Suppression of lncRNA GAS6-AS2 alleviates sepsis-related acute kidney injury through regulating the miR-136-5p/OXSR1 axis in vitro and in vivo

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\textbf{ABSTRACT}

Acute kidney injury (AKI) is a common complication of sepsis and increase morbidity and mortality. Long non-coding RNA (LncRNA) GAS6-AS2 was related to inflammation and apoptosis in different diseases by regulating miRNAs and downstream genes, but its role in AKI remains unclear. Thus, we speculated that GAS6-AS2 might function in sepsis-related AKI via regulating target genes. Here, LPS or CLP was used to establish in vitro or in vivo sepsis-related AKI model. The interactions between GAS6-AS2 and miR-136-5p, and miR-136-5p and OXSR1, were validated by luciferase reporter assay, RNA pull-down, or RIP assay. Cell apoptosis was determined by flow cytometry, Western blotting, or IHC. The kidney injury was evaluated by H&E staining. The expression of GAS6-AS2, miR-136-5p, and OXSR1 was determined by qRT-PCR or Western blotting. We found that GAS6-AS2 was up-regulated in LPS-treated HK2 cells and the CLP-induced rat model. In vitro, GAS6-AS2 knockdown decreased cleaved caspase-3 and bax expression and increased bcl-2 expression. The levels of TNF-α, IL-1β, and IL-6 were reduced by GAS6-AS2 down-regulation. GAS6-AS2 knockdown ameliorated oxidative stress in the cells, as indicated by the reduced ROS and MDA levels and the elevated SOD level. In vivo, GAS6-AS2 knockdown decreased urinary NGAL and Kim-1 levels and serum sCr and BUN levels, and H&E proved that the kidney injury was alleviated. GAS6-AS2 knockdown also reduced apoptosis, inflammation, and oxidation induced by CLP in vivo. Mechanically, GAS6-AS2 sponged miR-136-5p which targeted OXSR1. Overall, lncRNA GAS6-AS2 knockdown has the potential to ameliorate sepsis-related AKI, and the mechanism is related to miR-136-5p/OXSR1 axis.

\textbf{INTRODUCTION}

Sepsis is a syndrome characterized by infection-related physiological, pathological, and biochemical abnormalities [1]. Severe sepsis frequently causes multiple organ dysfunction, with the kidney being particularly vulnerable [2]. Therefore, acute kidney injury (AKI) is a common serious complication of sepsis. In septic patients, sepsis-related AKI frequently contributes to high morbidity and mortality [3] and is also a risk factor for chronic kidney disease progression [4]. Sepsis is the most common cause of AKI, and AKI from any source is connected with a higher risk of sepsis development [5]. Toxic molecules produced by sepsis in high concentrations were able to reach proximal renal tubular cells, causing kidney injury, inflammation, oxidative stress, renal cell apoptosis, and ultimately cell damage [6]. Unfortunately, since supportive therapy for sepsis-induced AKI is generally inefficient, AKI-related mortality remains high. Thus, in order to improve survival and possibly optimize treatment of sepsis-related AKI in the future, an intensive study of the underlying mechanisms of AKI is required.

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Sepsis-induced AKI is accompanied by abnormal changes in the expression and function of a number of genes, including RNAs and proteins. Long non-coding RNAs (lncRNAs) are important molecules that regulate gene expression in a variety of diseases, and lncRNA dysregulation is frequently associated with physiological abnormalities [7]. The important role of lncRNAs in sepsis has been well confirmed [8]. LncRNA GSA6-AS2 is a novel discovered lncRNA, which was mapped to 13q34 with the transcript length of 12,361 bp [9]. LncRNA GAS6-AS2 has been shown to act as an oncogene to promote the development of a number of tumors, such as hepatocellular carcinoma [10], breast cancer [11], osteosarcoma [12], non-small-cell lung cancer [9], bladder cancer [13], and melanoma [14]. In addition, existing results indicated that GAS6-AS2 was related to inflammatory response [15] and apoptosis [14], which are also the important features of septic AKI. However, the role and mechanism of GAS6-AS2 in sepsis-related AKI are unknown.

LncRNAs typically functioned as competing endogenous RNAs (ceRNAs) in diseases, sponging microRNAs (miRNAs) [16]. MiRNAs have been revealed to be involved in the development of a number of diseases, sponging microRNAs and regulating protein expression in a variety of diseases, such as cancer [9], bladder cancer [13], and melanoma [14]. In addition, existing results indicated that GAS6-AS2 was also an effective biomarker for sepsis-related AKI [17], which are also the important features of septic AKI. Recent experimental studies have confirmed that the effects of GAS6-AS2 on the expression and function of miR-136 in LPS-induced HK2 cells,

Material and methods

Cell culture and cell transfection

Both the HK2 and human embryonic kidney epithelial (HEK-293T) cell lines were purchased commercially from American Type Culture Collection (ATCC, Manassas, VA). HK2 cells were grown in RPMI-1640 medium (Thermo Fisher, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT). HEK-293T cells were grown in Dulbecco’s modified Eagle’s medium (Thermo Fisher, Waltham, MA) containing 10% FBS. All cells were cultured in an incubator at 37°C with 5% CO₂. HK2 cells were stimulated for 4 h with 1 μg/mL LPS (Sigma, St. Louis, MO) and then for 1 h with 5 mM ATP (Sigma St. Louis, MO).

Below are the shRNA sequences for GAS6-AS2. sh1: 5′-CTGTAGTACCTTTTTGTC-3′; sh2: 5′-CTGGGAATGTCC-3′, and sh3: 5′-GCTCCGATCATGAGTATCA-3′. The shRNA negative control (sh-NC, Vigene Biosciences, Rockville, MD) or shRNA GAS6-AS2 (Vigene Biosciences, Rockville, MD) was transfected to HK2 cells according to the manufacturer’s instruction using Lipofectamine 3000 (Invitrogen, Beijing, China) reagent. miRNA negative control mimic (miR-NC), miR-136-5p mimic, miRNA negative control inhibitor (miR-NC inhibitor), and miR-136-5p inhibitor were purchased commercially from JTS Scientific (Wuhan, China). shRNAs and miRNA inhibitors were cotransfected using Lipofectamine 3000 reagent following the manufacturer’s instruction.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from cells by RNAsimple Total RNA Kit (Tiangen, Sichuan, China) according to the protocol. cDNA was synthesized using the M-MLV reverse transcriptase (Tiangen, Sichuan, China). The primer sequences in this part were as follows: GAS6-AS2, forward: 5′-AAGAGGAGCGCATAACC-3′, reverse: 5′-ATCGTGTAACACGGT-3′; oxidative-stress responsive 1 (OXSR1), forward: 5′-GTGGGCAATCAAACGGTAAAAC-3′, reverse: 5′-TGATGGCATTGACTCATGGCT-3′; GAPDH, forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′, reverse: 5′-U6, forward: 5′-CTCGCTTCCGCTTTCACGTTG-3′, reverse: 5′-AGGTAGTCCGAGGTTGCT-3′; miR-136-5p, forward: 5′-ACACTCCAGCTGGAGCTCCATTGT-3′, reverse: 5′-CCAGTGACGGGTCCGAGGT-3′; U6, forward: 5′-CTCGCTTGCGACGACCA-3′, reverse: 5′-AAGCCTCCAGAGTATGTTGC-3′. This experiment was performed using SYBR Green (Solarbio, Beijing, China).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in cells were tested respectively by Human TNF alpha ELISA Kit (Abcam, Shanghai, China), Human IL-1 beta ELISA Kit (Abcam, Shanghai, China), and Human IL-6 ELISA Kit (Abcam, Shanghai, China) according to the protocol. The levels of TNF-α, IL-1β, and IL-6 in animal tissues were
detected by corresponding Rat TNF-α, IL-1β, and IL-6 kits (Abcam, Shanghai, China). In brief, the cells were added with 50 μL of Antibody Cocktail and incubated at room temperature for 1 h. Following 3 washes with Wash Buffer PT, 100 μL of TMB substrate was added to each well and incubated for 10 min. Following that, 100 μL of Stop Solution was used, and the results were read at 450 nm wavelength of ultraviolet.

To collect serum, blood samples from rats were centrifuged at 5000 g for 15 min. The extents of blood urea nitrogen (BUN) and serum creatinine (sCr) were determined using the BUN Detection Kit (StessMarq Biosciences, Victoria, Canada) and the Serum Creatinine Detection Kit (StessMarq Biosciences, Victoria, Canada), respectively. Rat urine samples were collected by centrifuging at 600 g for 5 min. The concentrations of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (Kim-1) concentrations in rat urine were determined using ELISA kits (Cusabio Biotech, Wuhan, China) according to the instructions.

**Oxidative stress detection**

The concentrations of reactive oxygen species (ROS), malondialdehyde (MDA), and superoxide dismutase (SOD) in cells and animal tissues were measured using the Reactive Oxygen Species Assay Kit (Beyotime, Jiangsu, China), Lipid Peroxidation MDA Assay Kit (Beyotime, Jiangsu, China), and Total Superoxide Dismutase Assay Kit (Beyotime, Jiangsu, China) following the manuals.

**Flow cytometry**

Cell apoptosis rates were measured using the Annexin V-FITC Apoptosis Staining and Detection Kit (Abcam, Shanghai, China). Cells were collected by centrifuging for 5 min at 800 rpm. The cells in each well were resuspended in 1xAnnexin V Binding Buffer to 10^5–10^6 cells/mL after being washed with ice-cold medium. The cell suspension was then incubated on ice for 10 min in the dark with 1 μL of Annexin V-FITC Conjugate and 12.5 μL of propidium iodide. Finally, the cell suspension was diluted to a volume of 250 μL with ice-cold 1xAnnexin V Binding Buffer and analyzed using BD FACScanto II flow cytometry (BD, Piscataway, NJ).

**Animal experiment**

Male Wistar rats (11–12 weeks, 350–370 g, Trophic Animal Feed High-Tech Co., Ltd, Nantong, China) were housed at 22°C and subjected to 12/12 light/dark cycles. According to the previous study [20], the animal model of sepsis-induced AKI was established by CLP. Rats were fasted for 12 h before modeling and anesthetized with 0.3% sodium pentobarbital (30 mg/kg, Solarbio, Beijing, China) intraperitoneal injection. The rats’ abdomens were dissected along the midline to separate the mesentery and cecum. Following that, the ileocecal valves of each rat were ligated, and the end of cecum was punctured twice with a needle. The excrrent was pushed out, and the cecum was eventually re-inserted into abdominal cavity. The cecum was exposed without perforation in the sham operation group. The recombinant lentivirus pLVTHM plasmid carrying negative control shRNA or GAS6-AS2 shRNA was transfected into 293T cells with Lipofectamine 3000. Following the construction of the CLP animal models, the rat tail veins were injected with lentivirus carrying GAS6-AS2 shRNA (1 × 10^9 TU) or negative control shRNA. After 48 h of CLP surgery, all rats were sacrificed, and tissues from the left kidney were removed for subsequent experiments. All experiments were carried out in accordance with Chinese legislation on the use and care of laboratory animals and approved by The First Hospital of Hebei Medical University institutional ethics committee (SYXK (JI)2018-008).

**Western blot**

RIPA buffer (CST, Danvers, MA) was used to extract total proteins in cells and animal tissues. The protein in each sample was then quantified using the Enhanced BCA Protein Assay Kit (Beyotime, Jiangsu, China). Then, each protein sample at same concentration was subject to SDS-PAGE and transferred onto PVDF membranes (Millipore, Burlington, MA). After blocking with 3% bovine serum albumin (BSA, Beyotime, Jiangsu, China), the membranes were incubated overnight at 4°C with corresponding primary antibodies. The primary antibodies used in this experiment were depicted as follows: Bax (1:2000, Abcam, Shanghai, China), Bcl-2 (1:2000, Abcam, Shanghai, China), cleaved caspase-3 (1:2000, Abcam, Shanghai, China), OXSR1 (1:2000, Abcam, Shanghai, China), cleaved caspase-3 (1:2000, Abcam, Shanghai, China), and β-actin (1:3000, Abcam, Shanghai, China). The membranes were incubated for 1.5 h at room temperature with corresponding secondary antibodies (1:5000, Boster, Wuhan, China) and visualized using ECL Chemiluminescence Detection Kit (Beyotime, Jiangsu, China).

**Luciferase reporter assay**

The luciferase activity was measured 48 h after transfection using Dual-Luciferase® Reporter Assay System
following the manufacturer’s protocol. HK2 cells were co-transfected with a wild-type (WT) or mutant (MUT) IncRNA or OXSR1 reporter plasmid and either a miR-136-5p mimic or a negative mimic control using Lipofectamine 3000 (Invitrogen, Waltham, MA). The luciferase activity of Renilla was used as a control.

**RNA pull-down assay**

Biotinylated miR-136-5p or a miRNA control were transfected into HK2 cells. After 24 h of transfection, the cells were lysed and incubated with streptavidin-coated magnetic beads (Ambion Life Technologies, Carlsbad, CA). The bead-biotin complex was pulled down, and the abundance of GAS6-AS2 in bound fractions was analyzed using qRT-PCR.

**RNA immunoprecipitation (RIP) assay**

The RIP assay was performed using Magna RIP RNA Binding Protein immunoprecipitation kit (Millipore, Burlington, MA) according to the instructions. The antibodies used for RIP assay, Anti-Argonaute 2 (AGO2) or negative control IgG, were purchased from Millipore. The coprecipitated RNA was measured by qRT-PCR.

**Hematoxylin and eosin (H&E) staining**

Rat kidney tissues were fixed with 4% paraformaldehyde, blocked in paraffin, and then cut into 5 μm-thick sections. Dehydration was accomplished using a set of alcohol, and conventional paraffin embedding was conducted. The kidney slice samples were then stained with H&E. The results were observed under a microscope (BX-42, Olympus, Tokyo, Japan).

**Immunohistochemistry (IHC)**

The 4 μm-thick sections of rat kidney tissues were deparaffinized and rehydrated with xylene and deescaled ethanol (100, 95, 80, and 50%). Antigen retrieval was conducted in 10 mmol sodium citrate buffer. To block endogenous peroxidase activity, the slices were incubated in 3% (v/v) hydrogen peroxide in methanol for 30 min. After being washed with phosphate-buffered saline with tween-20 (PBST), the sections were blocked in phosphate-buffered saline (PBS) containing 2% normal goat serum, 2% BSA, and 0.1% triton-X. The slices were then incubated with primary antibodies (anti-cleaved caspase-3, anti-F4/80, CST, Danvers, MA) overnight at 4 °C, followed by incubation with secondary antibody (goat anti-rabbit, Zhongshan Golden Bridge Inc., Beijing, China) for 1 h at room temperature. After the PBST washes (3 × 5 min), the slices were incubated with streptavidin peroxidase for 30 min at indoor temperature and rinsed with distilled water. DAB Kit was used to develop colors. The slices were counterstained with hematoxylin. PBS was used instead of the primary antibody to prepare a negative control.

**Statistical analysis**

GraphPad Prism version 8.0 was used to analyze the data. Two groups were compared using the unpaired Student’s t-test, and multiple groups were compared using the one-way ANOVA followed by Dunnett’s post hoc test. Mann–Whitney U test was used to analyze fold change in qRT-PCR. Data were presented as mean ± standard deviation (SD). p < 0.05 was considered statistically significant. Every experiment was repeated at least three times.

**Results**

**GAS6-AS2 is highly expressed in LPS-induced HK2 cells, and GAS6-AS2 knockdown attenuates apoptosis, inflammation, and oxidative stress in LPS-induced HK2 cells**

Initially, the level of GAS6-AS2 expression in sepsis-related AKI cells was investigated. In LPS-treated HK2 cells, GAS6-AS2 was highly expressed (Figure 1A). AKI development is associated with inflammatory responses, oxidative stress, and apoptosis in molecular and cellular pathways [21]. Therefore, we investigated the role of GAS6-AS2 in regulating apoptosis, inflammation, and oxidative stress in the cells by suppressing GAS6-AS2. Figure 1B demonstrates that sh-GAS6-AS2#1 exerted the best inhibiting effect in HK2 cells. As a result, sh-GAS6-AS2#1 was used in subsequent research. A flow cytometry experiment indicated that LPS was able to induce apoptosis in HK2 cells, which was then significantly inhibited by GAS6-AS2 knockdown (Figure 1C). The Western blotting assay confirmed this result. The apoptosis markers, cleaved caspase-3 and bax, were up-regulated by LPS in HK2 cells, and the anti-apoptotic protein, bcl-2, was notably down-regulated. However, GAS6-AS2 knockdown could significantly reverse these effects in the cells (Figure 1D). LPS stimulation remarkably promoted the production of inflammatory factors, such as TNF-α, IL-1β, and IL-6 in HK2 cells, whereas GAS6-AS2 knockdown significantly relieved the inflammatory response in the cells (Figure 1E–G). Furthermore, we discovered that LPS treatment increased the accumulation of ROS and MDA while decreased the
level of SOD in HK2 cells, whereas GAS6-AS2 knockdown could reverse these trends in the cells (Figure 1(H–J)). Our findings suggested that GAS6-AS2 was aberrantly up-regulated in LPS-induced HK2 cells, and that GAS6-AS2 depletion could alleviate the apoptosis, inflammation, and oxidative stress of sepsis-related AKI in the cells.

**GAS6-AS2 directly interacts with miR-136-5p**

To investigate the potential mechanism of GAS6-AS2 in the regulations of AKI in cells, the bioinformatics software StarBase was used to identify the underlying target miRNAs of GAS6-AS2. As shown in Figure 2(A), GAS6-AS2 contained a complementary sequence of miR-136-5p, implying that GAS6-AS2 might target miR-136-5p. To validate this prediction, dual-luciferase reporter, RNA pull-down, and RIP assays were performed. The dual-luciferase assay results showed that transfection of miR-136-5p mimic significantly suppressed the luciferase activity of WT GAS6-AS2 in HK2 cells, but had no effect on the luciferase activity of MUT GAS6-AS2 (Figure 2(B)). According to RNA pull-down assay, GAS6-AS2 was found to be more enriched in the miR-136-5p group than in the miRNA control group (Figure 2(C)). The RIP assay revealed that GAS6-AS2 and miR-136-5p were preferentially enriched in miRNA ribonucleoprotein complexes containing AGO2 compared with anti-IgG immunoprecipitates (Figure 2(D)). The results of RNA pull-down and RIP were validated using agarose gel electrophoresis (Figure 2(C, D)). To identify the relationship between GAS6-AS2 and miR-136-5p, the GAS6-AS2 shRNA was transfected into LPS-stimulated HK2 cells. LPS treatment notably declined miR-136-5p expression, while GAS6-AS2 knockdown significantly elevated miR-136-5p expression in
Our results demonstrated that GAS6-AS2 directly interacted and negatively regulated miR-136-5p. OXSR1 is identified as the target gene of miR-163-5p. According to the online software starbase, OXSR1 was predicted to be a target of miR-136-5p, and their potential binding sequence was depicted in Figure 3(A). The dual-luciferase reporter assay revealed that the luciferase activity of wild type (WT) OXSR1 3’UTR was significantly reduced after miR-136-5p transfection in HK2 cells, but the luciferase activity of MUT OXSR1 3’UTR was unaffected (Figure 3(A)). Furthermore, miR-136-5p inhibited OXSR1 expression in HK2 cells (Figure 3(B)), indicating an inverse correlation between miR-136-5p and OXSR1. To further investigate the regulation of miR-136-5p on OXSR1 in AKI, HK2 cells were transfected with miR-136-5p inhibitor. Figure 3(C) validated that OXSR1 is identified as the target gene of miR-163-5p.

### Figure 2. GAS6-AS2 targeted miR-136-5p.

A. The target gene of GAS6-AS2 toward miR-136-5p was predicted by Starbase, and the base complementary pairings between GAS6-AS2 and miR-136-5p were shown. The mutated sequences in GAS6-AS2 were highlighted in red. B. The luciferase reporter gene assay was performed in HK2 cells to verify the targeting relationship of GAS6-AS2 and miR-136-5p. Overexpression of miR-136-5p inhibited luciferase activity in cells, and the inhibition disappeared after the predicted GAS6-AS2 binding site was mutated. C. Agarose gel electrophoresis and RNA pull-down experiment was performed in HK2 cells. The biotinylated miR-136-5p (Bio-miR-136-5p) or miRNA control (miR-NC) was transfected into HK2 cells. qRT-PCR was applied to quantify the RNA levels of GAS6-AS2. D. Agarose gel electrophoresis and RIP assay was used to determine GAS6-AS2 and miR-136-5p RNA enrichment in the immunoprecipitated complex in HK2 cells. E. The expression levels of miR-136-5p in HK2 cells from different groups were detected by qRT-PCR. **p < 0.01 compared with miR-NC, anti-IgG, or con group. Error bars represent mean ± SD. *p < 0.05 compared with LPS + sh-NC group.
the introduction of miR-136-5p inhibitor reduced the miR-136-5p expression in HK2 cells. Additionally, silencing miR-136-5p dramatically elevated OXSR1 protein expression in HK2 cells (Figure 3(D)). These results suggested that miR-136-5p directly targeted and negatively regulated OXSR1.

**GAS6-AS2 regulates LPS-induced HK2 cell damage through miR-136-5p/OXSR1**

OXSR1 protein expression was remarkably enhanced in LPS-treated HK2 cells and notably lessened after GAS6-AS2 shRNA transfection, but this inhibiting effect was reversed in the cells after miR-136-5p inhibitor introduction (Figure 4(A)), demonstrating that GAS6-AS2 sponged miR-136-5p to regulate OXSR1 expression. Next, we investigated the apoptosis phenotype in LPS-treated HK2 cells and found that down-regulation of miR-136-5p markedly weakened the inhibitory effect of GAS6-AS2 knockdown on cell apoptosis (Figure 4(B)). Consistent with the flow cytometry results, Western blotting confirmed that miR-136-5p inhibitor transfection promoted apoptosis of HK2 cells transfected with GAS6-AS2 shRNA, as evidenced by increased cleaved caspase-3 and bax protein levels and decreased bcl-2 protein expression (Figure 4(C)). With the application of ELISA kits to detect the inflammatory response and oxidative stress in cells, we validated that GAS6-AS2 downregulation led to declined levels of inflammation and oxidative stress, while inhibition of miR-136-5p significantly reversed these effects, as demonstrated by elevated TNF-α (Figure 4(D)), IL-1β (Figure 4(E)), IL-6 (Figure 4(F)), ROS (Figure 4(G)), and MDA (Figure 4(H)) levels and decreased SOD (Figure 4(I)) level. It was concluded that GAS6-AS2 was able to regulate LPS-induced HK2 cell damage via miR-136-5p/OXSR1.

**Knockdown of GAS6-AS2 attenuates sepsis-associated AKI in vivo**

To assess the effect of GAS6-AS2 on sepsis-related AKI in vivo, we measured several general AKI-associated

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**Figure 3.** miR-136-5p targeted OXSR1. A. The presence of miR-136-5p binding sites in OXSR1 3’UTR was predicted by Starbase software. The base complementary pairing relationship between miR-136-5p and OXSR1 were depicted, and the mutated sequences in GAS6-A25 were shown in red font. The luciferase reporter gene assay was carried out in HK2 cells to verify whether miR-136-5p interacted with OXSR1. B. After inhibiting the level of miR-136-5p, the expression of OXSR1 protein was detected by Western blotting in HK2 cells. C. qRT-PCR was used to determine the level of miR-136-5p in HK2 cells after miR-136-5p inhibitor transfection. D. The expression level of OXSR1 protein in HK2 cells with miR-136-5p knockdown was determined by Western blotting. Error bars represent mean ± SD. **p < 0.01 compared with miR-NC or NC inhibitor group.
markers in rats, including urinary NGAL and Kim-1 and serum sCr and BUN. As expected, IHC and Western blot proved that the expression of NGAL and Kim-1 was elevated in the animal model with sepsis-related AKI, while knockdown of GAS6-AS2 effectively ameliorated these trends (Figure 5(A,B)). The levels of NGAL and Kim-1 in the urine of rats were significantly increased by CLP but dramatically reduced by GAS6-AS2 knockdown (Figure 5(C)). Meanwhile, CLP-induced septic AKI notably enhanced the levels of sCr and BUN in rat serum, while GAS6-AS2 knockdown markedly repressed these increases (Figure 5(C)). The impact of GAS6-AS2 depletion on AKI in animal model was assessed by H&E staining. The CLP + sh-NC group exhibited multitubular edema and coagulated necrosis, glomerular atrophy, and cystic dilatation. In contrast, the CLP + sh-SDH5 group had much less severe kidney damage and a lower injury score (Figure 5(D)). As expected, GAS6-AS2 knockdown notably elevated the level of miR-136-5p but restrained OXSR1 expression in vivo (Figure 5(E)). The cleaved caspase-3 level in renal tissues from each group, as determined by IHC, also showed that GAS6-AS2 depletion visibly reduced the apoptosis induced by CLP in vivo (Figure 5(F)). GAS6-AS2 down-regulation significantly inhibited the expression of pro-apoptotic proteins, cleaved caspase 3 and bax, and significantly promoted the expression of anti-apoptotic protein, bcl-2, suggesting that the apoptosis in CLP-treated rats was inhibited (Figure 6(A)). Moreover, we found that GAS6-AS2 knockdown significantly reduced inflammation in rats with AKI, as indicated by lower levels of TNF-α (Figure 5(G)), IL-1β (Figure 5(H)), and IL-6 (Figure 5(I)).
Figure 5. GAS6-AS2 knockdown reduced sepsis-related AKI in vivo. CLP-induced sepsis-related AKI were constructed in Wistar rats (n = 6), followed by corresponding treatment. The cecum in sham operation group was exposed without perforation. A, B. The expression of NGAL and Kim-1 in the renal tissues of rats from each group was assessed by IHC (Scale bar = 100 μm) and Western blot. C. The levels of NGAL and Kim-1 in the urine samples of rats and sCr and BUN in peripheral blood samples of rats were measured by corresponding ELISA kits. D. The rat kidney sections from different groups were stained with H&E and observed by a brightfield microscopy (Scale bar = 100 μm). The areas of damage are indicated by arrows. Histological scores of renal tissue injury were assessed by a blinded pathologist. E. qRT-PCR was used to measure the levels of GAS6-AS2, miR-136-5p, and OXSR1 in different groups of rat models. F. IHC was performed to detect cleaved caspase-3 levels in renal tissues of rats in different groups (Scale bar = 100 μm). G–I. The levels of TNF-α, IL-1β, and IL-6 in different groups of rat models were determined by ELISA. J. The levels of ROS, MDA, and SOD in different groups of rat models were detected by the kits. Error bars represent mean ± SD. **p < 0.01 compared with con group. *p < 0.05 compared with CLP + sh-NC group.
The IHC staining showed that GAS6-AS2 knockdown significantly decreased F4/80+ macrophages infiltration in renal tissues of CLP-treated rats (Figure 6B). The oxidative damage induced by CLP was strikingly alleviated in rat renal tissues by down-regulation of GAS6-AS2 (Figure 5J). These results indicated that GAS6-AS2 depletion could attenuate sepsis-related AKI in vivo.

Discussion

AKI is a severe and potentially fatal disease. Sepsis-mediated AKI exhibited a clear inflammatory response and oxidative stress, accompanied by significant apoptosis [22,23]. In HK-2 cells, LPS has been widely used to simulate AKI symptoms associated with sepsis [24]. Furthermore, CLP has been generally applied to induce severe sepsis, and AKI is demonstrated by a greater decline in renal microcirculation [25]. In this study, the role of GAS6-AS2 in the pathophysiological mechanism of sepsis-related AKI was explored. It has been demonstrated that IncRNA GAS6-AS2 was up-regulated in various cancer cells or tissues [9–13,26]. Here, GAS6-AS2 expression was found to be abnormally up-regulated in HK2 cells treated with LPS and renal tissues from sepsis AKI rats, which was consistent with previous results. Additionally, GAS6-AS2 is associated with the apoptosis of melanoma cells [14], hepatocellular carcinoma cells [10], and osteosarcoma cells [12], as well as inflammation [15]. We found that down-regulation of GAS6-AS2 could significantly relieve apoptosis, inflammatory response, and oxidative stress in renal epithelial cells, as well as reduce kidney pathology in AKI rats.

A series of evidences have indicated that sepsis promoted the release of inflammatory factors in renal tissues, which can lead to significant renal cell apoptosis and, as a result, AKI [27]. In chronic kidney disease rat models, increased TNF-α expression was closely related to enhanced renal inflammation [28]. The levels of IL-1β and IL-6 mRNA transcription in renal tubular epithelial cells of septic rats were significantly increased [29], and IL-6 knockout can ameliorate renal injury [30]. In this study, GAS6-AS2 depletion could dramatically reduce the inflammation in sepsis-related AKI cells and rats. Oxidative stress and subsequent oxidative damage play a major role in the occurrence and progression of AKI [31]. Multiple pathways for the production of excess ROS in the kidney have been identified as being associated with kidney disease [32]. In this study, we found that silencing GAS6-AS2 could notably decrease the oxidative stress in cells and animal models. Inflammation and oxidative stress can also accelerate cell apoptosis, exacerbating sepsis-related AKI. Our findings manifested that GAS6-AS2 knockdown could remarkably inhibit the apoptosis of LPS-induced cells and renal.
cells in CLP-induced rats. GAS6-AS2 was identified to be the upstream regulator for GAS6 [15]. GAS6 contains 4 epidermal growth factor-like domains and 2 laminin globular-like domains that contain the interaction sites for its TAM (Tyr03, Axl, and Mer) receptor tyrosine kinases [33]. The GAS6/TAM system is involved in a variety of pathophysiological processes, such as apoptosis inhibition [34,35], inflammation inhibition [36], and oxidative stress [37]. Thus, we speculated that GAS6-AS2 might alleviate sepsis-related AKI in this way. AKI is characterized by acute renal dysfunction [19]. Many protein products, such as NGAL and Kim-1, have been identified as novel markers in the early stage of AKI [38,39]. NGAL has prognostic value in predicting acute and chronic kidney disease and renal function deterioration in patients [40]. Kim-1 is difficult to be detected in healthy kidneys, but it is highly expressed in proximal tubule cells after ischemia and nephrotic injury [41]. Additionally, BUN and sCr are the biomarkers of renal dysfunction [42]. In our work, urinary NGAL and Kim-1 levels, as well as serum sCr and BUN levels, were sharply decreased after silencing GAS6-AS2, which was consistent with the histopathological alterations. Our results suggested that inhibiting GAS6-AS2 could ameliorate kidney injury and improve renal function.

In general, IncRNAs [43] target miRNAs to regulate the expression levels of downstream genes, thereby accelerating apoptosis and inflammation in diseases [8]. We proved for the first time in this experiment that IncRNA GAS6-AS2 could interact with miR-136-5p to perform its function. miR-136 was involved in inflammation and oxidative stress processes in different diseases. For instance, in human granulosa-like tumors, miR-136 mediated circle RNA 0118530 to regulate inflammatory response and oxidative stress [44]. Previous research also indicated that miR-136 overexpression reduced inflammatory cytokines and oxidative stress factors, as well as HK2 cell injury caused by hypoxia/reoxygenation condition, and circle RNA 0023404 triggered HK2 cell injury via sponging miR-136 [19]. Additionally, miR-136 has been proved to promote renal fibrosis in diabetic rats by suppressing tyrosine kinase SYK and TGF-β1/Smad3 pathway [45]. Here, down-regulation of GAS6-AS2 directly targeted and negatively modulated miR-136-5p to relieve HK2 cell injury and rat renal injury, which was consistent with prior researches.

MiRNAs bind to the 3’-UTR of target mRNAs, resulting in mRNA degradation and inhibition of mRNA protein translation [46]. We verified that miR-136-5p could directly target and bind to OXSR1 mRNA, and that OXSR1 expression was indeed negatively correlated with miR-136-5p in HK2 cells and rat renal tissues. OXSR1 is a serine/threonine kinase that regulates downstream kinases in response to environmental stress and is responsible for the co-transportation of ions in kidney [47]. Previous research revealed that OXSR1 level was higher in the serum of sepsis-related AKI patients compared to healthy individuals, and OXSR1 protein expression was strongly enhanced in LPS-stimulated HK2 cells compared to control cells [48]. In this study, OXSR1 expression was lifted in LPS-challenged cells and CLP-induced rats, which was in line with previous findings. However, the function and mechanism of OXSR1 in sepsis-related AKI are not well understood. OXSR1 is involved in cytoskeletal rearrangement and osmotic stress response, controlling cell proliferation and apoptotic death [49]. It could be a potential mechanism by which OXSR1 affects the apoptosis in renal injury. Prior studies have shown that OXSR1 is accountable for the phosphorylation of receptors expressed in lymphoid tissues (RELT) [50], and RELT activation promoted cleaved caspase-3 expression and induced human epithelial cell apoptosis in a new extrinsic manner [47]. RELT also recruits members of TNFR family, which in turn leads to the activation of key factors in different pathways, including oxidation-related p38 (p38 MAPK) pathway and inflammation-related p65 (NF-κB) pathway, both of which play crucial roles in the development of AKI [51].

Based on OXSR1 functions in apoptosis, inflammation, and oxidation, the beneficial effects of GAS6-AS2 knockdown on sepsis-related AKI could be attributed primarily to suppressing OXSR1 expression via negatively regulating miR-136-5p.

In conclusion, down-regulation of GAS6-AS2 could alleviate sepsis-induced AKI by reducing apoptosis, inflammatory response, and oxidative stress. Furthermore, we verified that GAS6-AS2 directly interacted with miR-136-5p to regulate OXSR1 synthesis, thereby modulating sepsis-related AKI injury. In this work, the experiments that have been carried out should be sufficient to draw a conclusion that inhibition of GAS6-AS2 had a protective effect on septic AKI. The purpose of cell experiment is to reveal the function and explore the corresponding mechanism, and in vivo experiment was also performed, which is in line with the idea of medical research: cell exploration first, then animal verification [52,53]. However, there are still some limitations to our study. The pathology of sepsis-induced AKI is relevant to multiple signaling pathways, the molecule network that correlated with GAS6-AS2 will be further investigated in the future, and more data from human samples of AKI patients should be analyzed. An unbiased analysis for micro-RNA seq would be more supportive element for this study. Moreover, transfection of GAS6-AS2 shRNA could not specifically target renal...
tissues, so whether the beneficial effect is mediated by kidney GAS6-AS2 is unclear in this article. Our study suggested that GAS6-AS2 might be a novel therapeutic target for the medicinal development of sepsis-related AKI.

**Author contributions**

Guangwei Ren, Liqin Mu, Xiuhong Hu, Baozhen Xu, and Yuping Li performed most of the experiments and analyzed data. Hongrui Cui and Zheli Niu designed the study and are guarantors of integrity of the entire study. Hongrui Cui, Guangwei Ren, Zheli Niu, and Liqin Mu prepared the manuscript.

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**Data availability statement**

All data generated or analyzed during this study are included in this published article.

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