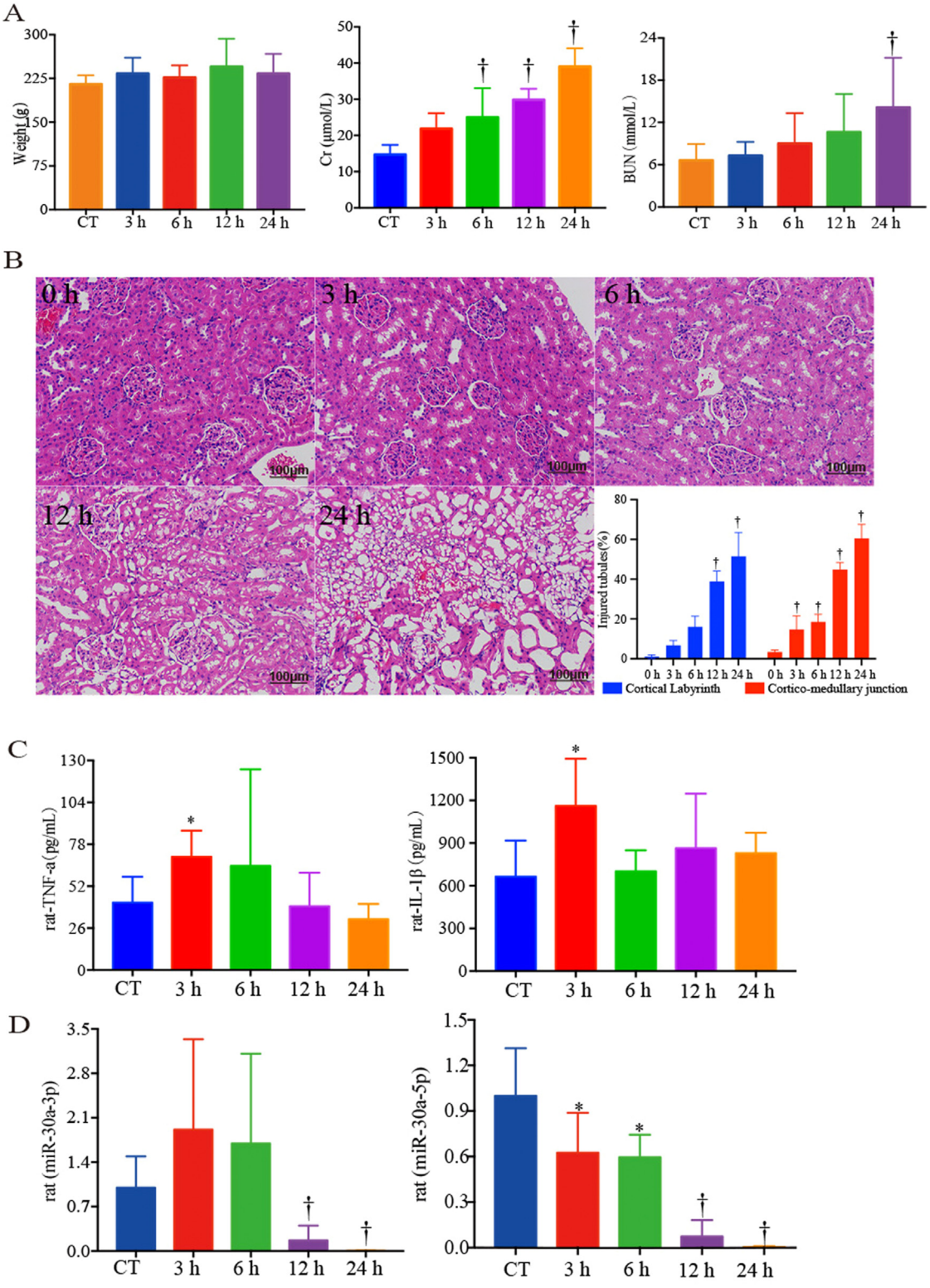

Figure 1. Changes in molecular expression levels and pathological manifestations in kidney tissues from SD rats after CLP operation. A: There was no significant difference in body weight among groups before model establishment. Cr and BUN levels increased gradually over time in each experimental group compared with the control group. B: HE staining (magnification, 200 ×). Pathological damage to renal tissue was observed to increase with operation duration. Acute tubular injury in the cortical labyrinth and corticomedullary junction was quantified by counting the number of injured tubules per 200 total tubules per region. C: TNF-α and IL-1β expression levels increased transiently and then decreased, as determined by ELISA. D: miR-30a (miR-30a-3p, miR-30a-5p) expression in rat kidney tissues gradually decreased with prolonged postoperative time, as determined by qRT-PCR; *U6* served as the internal reference RNA (n = 6 per group).
$^{*}P$ <0.05, $^{\dagger}P$ <0.01, compared with the control group.
BUN: Blood urea nitrogen; CLP: Cecal ligation and puncture; Cr: Creatinine; CT: Control; ELISA: Enzyme-linked immunosorbent assay; HE: Hematoxylin and eosin; IL-1β: Interleukin-1β; qRT-PCR: Real-time quantitative polymerase chain reaction; SD: Sprague-Dawley; TNF-α: Tumor necrosis factor-α.

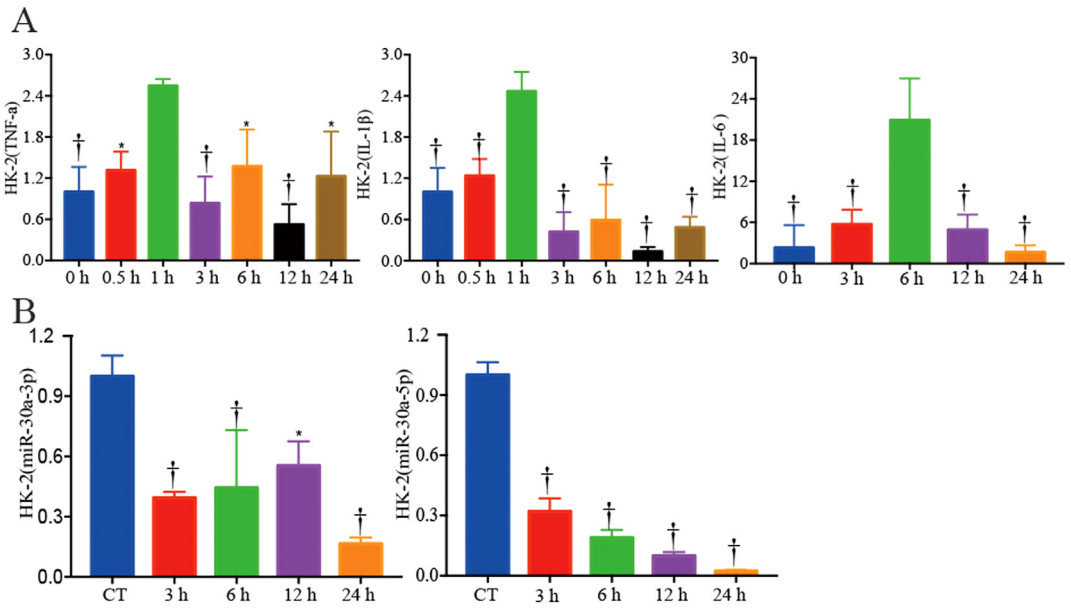

Figure 2. Molecular expression changes in HK-2 cells treated with LPS (10 μg/mL). A: qRT-PCR results showing decreased mRNA expression of the inflammation factors, *TNF-α, IL-1β, and IL-6*, in HK-2 cells after LPS treatment; *GAPDH* mRNA served as an internal reference (Table S4). $^{*}P$ <0.05, $^{\dagger}P$ <0.01, compared with the highest value group (n=3 per group.) B: miR-30a was significantly down-regulated at 3 h after LPS stimulation. $^{*}P$ <0.05, $^{\dagger}P$ <0.01, compared with the control group (n=3 per group).
CT: Control; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HK-2: Human Kidney 2; IL: Interleukin; LPS: Lipopolysaccharides; mRNA: Messenger RNA; qRT-PCR: Quantitative real-time polymerase chain reaction; TNF: Tumor necrosis factor.

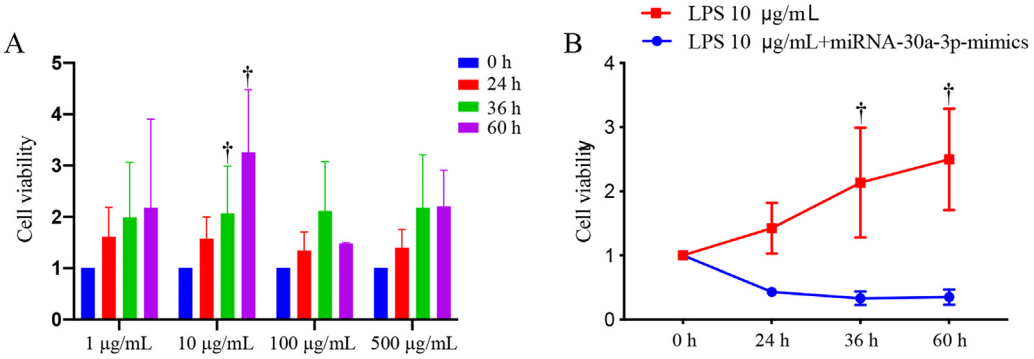

Figure 3. The effects of miR-30a on HK-2 cells proliferation following LPS-induced injury. A: LPS (1–500 μg/mL) stimulated HK-2 cells proliferation. B: miR-30a-3p inhibited the pro-proliferation effect of LPS. Stimulation with 10 μg/mL LPS significantly stimulated HK-2 cells proliferation; however, this proliferation was inhibited after miR-30a-3p-mimic transfection. $^{*}P$ <0.05, $^{\dagger}P$ <0.01, compared with the control group.
HK-2: Human Kidney 2; LPS: Lipopolysaccharides.

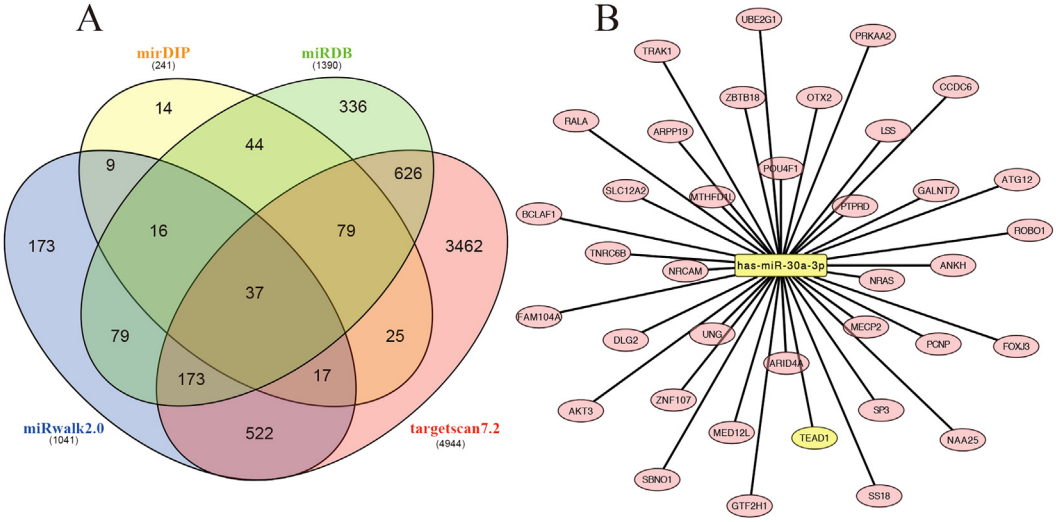

Figure 4. miR-30a-3p target gene prediction and screening. A: Four miRNA target analysis databases were searched for possible miR-30a-3p target genes, and intersecting prediction results were used to obtain 37 predicted targets. B: Thirty-seven predicted genes and selection of *TEAD1* for target gene verification analysis.
TEAD1: Transcriptional enhanced associate domain 1.

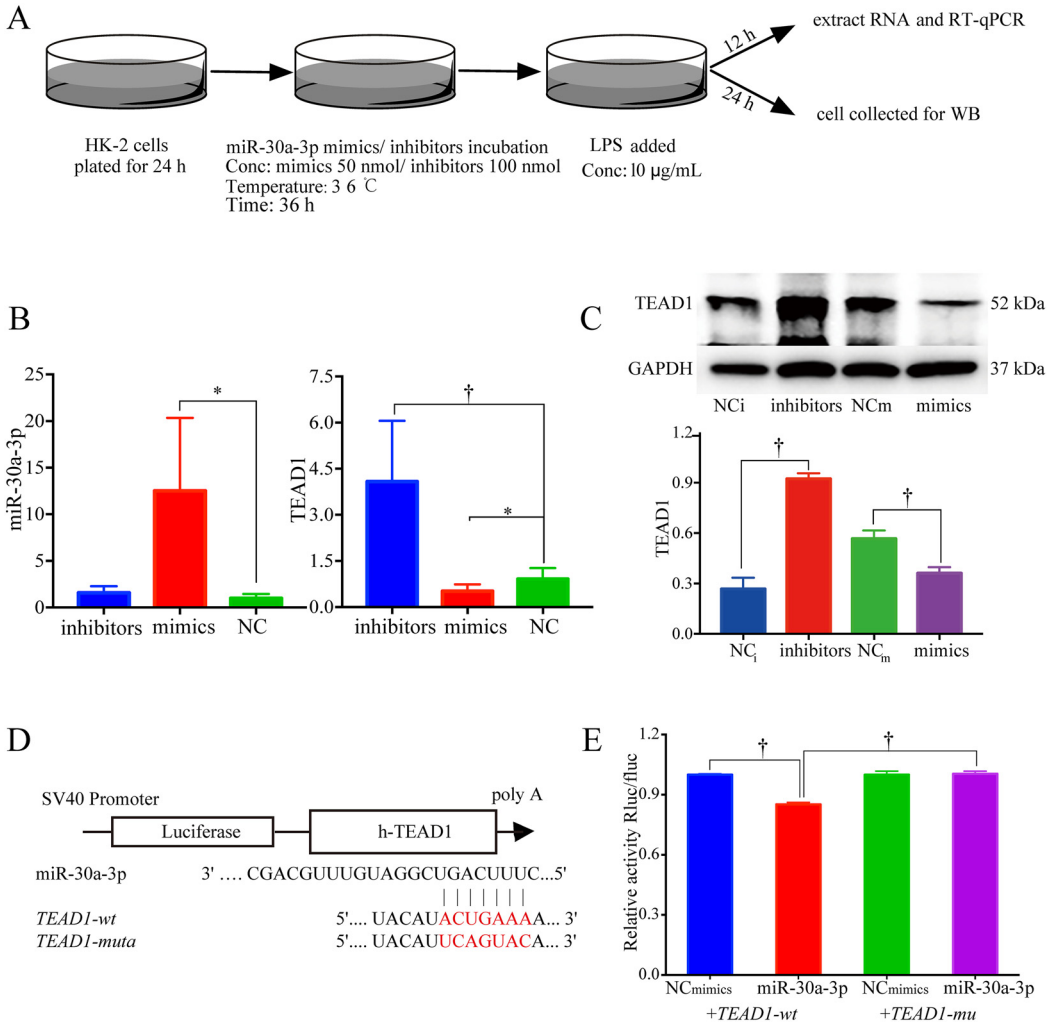

Figure 5. miR-30a-3p target gene validation. A: Flow diagram of miRNA-disrupted HK-2 cells treated with LPS; miR-30a-3p mimics and inhibitors were used at doses of 50 nmol and 100 nmol, respectively. After 36 h incubation, LPS was added to a fresh medium. B: mRNA expression levels of miR-30a-3p and *TEAD1* in each group after 12 h. Compared with the control group, miR-30a-3p was up-regulated in the mimics group (qRT-PCR, *U6* as the internal reference), *TEAD1* mRNA was down-regulated in the mimics group and up-regulated in the inhibitors group (qRT-PCR, *GAPDH* as the internal reference). C: *TEAD1* expression after LPS (10 μg/mL) treatment for 24 h. Western blot results showing that TEAD1 was up-regulated in the inhibitors group and down-regulated in the mimics group; GAPDH served as the internal reference. D: Indication of binding of hsa-miR-30a-3p to the *hsa-TEAD1* target site. *TEAD1-wt* contains a wild-type sequence complementary with the corresponding bases of miR-30a-3p. *TEAD1-muta* contains a mutant sequence, where corresponding bases are randomly mutated and will not pair with miR-30a-3p. E: Dual luciferase reporter assay. 293T cells were transfected with *TEAD1-wt* and *TEAD1-muta* plasmids. Subsequent transfection of miR-30a-3p mimics in cells containing *TEAD1-wt*-containing plasmids weakened the fluorescence intensity, while no difference in fluorescence was detected in the *TEAD1-muta* group. $^{*}P$ <0.05, $^{\dagger}P$ <0.01, compared with the control group (n=3 per group).
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HK-2: Human Kidney 2; LPS: Lipopolysaccharides; mRNA: Messenger RNA; NC: Negative control; NC_i: Negative control of inhibitors; NC_m: Negative control of mimics; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; *TEAD1*: Transcriptional enhanced associate domain 1.

LPS release from the cell wall of Gram-negative bacteria is a critical factor in causing sepsis or AKI, and inflammatory cytokines induced by LPS are reported to play an essential role in AKI pathogenesis.[24,25] LPS is a classical ligand for toll-like receptor 4 (TLR4), mediates TLR4-dependent signaling, and activates the NF-κB pathway, leading to increased expression of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Therefore, experimental models based on LPS induction are widely used in the study of sepsis-associated AKI.[26] The roles of TNF-α, IL-1β, and IL-6 in serum and kidney are characterized by LPS-induced AKI progression, and these cytokines are known to contribute to LPS-induced AKI.[26] There is increasing evidence suggesting that inhibition of NF-κB activation and these inflammatory factors can attenuate LPS-induced AKI and protect renal function.[27] We found that, in both the CLP-rat kidney tissue and LPS-HK-2 cells models, miR-30a began to be down-regulated in the early stage of sepsis-AKI, on a background of transiently increased TNF-α, IL-1β, and IL-6 (Figure 1 and 2).

Following LPS stimulation, we introduced chemically synthesized exogenous miR-30a-3p mimics by liposome transfection, the nucleotide sequence of which was complementary with miR-30a-3p-inhibited genes in HK-2 cells, to artificially increase the concentration of miR-30a-3p and complementary single-stranded small RNA in the cells. The transfection efficiency was verified by qRT-PCR, and the results showed that miR-30a-3p expression was significantly increased in the mimics group;

however, there was no significant change in the inhibitors group (Figure 5B), this is because miRNA inhibitors are single-stranded chemically modified enhanced oligonucleotides, which inhibit miRNA activity, but do not necessarily decrease or degrade its physical content. Further, the exogenously introduced miR-30a-3p mimics synergized with endogenous miR-30a-3p and bound with the 3′UTR of *TEAD1* to inhibit its expression at the transcriptional level, as verified by qRT-PCR. Conversely, miR-30a-3p inhibitors underwent complementary pairing with endogenous miR-30a-3p, leading to competitive suppression of the inhibitory effect of endogenous miR-30a-3p on *TEAD1* and significant *TEAD1* up-regulation in the inhibitor-treated group.

Furthermore, we verified the *TEAD1* expression changes at the protein level by western blot, and the results were consistent with the expression difference detected at the mRNA level (Figure 5C). To further verify the direct targeting relationship between miR-30a-3p and *TEAD1*, we also designed a dual luciferase reporter assay, and found that fluorescence intensity decreased after transfection of a plasmid containing wild-type *TEAD1* sequence into 293T cells after overexpression of miR-30a-3p (P <0.01), while in cells transfected with a plasmid expressing *TEAD* 3′UTR containing a mutation, fluorescence intensity remained unchanged after miR-30a-3p overexpression (P >0.05) (Figure 4). These findings suggest that miR-30a-3p inhibits *TEAD1* expression by directly targeting its 3′UTR. In addition, CCK-8 assays showed that treatment with different concentrations of LPS (1–500 μg/mL) could promote HK-2 cells proliferation. In contrast, cell proliferation was inhibited after transfection of miR-30a-3p-mimic (Figure 3), indicating that LPS may promote HK-2 cells proliferation through the miR-30a-3p pathway. Further, protein analysis found that miR-30a-3p up-regulation reduced *TEAD1* expression.

Conversely, *TEAD1* expression was up-regulated after miR-30a-3p inhibition, indicating that miR-30a-3p directly targets *TEAD1*. By inhibiting miR-30a-3p expression, *TEAD1* can be up-regulated during sepsis-AKI development. Furthermore, the Hippo signaling pathway can be targeted to regulate and influence cell proliferation and apoptosis.

Existing research indicates that direct targets of the Yes-associated protein (YAP)-TEAD complex, such as connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (CYR61), MYC, and Amphiregulin (AREG), can enhance the cell proliferation.[28,29] The YAP-TEAD complex is a core component of the Hippo signaling pathway, which is a regulator of cell growth, proliferation, homeostasis, stem cell function, and tissue regeneration, as well as a promising target for the treatment of cancer.[30–33] When HK-2 cells were stimulated with LPS, miR-30a-3p expression was down-regulated, leading to up-regulation of its direct target gene *TEAD1*, and may also induce up-regulation of target genes downstream of *TEAD1*, such as *CTGF, CYR61, MYC*, and *AREG*, ultimately promoting cell proliferation (Figure 6). LPS may promote HK-2 cells proliferation through the miR-30a-3p/*TEAD1* pathway.

While the function of the YAP-TEAD complex has been extensively characterized, only a few studies have examined YAP function as a regulator of gene expression via microRNAs.[34,35] A recent study revealed that YAP binds to the promoter and regulates the expression of miR-30a. YAP regulation of Schwann cell proliferation and death is mediated through miR-30a regulation of protein tyrosine phosphatase non-receptor type 13 (PTPN13) in Schwann cells.[34] Hence, there are complex mutual regulation relationships between miR-30a and the YAP-TEAD complex, which require further study and clarification. These regulatory mechanisms are not limited to specific disease environments, such as sepsis-AKI, nor do they only occur in Schwann cells.

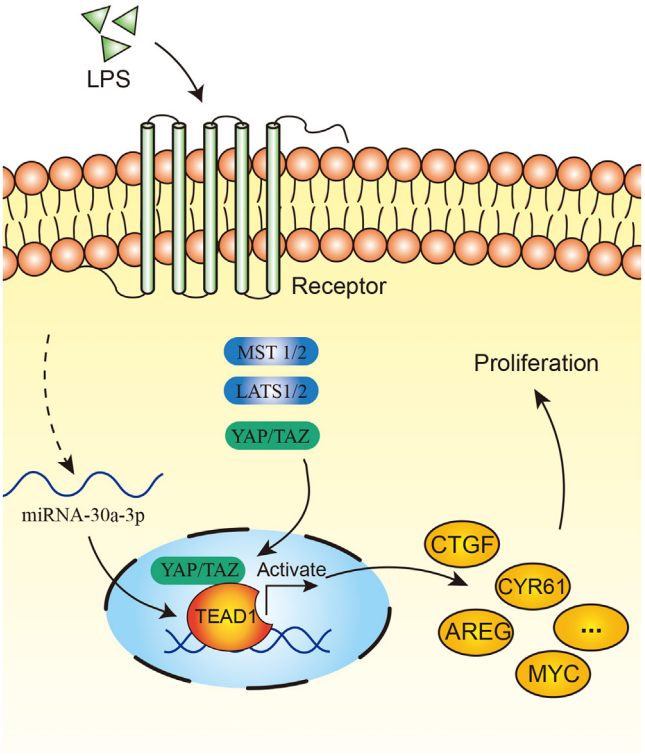

Figure 6. Expression of miRNA-30a-3p was down-regulated after LPS stimulation, subsequent increases in *TEAD1* levels may promote expression of downstream genes, leading to cell proliferation. Dotted arrows, negative regulation; solid arrows, positive regulation.
AREG: Amphiregulin; CTGF: Connective tissue growth factor; CYR61: Cysteine-rich angiogenic inducer 61; LATS1/2: Large tumor suppressor 1/2; LPS: Lipopolysaccharides; MST1/2: Macrophage stimulating 1/2; MYC: MYC proto-oncogene, bHLH transcription factor; TAZ: Transcriptional co-activator with PDZ-binding motif; *TEAD1*: Transcriptional enhanced associate domain 1; YAP: Yes-associated protein.

Conclusions

In a background of expression of inflammatory factors, including TNF-α, IL-1β, and IL-6, which were transiently increased in the sepsis-AKI model, miR-30a was down-regulated. Down-regulated miR-30a-3p may promote cell proliferation by targeting *TEAD1* in LPS-induced HK-2 cells, demonstrating its potential as a biomarker for early sepsis-AKI diagnosis. Further experiments are needed to verify the relationships between miR-30a and the YAP-TEAD complex and the underlying molecular mechanisms remain to be revealed.

Author Contributions

Junfeng Su: Conceptualization, Methodology, Visualization, Writing original draft. **Ying Wang:** Data curation, Formal analysis. **Jing Xie:** Methodology, Investigation. **Long Chen:** Soft-

ware, Validation. **Xinxin Lin:** Writing review & editing. **Jiandong Lin:** Resources, Supervision. **Xiongjian Xiao:** Project administration, Supervision.

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Ethics Statement

Animal handling and all experimental procedures were performed by applicable regulations and guidelines and approved by the Ethics Committee of Fujian Medical University, IACUC FJMU 2023-Y-0615.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

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

Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jointm.2023.08.004.

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